1   **Title:** Hybrid Songbirds are Deficient in Learning and Memory

2   **Running Title:** Hybrid Deficits in Learning and Memory

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10   **Author Contributions:** MAM and AMR conceived of the project idea, designed experiments,

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12   performed experiments, collected and analyzed data, and wrote the paper. AVH analyzed data

13   and edited the manuscript.

14

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23

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27   isolation, Hybrid breakdown

28

29   **Abstract:**

30   Identifying the phenotypes underlying postzygotic reproductive isolation is crucial for

31   fully understanding the evolution and maintenance of species. One potential postzygotic isolating

32   barrier that has not yet been examined is learning and memory ability in hybrids. Learning and

33   memory are important fitness-related traits, especially in scatter-hoarding species, where

34   accurate retrieval of hoarded food is vital for winter survival. Here, we test the hypothesis that

35   learning and memory ability can act as a postzygotic isolating barrier by comparing these traits

36   among two scatter-hoarding songbird species, black-capped (*Poecile atricapillus*), Carolina

37   chickadees (*Poecile carolinensis*), and their naturally occurring hybrids. In an outdoor aviary

38   setting, we find that hybrid chickadees perform significantly worse on an associative learning

39   spatial task and are worse at solving a novel problem compared to both parental species.

40   Deficiencies in learning and memory abilities could therefore contribute to postzygotic

41   reproductive isolation between chickadee species. Given the importance of learning and memory

42   for fitness, our results suggest that these traits may play an important, but as yet overlooked, role

43   in postzygotic reproductive isolation.

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Introduction

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48   A fundamental goal in evolutionary biology is to understand the origin and maintenance

49 of species. Natural hybridization – when distinct species mate and produce offspring of mixed

50 ancestry – provides a window into the process of species formation and can highlight the barriers

51 that reduce gene flow between species (Abbott et al. 2013). For example, hybridization often

52 results in the production of unfit hybrid offspring that are selected against (Coyne and Orr 2004).

53 This selection against hybrids, or postzygotic reproductive isolation, can maintain the integrity of

54 species boundaries despite ongoing gene flow (Servedio and Noor 2003). Alternatively,

55 hybridization can cause two species to fuse back into one as speciation moves in reverse (Taylor

56 et al. 2006). Finally, hybridization can stimulate evolutionary novelty through the formation of

57 completely new hybrid lineages (Gompert et al. 2006; Mallet 2007; Trier et al. 2014), or by

58 promoting the transfer of adaptive genetic material across species boundaries (Arnold 1997;

59 Grant and Grant 2010). Although the evolutionary outcomes of hybridization may vary widely,

60 they all influence the speciation process in some way. Therefore, identifying the mechanisms

61 underlying selection against hybrids and postzygotic isolation is crucial for fully understanding

62 how new species are formed and species barriers are maintained.

63   Two important fitness-related traits that have not been examined as potential postzygotic

64 isolating barriers are learning and memory. Learning has a well-established role in *prezygotic*

65 reproductive isolation, however (Verzijden et al. 2012; Dukas 2013). For example, many species

66 learn their mate preferences through sexual imprinting, where juveniles learn the phenotypes of

67 their parents and later select mates based on those phenotypes (Cate and Vos 1999; Verzijden et

68 al. 2008; Kozak et al. 2011). Species can also learn the traits that are themselves the targets of

69 mate choice, such as birdsong (Sorenson et al. 2003; Beecher and Brenowitz 2005). Despite a

70   demonstrated role for learning in prezygotic isolation, a potential role in postzygotic isolation has

71   not been adequately tested. A small number of studies have examined learning and memory

72   abilities in hybrids (Proops et al. 2009; Osthaus et al. 2013; Hoedjes et al. 2014), and in some

73   cases found that hybrids exhibited enhanced cognition compared to parental species (e.g. Proops

74   et al. 2009; Osthaus et al. 2013). However, these studies all examined cognition in hybrids

75   created by crossing domesticated species or inbred lines. In such cases, hybrid cognitive

76   performance may reflect enhanced heterozygosity and relief from inbreeding depression.

77   Therefore, it is difficult to generalize these results to cases of natural hybridization. Thus, the

78   potential role of learning and memory in postzygotic isolation remains relatively unexplored.

79   Here, our goal was to fill this gap by assessing learning and memory abilities in two songbird

80   species and their naturally occurring hybrids, for which learning and memory traits are important

81   for survival.

82   Learning and memory are important for fitness and survival in many species (e.g. Hollis

83   et al. 1997; Dukas and Bernays 2000; Maille and Schradin 2016), and this has been particularly

84   well demonstrated in species that are 'scatter-hoarders' (Vander Wall 1990; Shettleworth 1998).

85   Scatter-hoarding species cache (store) food items in hundreds to thousands of locations scattered

86   around their habitat. Individuals must then rely on cognitive traits, like learning and memory, to

87   accurately retrieve cached food at a later time (Pravosudov and Smulders 2010). The ability to

88   accurately retrieve caches is particularly important for winter survival, especially in harsh

89   environments where alternate sources of food are scarce or unpredictable (Pravosudov and

90   Clayton 2002). Furthermore, learning and memory traits in scatter-hoarders are both heritable

91   and variable among species, setting the stage for possible hybrid breakdown in these traits upon

92   interspecific hybridization (Brodbeck 1994; Roth et al. 2010, 2012; Pravosudov and Roth 2013).

93   Our two study species, the black-capped (*Poecile atricapillus*) and Carolina (*P.*

94   *carolinensis*) chickadee, are well-studied scatter-hoarding passerines that hybridize extensively

95   along a narrow hybrid zone stretching from New Jersey to Kansas (Bronson et al. 2005; Curry

96   2005; Taylor et al. 2014a; McQuillan and Rice 2015; **Fig. 1a**). These species are sister taxa

97   (Harris et al. 2014), and display ~6% mitochondrial DNA sequence divergence (Gill et al. 2005).

98   While selection against hybrid chickadees is strong at early life stages due to reduced hatching

99   success (Bronson et al. 2003, 2005), little is known about additional selection pressures on adult

100   hybrids (but see Olson et al. 2010).

