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Elevated Heterozygosity in Adults Relative to Juveniles Provides Evidence of Viability Selection on Eagles and Falcons

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Abstract

Viability selection yields adult populations that are more genetically variable than those of juveniles, producing a positive correlation between heterozygosity and survival. Viability selection could be the result of decreased heterozygosity across many loci in inbred individuals and a subsequent decrease in survivorship resulting from the expression of the deleterious alleles. Alternatively, locus-specific differences in genetic variability between adults and juveniles may be driven by forms of balancing selection, including heterozygote advantage, frequency-dependent selection, or selection across temporal and spatial scales. We use a pooled-sequencing approach to compare genome-wide and locus-specific genetic variability between 74 golden eagle (*Aquila chrysaetos*), 62 imperial eagle (*Aquila heliaca*), and 69 prairie falcon (*Falco mexicanus*) juveniles and adults. Although genome-wide genetic variability is comparable between juvenile and adult golden eagles and prairie falcons, imperial eagle adults are significantly more heterozygous than juveniles. This evidence of viability selection may stem from a relatively smaller imperial eagle effective population size and potentially greater genetic load. We additionally identify ~2000 single-nucleotide polymorphisms across the 3 species with extreme differences in heterozygosity

between juveniles and adults. Many of these markers are associated with genes implicated in immune function or olfaction. These loci represent potential targets for studies of how heterozygote advantage, frequency-dependent selection, and selection over spatial and temporal scales influence survivorship in avian species. Overall, our genome-wide data extend previous studies that used allozyme or microsatellite markers and indicate that viability selection may be a more common evolutionary phenomenon than often appreciated.

Keywords: balancing selection, heterozygote advantage, immune function, olfaction, pooled sequencing, raptors

Genetic variability can confer a positive effect on survivorship through either genome-wide or locus-specific effects. Genome-wide heterozygosity may act as a proxy for an individual's inbreeding coefficient, meaning that decreased heterozygosity would indicate the expression of deleterious, recessive alleles and an associated fitness cost (Hansson and Westerberg 2002; Balloux et al. 2004). Alternatively, genetic variation associated with specific loci may influence fitness (i.e., heterozygote advantage; Frydenberg 1963; Mitton 1997; Lynch and Walsh 1998; Hansson and Westerberg 2002). One way of testing for a relationship between genetic variability and survivorship is by identifying evidence of viability selection. Viability selection occurs when homozygosity has a negative effect on survival to adulthood, resulting in a more heterozygous adult population relative to juveniles (Clegg and Allard 1973; Ledig et al. 1983; Cohas et al. 2009; Lampila et al. 2011). For example, Cohas et al. (2009) genotyped alpine marmots (*Marmota marmota*) at 16 microsatellite markers and determined that adults were more heterozygous than juveniles. The authors used these data to argue that a 10% increase in heterozygosity was synonymous with a 13% increased likelihood of survival to adulthood by juveniles (i.e., there was evidence of heterozygote advantage).

Genetic variation can also be maintained within a population by forms of balancing selection other than heterozygote advantage. Negative frequency-dependent selection, for example, favors traits and their associated alleles when they are rare; decreasing the likelihood that more common alleles will become fixed within the population (Asmussen and Basnayake 1990; Browne and Karubian 2016; Ayala and Campbell 2018). Selection varying in space and time or between sexes and life stages can also maintain genetic diversity at a given locus (Hedrick 2012; Bergland et al. 2014).

Although data from allozymes and microsatellite genotypes can be associated with fitness (Chapman et al. 2009), high-density single-nucleotide polymorphisms (SNPs) should estimate genome-wide heterozygosity more accurately (DeWoody and DeWoody 2005; Miller et al. 2014a), enhancing our ability to detect the relationship between variability and fitness. A genome-wide approach considers thousands of SNPs across the genomes (as opposed to tens of conventional markers), so the probability of identifying a locus linked to a fitness-influencing gene is significantly increased (although, so too is the risk of false positives). Pooled sequencing, in particular, has been used to identify the genetic underpinnings of polymorphic phenotypes within species, including the right and left asymmetric foraging behavior in scale-eating fish (Raffini et al. 2017) and color morphs in birds and butterflies (Neethiraj et al. 2017). It has also been used to elucidate how species adapt to new habitats (e.g., adaptations associated with osmoregulation and metabolism in steelhead trout introduced to a freshwater environment; Willoughby et al. 2018) and to identify genomic regions in copepods that contribute to hybrid inviability and reproductive isolating barriers (Lima and Willett 2018). Perhaps most relevant to this study, pooled sequencing has been used to identify signatures of balancing selection, namely in the forms of SNPs that oscillate in frequency over time or trans-species polymorphisms (Bergland et al. 2014).

