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 a New Zealand freshwater snail species 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 morphologically 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.

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

We investigate how shifts in reproductive mode (here, sexual to asexual reproduction) alter the selective regime experienced by traits that are used only for sexual reproduction. Our particular focus is on the nature and extent of the loss of “maleness” in asexual lineages where females reproduce without any need for males. In this scenario, males, and the genes underlying male-specific traits, are presumably extraneous (Carson *et al.* 1982). This is the basis for the expectation that in these asexual lineages, traits needed only for sexual reproduction (“sex-specific traits”; *e.g.*, genes used only for meiosis or male-specific traits like sperm) (Schurko & Logsdon 2008; Tvedte *et al.* 2017) will experience a shift from purifying to relaxed selection at the onset of asexuality. If maintenance of these now-neutral sex-specific traits is not otherwise costly, and if the genes underlying the sex-specific traits are not 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). If maintenance of these traits does involve cost, selection favoring trait loss in the
67 asexual lineage can contribute to the rate of trait degeneration. These expectations have been
68 evaluated with respect to sexual signaling in bush crickets (Lehmann *et al.* 2007) and wasps
69 (Pannebakker *et al.* 2005), sperm-storage organs and fertilization success of males produced by
70 otherwise all-female asexual lineages in stick insects (Schwander *et al.* 2013), and sperm formation in
71 oribatid mites (Taberly 1988; Maraun 2003; Heethoff 2007). While these studies indicate that transitions
72 to asexuality often but not always (*e.g.* Lehmann *et al.* 2007) underlie sex-specific trait degeneration,
73 the conclusions that can be drawn are limited by the inability to directly compare the sexual and asexual
74 forms (*e.g.*, sexuals and asexuals are allopatric, are different species, or the asexuals are hybrids).

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

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

88 We are able to overcome both of these barriers by taking advantage of the occasional production of
89 male offspring by obligately asexual *Potamopyrgus antipodarum* (Neiman *et al.* 2012). This New Zealand
90 freshwater snail provides a powerful means of directly evaluating the impact of shifts in reproductive
91 mode because phenotypically and ecologically similar obligately sexual and obligately asexual snails
92 regularly coexist in natural populations (Lively 1987; Neiman *et al*. 2011), enabling direct comparisons
93 between sexual and asexual individuals, genomes, and populations. Asexual *P. antipodarum* are the
94 product of multiple separate transitions from sexual conspecifics, allowing us to treat distinct asexual
95 lineages as replicated experiments into asexuality (Neiman *et al.* 2011; Paczesniak *et al.* 2013). The
96 polyploidy (triploidy and tetraploidy; Wallace *et al*. 1992; Neiman *et al*. 2011) of asexual *P. antipodarum*
97 also allows us to use comparisons between the two asexual ploidy levels and the diploid sexuals to study
98 the effects of ploidy elevation.

99 What makes *P. antipodarum* particularly well suited for this study is that multiple asexual female
100 lineages occasionally (~1.5% of all offspring, depending on the asexual lineage involved; Neiman *et al.*
101 2012) produce males, perhaps *via* loss of sex chromosomes during oogenesis (Soper *et al.* 2013). We
102 leverage this unusual phenomenon to characterize morphological variation of sperm in sexually *vs.*
103 asexually produced males, with the expectation that the latter have experienced a relatively recent
104 history of relaxed selection on male-specific traits and genes. A general pattern of distinct phenotypes
105 and, in particular, higher variation across sperm phenotypes from asexual *vs.* sexual males would be
106 consistent with this prediction.

107 While we have been implicitly assuming that male genotype drives sperm phenotype (Hecht 1998), we
108 also took advantage of the fact that eukaryotic cell size and nuclear genome content are typically
109 positively correlated (Gregory 2001; Jovtchev *et al.* 2006; Beaulieu *et al.* 2008) - including in sperm cells
110 (Cui 1997; Gallardo *et al.* 1999; van Munster *et al.* 1999)- to establish predictions that can also enable us
111 to decouple effects of nuclear genome DNA content (which accounts for both ploidy elevation and

112 diploid genome size changes) from those of transitions to asexuality. In particular, we expected that the
113 mean size of sperm heads will increase with increasing nuclear genome content (regardless of
114 reproductive mode), but that increased nuclear genome DNA content will not be associated with
115 increased sperm tail length because the tail does not contain nuclear DNA. In other words, dual main
116 effects of nuclear DNA content and reproductive mode on sperm morphology in *Potamopyrgus* should
117 manifest as consistently larger sperm heads (nuclear DNA content) along with consistently higher
118 variance in both sperm heads and tails in the asexually produced males.

119 *Materials and Methods*

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

121 *Potamopyrgus antipodarum* used in this study were collected either from New Zealand lake populations
122 (7 lakes; 21 sexual males) or from individual and separately derived asexual lineages (8 lineages; 24
123 asexual males). The latter are recently descended (<5 generations) from asexual snails collected from
124 New Zealand lake populations that were subsequently cultured in our laboratory at the University of
125 Iowa. All snails were housed in plastic 10L tanks filled with oxygenated carbon-filtered tap water in a
126 16C room with a 16:8-hr light:dark cycle and fed dried *Spirulina* algae *ad libitum* (following Zachar &
127 Neiman 2013). We focused our search for asexual males in lineages that had been previously
128 characterized as relatively likely to harbor asexual males (Neiman *et al.* 2012) and asexual lineages
129 descended from lake populations that population genetic studies (Paczesniak *et al.* 2013) demonstrated
130 harbor asexual subpopulations that represent separate *in situ* transitions from sexual to asexual
131 reproduction. For the sexual males, we used field collections from lakes that were known to contain
132 relatively high frequencies (>10-20%) of sexual males (Neiman *et al.* 2011).