101   Learning and memory abilities in chickadees are heritable and shaped by natural

102   selection. For example, black-capped chickadees inhabiting harsh winter environments, where

103   selection is expected to favor enhanced cognition, cache more food and display significantly

104   better spatial learning, memory, and problem-solving abilities than birds from mild environments

105   (Clayton and Pravosudov 2002; Roth et al. 2010, 2012). In addition, chickadees from these harsh

106   environments possess significantly more hippocampal neurons (the area of the brain associated

107   with spatial memory), and display higher levels of hippocampal neurogenesis than mild-

108   environment birds (Roth and Pravosudov 2009; Roth et al. 2011). These behavioral and

109   hippocampal differences remain when birds from the divergent environments are raised under

110   common laboratory conditions, suggesting a heritable genetic basis (Roth et al. 2010, 2012).

111   Carolina chickadees are also prodigious scatter-hoarders (Lucas and Zielinski 1998; Lucas et al.

112   2006), and one might expect learning and memory to be important traits for survival, especially

113   at the northern portion of their range where winters can be harsh and hybridization occurs (**Fig.**

114   **1a**).

115   In this study, we test the hypothesis that learning and memory have the potential to

116   function in postzygotic reproductive isolation by comparing the learning and memory abilities of

117   wild-caught black-capped, Carolina, and hybrid chickadees from a hybrid zone population.

118   Specifically, we measure individual performance on an associative learning spatial task and a

119   novel problem-solving test. If hybrids perform worse in these tests of learning and memory

120   compared to one or both parental species, then these traits may represent a novel postzygotic

121   isolating barrier. In contrast, if hybrids display equal or superior abilities compared to parental

122   species, then learning and memory are unlikely to act as postzygotic barriers in this system, and

123   may even provide adaptive benefits to hybrids.

124

125   **Materials and Methods**

126   *Field Methods and Aviary Housing*

127   We caught black-capped, Carolina, and hybrid chickadees (total n=50) using mist nets at

128   bird feeders or using song playback over the course of 13 months (Jan 2016 –Feb 2017),

129   excluding most of the breeding season (April-May). We caught birds at two geographically

130   proximate sites within the hybrid zone in eastern Pennsylvania (20 km apart; Lehigh University

131   40°36'5.19"N, 75°21'34.08"W; Jacobsburg State Park 40°47'3.97"N, 75°17'34.67"W) (**Fig. 1a**).

132   Upon capture, we banded, measured, and weighed each bird, and took a blood sample for

133   ancestry (McQuillan et al. 2017) and sex determination (Griffiths et al. 1998). We then

134   transported each bird by car to an outdoor aviary at Lehigh University, where birds were housed

135   individually in aviary compartments measuring $1.5 \times 2 \times 2.5$ m. Birds were visually, but not

136   aurally isolated from each other. We covered the outside of the aviary with house wrap to

137   prevent birds from seeing out, while still allowing ambient light to pass through. All behavioral

138   testing took place in home aviary compartments. We provided birds with *ad libitum* sunflower

139   seeds, pine nuts, and vitamin-supplemented water. We used wax worms, a highly desirable food

140   item, in behavioral trials (see below). We outfitted each aviary compartment with 10 small

141   rubber caching pockets (each 2.5 × 4 cm) that were accessible by perches. We did this so that

142   birds would acclimate to caching and retrieving food from these pockets, which were similar to

143   those used in the associative learning spatial task, described below. After behavioral testing was

144   complete (birds spent an average of 3 weeks in captivity), we released birds at the point of

145   capture. All procedures were approved by Lehigh University's Institutional Animal Care and

146   Use Committee (Protocol #175).

147

148   *Genetic Determination of Species Ancestry*

149   Because black-capped, Carolina, and hybrid chickadees are morphologically similar and

150   song is not a reliable species-identifier within the hybrid zone (Kroodsma et al. 1995), we

151   utilized genetic tools to assign ancestry to each bird. Briefly, we genotyped all birds at 10

152   species-diagnostic single nucleotide polymorphism (SNP) markers (McQuillan et al. 2017).

153   Because chickadees in our population are highly admixed and include pure-species, F1, as well

154   as advanced-generation and backcrossed hybrids (McQuillan et al. 2017), we used

155   STRUCTURE (Hubisz et al. 2009) to estimate admixture proportions and assign ancestry

156   categories for each bird. To do this, we combined the genotypes of our test subjects with a larger

157   dataset containing genotypes of individuals (N=301) from multiple Pennsylvania hybrid-zone

158   populations, as well known pure-species individuals from allopatric populations of both species

159   (New York and Louisiana, USA). We ran STRUCTURE on this larger dataset, using the same

160   program settings as those used in McQuillan et al. 2017 (**Fig. S1**). Birds with admixture values

161   that fell within the average 90% credible interval of the known pure individuals in the dataset

162   were classified as either a pure black-capped or Carolina individual. In contrast, birds with

163   admixture values outside of the average 90% credible interval for the known parentals were

164   considered hybrids.

165

166   *Associative Learning Spatial Task*

167   We subjected birds to an associative learning spatial task (following Roth et al. 2012).

168   We used a large plywood 'caching array,' measuring 1.25×0.6 m with 60 rubber pockets

169   identical to those used during acclimation, and accessible by perches. The contents of each

170   pocket could be concealed by placing a white, craft 'pom-pom' ball over the opening, which

171   birds had to remove in order to investigate the pocket's contents (**Fig. 1b**). All birds were able to

172   remove the balls from the pockets. On the first day of the test, we placed a wax worm in one of

173   the 60 pockets (chosen randomly for each bird), covered it and all other pockets with the balls,

174   introduced the array to the bird's aviary compartment, and allowed the bird to remove the pom-

175   pom balls and find the worm. For the next 9 days, once per day, we presented the same caching

176   array to the bird with all pockets covered and a wax worm concealed in the same pocket. Care

177   was taken to hang the caching array on the aviary wall in the same place each day. Each day, we

178   recorded the number of inspections (defined as the number of pom-pom balls removed) required

179   to successfully locate the worm. We tested 11 black-capped, 8 Carolina, and 10 hybrid

180   chickadees on this task.

181   Because some of the birds we tested had been used previously to collect pilot data for

182   another experiment using the caching array (total n=10; 2 black-capped, 5 Carolina, 3 hybrid),

183   we tested for an effect of this prior experience on performance on the associative learning spatial

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184   task. We found a marginally non-significant interaction between this prior experience and testing

185   day (GLMM $\chi^2(1)$=3.815, p=0.051). Although not statistically significant, we sought to eliminate

186   any potential influence of prior experience on our results by limiting our subsequent analysis to

187   days 4 through 10 (hereafter referred to as testing days 1 through 7). During this time window,

188   the performance of birds with and without prior caching array experience converged (**Fig. S2**;

189   GLMM; prior experience × testing day, $\chi^2(1)$=0.370, p=0.543; prior experience main effect,

190    $\chi^2(1)$=1.692, p=0.193). Importantly, limiting our analysis to this time window did not

191   qualitatively change our results.