We used pooled sequencing of raptor genomes to systematically assess the extent of viability selection within 2 related but distant avian lineages (Aquila within Accipitridae and Falco within Falconidae; Prum et al. 2015). We chose these species because of preliminary evidence of viability selection at 162 golden eagle SNPs and because of genetic continuity (i.e., lack of structure) across sampling locations (Rudnick et al. 2008; Doyle et al. 2016; Doyle et al. 2018). In this study, we extend previous marker-based work on viability selection to genome-wide sequencing. We begin with a modeling exercise to provide context; exploring how starting allele frequencies and the strength of viability selection will interact to influence the difference in heterozygosity between cohorts. We then generate empirical data sets in golden eagles (*Aquila chrysaetos*), imperial eagles (*Aquila heliaca*), and prairie falcons (*Falco mexicanus*). Subsequently, we use our sequencing data to test for differences in genome-wide heterozygosity between golden eagle, imperial eagle, and prairie falcon nestlings and adults. Finally, we identify specific SNPs with extreme differences in heterozygosity between juveniles and adults, where variation is likely maintained by viability selection.

Methods

Expected Heterozygosity Differences Between Age Cohorts

To explore how viability selection might influence expected allele frequencies and under which conditions viability selection is detectable (specifically using a pooled-sequencing approach, see below), we construct a forward-time, agent-based model. Our model was designed to simulate changes in heterozygosity under viability selection by heterozygote advantage on a single gene over a single reproductive event. It was initiated with 1000 adults that were randomly assigned sex and genotypes at a single, biallelic locus with alleles "A" and "B." The initial allele frequency of the "A" allele was varied across runs (ranged from 0.00 to 0.95), but genotypes were assigned such that they were consistent with Hardy–Weinberg Equilibrium. After initialization, adults were randomly mated with replacement, and each pair produced a single offspring until the number of offspring was equal to the number of adults. Nestling genotypes were determined from the parental genotypes following Mendelian inheritance patterns. Finally, we applied a viability selection function to our population of nestlings following one of 2 patterns in any

particular run. We simulated viability selection by heterozygote advantage by randomly removing homozygous nestlings (AA and BB) with a probability that we varied from 0.05 to 0.95. We additionally modeled directional selection to determine how differences in heterozygosity between cohorts might compare to that of the heterozygote advantage scenario. Directional selection on the initial cohort might best mimic other forms of balancing selection acting on a single generation of individuals (e.g., selection varying in time; Hedrick 2012; Bergland et al. 2014). We simulated directional selection by removing nestlings with one homozygous genotype (AA), as well as heterozygous nestlings (AB), with a probability that was again varied from 0.05 to 0.95 in any particular run. To assess the effects of the viability selection protocols, we computed the difference in expected heterozygosity (estimated from allele frequencies as is done with pooled-sequencing data) in the final nestling cohort compared to initial cohort of adults.

Field Sampling

Blood and tissue samples were collected throughout the course of several long-term monitoring programs (see Table 1 and Rudnick et al. 2005; Katzner et al. 2015; Doyle et al. 2016; Doyle et al. 2018 for additional detail). Briefly, golden eagle and prairie falcon adults were sampled following capture with nets (Doyle et al. 2014) or a mortality event (Katzner et al. 2015). Adult prairie flacons were captured with dho-gaza nets using a live, non-releasable great horned owl (Bubo virginianus) as a decoy (Bloom et al. 2007). Golden eagle, imperial eagle, and prairie falcon nestlings were sampled directly in the nest prior to fledging. We collected blood samples by venipuncture of the brachial vein (Doyle et al. 2014) and muscle samples directly from carcasses (Katzner et al. 2015). We noninvasively sampled adult imperial eagles by collecting naturally molted feathers from beneath nests (Rudnick et al. 2005).

