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Temporal genetic stability in natural populations of the waterflea Daphnia magna in response to strong selection pressure

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1 **Temporal genetic stability in natural populations of the waterflea *Daphnia magna* in**
2 **response to strong selection pressure**
3

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22 **Abstract**

23 Studies monitoring changes in genetic diversity and composition through time allow a unique
24 understanding of evolutionary dynamics and persistence of natural populations. However, such
25 studies are often limited to species with short generation times that can be propagated in the
26 laboratory or few exceptional cases in the wild. Species that produce dormant stages provide
27 powerful models for the reconstruction of evolutionary dynamics in the natural environment. A
28 remaining open question is to what extent dormant egg banks are an unbiased representation of
29 populations and hence of the species' evolutionary potential, especially in presence of strong
30 environmental selection. We address this key question using the water flea *Daphnia magna*,
31 which produces dormant stages that accumulate in biological archives over time. We assess
32 temporal genetic stability in three biological archives, previously used in resurrection ecology
33 studies showing adaptive evolutionary responses to rapid environmental change. We show that
34 neutral genetic diversity does not decline with the age of the population and it is maintained in
35 presence of strong selection. In addition, by comparing temporal genetic stability in hatched and
36 unhatched populations from the same biological archive, we show that dormant egg banks can be
37 consulted to obtain a reliable measure of genetic diversity over time, at least in the multi-decadal
38 time frame studied here. The stability of neutral genetic diversity through time is likely mediated
39 by the buffering effect of the resting egg bank.

40

41 **Introduction**

42 Understanding how environmental change affects the genetic composition of populations over
43 time is critical for gauging how species respond and persist to environmental change. However,
44 excluding a few exceptional cases of long-term studies (e.g. Galapagos Darwin finches, Grant &
45 Grant 2002) the processes underpinning evolutionary dynamics through time often remain
46 elusive. Because of logistic difficulties associated with accessing temporal samples, changes in
47 genetic composition in response to environmental change are more frequently studied in spatial
48 (synchronic) rather than temporal (allochronic) settings. Most studies analysing temporal
49 dynamics involve experimental evolution in the laboratory or controlled mesocosm experiments
50 (Barrick *et al.* 2009; Blount *et al.* 2012; Kawecki *et al.* 2012) with exceptional studies that
51 reconstruct evolution of natural populations using transplant experiments in the wild (Reznick *et*
52 *al.* 1997). For species that cannot be easily manipulated experimentally, or for which temporal
53 samples are inaccessible, the ‘space-for-time’ substitution (Fukami & Wardle 2005) is
54 frequently adopted as a surrogate to study long-term evolutionary dynamics. Space-for-time
55 analyses assume that two different conditions at two points in space can be treated as though they
56 are in the same region at two different time points. This approach has its limitations as rates of
57 adaptation at different spatial scales can differ compared to temporal variation in the same
58 population evolving in time (Merila & Hendry 2014). It is, however, the only possible approach
59 when temporal samples are not accessible. A powerful alternative that allows studying
60 evolutionary dynamics in natural populations over time is the analysis of genetic change in
61 species producing dormant stages. Zooplankters are among the species that adopt this strategy. In
62 these species, dormant stages are early stage embryos that escape environmental hardships by
63 arresting development to remain dormant and protected from the elements by a resistant capsule.

64 These dormant stages become buried in the sediment at the bottom of lakes, remaining viable for
65 decades, centuries or more (Frisch *et al.* 2014; Yashina *et al.* 2012) and providing a powerful
66 resource to generate long-term data in natural systems (Bidle *et al.* 2007; Frisch *et al.* 2014;
67 Härnström *et al.* 2011; Orsini *et al.* 2013b). Freshwater crustaceans are a group of organisms for
68 which the practise of 'resurrection ecology', the study of individuals and populations hatched
69 from dormant stages retrieved from dated lake sediments (Kerfoot *et al.* 1999), has been widely
70 applied to study micro-evolutionary responses to environmental change, mostly via the analysis
71 of phenotypic traits or genotypic trait values (e.g. Cousyn *et al.* 2001; Decaestecker *et al.* 2007;
72 Frisch *et al.* 2014; Hairston *et al.* 1999; Stoks *et al.* 2016; Weider & Pijanowska 1993). These
73 studies have provided important insights into micro-evolutionary responses to environmental
74 change in natural populations through time. However, the study of temporal changes in genetic
75 diversity has been largely neglected, and the few studies that addressed changes in genetic
76 diversity over time used a small set of genetic markers and thus had limited power (Cousyn *et al.*
77 2001).
78 The increasing availability of genetic tools provides great promise for the use of dormant stages
79 extracted from biological archives to study evolutionary responses to environmental change
80 (Orsini *et al.* 2013b). Yet, it has been questioned whether dormant egg banks that are recovered
81 from layered sediments are representative of the genetic diversity and composition of the
82 populations during historical times (Jankowski & Straile 2003), especially in presence of strong
83 selection pressure. It is possible that with increasing age of the sediment non-random mortality
84 of the eggs might result in biased estimates of genetic diversity and composition. With increasing
85 age the hatching success of the dormant stages may be reduced, and as a result genetic assays are
86 often based on relatively small numbers of individuals. Hence, an important methodological

87 issue to be addressed is the minimum threshold sample size required to represent the genetic
88 diversity of dormant populations.
89 The analysis of temporal genetic stability in layered dormant egg banks allows a retrospective
90 assessment of environmental impacts on genetic diversity. A key question is whether neutral
91 genetic diversity in natural populations is reduced following strong environmental selection
92 pressure leaving a long-term signature of reduced genetic diversity. Another important aspect is
93 to assess whether genetic drift has a strong impact on the genetic composition of populations,
94 which would result in significant allele frequency differences between years and an overall
95 reduced allelic richness over time, limiting the ability of populations and species to cope with
96 selection pressure.
97
98 To address the conceptual and methodological issues outlined above, we analyze changes in
99 temporal genetic diversity of three biological archives containing layered dormant egg banks of
100 *D. magna*, which we know have experienced strong selection pressure. *Daphnia* arguably has
101 one of the best-understood ecologies of any animal, primarily because of its central role in food-
102 webs of inland water habitats and its amenability as ecological model system (Altshuler *et al.*
103 2011; Miner *et al.* 2012). *D. magna* is a cyclical parthenogenetic zooplankter, with a life cycle
104 alternating between asexual clonal reproduction in favorable environmental conditions and
105 sexual reproduction in the presence of deteriorating environmental conditions. When
106 environmental conditions deteriorate the asexual production of males and the production of
107 sexual eggs is induced. The resulting early stage embryos arrest their development remaining
108 dormant and protected from the environment within a resistant capsule (ephippium) until
109 favorable environmental condition induce hatching.

110 Using microsatellite (up to 72) and SNP (up to 840) markers, we measure neutral genetic
111 diversity changes over time in the three biological archives mentioned above. By comparing
112 genetic diversity before and after a well-documented and strong environmental shift, we test
113 whether genetic adaptation to a strong environmental change leads to genetic erosion due to
114 severe bottlenecks. If strong selection pressure determines genetic erosion, we expect a
115 consistent decline in genetic diversity in the three archives following environmental selection. A
116 directional decline in genetic diversity from old to recent (sub)populations along a sedimentary
117 archive would indicate loss of genetic diversity through genetic drift. Conversely, if loss of
118 genetic diversity occurs with aging of the dormant (sub)populations-from recent to old layers-,
119 we expect a consistent decline in genetic diversity with age in the three archives. In addition to
120 testing the hypotheses outlined above, we test whether hatched (sub)populations from the
121 dormant egg bank are an unbiased sample of the dormant egg bank by comparing both genetic
122 diversity and composition of the hatched (sub)populations with that of the unhatched egg bank in
123 one of the biological archives. Finally, we determine the threshold sample size and marker set
124 required to assess genetic diversity in *D. magna* populations using a rarefaction analysis on the
125 (sub)populations from the biological archives and on a set of 19 populations with relatively large
126 sample size and previously used in a population genomic study (Orsini *et al.* 2012). With this
127 approach we identify the threshold sample size needed to capture changes in genetic diversity in
128 our study species both in space and over multi-decadal time spans. We also identify the
129 combination of sample sizes and number of markers appropriate to describe neutral genetic
130 diversity in our study species. Our study shows that dormant egg banks can be consulted to
131 obtain a reliable measure of genetic diversity over time, at least in the multi-decadal time frame
132 studied here employing a combination of reasonably small number of markers and sample sizes.