192

193   *Novel Problem-Solving Test*

194   To evaluate each bird's innovativeness and general learning ability, we assessed each

195   bird's ability to solve a reward-based, novel problem (following Roth et al. 2010). The problem-

196   solving test required birds to physically move a circular nylon washer with a transparent coating

197   (3.3 cm diameter, 6 g, ~1/2 the mass of the bird) from covering a 1.75 cm well in a wooden

198   block (15 × 10 × 4 cm) that contained a waxworm (**Fig. 1c**). The test was designed so that birds

199   could see through the circular washer and recognize that a food reward was concealed

200   underneath, but the reward could only be retrieved by sliding the washer to the side. On the day

201   of the problem-solving test, we deprived birds of food for one hour in the morning, after which

202   we introduced the problem-solving apparatus to the bird's home compartment. We recorded

203   whether or not the bird was able to solve the problem (by moving the washer and recovering the

204   worm) in a one-hour maximum time-frame. We conducted a second test in the afternoon, with

205   the two tests spaced by at least 4 hours. We tested 14 black-capped, 6 Carolina, and 16 hybrid

206   chickadees (total n=36) on this problem-solving test. Included in this group of 36 birds were 15

207   birds that had previously performed the associative learning spatial task.

208   All birds were habituated to finding wax worms in the well of the problem-solving

209   apparatus and to the presence of the nylon washer, which was mounted adjacent to the well

210   during habituation (>40 hours of habituation time per bird). To control for motivation and the

211   birds' willingness to feed from the problem-solving apparatus, we conducted a pre-trial control

212   in the afternoon of the day preceding the problem-solving test. For the pre-trial, we introduced

213   the problem-solving apparatus to the bird with an un-concealed wax worm and the nylon washer

214   mounted adjacent to the well. We measured the latency in seconds for the bird to land on the

215   apparatus and take the worm. Birds were also food deprived for one hour prior to this pre-trial

216   control.

217

218   *Statistical Analysis*

219   To test for performance differences among ancestry groups in the associative learning

220   spatial task, we fit generalized linear mixed models (GLMMs) using maximum likelihood to our

221   data. We specified log link functions in our models and used the 'lme4' package in R, version

222   3.4.1 (R Core Team, 2017; Bates et al. 2015). Specifically, we fit GLMMs with 'score' (the

223   number of pom-pom balls removed before the bird recovered the worm) as our response

224   variable. We included the following independent variables as fixed effects, including all possible

225   interactions: Ancestry (black-capped, Carolina, or hybrid), sex, testing day, and season (spring,

226   fall, or winter). In order to account for the repeated-measures nature of our dataset (i.e. we

227   measured each bird's performance once per day for multiple days), we specified a random

228   intercept, as well as a random slope for each bird across testing days in our model. Once we had

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229   constructed this 'full' model, we performed a step-wise model simplification procedure by

230   removing the least significant variable, starting with the highest order interactions. After variable

231   removal, we performed likelihood ratio tests (LRT) to compare models with and without each

232   focal term. If the simplified model explained significantly less variation in the response variable,

233   then the focal term was retained. We continued this process until we were left with a 'best-fit'

234   model, which contained only those fixed effects that were significant predictors, according to the

235   likelihood ratio tests. If a variable was found to be a significant predictor, we assessed post-hoc

236   pairwise contrasts of the levels within that factor using least-square means (LSM) in the R

237   package 'lsmeans' (v. 2.27-2; Lenth 2016).

238   To test for differences among the ancestry groups in ability to solve the novel reward-

239   based problem, we compared the proportion of individuals from each ancestry group that were

240   able to successfully solve the problem at least once (of two total trials), using Fisher's exact tests.

241

242   **Results**

243   For the associative learning spatial task, hybrids performed worse across the testing

244   period than either pure species. Our best-fit GLMM was significant overall (LRT compared to

245   null model, $\chi^2(4)=31.10$, p<0.001), and contained ancestry ($\chi^2(2) = 8.11$, p=0.017), sex

246   ($\chi^2(1)=8.32$, p=0.004), and testing day ($\chi^2(1)=17.62$, p<0.001) as fixed effects. Our post-hoc

247   pairwise comparisons indicated that hybrids required more inspections to recover the wax worm

248   across the testing period than pure black-capped (estimated LSM contrast=0.437, p=0.033) and

249   Carolina (LSM contrast=0.525, p=0.018) birds (**Fig. 2**). There was no significant difference in

250   performance between the two pure-species groups (LSM contrast= 0.088, p=0.890).

251   Additionally, male birds made significantly fewer inspections to recover the wax worm, on

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252   average, than female birds (LSM contrast= 0.503, p=0.002). The significant effect of testing day

253   on test performance indicated that learning occurred across all ancestry groups, with birds

254   requiring fewer inspections to recover the food reward as the test progressed. Interestingly,

255   female hybrids were the worst performers on the associative learning spatial task (**Fig. 3**).

256   Although this sex × ancestry interaction was not retained as a significant predictor in the best-fit

257   model, likely due to low power, it suggests the intriguing possibility that hybrid cognition may

258   follow Haldane's Rule (see discussion).

259   Hybrids were also worse at problem solving. In the novel problem-solving test, 13/14

260   (93%) black-capped, 6/6 Carolina (100%), and 10/16 (62.5%) hybrids were able to solve the

261   problem at least once (**Fig. 4a**). These differences in success were marginally non-significant

262   (Fisher's exact test, p=0.054). However, the lack of statistical significance may be due to a lack

263   of power from the relatively small Carolina chickadee sample size. Because we were most

264   interested in testing hybrid cognitive abilities relative to pure species, and because the black-

265   capped and Carolina chickadees exhibited similar rates of success (**Fig. 4a**), we combined black-

266   capped and Carolina birds into a single, pure-species category and compared it to the hybrid

267   group. When analyzed in this way, hybrids were significantly less likely to solve the problem

268   than pure-species individuals across the two trials (**Fig. 4b;** p=0.0298, Fisher's exact test; 62.5%

269   vs. 95% success, respectively). All birds that solved the problem during the first trial also

270   successfully solved in the second trial. There were no significant differences between ancestry

271   groups in the latency to take the worm in the pre-trial control, indicating that all birds were

272   equally motivated to feed from the problem-solving apparatus (Kruskal-Wallis $\chi^2(2)$=2.78,

273   p=0.248). Additionally, differences among the ancestry groups in problem-solving ability cannot

274   be explained by differences in body mass (ANOVA F(2,32)=1.469, p=0.245; **Fig. S3**).