Whole-Genome Sequencing and Annotation

Bioinformatic pipelines appropriate to this study require both 1) paired-end (PE) reads sequenced from pools of DNA extracted from multiple individuals and 2) reference genomes to which pooled-sequencing PE reads can be mapped (Schlötterer et al. 2014). Golden eagle and prairie falcon genome assemblies (made up of scaffolds 10 kb and greater in size) were downloaded from GenBank (Accession: GCA_000766835.1; Van Den Bussche et al. 2017) and Dryad (doi:10.5061/dryad.8b0s04t; Doyle et al. 2018). To generate an imperial eagle genome assembly, we conducted one lane of PE sequencing and one lane of mate-paired (MP) sequencing using an Illumina HiSeq2500 that produced read lengths of 100 bp. We used Trimmomatic 0.35 (Bolger et al. 2014) to remove adaptors and discard low-quality bases as in Doyle et al. (2018). We then used ABySS 1.5.2 (Simpson et al. 2009) to conduct several preliminary assemblies of PE and MP reads, using k-mer lengths ranging from 41 to 61. MP reads were used only during the scaffolding step and a minimum of 10 pairs of reads were required to join 2 contigs. We determined that a k-mer length of 61 produced the best assembly by considering both N50 values and the length of the longest scaffold.

Golden eagle and imperial eagle scaffolds greater than 10 kb were annotated using the MAKER 2.31.9 pipeline (Cantarel et al. 2008), following Doyle et al. (2014) and Doyle et al. (2018). We used Repeat-Masker 4.0.7 to identify and mask stretches of repetitive DNA, BLAST 2.3.0 to align avian expressed sequence tags (ESTs) and proteins to the genome and SNAP 0.15.4 (Korf 2004) to generate ab initio gene predictions, while InterProScan 5.25–64.0 was used to identify putative protein domains. Prairie falcon gene annotations were downloaded from Dryad as described above (Doyle et al. 2018). InterProScan 5.25–64.0 was additionally used to assign gene ontology (GO) terms to all genes.

Pooled Sequencing

Previous work on golden eagles sampled in California, USA, imperial eagles in Kostanay Olbast, Kazakhstan, and prairie falcons in California indicate that samples are each from a single population (i.e., there is genetic homogeneity across the sampling areas; Rudnick et al. 2008; Doyle et al. 2016; Doyle et al. 2018). We extracted DNA from blood, bone, and muscle samples collected from 62–74 golden eagles, imperial eagles, and prairie falcons in total (Table 1), again using an ammonium acetate protocol (Rudnick et al. 2005). In almost all cases, DNA was extracted from a single sample from a known individual. In cases where multiple sibling chicks were present at a nest, we extracted DNA from only one of the related individuals. Adult imperial eagle DNA, however, was extracted from the tips of feathers collected from beneath nests (Rudnick et al. 2007). We used DNA from a single feather from a male and a single feather from a female collected from below each nest, as only a single breeding pair is ever found in imperial eagle territories (Rudnick et al. 2005). We used Fridolfsson and Ellegren's (1999) method to assign sex to each sample using molecular markers. Samples sizes were roughly equal for male nestlings, female nestlings, male adults, and female adults of each species (Supplementary File 1). We quantified DNA concentration using a Qubit fluorometer (Life Technologies) and pooled equal amounts of DNA from each individual of each species × cohort (nestling or adult) × sex group (male or female). Each of these 12 pools were used to prepare a PoolSeq library following the Illumina TruSeq DNA PCR-Free Sample Preparation Guide (Illumina Inc., San Diego, CA). Covaris AFA® Technology was used to generate ~400 bp insert sizes. The 4 libraries associated with each species (i.e., adult male, adult female, juvenile male, and juvenile female) were sequenced together on a single lane using an Illumina HiSeq2500 (i.e., we performed 3 lanes of pooled sequencing in total).