133 We identified males by turning each individual snail on its back (*i.e.*, foot up) under a dissecting
134 microscope and then carefully looking for the penis, evident on the right side of the body of adult male

135 snails while the snail is attempting to right itself. We used snails from field collections (sexed a total of
136 1370 snails) or lineages (sexed a total of 4505 snails) that were large enough (>/=3mm shell length) to
137 have reached sexual maturity (*e.g.*, Larkin *et al.* 2016). Because we did not find any asexual males in field
138 collections despite evaluating > 380 field-collected male *P. antipodarum* from eight lake populations
139 (unsurprising in light of the scarcity of these males amongst all asexual *P. antipodarum*; Neiman *et al.*
140 2011, 2012), we had to compare field-collected sexual individuals to laboratory-reared asexual males.
141 Because environmental conditions can influence sperm morphology (Perez-Crespo & Guitierrez-Adan
142 2008; Immler *et al.* 2010; Bakos *et al.* 2011), we addressed whether rearing conditions had an effect on
143 sperm morphology by comparing 12 sperm from each of three males from a lab-reared sexual lineage to
144 12 sperm from each of three wild-caught sexual males from the same lake of origin (Alexandrina) as the
145 lab-reared sexuals. There were no significant differences between lab-reared and field-collected males
146 in any of the traits that we measured (Supp Figure 1A). A principal component analysis demonstrated
147 nearly complete overlap between wild-caught and lab-reared snails when plotting the first two principal
148 components (explaining 77.7% of all variation) of sperm traits (Supp Figure 1B), suggesting that field *vs*.
149 lab rearing environments are not likely to provide a major source of variation in sperm morphology.

150 We used a Becton Dickinson ARIA II flow cytometer on dissected head tissue (following Bankers *et al.*
151 2017) to assign ploidy (diploid, triploid, tetraploid) and, thus, reproductive mode (diploid = sexual;
152 triploid, tetraploid = asexual; Wallace 1992; Neiman *et al*. 2011) to each male. The overall incidence of
153 males in asexual lineages was ~1% (59/5875), and asexual lineages varied in the relative frequency of
154 male production (Supp Table 1). Both observations regarding male frequency in asexual lineages are
155 consistent with a similar survey in Neiman *et al*. (2012). Because our experimental design goal was to
156 replicate males three times within each asexual lineage that we used, and because we did not want to
157 perform comparisons involving variation with unbalanced sample sizes, we haphazardly excluded 35
158 males that either represented a lineage in which more than three males were found (13 asexual

159 lineages) or were from a lineage in which fewer than three males were found (six asexual lineages). This
160 left us with a total of 24 asexually produced male *P. antipodarum* for our study. For sexual males, we
161 used the first three haphazardly selected sexual males that we identified per each of seven field
162 collections, for a total of 21 sexual male *P. antipodarum*.

163 Hereafter, we use "set" to indicate one three-male replicate from each unique lineage or lake of origin
164 and ploidy combination: our diploid *P. antipodarum* sample represents seven male sets (each from a
165 different field collection), triploid *P. antipodarum* are represented by six male sets (each from a different
166 asexual lineage and lake of origin), and tetraploid *P. antipodarum* are represented by two male sets
167 (from two different asexual lineages, each from a different lake), for a total of 15 *P. antipodarum* sets
168 and 45 males (Figure 1).

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

177 **Sperm extraction and imaging**

178 We used forceps to extract the bodies of male snails from their shells and then dissected away the
179 sperm duct, which transports sperm and seminal fluid from the testis to the penis. We centered the
180 sperm duct tissue on a glass microscope slide and added ~5µl of DAPI staining solution (following Soper
181 *et al.* 2013). We used repeated pipetting of this solution to release sperm from the ruptured duct and

182 distribute the sperm across the slide. We then mounted the slide on an Olympus IX71 fluorescence
183 microscope and used the DAPI filter to locate the fluorescent sperm heads and the polarized filter to
184 photograph sperm for subsequent analysis. We haphazardly selected and then photographed a
185 minimum of 12 whole sperm cells for every male. We chose 12 sperm because the amount of variation
186 around the mean when including an increasing amount of sperm measurements asymptotes at ~10
187 sperm in our rarefaction analysis (see Supp Figure 2). We excluded sperm cells that were entangled with
188 other sperm or were obscured by non-sperm cellular detritus. We used sperm from at least three males
189 (one male set) for each unique lake and ploidy combination, for a total of at least 36 sperm per set.

190 **Phenotype measurements**

191 We imported photographs into ImageJ (Rueden *et al.* 2017) and measured head length (HL; head tip to
192 head base), head width (HW; width at midpoint of head), head circumference (HC; perimeter of head),
193 and tail length (TL; length from head base to tail end) for each sperm cell. We used our consistent
194 observations of a number of qualitatively distinct phenotypic classes of sperm abnormalities to sort
195 sperm into "normal" (most common phenotype of smooth cylindrical head and smooth tail of consistent
196 width; 79% of all sperm) *vs.* one of six "abnormal" ultrastructural categories: >1 tail, distorted neck,
197 oblong head, tail bump, two heads, wrinkled head (*e.g.*, Menkveld *et al.* 1990). This approach allows us
198 to distinguish large-scale differences in complex sperm morphology that might not be evident from our
199 measurements of individual sperm traits.