Discussion

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276   Overall, our results suggest that hybrid chickadees are deficient in learning and memory

277   traits relative to their pure-species counterparts. Hybrid birds performed worse on average than

278   pure-species individuals at an associative learning spatial task (**Figs. 2,3**), and were less likely to

279   solve a reward-based novel problem than pure-species birds (**Fig. 4**). These results suggest a role

280   for learning and memory in postzygotic isolation, which has not been examined previously. Our

281   behavioral results likely have real-world implications for scatter-hoarding chickadees in nature,

282   as learning and memory are important fitness-related traits in these species.

283   Accurate retreival of cached food is crucial for winter survival, and natural selection

284   shapes the cognitive traits necessary to achieve this (Pravosudov and Roth 2013). Previous work

285   uncovered heritable variation in caching-related learning and memory traits between populations

286   of black-capped chickadees from different environments (Pravosudov and Clayton 2002; Roth et

287   al. 2010, 2012). These results suggest that learning and memory traits in chickadees are

288   important for survivial, potentially under genetic control, and shaped by natural selection. We

289   extended this line of reasoning by examining the learning and memory abilites of hybrid

290   chickadees, and comparing them against pure-species individuals from the same environment.

291   The fact that hybrid chickadees display inferior learning and memory abilities relative to pure-

292   species birds suggests that hybrids may suffer a fitness disadvantage in nature, particularly in

293   their ability to accurately retreive cached food. There are multiple possible explanations for this

294   deficiency in hybrids.

295   One possible explanation for hybrid deficits in our cognitive tests is the accumulation of

296   genetic incompatibilities among loci underlying learning and memory traits in hybrid genomes.

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297   Genetic incompatibilites arise when alleles at two or more loci diverge in geographically isolated

298   species, and recombine in hybrid genomes upon secondary contact (Dobzhansky 1936; Muller

299   1942). If genes underlying learning and memory traits have diverged in black-capped and

300   Carolina chickadees, negative genetic interactions between these loci could occur in hybrids,

301   leading to breakdown in these traits. One ubiquitous outcome of hybrid genetic incompatibilities

302   is Haldane's Rule, which states that within hybrid offspring, the heterogametic sex (i.e. the sex

303   possessing two different sex chromosomes) is more likely to be absent, rare, or sterile (Haldane

304   1922). This rule applies whether males are heterogametic, as in mammals and *Drosophila*, or

305   whether females are heterogametic, as in birds and Lepidoptera. Haldane's Rule has been upheld

306   in virtually all taxa that have been surveyed (Orr 1997; Delph and Demuth 2016). Of the

307   multiple explanations for Haldane's Rule, all are genetic in nature. The explanation with the

308   most support, termed 'dominance theory' (Turelli and Orr 1995), posits that the heterogametic

309   sex (females in birds) will be negatively affected by any and all incompatibility loci located on

310   the sex chromosmes, regardless of whether the incompatible alleles act dominantly or

311   recessively. In contrast, the homogametic sex (males in birds) will only be negatively affected by

312   the subset of sex-linked incompatibility loci that act in a relatively dominant fashion.

313   Consistent with Haldane's Rule, our results on the associative learning spatial task

314   suggest that female hybrids have the worst memories (**Fig. 3**). If hybrid females are less able to

315   accurately retrieve cached food in nature, then their ability to survive particularly harsh winter

316   conditions may be reduced. Interestingly, Bronson et al. (2005) found a distinct paucity of hybrid

317   female chickadees along a hybrid zone transect in Ohio. The authors concluded that female

318   hybrids may have reduced viability. Our results provide a potential explanation for this result.

319   Furthermore, a genomic analysis of introgression patterns across the chickadee hybrid zone in

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320   Pennsylvania found that a majority of loci putatively underlying reproductive isolation are

321   located on the Z chromosome (in birds, females are heterogametic ZW, while males are

322   homogametic ZZ; Taylor et al. 2014b). This result would predict that female hybrid chickadees

323   are likely to experience greater negative effects from sex-linked genetic incompatibility loci than

324   male hybrids. In contrast, we did not discover an effect of sex in the novel problem solving test:

325   Of the six hybrids that were unable to solve the problem, three were male and three were female.

326   Another possible explanation for our results is that hybrid chickadees are not deficient in

327   their learning and memory abilities, but rather are deficient in some other respect, such as their

328   general health or body condition. In other words, worse performance on our cognitive tests may

329   be an artifact of reduced overall hybrid quality. For example, Turissini et al. (2017) found that

330   lab-generated *Drosophila* hybrids were less capable of finding food than their pure-species

331   parents. If hybrid chickadees are also worse foragers than pure-speices individuals, impaired

332   cognitive performance may simply result from the fact that hybrids have a lower nutritional state.

333   In rats, nutritional stress leads to anatomical defects in the hippocampus, which is associated

334   with reduced performance on spatial memory tasks (Jordan et al. 1981; Levitsky and Strupp

335   1995). However, we see no evidence that hybrid chickadees are of lower body condition than

336   pure-species birds in our hybrid zone transect as a whole, or in the subset of birds used for this

337   study (**Fig. S4**).

338   Related to this point, it is also possible that deficient learning and memory in hybrids

339   could result from overall metabolic dysfunction. Because the brain is a metabolically costly

340   organ, breakdown in hybrid metabolism could result in an associated breakdown in learning and

341   memory. In an Ohio hybrid zone transect, hybrid chickadees display higher mass-corrected basal

342   metabolic rates compared to both parental species, suggesting less efficient hybrid metabolism

343   (Olson et al. 2010). Similar results have been found in hybrid flycatchers (Mcfarlane et al. 2016).

344   Future work should aim to disentangle the possible consequences of metabolic breakdown on

345   learning and memory from other neurological causes for breakdown in these traits. More

346   generally, future work should examine the relative contributions of genetic/epigenetic factors

347   versus plastic responses to the environment on hybrid cognition. For example, hybrids may

348   experience the environment differently than pure-species individuals, and this difference in life

349   experience could affect cognitive performance. This could be controlled for by hand-rearing

350   pure-species and hybrid individuals under identical laboratory conditions and subjecting them to

351   learning and memory tests as adults (as in Roth et al. 2010, 2012).