133 **Materials and methods**

134 ***Source material***

135 The material used in this study consisted of three sedimentary archives from which several
136 (sub)populations of *D. magna* were sampled and of 19 populations isolated from 19 ponds
137 distributed in the landscape along orthogonal gradients of selection (Table S1) (Orsini *et al.*
138 2012). Hereafter, we use the term population when referring to populations from the spatial
139 survey, hence populations in the landscape; we use the term (sub)populations when referring to
140 temporally sampled populations along the sedimentary archives. The sedimentary archives were
141 sampled using a piston corer or a Plexiglas tube of 6cm of diameter. After sampling, the cores
142 were brought to the laboratory where they were sliced in incremental intervals of 0.5 or 1cm.
143 Each layer was then stored separately in the dark at 4°C. A few grams of sediment were collected
144 from 10 to 15 depths for radiolabeling and dating using established techniques (Appleby *et al.*
145 1986). Based on radio dating or using fractions of organic material and assuming constant
146 sedimentation rates (e.g. Oud Heverlee Zuid; Cousyn *et al.* 2001) the year of each layer and
147 hence of the dormant eggs isolated from each layer were established. By aligning the chronology
148 of each core with the known history of the lake where the core was sampled, we could identify
149 how populations respond to major ecological shifts in the lakes.

150 *Core OH (Oud Heverlee, Belgium)* (50°50' 13.12"; 4°39' 48.87"E). This core was
151 sampled in 1997 from a shallow artificial pond established in 1970 for fish culture and spans 23
152 years (Cousyn *et al.* 2001). The material obtained from this biological archive has been used in
153 one of the first resurrection ecology studies showing adaptive responses of the crustacean *D.*
154 *magna* to fish predation (Cousyn *et al.* 2001). The same resurrected (sub)populations from this
155 archive were used more than a decade later to identify genome-wide signatures of selection

156 induced by vertebrate predators (Orsini *et al.* 2012). During the first three years after its creation,
157 the pond was stocked annually with a low number of benthivorous fish. From 1973 to the early
158 1980s, a very high biomass of planktivorous fish (300 kg/ha) was stocked each year. The amount
159 of stocked fish subsequently was reduced and varied from the mid-1980s until 1993 when fish
160 stocking ceased. Based on changes in fish predation pressure, we thus differentiate three main
161 periods: before fish stocking (1970–1972; Bottom, low predation); intense fish stocking (1976–
162 1979, Middle; high predation); and after relaxing fish stocking (ca. 1988, Top; relaxed predation;
163 see also (Cousyn *et al.* 2001; Stoks *et al.* 2016). For each of these time periods 12 *D. magna*
164 dormant eggs were hatched following standard protocols (Marcus 1990; Onbe 1978) and clonal
165 lineages established for a total of 36 distinct genotypes.

166 *Core OM2 (Oude Meren 2, Belgium)* (50°51'51.61", 4°51' 48.98"E). This core was
167 obtained in 2006 from a shallow artificial pond in Belgium. Material from this biological archive
168 was used to document host-parasite co-evolutionary dynamics (Decaestecker *et al.* 2007) and,
169 later, to identify genome-wide signatures of selection caused by the endoparasite *Pasteuria*
170 *ramosa* (Orsini *et al.* 2012). The OM2 core spans approximately 40 years, sampled over 8
171 depths. A total of 68 distinct genotypes were hatched and established as clonal lines from this
172 sedimentary archive. For the purpose of the current study the 8 sampled depths are considered
173 (sub)populations, each spanning approximately 5 years.

174 *Core LR (Lake Ring, Denmark)* (55° 57' 51.83'' N, 9° 35' 46.87'' E). This core was
175 obtained in 2004 from a shallow pond in Denmark. The pond was pristine until the late 1950s
176 when agricultural runoff and sewage inflow from a nearby town initiated symptoms of severe
177 eutrophication. The sewage inflow was diverted from the lake in the late 1970s, leading to the
178 partial recovery of the lake from eutrophication starting from the 1980s. Hence, the

179 eutrophication history of the lake consists of four periods: pristine conditions (prior to 1950),
180 severe eutrophication (1960-1970), a transition phase following the diversion of sewage inflow
181 (after 1985) and a return to clear-water conditions (after 1999) (Michels 2008). To analyze
182 changes in genetic diversity and composition through time of this lake we genotyped both
183 dormant and hatched (sub)populations covering 50 years of history encompassing three lake
184 phases: the eutrophic, transition and clear-water phase. The samples were grouped in four
185 temporal equally-spaced (sub)populations, each representing a time period of 6 years to enable a
186 fine-grained analysis. The dating of this sediment core was conducted using a classic
187 radioisotope approach (Appleby 2001) in 2015. We analyzed 96 genotypes from hatched
188 dormant eggs and 48 genotypes from unhatched dormant eggs.

189 *Spatial survey (Belgium)*. This dataset comprises 19 populations of *D. magna* previously
190 sampled by hatching dormant eggs from the surface sediment layers (the most recent 3 to 5
191 years) of 19 ponds in Belgium. Clonal lines were established for each genotype and used in a
192 study to assess adaptive responses to three environmental stressors: fish predation, land use and
193 parasite infection (Orsini *et al.* 2012). The sample sizes of these 19 populations range between
194 12 and 51 genetically distinct genotypes. They are used in this study, in addition to the
195 (sub)populations isolated from the sediment cores, to identify the threshold sample size needed
196 to represent genetic diversity in *D. magna* and to identify the combination of sample sizes and
197 marker sets required to reliably estimate genetic diversity in the study species.
198

199 *Genetic markers*

200 We quantified changes in genetic diversity over time within the three biological archives
201 described in the previous section. As we focus on neutral genetic variation, our first action was to

202 identify a set of neutral loci among the ones used. A previous study on populations from the
203 spatial survey, the OH and OM2 cores (Orsini *et al.* 2012) adopted an outlier approach
204 (Beaumont 2005; Beaumont & Balding 2004) to identify loci putatively under selection for three
205 environmental stressors known to induce evolutionary responses in *D. magna* (Orsini *et al.*
206 2012). The populations from the spatial survey were sampled along three orthogonal gradients of
207 selection represented by land use, fish predation, and parasite infection by the endoparasite
208 *Pasteuria ramosa*. To identify outlier loci, populations diverging at only one stressor while
209 identical with respect to the other two stressors were contrasted in multiple pairwise
210 comparisons. (Orsini *et al.* 2012). Outlier loci identified in the spatial survey were then validated
211 using temporal surveys. For these temporal surveys (sub)populations resurrected from different
212 time periods along three sediment cores with known history of exposure to one of the three
213 stressors investigated in the spatial survey, including the OH and OM2 cores in this study, were
214 contrasted in an outlier analysis. Hence, for each stressor a spatio-temporal analysis of multiple
215 pairwise population comparison was conducted. Only outliers found across multiple spatio-
216 temporal pairwise comparisons were retained as putatively under selection. The set of neutral
217 loci used here consisted of loci that were not associated with any of the environmental stressors
218 previously analyzed. The OH core was also genotyped with SNP markers for this study using a
219 SNP-chip (NimbleGen, Roche) developed to construct a high-density linkage map in *D. magna*
220 (Routtu *et al.* 2014). The NimbleGen (Roche) array contained probes that interrogated 1,324
221 SNPs, and included the SNPs previously used to genotype the OH and OM2 cores (Orsini *et al.*
222 2012). After excluding loci with low amplification success (loci with more than 30% failings),
223 950 SNPs were retained. A total of 873 SNPs were polymorphic in the samples of the OH core
224 and were used for downstream analyses. Following Orsini *et al.* (2012), we performed a genome

225 scan analysis on the 873 SNPs using Fdist (Beaumont & Nichols 1996) implemented in Lositan
226 (Antao *et al.* 2008; Beaumont & Nichols 1996) and contrasted the three time periods (B, M, T) in
227 all possible pairwise combinations, identifying outlier loci putatively under selection for fish
228 predation. This exercise allowed us to identify 840 neutral SNP loci, which we used for
229 downstream analyses (Table S1).

230 The LR core was genotyped here for the first time at 45 microsatellite loci, representing a
231 subset of the loci used for genotyping the OH and OM2 cores (Table S1). The core was
232 subdivided in four (sub)populations encompassing 6-years each and covering a total time period
233 of 50 years. From each (sub)population both hatched *D. magna* and unhatched dormant eggs
234 were genotyped. To identify outlier loci putatively linked to eutrophication, we adopted the same
235 approach of Orsini *et al.* (2012) and contrasted (sub)populations from the eutrophic, transition
236 and clear water phases in pairwise combinations from both the hatched and unhatched
237 (sub)populations. We retained 41 neutral loci, which we used for downstream analyses.
238

239 *Change in genetic diversity over time*

240 To assess temporal stability in genetic diversity, we compared population genetic diversity
241 indices among time periods [(sub)populations] within sediment cores. We quantified observed
242 and expected heterozygosity (H_o and H_e), and allelic richness (AR) using the diveRsity package
243 in R (Keenan *et al.* 2013).