200 **Statistical analyses**

201 We used a RAxML tree (CIPRES V. 3.1) generated from Illumina paired-end whole-genome sequencing
202 data (70,437 SNPs) from 26 *P. antipodarum* (10 sexual individuals, 16 asexual individuals (11 triploid and
203 five tetraploid)) to provide a phylogenetic framework needed to control for potential effects of
204 phylogenetic relationship on sperm phenotype (Supp Figure 3). This tree also demonstrates that we

205 have likely captured a broad sample of sexual and asexual *P. antipodarum* genetic diversity, including a
206 number of separately derived asexual lineages. We used this tree in Phylosignal (Keck *et al.* 2016), an R
207 package that tests for phylogenetic signal, to assess whether sperm morphology was determined at
208 least in part by shared ancestry. We attached mean trait values for each of the sperm traits measured
209 below to a subsampled phylogenetic tree, which represented directly (same asexual lineage) or
210 indirectly (shared lake of origin, justified by multiple lines of evidence for a predominant effect of source
211 population on *P. antipodarum* genotype, *e.g.*, Dybdahl & Lively 1995; Paczesniak *et al.* 2013; Bankers *et*
212 *al.* 2017) nine of the 15 *P. antipodarum* sets from which we sampled males (*i.e.*, sets with both genetic
213 data and sperm measurements). We did not find any significant phylogenetic correlations underlying
214 these sperm traits (Supp Figure 4). Together, the outcome of these analyses indicate that phylogenetic
215 relatedness is not a main driver of variation in sperm morphology. We also used hierarchical clustering
216 of sperm morphology data to produce a cladogram that we used to assess whether phylogenetic
217 relatedness is a main source of across-lineage/lake sperm variation (Figure 2, Supp Figure 5).

218 We then used the means calculated from the 12 sperm from each individual male for each trait (HL, HW,
219 HC, TL) in the general linear model and Tukey posthoc pairwise comparison analysis framework as
220 applied in SPSS 24 to assess whether the samples were analytically distinguishable across the
221 comparison categories of nuclear genome DNA content, ploidy, and reproductive mode. Finally, we used
222 Linear Discriminate Analysis (LDA) to assess whether and how ploidy level affects sperm morphology.
223 This analysis approach minimizes within-group variance and maximizes across-group variance to find
224 linear combinations of variables that best explain the data. All statistical analyses were implemented in
225 R 3.4.1 (R Core Team 2013) or SPSS 24.0 (IBM Corp. 2016)

226 *Results:*

227 **Sperm morphology is primarily driven by nuclear genome content and reproductive mode**

228 We evaluated a total of 750 sperm from each of 57 male snails (21 diploid sexuals, 18 triploid asexuals,
229 six tetraploid asexuals, nine *P. estuarinus*, three *H. pupoides*) (Figure 1), derived from 12 lakes and three
230 estuaries of origin as well as three species. Our analyses revealed extensive qualitative (Supp Figure 6)
231 and quantitative variation (Supp Table 2, Supp Figure 7) in sperm morphology. Multi-dimensional
232 hierarchical clustering of primary sperm traits (HL, HW, HC, TL) suggested that reproductive mode is a
233 primary driver of sperm morphology in *P. antipodarum* (Figure 2). This conclusion is bolstered by the
234 fact that diploid sexual *P. antipodarum* sperm clustered with the two diploid sexual outgroup species
235 instead of with the asexual polyploid conspecifics.

236 We next used comparisons across the three ploidy levels represented by *P. antipodarum* and sperm
237 from the diploid outgroup species *P. estuarinus* and *H. pupoides* to decouple effects of nuclear genome
238 DNA content from ploidy and reproductive mode on sperm morphology. These comparisons revealed
239 that nuclear genome DNA content seems to have a major effect on head shape, with head shape metrics
240 increasing consistently with nuclear genome content. Nevertheless, that nuclear genome DNA content
241 *per se* is not the sole driver of sperm morphology in these snails is demonstrated by several lines of
242 evidence. First, sperm produced by *H. pupoides* and *P. estuarinus*, which seem to share the same
243 relatively small nuclear DNA content (Logsdon *et al*. 2017), were nevertheless morphologically distinct
244 (Figure 2, Supp Figure 9). Second, head width and tail length were statistically indistinguishable between
245 triploid and tetraploid *P. antipodarum* (Supp Figure 7). Third, the CVs for triploid and tetraploid *P.*
246 *antipodarum* are statistically indistinguishable for all head-related traits (Supp Figure 8). Fourth, while
247 tail length is similar across different nuclear DNA contents in *P. antipodarum*, the coefficient of variation
248 of our tail length estimates is significantly increased in triploid (CV mean = 12.2) and tetraploid (CV mean
249 = 14.9) sets relative to diploids (CV mean = 4.7) (Supp Figure 8). Finally, our linear discriminant analyses
250 demonstrate that the three different *P. antipodarum* ploidies partially overlap each other in
251 morphometric space (in particular, triploid-produced sperm share space with both diploid and

252 tetraploid-produced sperm) (Figure 3). We also detected relatively minor evidence for ploidy effects on
253 sperm trait means and variances: the LDA revealed ploidy-based clustering in *P. antipodarum* (Figure 3),
254 and the relative magnitude of change between measurements of *P. antipodarum* head length and head
255 circumference across ploidy levels show a pattern consistent with a positive effect of ploidy-driven
256 variation of nuclear genome DNA content on sperm head size.