Table 1. Locations, years during which sampling occurred, and sample type associated with DNA pooled for adult male, adult female, juvenile male, and juvenile female raptors

Species	Sampling location	Sex/cohort	Years	Sample type	Individuals per pool
Golden eagle	California, USA	Adult	2012–2016	Blood, bone, feather, and muscle	38
		Juvenile	2004–2016	Blood, growing feathers, and muscle	36
Imperial eagle	Kostanay Olbast, Kazakhstan	Adult	2006–2007	Feathers	34
		Juvenile	2006–2007	Growing feathers	28
Prairie falcon	California, USA	Adult	2002–2008	Blood	27
		Juvenile	2006–2014	Blood	42

Estimation of Genome-Wide and Locus-Specific Differences in Expected Heterozygosity

We used Trimmomatic 0.35 (Bolger et al. 2014) to remove adaptors and discard low-quality bases from pooled-sequencing reads. Reads were scanned using a 4-bp window and cut whenever the average phred quality score dropped below 20. Reads less than 40 bases long were subsequently discarded (adapted from Kofler et al. 2011a; Delmore et al. 2015). High-quality reads were mapped to the relevant genome using BWA 0.7.13 (Li and Durbin 2009). We subsequently used Picard to merge male and female reads within each life-stage group (i.e., nestling or adult) to increase coverage as well as remove duplicate reads. Samtools (Li et al. 2009) was used to remove ambiguously mapped reads (e.g., reads with mapping quality below 20) and generate an mpileup file. PoPoolation2 (Kofler et al. 2011b) was used to filter indels, identify SNPs, and calculate allele frequencies. When calculating allele frequencies, we discarded SNPs with a minor allele frequency below 2, a minimum coverage below 15 for either cohort, or a maximum coverage greater than 99 (Kofler et al. 2011a).

We compared genome-wide estimates of expected heterozygosity in adults and nestlings using a resampling procedure that was stratified across scaffolds to reduce the effects of linkage on our estimates. For each species, we identified a random subset of 80% of all scaffolds and then randomly selected a single SNP from each of these scaffolds. We then calculated the genomic difference in expected heterozygosity in adults and nestlings by averaging across the selected SNPs. After repeating this procedure 1000 times, we used the distribution of expected heterozygosity differences to estimate a mean genomic difference in expected heterozygosity in adults compared to nestlings, as well as the 95% confidence interval (CI) around the mean.

To evaluate whether the resulting estimate was an artifact of low coverage or differences in read depth between juvenile and adult pools, we repeated our analyses with downsampled reads. We used the "target-coverage" option provided by PoPoolation2 to 1) generate a subset of SNPs with coverage of at least 22 for both cohorts and 2) reduce coverage to 22 for each SNP by randomly sampling bases. We then once again used the resampling procedure described above to compare genome-wide estimates of expected heterozygosity in adults and nestlings.

We used custom bash scripts to calculate expected heterozygosity at each locus for nestlings and adults and calculate the absolute difference in heterozygosity between nestlings and adults at each locus. We subsequently z-transformed the distribution of the absolute differences in heterozygosity (autoscaling the mean and standard deviation [SD]) and extracted outliers at the tail of the distribution (i.e., 3 SDs from the mean; Axelsson et al. 2013). We used BEDtools (Quinlan and Hall 2010) to identify genes associated with the golden eagle, imperial eagle, and prairie falcon outlier SNPs. We used topGo (Alexa et al. 2006) to test for gene enrichment in the genes with outlier SNPs relative to all genes present in the genome. More specifically, we used the "weight01" algorithm to account for the gene ontology hierarchy and reduce false positives (Alexa et al. 2006). Finally, OrthoMCL (Li et al. 2003) was used to identify which of the golden eagle, imperial eagle, and prairie falcon genes associated with outlier SNPs represent orthologs. These genes are similar in sequence due to common ancestry and presumably would retain similar function(s).

We additionally used BEDtools to identify all polymorphic SNPs associated with genes (including exons, introns, and 5' and 3' untranslated regions [UTRs] if included in the annotation). For any genes with at least 8 polymorphic SNPs, we calculated average absolute differences in heterozygosity (between nestlings and adults) using custom bash and R scripts. We z-transformed the distribution of the average absolute differences in heterozygosity and again extracted outliers at the tail of the distribution. OrthoMCL (Li et al. 2003) was once again used to identify which of these genes represent orthologs in the golden eagle, imperial eagle, and prairie falcon genomes.