244 Partition of genetic diversity among (sub)populations within sediment cores was assessed with a
245 two-level analysis of molecular variance (AMOVA) using Arlequin (Excoffier *et al.* 2005), on
246 microsatellite and SNP markers separately. The two hierarchical levels were within and among

247 (sub)populations sampled along the sediment cores. This analysis was performed separately for
248 the three cores.
249
250 *Comparing hatched and non-hatched populations*
251 If dormant stages are non-randomly resilient to hatching cues, the estimates of genetic diversity
252 obtained on hatched (sub)populations may differ from the ones based on dormant egg banks. To
253 assess whether this bias exists, we compared population genetic diversity and structure in
254 hatched and unhatched *D. magna* (sub)populations from Lake Ring over a period of 50 years.
255 Heterozygosity and allelic richness were quantified in both data sets. Moreover, the partition of
256 genetic diversity (AMOVA, Excoffier *et al.* 2005) among the (sub)populations along the
257 sediment core was assessed on both the hatched and unhatched (sub)populations using the
258 hierarchical levels described above.
259 Changes in genetic structure based on individuals were assessed using STRUCTURE
260 (Falush *et al.* 2003; Pritchard *et al.* 2000). After testing different parameter settings the following
261 were used based on the stability of the MCMC parameters: 1,000,000 burn-in period, 100,000
262 MCMC iterations, uncorrelated loci and admixture model using population information as *prior*.
263 Different prior of K were tested, ranging from 1 to 10 in triplicates. To estimate the number of
264 clusters identified by STUCTURE we used the Evanno method (Evanno *et al.* 2005)
265 implemented in HARVESTER (Earl & vonHoldt 2012). To estimate whether the allelic profiles
266 between the hatched and unhatched (sub)populations were comparable we studied the allelic
267 composition of 6 microsatellite loci randomly chosen from the set used in this study. The
268 comparison of allelic profiles was performed after standardizing the hatched (sub)populations to

269 the smallest sample size of the unhatched (sub)populations. The standardization was performed
270 using the resampling with replacement approach described in the following section.

271

272 *Threshold sample size to capture genetic diversity changes in D. magna*

273 To assess the threshold sample size needed to obtain an unbiased estimate of genetic diversity
274 changes in *D. magna* populations we performed a rarefaction analysis by drawing random sub-
275 samples with replacement (Luikart *et al.* 2010) from each (sub)population extracted from the
276 three biological archives using the largest available markers set. At each random draw, 5
277 individuals were removed from the total set of individuals until a minimum sample size of 5
278 individuals was reached; additionally sample sizes smaller than 5 individuals were tested (N= 2,
279 3, 4). This analysis was also conducted on the set of 19 populations from the spatial survey,
280 which offer a large independent set of populations with, on average, larger sample sizes (Orsini
281 *et al.* 2012). Rarefaction curves were obtained for the key population genetic indices: H_o, H_e, and
282 AR. These rarefaction curves allowed us to identify changes in key population genetic
283 parameters as a function of sample size and to identify the threshold sample size minimally
284 required to estimate genetic diversity in *D. magna* populations.

285 For species with limited genetic resources, the number of molecular markers available can be
286 small. To assess the optimal combination of markers and sample sizes needed to estimate genetic
287 diversity in our study species we performed a rarefaction analysis in which genetic diversity
288 indices were calculated with different number of markers and sample sizes. For this analysis we
289 performed random resampling of markers with replacement on the subsamples of the rarefaction
290 analysis described above, effectively measuring population genetic parameters on all
291 combinations of sample sizes and number of markers to a minimum sample size of 2 individuals

292 and 10 markers. This analysis was conducted on three random populations from the spatial
293 survey that had sufficiently large sample sizes and on the hatched and unhatched
294 (sub)populations of Lake Ring. We used a total of 70 microsatellites and 40 SNPs for the three
295 populations from the spatial survey and 40 microsatellites for the (sub)populations from Lake
296 Ring.
297
298 **Results**

299 *Change in genetic diversity over time*
300 The results from multiallelic (microsatellites) and biallelic (SNPs) markers were congruent,
301 reflecting stable genetic diversity over time. All (sub)populations, with the exception of two in
302 the OM2 core (D4 and D5 in the SNPs analysis), were in Hardy-Weinberg equilibrium.
303 Heterozygosity and allelic richness within marker type were stable over time in all three
304 biological archives (Fig 1A-C, Table S2).
305 The AMOVA analysis also showed congruent results for microsatellite and SNP markers. For all
306 cores the largest fraction of molecular variance was at the within (sub)population level (Table 1),
307 consistent with results of previous studies on *D. magna* (Orsini *et al.* 2012; Orsini *et al.* 2013c).
308 In line with these previous results, the proportion of molecular variance among (sub)populations
309 was small but significant. This result was observed for both multiallelic and biallelic markers.
310
311 *Comparing hatched and non-hatched populations*
312 Heterozygosity did not differ significantly between the hatched and unhatched (sub)populations
313 in the LR core (Fig 1A, LR) (t-test, P=0.54). Allelic richness between the two sets of
314 (sub)populations, calculated after standardizing the (sub)populations to the smallest sample size,

315 did not significantly differ (t-test, P=0.55). The standardization was performed by randomly
316 resampling the hatched (sub)populations with replacement to a sample size identical to the
317 unhatched (sub)populations using the resampling with replacement approach described in the
318 methods section.

319 In both hatched and unhatched populations the amount of molecular variance at the within and
320 among (sub)population levels as quantified by the AMOVA was comparable. More specifically,
321 a large proportion of the molecular variance was explained at within (sub)population level and a
322 smaller yet significant proportion was explained at among (sub)populations level. This result
323 reflects the one obtained for the other two sediment cores studied here (Table 1) and the results
324 obtained in previous studies (Orsini *et al.* 2013a; Orsini *et al.* 2012).The STRUCTURE analysis
325 identified comparably low genetic structure in the hatched and unhatched population sets (Fig 2).
326 The Evanno method identified 7 clusters in the hatched (sub)populations and 6 in the unhatched
327 (sub)populations (Table S3). The STRUCTURE plots corresponding to these clusters show that
328 no obvious changes occurred over time in Lake Ring at neutral microsatellite loci. The allelic
329 profiles between hatched and unhatched (sub)populations were congruent. Moreover, the alleles
330 with high frequency are the same in the hatched and unhatched (sub)populations (Fig. S1).

331

332 *Threshold sample size to capture genetic diversity changes in D. magna*

333 This analysis aimed at identifying the optimal combination of sample size and number of
334 markers to capture genetic diversity in our populations. Not surprisingly, this analysis showed
335 that genetic diversity indices display different levels of sensitivity to the combinations of these
336 key parameters. More specifically, for sample sizes larger than 5 individuals, estimates of
337 heterozygosity were robust across the marker sets and sample sizes (Fig. 3 and Fig. S2).

338 Conversely, allelic richness was more sensitive to small sample sizes (Fig. 3 and Fig. S2)
339 especially when small markers sets were genotyped (Fig.4 and Fig. S3). In summary, our
340 analysis suggests that for heterozygosity and allelic richness a sample size of 5-10 individuals
341 genotyped at 20 markers or more provides unbiased estimates of genetic diversity in our study
342 species (Fig.4 and S3; Table S4).

343

344 **Discussion**

345 *Temporal stability of genetic diversity*

346 Temporal stability of genetic diversity is important because it determines the evolutionary
347 potential of natural populations and their ability to persist in the face of environmental change.
348 Studies of temporal changes conducted on a wide array of species (*e.g.* Alasaad *et al.* 2011;
349 DeFaveri & Merila 2015; Goetze *et al.* 2015; Larsson *et al.* 2010; Tessier & Bernatchez 1999;
350 Welch *et al.* 2012) report genetic stability and no reduction in genetic diversity over time.
351 However, as most of these studies are limited in the number of years or generations studied or in
352 the number of genetic markers used, the power to detect changes in genetic diversity is often
353 limited. Here, we use layered dormant egg banks to reconstruct genetic diversity in natural
354 populations over periods spanning several decades (20 to 50 years) in an organism with a cyclic
355 parthenogenetic life cycle and a generation time of approximately 14 days. In addition, we assess
356 genetic diversity through time using multi- and bi-allelic markers.