257 With respect to the latter point, the magnitude of fold difference in sperm head size traits is the lowest
258 in the triploid/tetraploid comparison (*i.e.*, a 33% increase in nuclear genome DNA content), and the
259 highest in the diploid/tetraploid comparison (*i.e.*, a 100% increase in nuclear genome DNA content),
260 with the diploid/triploid values in between (*i.e.*, a 50% increase in nuclear genome DNA content). This
261 pattern is as expected in a situation where sperm heads increase in size as nuclear DNA content
262 increases, but only in proportion to the proportional increase in nuclear DNA content (Supp Figure 10).

263 Finally, as predicted under a scenario where the absence of sex translates into relaxed selection, CV-
264 based analyses indicated that sperm produced by diploid male *P. antipodarum* harbors significantly
265 lower variance (*e.g.*, HL mean = 4.48µm, SD = 0.09) than sperm produced by triploid (HL mean = 5.29µm,
266 SD = 0.32) and tetraploid (HL mean = 5.99µm, SD = 0.22) males (Supp Figure 8).

267 **Asexual *P. antipodarum* often produce qualitatively abnormal sperm**

268 Visual inspection of *P. antipodarum* sperm revealed substantial variation. Most (N=429; 76%) of these
269 sperm had an overtly normal phenotype, represented by a smooth and cylindrical head and a tail of
270 consistent width from the head base to tip (Supp Figure 6A). We sorted the most common deviations we
271 observed from this normal phenotype into six discrete categories that emerged during our imaging and
272 analysis stages: >1 tail (3.5% of all sperm), distorted neck (2.5%), oblong head (1.5%), tail bump (10%),
273 two heads (1%), and wrinkled head (6.5%) (see Supp Figure 6B-D). There was markedly higher incidence
274 of abnormal sperm produced by triploid (N=95; 43% of all triploid sperm) and tetraploid (N=24; 27%) *P.*

275 *antipodarum* relative to the sperm produced by diploid *male P. antipodarum* (N=16; 6%) (Fisher's exact
276 test $p = < 0.001$) (Figure 4). Misshapen heads were the only abnormality shared across all three ploidies,
277 while the most common abnormality, tail bump, is restricted to triploid asexual *P. antipodarum*.

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

279 We used a principal component analysis to visualize and compare multivariate sperm phenotypes across
280 sexual *P. antipodarum* and the two closely related sexual outgroup species. This analysis revealed a
281 distinct species-level signal, with no overlap between the clusters of sperm data corresponding to each
282 species (Supp Figure 9). Our general linear model and Tukey posthoc analyses also demonstrated
283 statistically significant differences in each of the four individual sperm traits amongst these three species
284 (Supp Table 2).

285 *Discussion*

286 We used the powerful *Potamopyrgus antipodarum* system to perform a direct comparison of male-
287 specific traits across otherwise similar male snails that differ in ploidy level and reproductive mode. Our
288 study is especially novel in comparing sperm phenotypes across reproductive modes as well as ploidy
289 level and diploid nuclear genome sizes, allowing us to at least in part decouple effects of polyploidy and
290 genome size from those of asexuality.

291 *Nuclear DNA content affects sperm head size, while reproductive mode affects sperm variation*

292 We used comparisons across the three different *P. antipodarum* ploidies and the three different diploid
293 sexual taxa included in our study to assess the extent to which increased nuclear genome DNA content
294 in the asexual *P. antipodarum*, *versus* asexuality *per se*, might affect sperm morphology. That asexual-
295 produced sperm are generally larger, and that these size differences to some extent scale with ploidy
296 and nuclear genome DNA content (Supp Figure 7), is consistent with the former possibility. Even so, the

297 significant differences in sperm trait values (*e.g.*, head length) between *P. estuarinus* and *H. pupoides*,
298 which have virtually identical genome sizes (Logsdon *et al.* 2017), and the significantly increased tail
299 length variation in asexuals indicates that other factors besides nuclear genome content (including but
300 not necessarily limited to reproductive mode) are also important in determining sperm morphology. The
301 different outcomes between the analyses of head *vs*. and tail-related traits also provide a strong line of
302 evidence in support of our conclusion that both nuclear genome DNA content and reproductive mode
303 contribute to *P. antipodarum* sperm morphology. In particular, we found that sperm head trait values
304 (*e.g.*, mean HL diploid = 4.48 µm, SD = 0.09; triploid = 5.29 µm, SD = 0.32; tetraploid = 5.99 µm, SD
305 =0.22) but not tail length (mean tail length diploid = 128.6 µm, SD = 3.34; triploid = 137.73, µm SD =
306 22.05; tetraploid = 137.52 µm, SD = 12.99) increase with genome size. This result is as predicted if
307 nuclear genome DNA content affects the sperm nucleus size, which is housed in the sperm head.
308 The fact that the CVs are distinctly higher for both head and tail traits in the asexual *vs*. sexual males
309 (*e.g.*, CV mean HL diploid = 3.2, SD = 0.92; triploid = 5.8 SD, = 1.98; tetraploid = 5.5, SD = 1.66; CV mean
310 TL diploid = 4.7, SD = 3.52; triploid = 12.2, SD = 10.41; tetraploid = 14.9, SD = 7.90), along with the
311 pronounced increase of incidence of tail abnormalities in asexuals relative to sexuals, also points to a
312 distinct role for reproductive mode. In particular, the higher CVs across both heads and tails in asexual
313 males are as expected if relaxed selection on sperm traits is driving a higher rate of morphological
314 evolution.
315 We can draw parallels and contrasts with the few other ploidy-focused sperm studies of which we are
316 aware: Alavi *et al.* (2013) showed that tetraploid European weatherfish (*Misgurnus fossilis*) produce
317 sperm that are 4.8% larger than sperm produced by their triploid counterparts, and Flajshans *et al.*
318 (2008) found that triploid male Prussian carp (*Carassius gibelio*) make sperm with higher volume than
319 sperm generated by diploid and tetraploid males. Considered together along with these two earlier

320 studies, we can conclude that nuclear genome DNA content is not the sole driver of the patterns we
321 observed in *P. antipodarum*.