352   The fact that hybrids are less able to solve a novel problem than pure-species birds (**Fig.**

353   **4**) also likely has real-world implications in this system. In unpredictable environments, animals

354   must either invent new behaviors, or flexibly adjust established behaviors in order to solve novel

355   problems (Reader and Laland 2003). The ability to solve novel problems is often used as a

356   measure of an animal's ability to innovate (e.g. Morand-Ferron et al. 2011; Cauchard et al.

357   2013). Environmental perturbations as a result of climate change will add to the unpredictability

358   of many environments, perhaps increasing selective pressure for innovativeness and problem-

359   solving abilities. If hybrids are less able to solve problems and innovate than their pure-species

360   counterparts, this may represent a selective disadvantage in nature. As the frequency of

361   hybridization is expected to rise under climate change (Chunco 2014), future work should

362   examine hybrid problem-solving abilities in other systems inhabiting unpredictable

363   environments.

364   Identifying the barriers that prevent gene flow between closely related species is crucial

365   in order to understand how new species arise. Much work on postztgotic isolation has focused on

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366   identifying the genetic underpinnings of hybrid sterility and inviability, often in model laboratory

367   systems. However, less attention has been paid to maladaptive behaviors in hybrids that

368   potentially contribute to reproductive isolation (but see Noor 1997a; Delmore and Irwin 2014;

369   Schmidt and Pfennig 2016). Here, we have shown evidence that naturally occurring hybrids are

370   deficient in learning and memory abilities relative to their pure-species parents. This may be an

371   important and widespread, but as yet overlooked, source of postzygotic reproductive isolation.

372   One additional goal of future studies should be to evaluate the degree to which hybrids display

373   these deficiencies in their natural environments (sensu Croston et al. 2016). Given the

374   widespread nature of hybridization (Mallet 2005), examining potential behavioral sources of

375   postzygotic isolation is paramount for a complete understanding of the speciation process.

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389 **References**

390 Abbott, R., D. Albach, S. Ansell, J. W. Arntzen, S. J. E. Baird et al. 2013. Hybridization and
391   speciation. J. Evol. Biol. 26:229–246.
392

393 Arnold, M. L. 1997. Natural hybridization and evolution. Oxford University Press, Oxford.
394

395 Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models
396   using lme4. J. Stat. Softw. 67(1):1-48.
397

398 Beecher, M. D., and E. A. Brenowitz. 2005. Functional aspects of song learning in songbirds.
399   Trends Ecol. Evol. 20:143–149.
400

401 Brodbeck, D. R. 1994. Memory for spatial and local cues: A comparison of a storing and a
402   nonstoring species. Anim. Learn. Behav. 22:119–133.
403

404 Bronson, C. L., T. C. Grubb, and M. J. Braun. 2003. A test of the endogenous and exogenous
405   selection hypotheses for the maintenance of a narrow avian hybrid zone. Evolution 57:630–
406   637.
407

408 Bronson, C. L., T. C. Grubb, G. D. Sattler, and M. J. Braun. 2005. Reproductive success across
409   the black-capped chickadee (Poecile atricapillus) and Carolina chickadee (P. carolinensis)
410   hybrid zone in Ohio. Auk 122:759-772.
411

412 Cate, C. T., and D. R. Vos. 1999. Sexual imprinting and evolutionary processes in birds: A
413   reassessment. Adv. Study Behav. 28:1–31.
414

415 Cauchard, L., N. J. Boogert, L. Lefebvre, F. Dubois, and B. Doligez. 2013. Problem-solving
416   performance is correlated with reproductive success in a wild bird population. Anim. Behav.
417   85:19–26.
418

419 Chunco, A. J. 2014. Hybridization in a warmer world. Ecol. Evol. 4:2019–2031.
420

421 Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.
422

423 Croston, R., D. Y. Kozlovsky, C. L. Branch, T. L. Parchman, E. S. Bridge, and V. V.
424   Pravosudov. 2016. Individual variation in spatial memory performance in wild mountain
425   chickadees from different elevations. Anim. Behav. 111:225–234.
426

427 Delmore, K. E., and D. E. Irwin. 2014. Hybrid songbirds employ intermediate routes in a
428   migratory divide. Ecol. Lett. 17:1211–1218.
429

430 Dukas, R., and E. A. Bernays. 2000. Learning improves growth rate in grasshoppers. Proc. Natl.
431   Acad. Sci. U. S. A. 97:2637–2640.
432

433   Dukas, R. 2004. Evolutionary biology of animal cognition. Annu. Rev. Ecol. Evol. Syst. 35:
434   347–374.
435
436   Curry, R. 2005. Hybridization in chickadees: much to learn from familiar birds. Auk 122:747–
437   758.
438
439   Delph, L. F., and J. P. Demuth. 2016. Haldane's rule: Genetic bases and their empirical support.
440   J. Hered. 107:383–391.
441
442   Dobzhansky, T. 1936. Studies on hybrid sterility. II. Localization of sterility factors in
443   *Drosophila pseudoobscura* hybrids. Genetics 21:113–135.
444
445   Gill, F. B., B. Slikas, and F. H. Sheldon. 2005. Phylogeny of titmice (paridae): II. Species
446   relationships based on sequences of the mitochondrial cytochrome-b gene. Auk 122(1):121-
447   143.
448
449   Gompert, Z., J. A. Fordyce, M. L. Forister, A. M. Shapiro, and C. C. Nice. 2006. Homoploid
450   hybrid speciation in an extreme habitat. Science 314:1923–1925.
451
452   Grant, P. R., and B. R. Grant. 2010. Conspecific versus heterospecific gene exchange between
453   populations of Darwin's finches. Phil. Trans. R. Soc. B 365:1065–1076.
454
455   Griffiths, R., M. C. Double, K. Orr, and R. J. G. Dawson. 1998. A DNA test to sex most birds.
456   Mol. Ecol. 7:1071-1075.
457
458   Haldane, J. B. S. 1922. Sex ratio and unisexual sterility in hybrid animals. J. Genet. 12:101-109.
459
460   Harris, R. B., M. D. Carling, and I. J. Lovette. 2014. The influence of sampling design on species
461   tree inference: a new relationship for the New World chickadees (Aves: Poecile). Evolution
462   68:501–13.
463
464   Hoedjes, K. M., H. M. Smid, L. E. M. Vet, and J. H. Werren. 2014. Introgression study reveals
465   two quantitative trait loci involved in interspecific variation in memory retention among
466   Nasonia wasp species. Heredity 113:542–550.
467
468   Hollis, K. L., V. L. Pharr, M. J. Dumas, G. B. Britton, and J. Field. 1997. Classical conditioning
469   provides paternity advantage for territorial male Blue Gouramis (Trichogaster trichopterus).
470   J. Comp. Psychol. 111:219–225.
471
472   Hubisz, M. J., D. Falush, M. Stephens, and J. K. Pritchard. 2009. Inferring weak population
473   structure with the assistance of sample group information. Mol. Ecol. Resour. 9:1322–1332.
474
475   Jordan, T. C., S. E. Cane, and K. F. Howells. 1981. Deficits in spatial memory performance
476   induced by early undernutrition. Dev. Psychobiol. 14:317–325.
477