357 Our results show no apparent genetic erosion in *D. magna* populations with time (from old to
358 recent populations), even following a period of strong selection pressure. In the time periods
359 studied here, considering the short generation time of the waterflea, strong differential mortality

360 or genetic drift can occur. Lack of genetic erosion with time suggests that genetic drift and
361 selection have no detectable impact on neutral genetic diversity in the populations and over the
362 time scales studied. Our results show no reduction in genetic diversity as the egg banks age
363 (from recent to old populations), at least over the multi-decadal time covered by our study. This
364 result shows that there is no reduction in genetic diversity because of differential mortality of old
365 dormant eggs, and hence, indicates that dormant egg banks can be reliably used to study genetic
366 changes in natural populations. Genetic stability in a zooplankter such as *Daphnia* is striking
367 given that there are many reports of strong shifts in clonal composition during the growing
368 season in active *Daphnia* populations (De Meester *et al.* 2006; Hebert 1974; Lynch 1984) and of
369 clonal erosion as the growing season progresses (Vanoverbeke & De Meester 2010). Moreover,
370 while the census population size of *Daphnia* populations is typically very large, several studies
371 have estimated the effective population size (N_e) of natural populations of this cyclical
372 parthenogen to be rather small (Hamrova *et al.* 2011; Orsini *et al.* 2013a; Orsini *et al.* 2012) with
373 even more reduced N_e in populations with higher turnover (Walser & Haag 2012). Small N_e can
374 potentially lead to genetic drift (Hartl & Clark 2007). If genetic drift occurred, it would be visible
375 as a significant allele frequency difference between years (e.g. Charlier *et al.* 2012) and reduced
376 allele richness and heterozygosity over time.We do not observe these patterns and observe
377 instead stability in neutral genetic variation, even though earlier findings show clear signature of
378 selection and hence reduced diversity in loci under selection or linked to genes under selection in
379 two of the biological archives studied here (Orsini *et al.* 2012). This suggests that whereas
380 environmental selection pressure can impact target loci or genomic regions, neutral genetic
381 diversity, and possibly effective population size, is conserved over time even in presence of
382 strong selection pressure.

383 Genetic stability is likely maintained by the buffering effect of the resting egg bank, a
384 concept put forward in the late 1980's as a mechanism that zooplankton adopts to survive
385 environmental hardship (Hairston *et al.* 1999). Similarly, in a community context, the buffering
386 effect of the dormant egg bank has been used to explain species diversity (storage effect, Caceres
387 1997; Chesson & Warner 1981). A simulation study demonstrated that the buffering effect of
388 dormant individuals mitigates if not eliminates the impact of chaotic environmental dynamics
389 (Lalonde & Roitberg 2006), helping species with dormant stages to better cope with chaotic
390 environments (environments that change unpredictably). The temporal stability in neutral genetic
391 diversity we observe here, even in presence of strong selection pressure as opposed to a clear
392 signature of selection on specific loci or regions of the genome, suggest that the dormant egg
393 bank may act as buffer of genetic diversity. This hypothesis is supported by the knowledge that
394 strong changes in the environment occurred in all three habitats and the observation that in two
395 of the three studied biological archives earlier resurrection ecology studies have revealed rapid
396 and adaptive evolutionary responses of the *Daphnia* population to environmental change
397 (Cousyn *et al.* 2001; Decaestecker *et al.* 2007; Michels 2008; Pauwels *et al.* 2010 ; Stoks *et al.*
398 2016). Furthermore, a population genomic study of the same two biological archives identified
399 signatures of selection and shifts in genetic composition in selected regions of the genome in
400 response to the same environmental changes (Orsini *et al.* 2012). Hence, the absence of genetic
401 erosion at neutral markers was observed whilst significant change in genotypic trait values and
402 markers under selection was documented in response to environmental change.

403 The study of temporal genetic stability in species that produce dormant stages is in its
404 infancy, our study being the first to formally address this issue using three sedimentary archives.
405 The patterns we observed are repeatable across archives exposed to different environmental

406 stress providing support that our observations are not population- specific. However, the absence
407 of similar studies in other taxa, or even other *Daphnia* species, does not allow us to conclude
408 whether the trends observed in this study can be generalized. Further studies on temporal genetic
409 stability in species producing dormant stages will be of critical importance to conclude whether
410 the patterns we observed in *D. magna* can be extended to other species or taxa.

411 We show that genetic diversity changes and allelic composition through time in one of
412 the sedimentary archives are comparable between hatched and unhatched (sub)populations,
413 suggesting that there is no bias in genetic diversity and composition of the hatched
414 (sub)populations as compared to the dormant ones. These results indicate that the use of
415 unhatched dormant stages could be embraced in allochronic studies without introducing bias in
416 the study of evolutionary responses to selection pressure. However, we acknowledge that a
417 comparative analysis of hatched and unhatched (sub)populations should be conducted on
418 additional archives for the same species and possibly for other species before we can confidently
419 conclude that the patterns observed for Lake Ring are common to other populations and/or
420 species. If the patterns observed in the sedimentary archive of Lake Ring are observed in other
421 archives, this critically demonstrates that dormant stages can be used in place of hatched
422 individuals when decreasing hatching success hampers the application of resurrection ecology.
423 Limited hatching success with increasing age of the dormant eggs is an acknowledged limitation
424 of resurrection ecology and one of the main reasons why most studies are limited to the recent
425 past (but see Frisch *et al.* 2014 for an exceptional study).

426 We show that when a relatively high number of molecular markers (from 41 to 840) is
427 employed, a sample size of five to ten distinct genotypes is sufficient to obtain good estimates of
428 genetic diversity. We also show that for smaller markers sets (20 loci) a sample size of 10-15

429 individuals reliably estimates population genetic diversity. These finding have important
430 implication for resurrection ecology studies in which the number of hatchlings tends to decrease
431 with the age of the sediment. It is also relevant for studies directly measuring genetic diversity of
432 the egg bank, when limited number of dormant eggs can be retrieved. The amount of sediment
433 that can be retrieved from natural habitats is generally not limiting in temperate regions but can
434 be logistically challenging when sampling polar or high altitude lakes. In these circumstances
435 sediment cores of small diameter (e.g. 6 cm or less) are generally used, and hence limited
436 material can be retrieved per layer of sediment. Our findings have also important implications for
437 species that have limited genetic resources. Our results suggest that a limited number of
438 individuals can reliably represent the genetic pool of temporal (sub)populations and that a
439 combination of 15 individuals genotyped at 20 markers reliably estimates genetic diversity in
440 space and time. Whether this applies to other species remains to be seen. If these results will be
441 confirmed in other species producing dormant stages, evolutionary and population genetic
442 studies can extend beyond the decadal time scale analyzed here and potentially enable
443 paleogenomic studies over centuries and millennia.
444

445 *The use of biological archives in evolutionary applications*

446 Temporally spaced DNA samples offer a unique opportunity to study genetic changes in
447 response to changes in the environment. By comparing the genetic composition of a population
448 before and after a well-documented environmental change, it is possible to track changes in
449 allele frequencies for a retrospective 'real time' assessment of genetic impacts. To date, very few
450 studies were able to track genetic changes in natural populations associated with environmental
451 change using genetic markers (Frisch *et al.* 2014; Larsson *et al.* 2010; Welch *et al.* 2012;

452 Yashina *et al.* 2012). Genomic tools applied to dormant eggs open up possibilities for
453 reconstructing the evolutionary history of natural populations over hundreds of years (Frisch *et*
454 *al.* 2014; Mergeay *et al.* 2007; Orsini *et al.* 2013b; Orsini *et al.* 2012). With the advent of third
455 generation sequencing technologies, which enables the sequencing of genomes and
456 transcriptomes from just a few cells (Dey *et al.* 2015), more technical limitations are being lifted,
457 enabling the application of 'omics' technologies to limited and degraded material, unthinkable
458 until few years ago. The power of performing 'omics' studies on dormant stages is further
459 amplified by the fact that for *D. magna* and possibly other crustaceans the sample size required
460 to represent genetic diversity of temporal populations is small, being in the order of 10 distinct
461 genotypes, as our rarefaction analysis demonstrates. Similar studies in other taxa will allow us to
462 confirm whether such small sample sizes can be applied widely in population genetics of species
463 with dormant stages.
464

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473

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624

625 **Data accessibility**

626 SNP and microsatellites genotypes for populations from the spatial survey are deposited in the

627 DRYAD databank at the following entries: http://dx.doi.org/10.5061/dryad.384rr593.2/2.2 and

628 http://dx.doi.org/10.5061/dryad.384rr593.2/7.2.