322 *Asexual* P. antipodarum *are characterized by abnormal sperm*

323 We found evidence for a major effect of sexual *vs*. asexual reproduction on sperm morphology, with
324 sperm produced by asexual males harboring substantially more variation and much more likely to
325 feature large-scale structural abnormalities (Figure 3, 4). That a substantial fraction of asexual-produced
326 sperm is abnormal is consistent with a scenario where male-specific traits are no longer useful in an
327 asexual lineage. The abnormal phenotypes that often characterized sperm from asexual male *P.*
328 *antipodarum* do appear to be affected in part by ploidy: sperm from triploid asexual males spanned a
329 wider range of abnormal phenotypes and were more than twice as likely to be abnormal than sperm
330 from tetraploid counterparts. Cytogenetic characterization of some components of spermatogenesis in
331 diploid sexual *vs.* triploid asexual *P. antipodarum* have demonstrated that sperm production in the latter
332 is associated with aberrant meiotic pairing (Soper *et al.* 2013). Meiosis is disrupted when odd number of
333 chromosomes pair (Gupta 1985; Comai 2005; Henry *et al.* 2005), hinting that meiotic disruption and the
334 across-sperm variation in genome size and gene content that this disruption might cause is one possible
335 explanation for the differences between triploid and tetraploid-produced sperm in *P. antipodarum*.

336 There are at least two other non-mutually exclusive potential explanations for the increased frequency
337 of production of abnormal sperm in asexual *vs*. snails. One possibility is mutation accumulation in the
338 genes underlying sperm production, which are expected to be under relaxed selection in all (or nearly
339 all)-female asexual populations (Schurko & Logsdon 2008; Lahti *et al.* 2009). Under this scenario, genes
340 that influence sperm traits in asexuals should be accumulating mutations at a higher rate than sexuals.
341 This mutation accumulation process would be evidenced by the degeneration and eventual
342 pseudogenization of genes and traits related to sperm function in asexual populations. Second, it is

343 possible that the increased incidence of sperm abnormalities in asexuals is affected by the higher
344 incidence of aneuploidy in the asexually produced males (Neiman *et al.* 2011, Soper *et al.* 2013). While
345 we cannot formally exclude this possibility, we would expect that aneuploidy-linked deformities would
346 also be evident in other aspects of asexual male phenotype. Counter to this expectation, asexual male *P.*
347 *antipodarum* are to the extent that we know behaviorally and morphologically indistinguishable from
348 sexual males (*e.g.*, Soper *et al.* 2015) except in their sperm traits.

349 The fact that the male offspring occasionally produced by female asexual *P. antipodarum* are still
350 investing in sperm suggests that the time period over which the genes underlying sperm production
351 have been subject to relaxed selection is relatively short and/or that selection favoring the loss of this
352 trait is weak. The latter would not be surprising in light of the fact that male production is quite rare
353 (~<1.5% of all offspring) in asexual *P. antipodarum*. A powerful test of the former could come from
354 comparisons of males produced by different asexual *P. antipodarum* lineages, which are often recently
355 derived (Paczesniak *et al.* 2013) but also vary in the time since derivation from sexual *P. antipodarum*
356 (Neiman *et al.* 2005). If time since derivation to asexuality is a key determinant of the extent of male
357 trait degeneration, we predict that males from older asexual lineages should tend to produce sperm that
358 harbors relatively high variance and is abnormal at a higher frequency than sperm produced by males
359 from younger asexual lineages.

360 While we cannot formally exclude the possibility that ploidy elevation *per se* is a major driver of sperm
361 abnormality, the fact that diploid male sexual *P. antipodarum* produce normal sperm at a similar
362 frequency to the two outgroup species suggests that nuclear genome DNA content alone does not
363 account for the high frequency of sperm abnormalities observed in asexual male *P. antipodarum*. The
364 wide sperm phenotype variation that we observed across asexual males also hints at a process (*i.e.*
365 mutation accumulation) that is stochastic compared to a direct consequence of ploidy elevation.

366 Summary & Conclusion

367 Together, our results indicate that asexual male *P. antipodarum* are likely experiencing decay of male-
368 specific traits, highlighting the likelihood that transitions to asexual reproduction results in striking
369 differences in selective regimes between sexual and asexual populations. This finding is of direct
370 relevance to evaluating how relaxed selection influences phenotypes in natural populations and
371 suggests that transitions back to sexual reproduction in asexual populations are likely to face challenges
372 associated with degeneration of sex-specific traits. Critical next steps include the characterization of
373 molecular evolution of sperm-related genes in sexual *vs.* asexual populations and assessment of
374 whether older asexual populations have experienced a relatively high level of trait decay.