478 Kozak, G. M., M. L. Head, and J. W. Boughman. 2011. Sexual imprinting on ecologically
479   divergent traits leads to sexual isolation in sticklebacks. Proc. R. Soc. B Biol. Sci. 278:2604–
480   2610.
481
482 Kroodsma, D., D. Albano, P. Houlihan, and J. Wells. 1995. Song development by black-capped
483   chickadees (Parus atricapillus) and Carolina chickadees (P. carolinensis). Auk 112:29–43.
484
485 Lenth, R. V. 2016. Least-Squares Means: The R Package lsmeans. J. Stat. Softw. 69.
486
487 Levitsky, D., and B. Strupp. 1995. Malnutrition and the brain: Changing concepts, changing
488   Concerns1. J Nutr. 125:2212–2220.
489
490 Lucas, J. R., and D. L. Zielinski. 1998. Seasonal variation in the effect of cache pilferage on
491   cache and body mass regulation in Carolina chickadees: What are the trade-offs? Behav.Ecol.
492   9:193–200.
493
494 Lucas, J. R., T. M. Freeberg, J. Egbert, and H. Schwabl. 2006. Fecal corticosterone, body mass,
495   and caching rates of Carolina chickadees (Poecile carolinensis) from disturbed and
496   undisturbed sites. Horm. Behav. 49:634–43.
497
498 Maille, A., and C. Schradin. 2016. Survival is linked with reaction time and spatial memory in
499   African striped mice. Biol. Lett. 12:20160346.
500
501 Mallet, J. 2005. Hybridization as an invasion of the genome. Trends Ecol. Evol. 20:229–37.
502
503 Mallet, J. 2007. Hybrid speciation. Nature 446:279–283.
504
505 Mcfarlane, S. E., P. M. Sirkiä, Å. Murielle, and A. Qvarnström. 2016. Hybrid Dysfunction
506   Expressed as Elevated Metabolic Rate in Male Ficedula Flycatchers. PLoS ONE 11(9):
507   e0161547.
508
509 McQuillan, M. A., and A. M. Rice. 2015. Differential effects of climate and species interactions
510   on range limits at a hybrid zone: potential direct and indirect impacts of climate change.
511   Ecol. Evol. 5(21):5120-5137.
512
513 McQuillan, M. A., A. V. Huynh, S. A. Taylor, and A. M. Rice. 2017. Development of 10 novel
514   SNP-RFLP markers for quick genotyping within the black-capped (Poecile atricapillus) and
515   Carolina (P. carolinensis) chickadee hybrid zone. Conserv. Genet. Resour. 9:261–264.
516
517 Morand-Ferron, J., E. F. Cole, J. E. C. Rawles, and J. L. Quinn. 2011. Who are the innovators? A
518   field experiment with 2 passerine species. Behav. Ecol. 22:1241–1248.
519
520 Muller, H. J. 1942. Isolating mechanisms, evolution and temperature. Pp. 71–125 in T.
521   Dobzhansky, ed. Biological Symposia. The Jaques Cattell Press.
522

Noor, M. A. F. 1997. Genetics of sexual isolation and courtship dysfunction in male hybrids of Drosophila pseudoobscura and Drosophila persimilis. Evolution 51:809–815.

Olson, J. R., S. J. Cooper, D. L. Swanson, M. J. Braun, and J. B. Williams. 2010. The relationship of metabolic performance and distribution in black-capped and Carolina chickadees. Physiol. Biochem. Zool. 83:263–275.

Orr, H. A. 1997. Haldane's rule. Annu. Rev. Ecol. Syst. 28:195-218.

Osthaus, B., L. Proops, I. Hocking, and F. Burden. 2013. Spatial cognition and perseveration by horses, donkeys and mules in a simple A-not-B detour task. Anim. Cogn. 16:301–305.

Pravosudov, V. V., and N. S. Clayton. 2002. A test of the adaptive specialization hypothesis: Population differences in caching, memory, and the hippocampus in black-capped chickadees (Poecile atricapilla). Behav. Neurosci. 116:515–522.

Pravosudov, V. V., and T. C. Roth. 2013. Cognitive Ecology of Food Hoarding: The Evolution of spatial memory and the hippocampus. Annu. Rev. Ecol. Evol. Syst. 44:173–193.

Pravosudov, V. V., and T. V. Smulders. 2010. Integrating ecology, psychology and neurobiology within a food-hoarding paradigm. Philos. Trans. R. Soc. B Biol. Sci. 365:859–867.

Proops, L., F. Burden, and B. Osthaus. 2009. Mule cognition: A case of hybrid vigour? Anim. Cogn. 12:75–84.

R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Reader, S. M. & Laland, K. N. 2003. Animal innovation: an introduction. Oxford University Press, Oxford.

Roth, T. C., and V. V. Pravosudov. 2009. Hippocampal volumes and neuron numbers increase along a gradient of environmental harshness: a large-scale comparison. Proc. Biol. Sci. 276: 401–405.

Roth, T. C., L. D. LaDage, and V. V. Pravosudov. 2010. Learning capabilities enhanced in harsh environments: a common garden approach. Proc. Biol. Sci. 277:3187–3193.

Roth, T. C., L. D. LaDage, and V. V Pravosudov. 2011. Variation in hippocampal morphology along an environmental gradient: controlling for the effects of day length. Proc. Biol. Sci. 278:2662–2667.

Roth, T. C., L. D. LaDage, C. A. Freas, and V. V. Pravosudov. 2012. Variation in memory and the hippocampus across populations from different climates: a common garden approach. Proc. Biol. Sci. 279:402–410.

21

568

569 Schmidt, E. M., and K. S. Pfennig. 2016. Hybrid female mate choice as a species isolating
570   mechanism: environment matters. J. Evol. Biol. 29:865–869.