Additional Evidence of Selection

To determine which of our outlier SNPs represent the best targets for future analyses of viability selection, we considered 2 additional forms of evidence of selection. First, we identified polymorphisms by sequence in golden and imperial eagles to help determine if loci under viability selection might also represent trans-species polymorphisms (although we note that confirmation would require additional phylogenetic analyses; Ejsmond et al. 2018). Trans-species polymorphisms are typically indicative of balancing selection that has maintained genetic variation across species boundaries, sometimes for tens of millions of years (Klein et al. 1998; Leffler et al. 2013; Bergland et al. 2014; Martin and Vinkler 2015). We mapped all reads from golden and imperial eagles to the golden eagle genome. We subsequently followed the pipeline described above, with the exception of requiring a minimum coverage of 10 when calculating allele frequencies. We used custom bash scripts to extract loci with the same polymorphism by sequence (e.g., C to T polymorphism occurred in both species) and a minimum minor allele count of 4 in each species. We used a minor allele count of 4 to account for a potential increase in sequencing errors associated with increased coverage when reads from each species are aligned as a single pool. We identified polymorphisms by sequence that were also identified as outlier SNPs and used BEDtools (Quinlan and Hall 2010) to identify genes associated to these markers.

Next, we additionally used PoPoolation (Kofler et al. 2011a) to calculate Tajima's *D* (Tajima 1989) over nonoverlapping 20- and 100-kb windows across the golden eagle genome, once again using merged juvenile and adult pooled reads. We chose the golden eagle genome for this initial analysis as it is the most contiguous of the 3 raptor genomes (Supplementary File 2), allowing us to consider a relatively greater number of 20- and 100-kb windows. When calculating Tajima's *D*, we required a minor allele count of at least 2 to include a polymorphic site. Windows with <50% coverage of at least 15 reads and fewer than 99 reads per site were discarded. An excess of high-frequency polymorphisms results in a positive Tajima's *D* value and suggests balancing selection (although recent bottlenecks can also result in positive Tajima's *D* values; Tajima 1989). We again z-transformed the distribution Tajima's *D* values and extracted positive outliers at the tail of the distribution. We used BEDtools to identify outlier SNPs and genes that overlapped with these windows.

Results

Expected Heterozygosity Differences Between Age Cohorts

We used simulations to understand how initial allele frequency and intensity of viability selection would impact our ability to detect changes in heterozygosity between adults and nestlings. We found that, under conditions of both heterozygote advantage and

directional selection, detecting viability selection when allele frequencies at a biallelic locus are similar is very difficult. Under heterozygote advantage conditions, even a mortality probability of 0.95 in homozygous individuals did not result in large heterozygosity changes (Figure 1A). Similarly, mortality probabilities less than 0.85 in directional selection conditions resulted in very small (i.e., near 0) changes in heterozygosity (Figure 1B). However, when mortality probability neared 0.95, heterozygosity changes of up to 0.4 occurred, suggesting that extreme selection events under directional selection conditions would be detectable even when allele frequencies are similar. When initial allele frequencies between the "A" and "B" allele were not similar (i.e., either "A" or "B" is rare), we found that heterozygosity changes >0.20 should be expected when at least 50% of homozygous nestlings are removed under both heterozygote