375

376 *Acknowledgments*

377 We gratefully acknowledge help in snail collections from Mike Winterbourn, Mary Morgan-Richards,
378 Katelyn Larkin, Kyle McElroy, Jeremy Richardson, Laura Bankers, and Kaitlin Hatcher. We thank Sarit
379 Smolikove for microscope use, Julie Meachen for statistical advice, and Deanna Soper for assistance in
380 sperm extraction protocols. Three anonymous reviewers provided very helpful feedback. The ploidy
381 identification data presented herein were obtained at the Flow Cytometry Facility, which is a Carver
382 College of Medicine/Holden Comprehensive Cancer Center core research facility at the University of
383 Iowa. The Facility is funded through user fees and the generous financial support of the Carver College
384 of Medicine, Holden Comprehensive Cancer Center, and Iowa City Veteran's Administration Medical
385 Center. This project was funded by NSF-MCB 1122176 and NSF DEB-1753851

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529

530 *Figure Legends*

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

538

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

542

543 Figure 3. Linear discriminant function analysis of transformed sperm traits (head length, head width,
544 head circumference, tail length) for diploid, triploid, and tetraploid *P. antipodarum*. Histogram
545 represents the cumulative numbers of sperm in each bin for LD1. Arrows represent each variable (HL,
546 HW, HC, TL), length of arrows represents degree of discrimination, and arrow angle is the direction of
547 the variables in 2-D space. Shaded area encompasses 95% of data.

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

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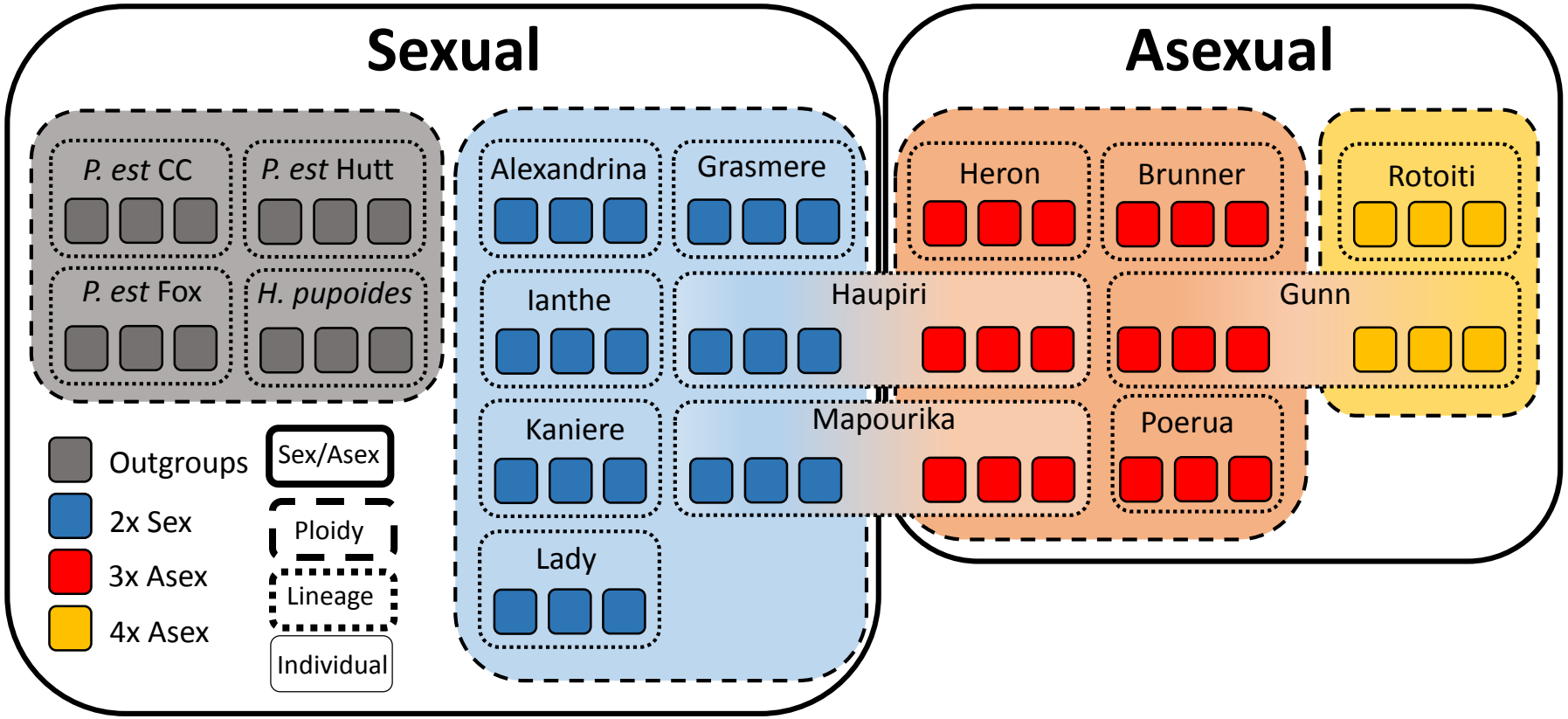