571

572 Servedio, M. R., and M. A. F. Noor. 2003. The role of reinforcement in speciation: theory and
573   data. Annu. Rev. Ecol. Evol. Syst. 34:339–364.

574

575 Shettleworth, S. J. 1998. Cognition, evolution, and behavior. Oxford University Press, Oxford.

576

577 Sorenson, M. D., K. M. Sefc, and R. B. Payne. 2003. Speciation by host switch in brood parasitic
578   indigobirds. Nature 424:928–931.

579

580 Taylor, E. B., J. W. Boughman, M. Groenenboom, M. Sniatynski, D. Schluter, and J. L. Gow.
581   2006. Speciation in reverse: morphological and genetic evidence of the collapse of a three-
582   spined stickleback (Gasterosteus aculeatus) species pair. Mol. Ecol. 15:343–55.

583

584 Taylor, S. A., T. A. White, W. M. Hochachka, V. Ferretti, R. L. Curry, and I. Lovette. 2014a.
585   Climate-Mediated Movement of an Avian Hybrid Zone. Curr. Biol. 24:671–676.

586

587 Taylor, S. A., R. L. Curry, T. A. White, V. Ferretti, and I. Lovette. 2014b. Spatiotemporally
588   consistent genomic signatures of reproductive isolation in a moving hybrid zone. Evolution
589   68:3066–3081.

590

591 Trier, C. N., J. S. Hermansen, G.-P. Sætre, and R. I. Bailey. 2014. Evidence for mito-nuclear and
592   sex-linked reproductive barriers between the hybrid Italian sparrow and its parent species.
593   PLoS Genet. 10: e1004075.

594

595 Turelli, M., and H. A. Orr. 1995. The dominance theory of HALDANE's rule. Genetics 140:
596   389–402.

597

598 Turissini, D. A., A. A. Comeault, G. Liu, Y. C. G. Lee, and D. R. Matute. 2017. The ability of
599   Drosophila hybrids to locate food declines with parental divergence. Evolution 71:960–973.

600

601 Vander Wall, S. B. 1990. Food hoarding in animals. The University of Chicago Press, Chicago,
602   IL.

603

604 Verzijden, M. N., R. E. M. Korthof, and C. Ten Cate. 2008. Females learn from mothers and
605   males learn from others. The effect of mother and siblings on the development of female
606   mate preferences and male aggression biases in Lake Victoria cichlids, genus Mbipia. Behav.
607   Ecol. Sociobiol. 62:1359–1368.

608

609 Verzijden, M. N., C. Cate, M. R. Servedio, G. M. Kozak, J. W. Boughman, and E. I. Svensson.
610   2012. The impact of learning on sexual selection and speciation. Trends Ecol. Evol. 27:511–
611   519.

612

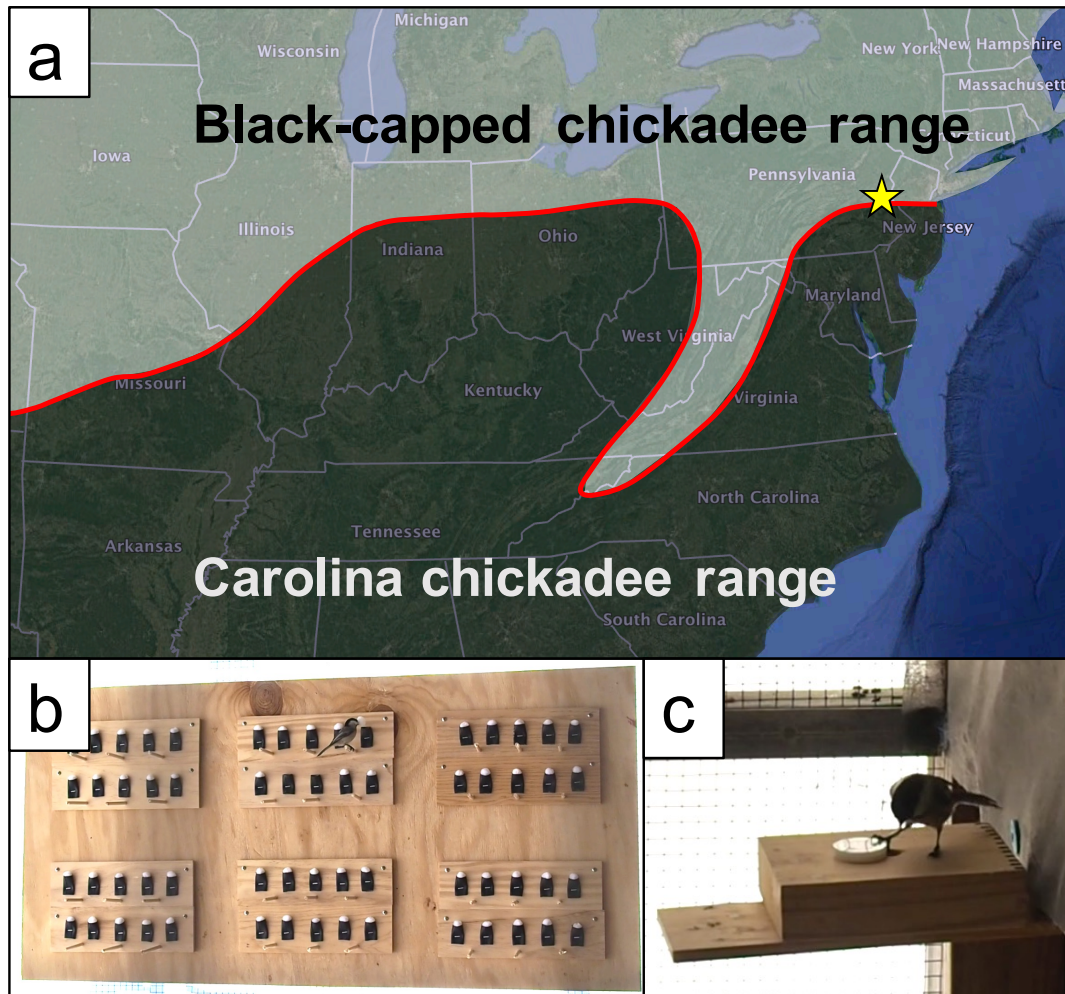613　**Figures**

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615　**Figure 1**. (A) Approximate location of black-capped and Carolina chickadee hybrid zone shown in red. Sampling

616　location for this study in eastern Pennsylvania is shown as yellow star. (B) Chickadee interacting with 'caching