629 Microsatellites genotypes for the OH core are deposited in the DRYAD databank at the

630 following entry: http://dx.doi.org/10.5061/dryad.384rr593.2/10.2.

631 SNP genotypes for the OH core and microsatellite genotypes for LR, hatched and unhatched, are

632 deposited in the DRYAD databank at: doi:10.5061/dryad.p1k64

633 SNP and microsatellites genotypes for the OM2 core are deposited in the DRYAD databank at

634 the following entries: http://dx.doi.org/10.5061/dryad.384rr593.2/3.2 and

635 http://dx.doi.org/10.5061/dryad.384rr593.2/8.2.

636

637 **Authors' contribution:**

638 LO and LDM conceived the study; HM and MCC generated and analyzed the data on Lake Ring;
639 AC performed the rarefaction analysis; KIS contributed to data analysis; KWT and MEP
640 generated SNP chip data for the OH core; LO generated and analyzed the data and wrote the
641 paper. All authors contributed to the final editing of the paper.

642

643 **Table 1. AMOVA analysis.** Partitioning of genetic variance within and among (sub)populations
644 within cores estimated with an Analysis of Molecular Variance. The two hierarchical levels used
645 in the analysis are (1) among the (sub)populations along each sediment core and (2) within
646 (sub)population. Statistically significant values (*, P < 0.001) are based on permutation tests
647 (10,000 permutations). For the LR core the AMOVA results are shown for both for the hatched
648 and unhatched populations at microsatellite loci; for the other cores results are shown for
649 microsatellite and SNP markers.

650

	Among (sub)populations	Within (sub)populations
OH core		
Neutral μsat	4.82*	95.18*
Neutral SNPs	1.04*	98.96*
OM2 core		
Neutral μsat	2.94*	97.06*
Neutral SNPs	1.84*	98.16*
LR core		
Neutral μsat (Hatched)	1.08*	98.92*
Neutral μsat (Unhatched)	1.75*	98.25*

651

652

653 **Figure 1. Genetic diversity indices.** Variation at genetic diversity indices in the three
654 sedimental archives. (A) Hatched and unhatched (sub)populations from Lake Ring genotyped at
655 microsatellite markers. Populations LR12_15, LR8_11, LR5_7, LR0_4 represent different
656 (sub)populations from old to recent. (B) (sub)populations form Oud Heverlee (OH) and (C)
657 Oude Meren (OM2) ponds genotyped at microsatellite and SNP markers displayed from old to
658 recent as follows: B=bottom, M=median, T=top for OH; and D1-8 = depth 1 to 8 for OM2.
659 Observed heterozygosity (Ho), expected heterozygosity (He), and allelic richness (AR) are
660 shown.

661

662 **Figure 2. STRUCTURE analysis.** Population genetic structure changes are shown for hatched
663 and unhatched populations of LR (Lake Ring). The color code for the alleles is randomly
664 generated, hence identical colors may represent different alleles in different runs. Horizontal
665 lines define (sub)populations, each spanning 6 years.

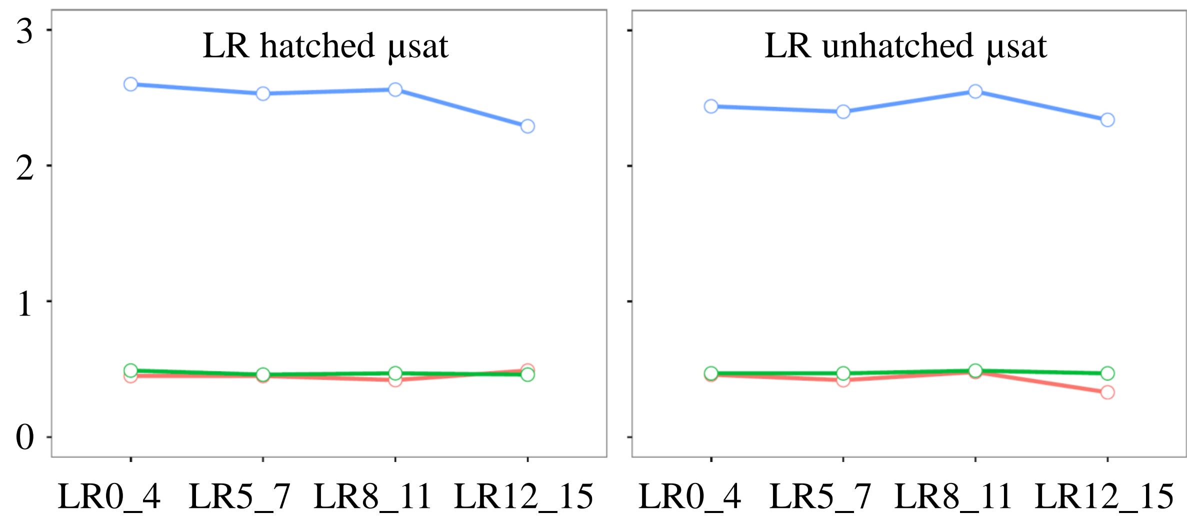
666

667 **Figure 3. Rarefaction curves for sample sizes.** Rarefaction curves are shown for
668 heterozygosity (H_o and H_e) and allelic richness (AR) in (sub)populations from (A) Lake Ring, for
669 hatched and unhatched populations, (B) Oude Heverlee (OH core), and (C) Oude Meren (OM2
670 core). Samples from LR were genotyped at microsatellite loci whereas samples from the other
671 two cores were genotyped at microsatellite and SNP markers. The rarefaction curves were
672 obtained by randomly resampling sample size with replacement to a minimum sample size of
673 two individuals. The rarefaction curves for the populations from the spatial survey confirming
674 the patterns observed in the temporal (sub)populations are shown in Figure S2.

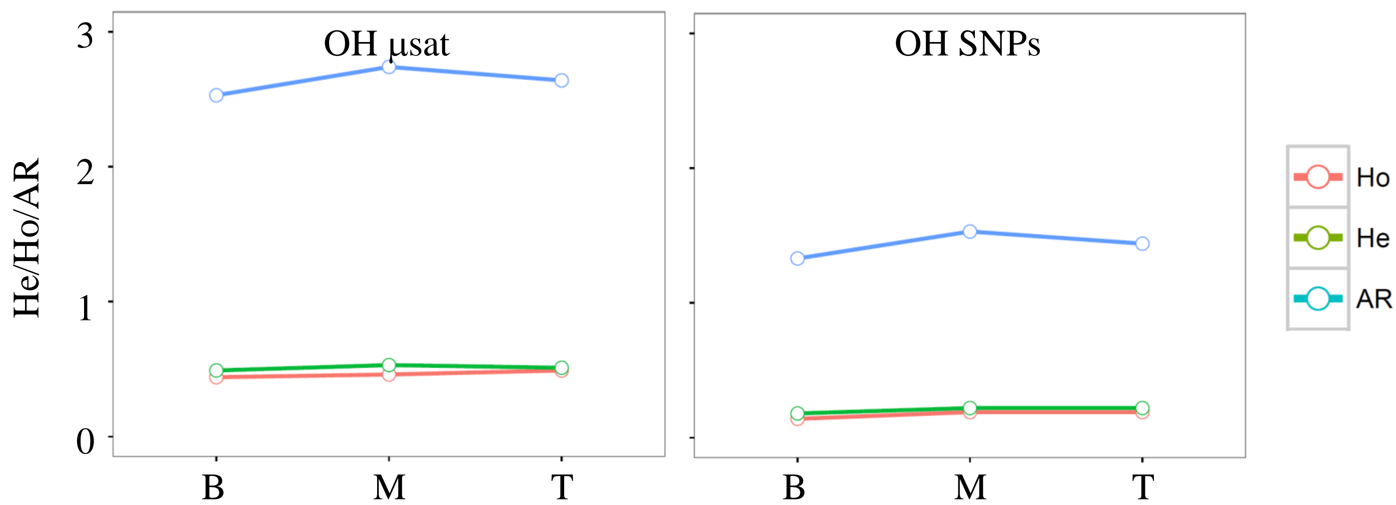
675 **Figure 4. Rarefaction curves for markers sets.** Rarefaction curves are shown for
676 heterozygosity (H_o and H_e) and allelic richness (AR) for three populations from the spatial
677 survey genotyped at microsatellites (A) and SNPs (B). The different markers sets, randomly
678 resampled with replacement to a minimum number of 10 markers, were tested on rarefied sample
679 sizes to a minimum sample size of two individuals. These sample sizes are the rarefied
680 subsamples in Fig S2. The rarefaction curves calculated for the (sub)populations from Lake Ring
681 are shown in Figure S3.