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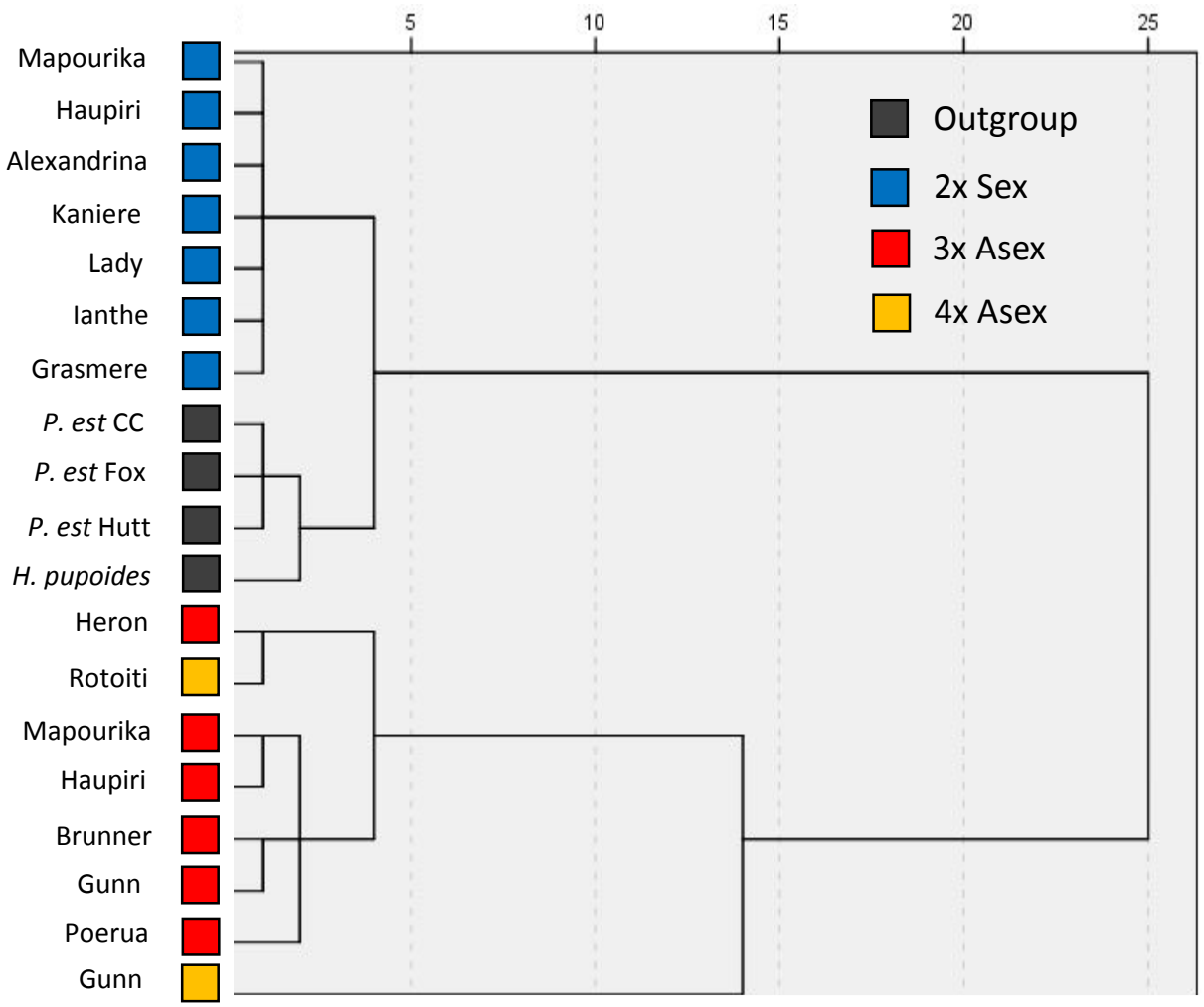

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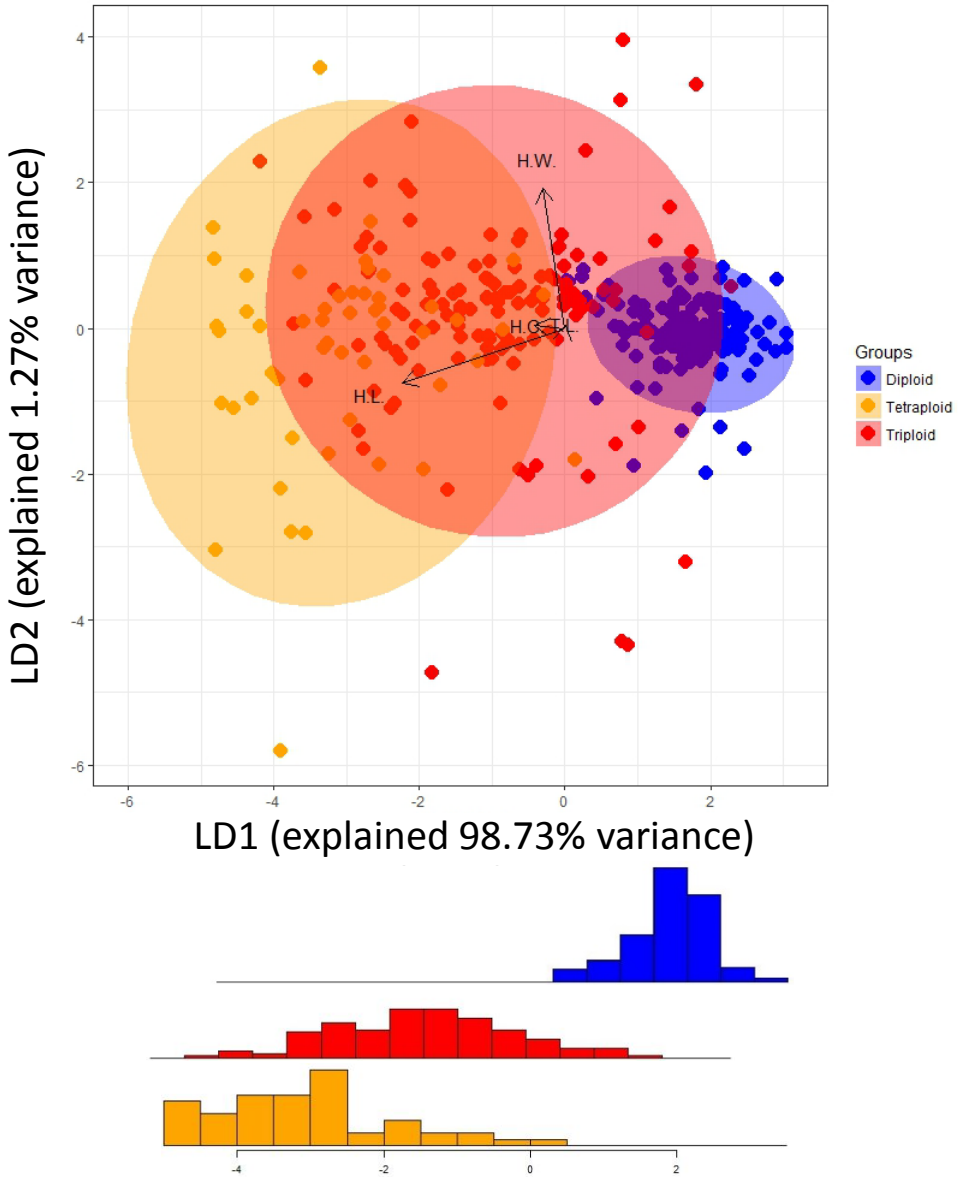