617　array' used in associative learning spatial task. (C) Chickadee performing novel problem-solving test.
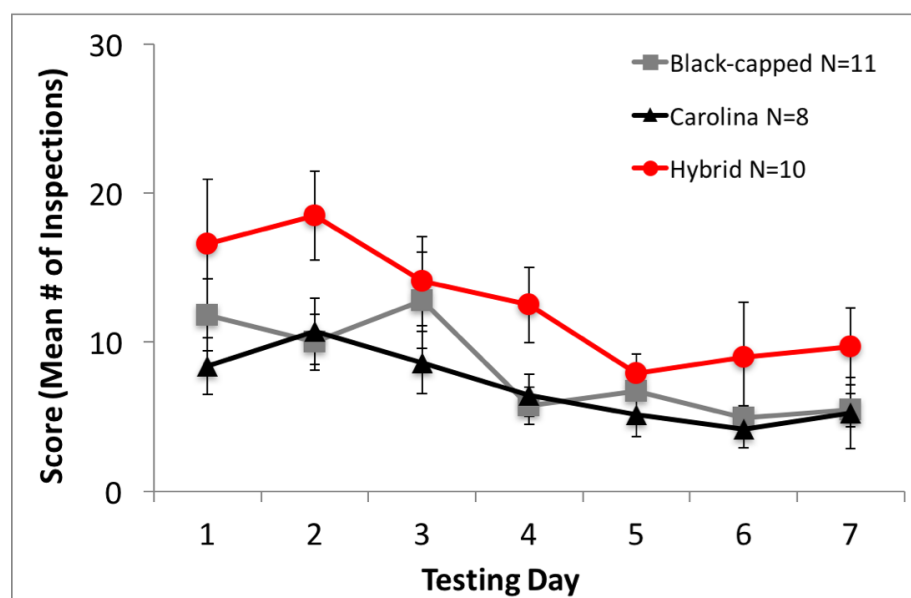
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620 **Figure 2**. Associative learning spatial task results, showing performance over the testing period for the three

621 ancestry groups. Hybrid chickadees require significantly more inspections, on average, than both pure-species
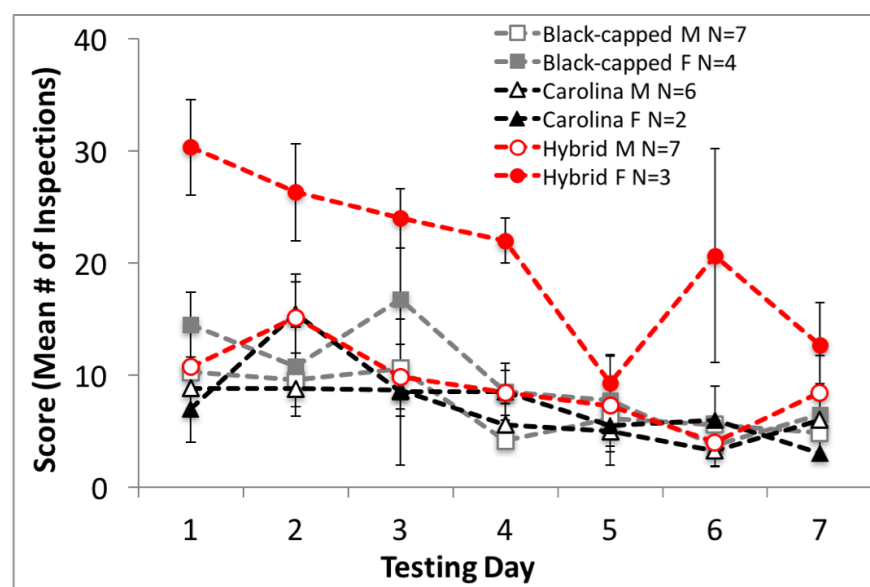
622 groups. Data points denote mean ± SEM.

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625 **Figure 3**. Performance on the associative learning spatial task split by ancestry and sex. Hybrid females are the

626 worst performers on associative learning spatial task (red dashed line, closed circles). Data points denote mean ±
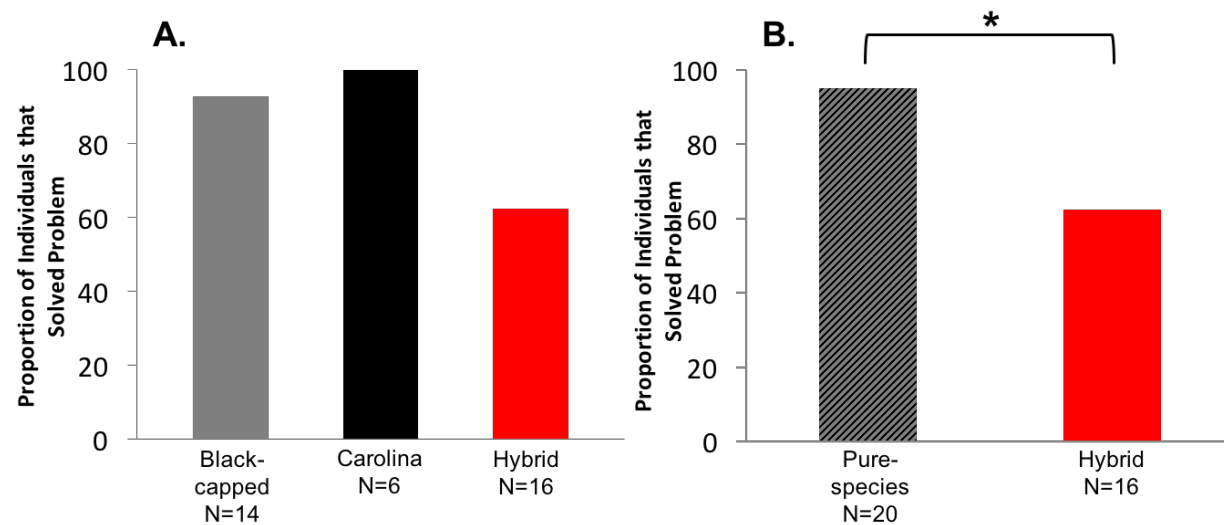
627 SEM.

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630 **Figure 4.** Novel problem-solving results. (A) Proportion of individuals from each ancestry group that were able to

631 solve the problem at least once (of two total trials). Hybrids show a non-significant trend towards being less likely to

632 solve the problem (Fisher's exact test, p=0.054). (B) Hybrids are significantly less likely to solve the problem

633 compared to a combined pure-species group (Fisher's exact test, p=0.0298).

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