682

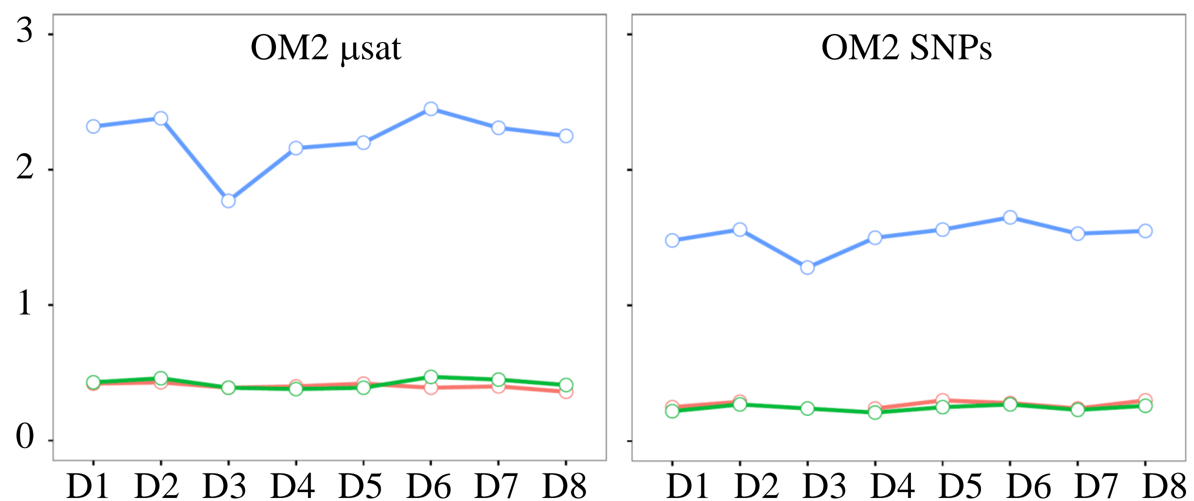
A



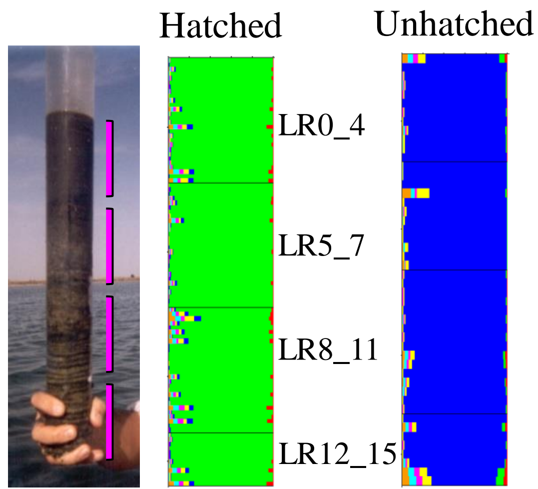
B

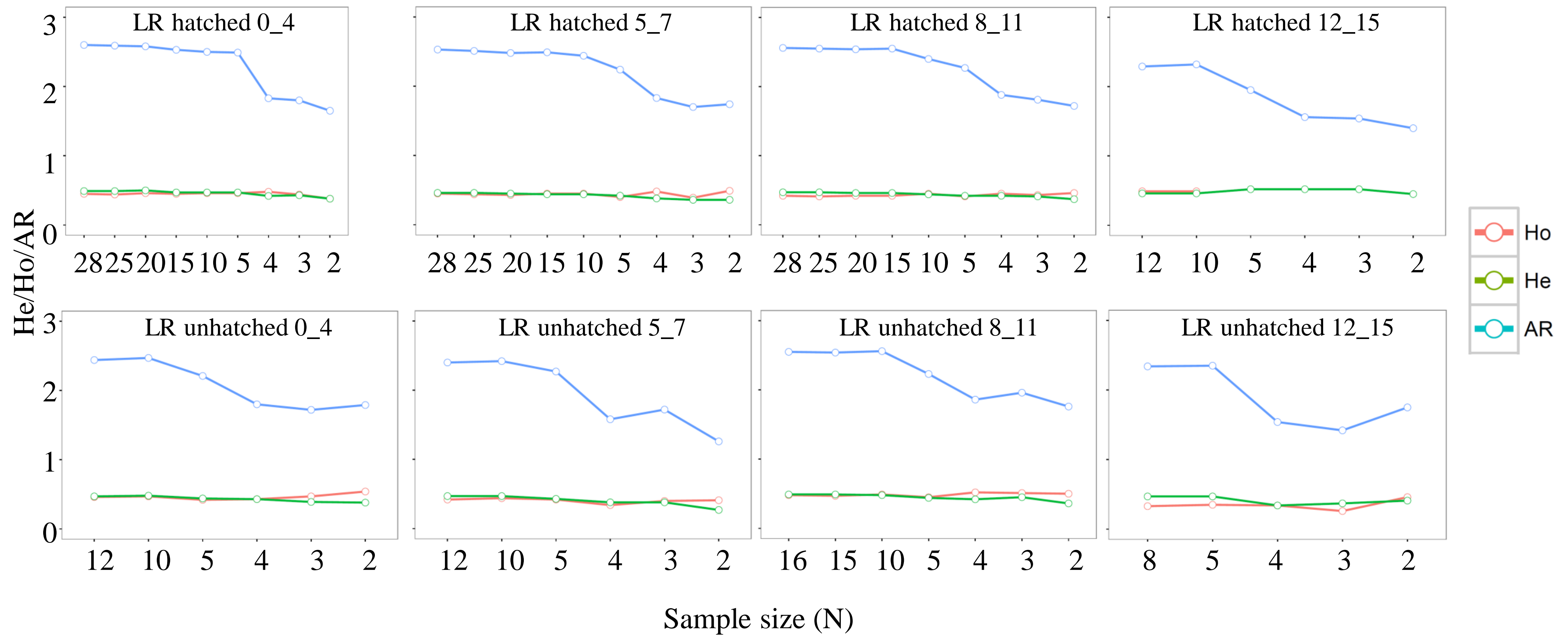


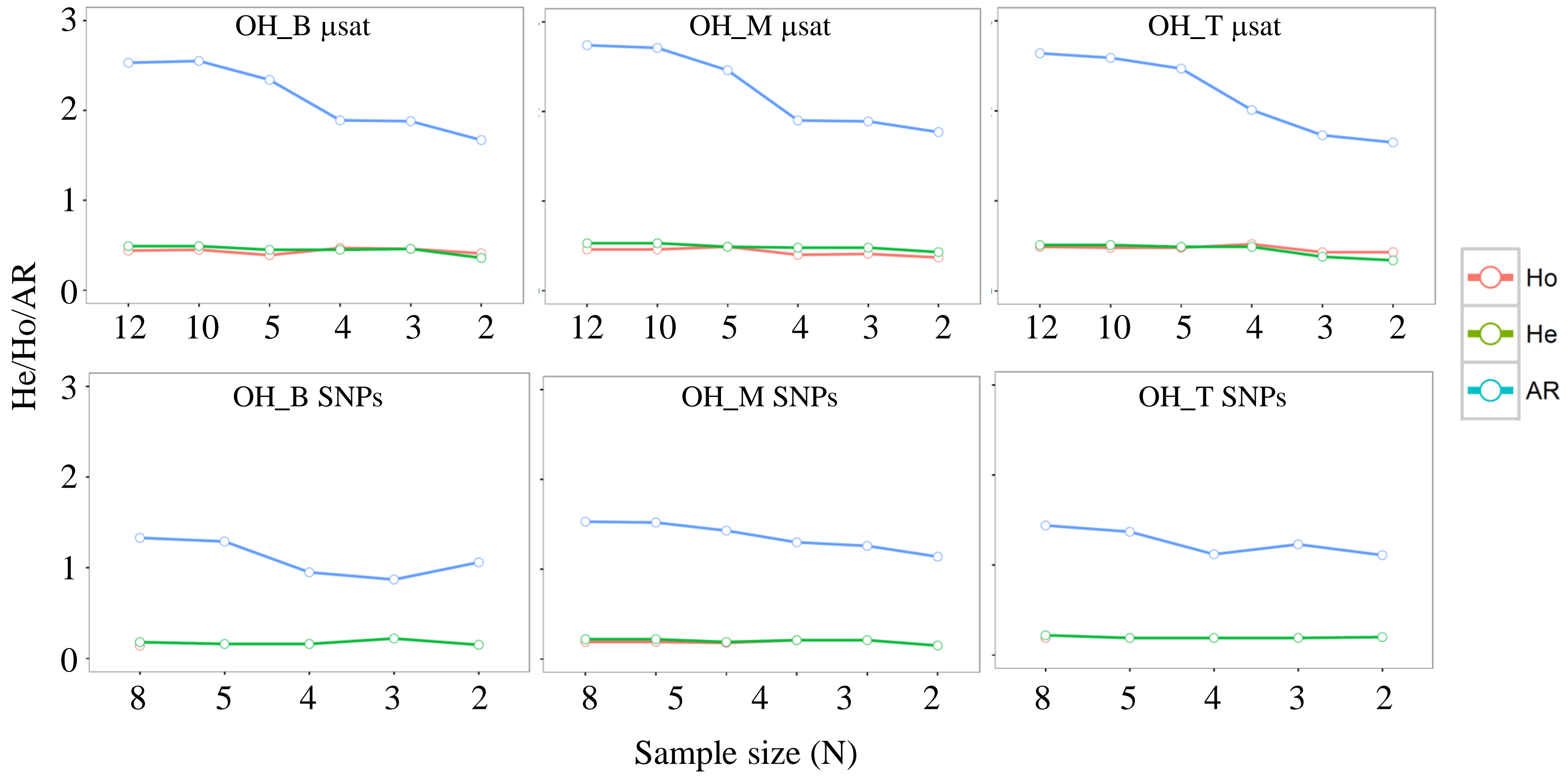
C

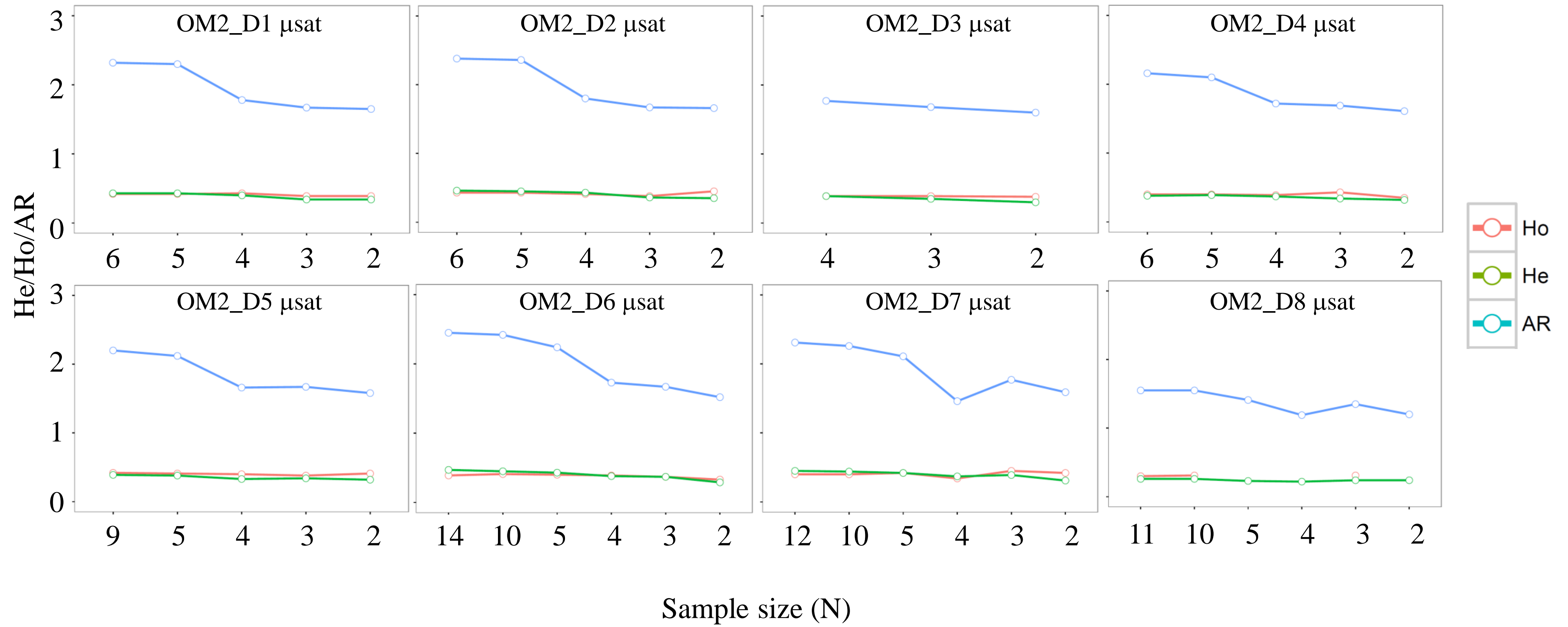


(sub)population

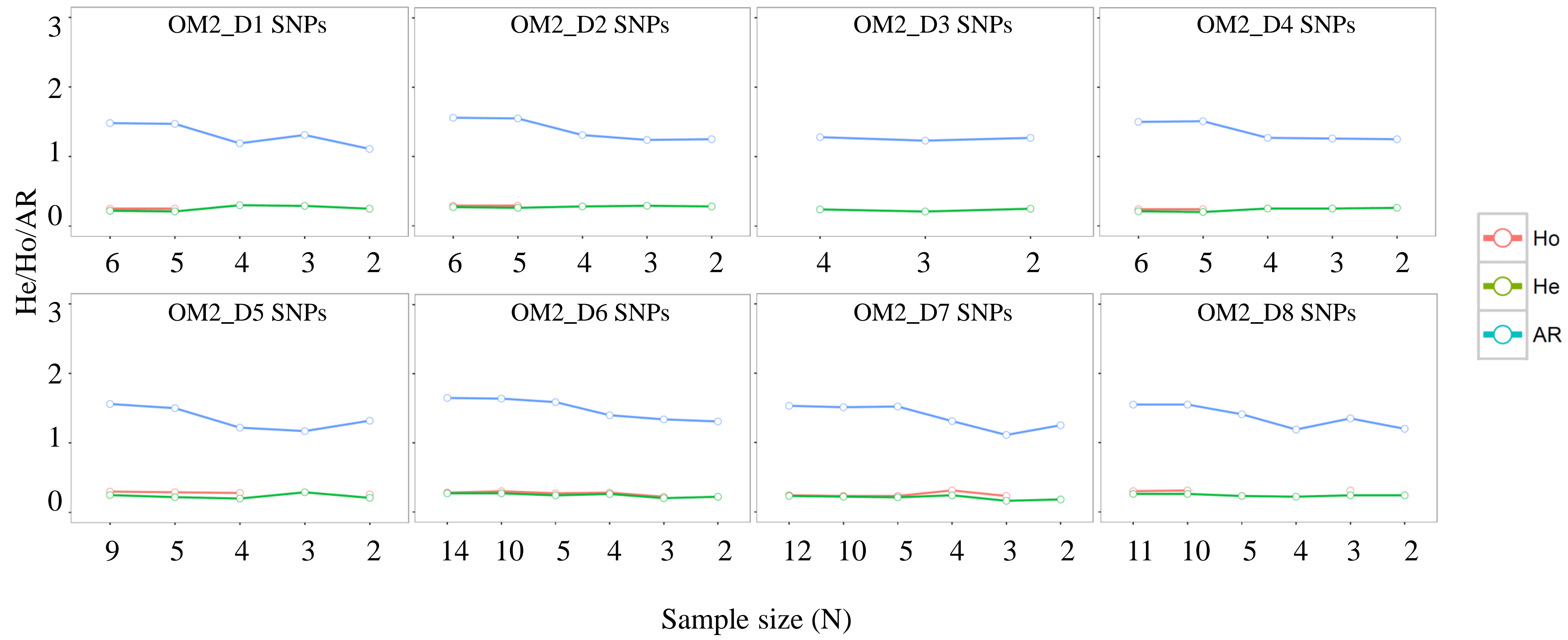

Hatched
Unhatched
LR0_4
LR5_7
LR8_11
LR12_15