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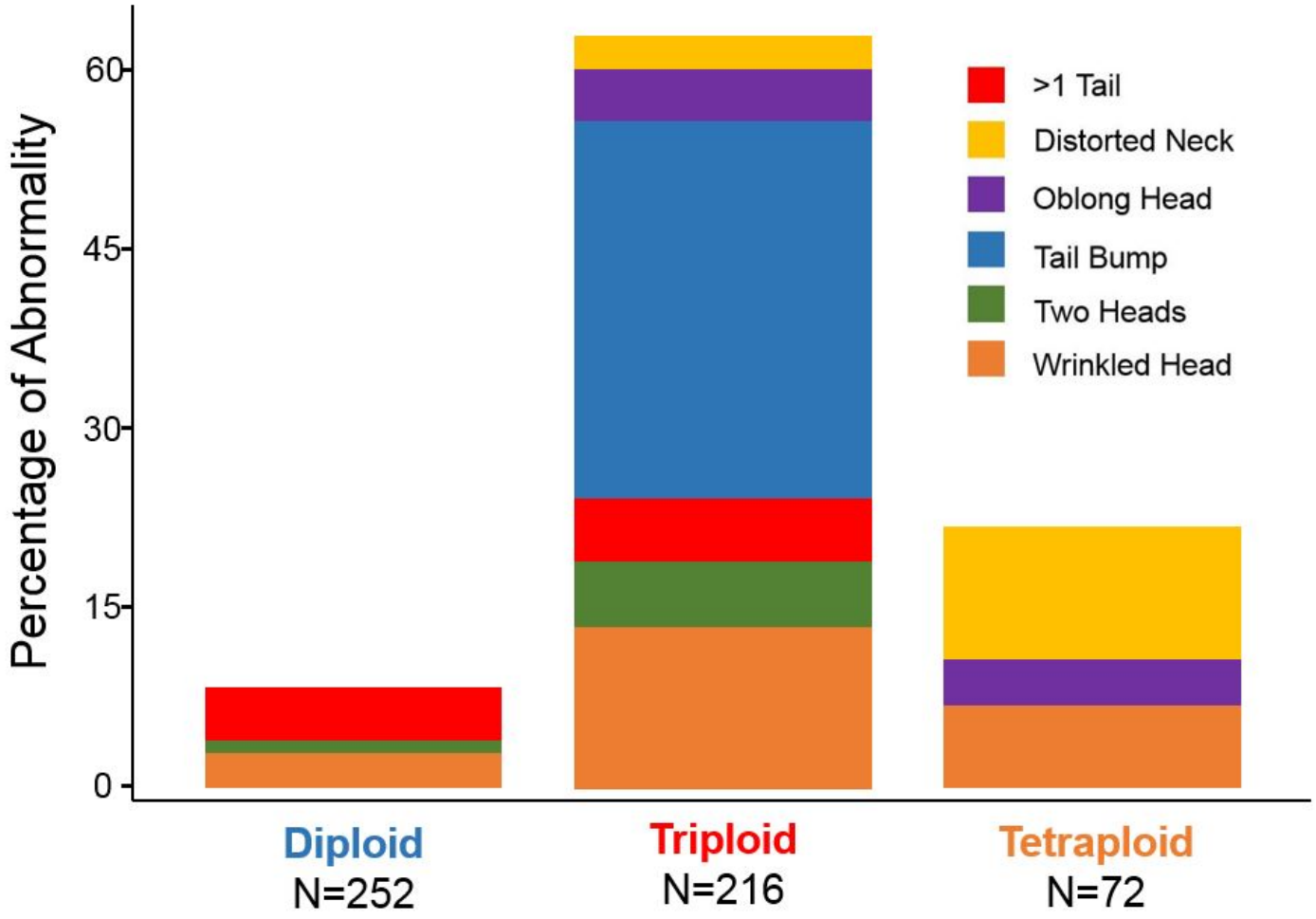

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