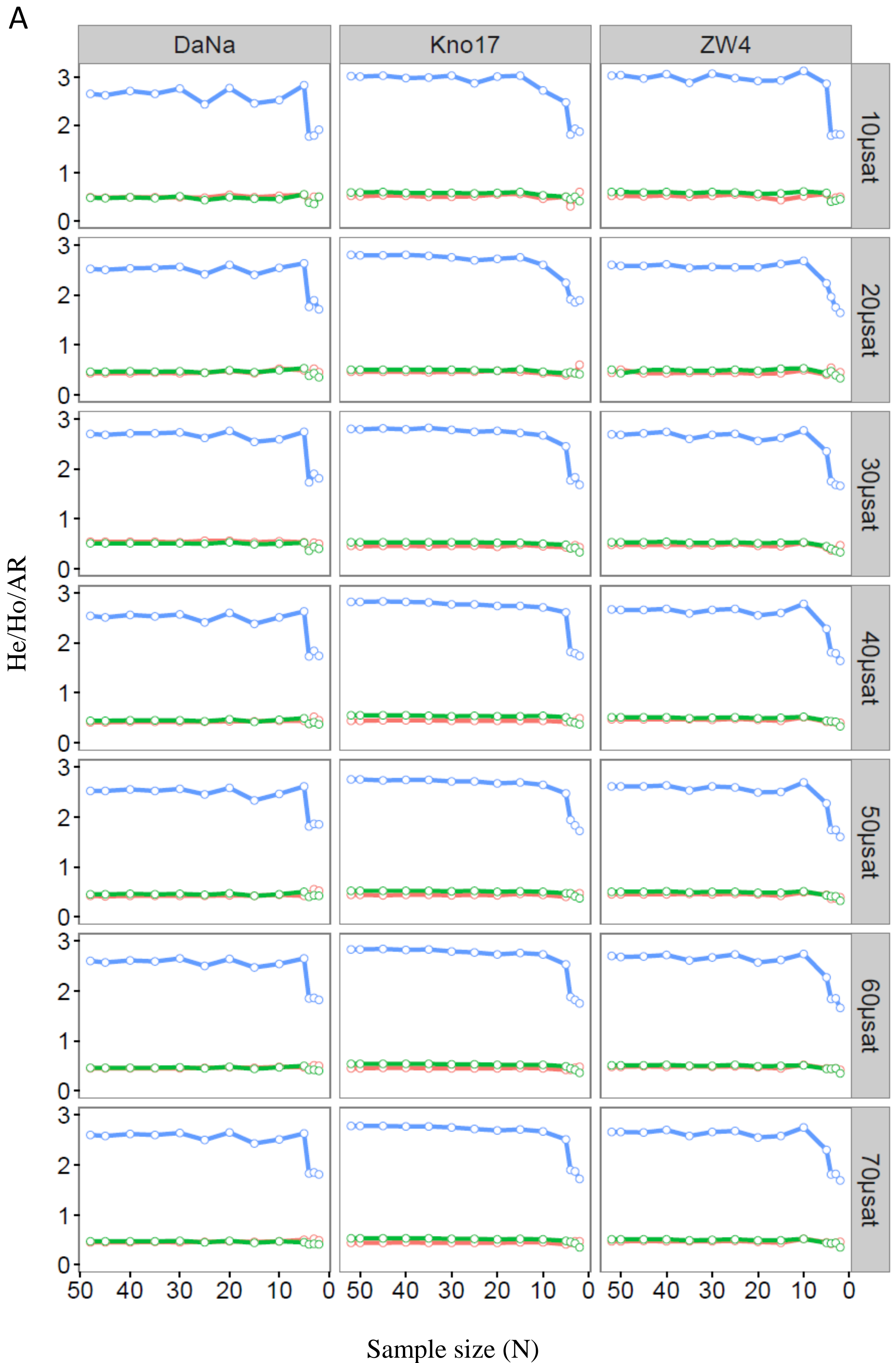

A
LR hatched 0_4
LR hatched 5_7
LR hatched 8_11
LR hatched 12_15
LR unhatched 0_4
LR unhatched 5_7
LR unhatched 8_11
LR unhatched 12_15
He/Ho/AR
Sample size (N)
Ho
He
AR

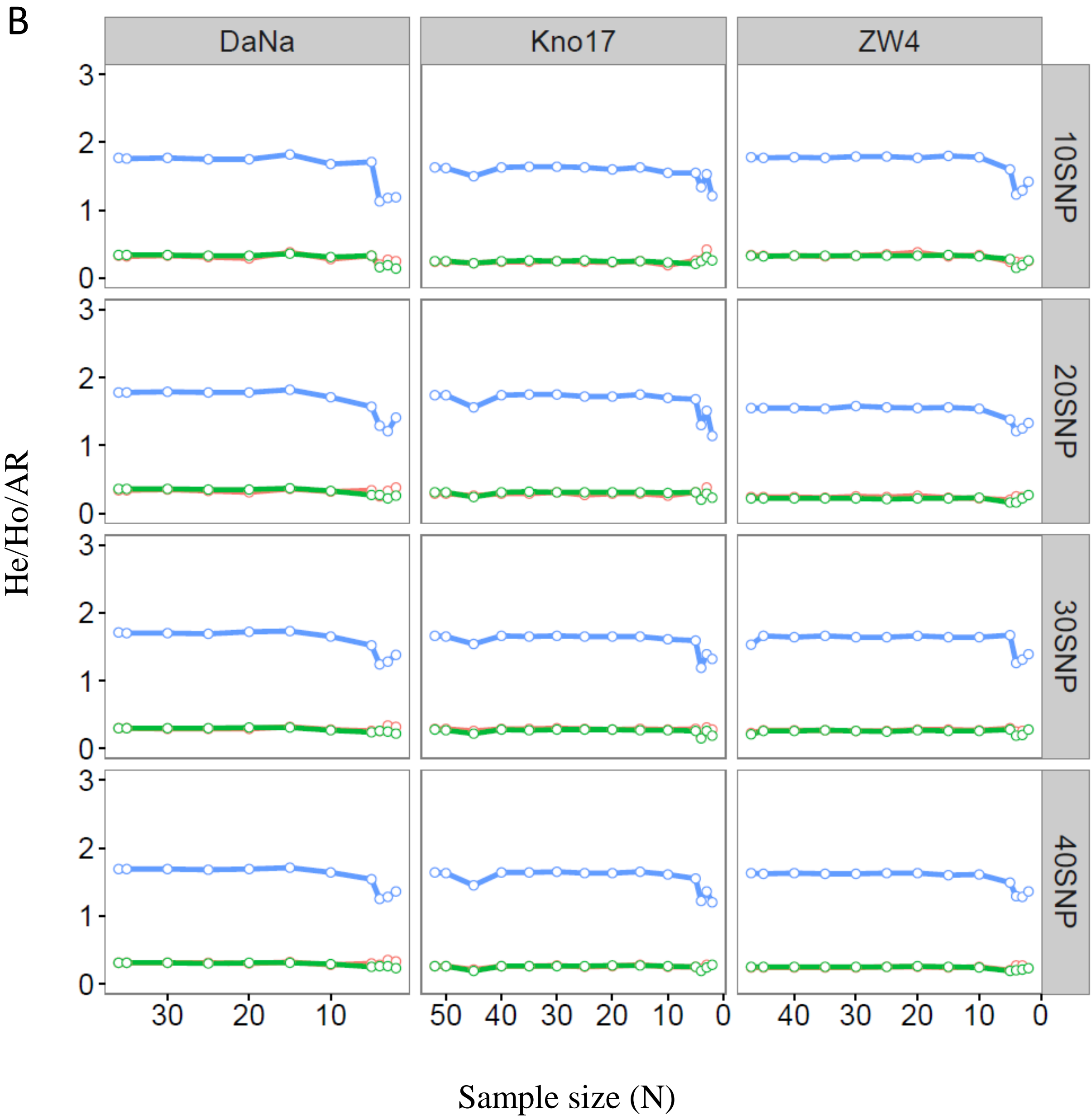

B
He/Ho/AR
OH_B µsat
OH_M µsat
OH_T µsat
OH_B SNPs
OH_M SNPs
OH_T SNPs
3
2
1
0
12
10
5
4
3
2
8
Sample size (N)
Ho
He
AR


C
He/Ho/AR
OM2_D1 µsat
OM2_D2 µsat
OM2_D3 µsat
OM2_D4 µsat
OM2_D5 µsat
OM2_D6 µsat
OM2_D7 µsat
OM2_D8 µsat
Ho
He
AR
Sample size (N)

C continued




A
DaNa
Kno17
ZW4
10µsat
20µsat
30µsat
40µsat
50µsat
60µsat
70µsat
He/Ho/AR
Sample size (N)
0
1
2
3
50
40
30
20
10
0


B
DaNa
Kno17
ZW4
10SNP
20SNP
30SNP
40SNP
He/Ho/AR
Sample size (N)