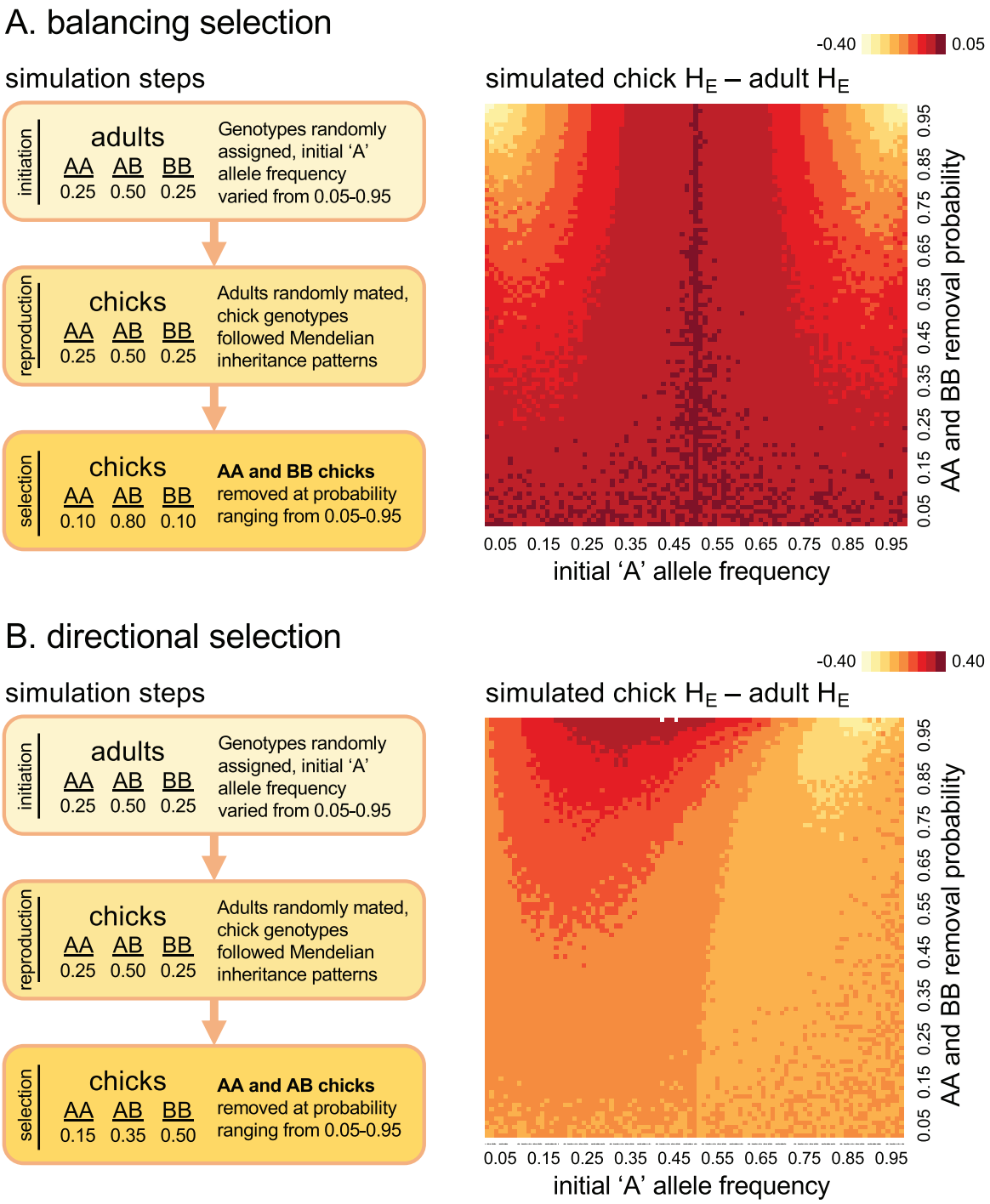

Figure 1. Diagrammatic outline of viability selection simulation and simulated change in heterozygosity due to viability selection. We simulated the effects of (**A**) balancing selection and (**B**) directional selection conditions when considering how viability selection effects heterozygosity of nestlings compared to adults. Under both conditions, the heatmap displays the range of heterozygosity differences we observed while varying initial allele frequencies and the strength of viability selection. Note that the scale for heterozygosity differences between nestlings and adults is different in the 2 heatmaps.

advantage and directional selection situations (Figure 1). Overall, our simulations suggest that viability selection by heterozygote advantage (balancing selection) results in no change or increased expected heterozygosity in the postselection nestlings when compared to the initial adult cohort. However, a large decrease in nestling heterozygosity is expected under directional selection conditions when initial allele frequency of the unfavorable allele (i.e., the allele selected against) is small. In contrast, an increase in postselection nestling heterozygosity is expected under directional selection conditions when the unfavorable allele is initially common.

Whole-Genome Sequencing and Assembly of the Imperial Eagle

We generated 102 Gb of raw sequence data from the single imperial eagle, including 46.0 Gb (455 318 054 total reads) from the PE library and 56.2 Gb (556 638 802 total reads) from the MP library. Following quality control measures, 45.5 Gb (452 742 882 total reads) and 34.2 Gb (385 035 378 total reads) were available for assembly. Our draft imperial eagle nuclear genome assembly includes 7813 scaffolds greater than 2000 bp. These scaffolds had an N50 of 2331 kb and the longest scaffold was 11 630 kb in length. All 3 raptors genomes have between 470 and 2648 scaffolds greater than 10 kb in length, 16 003 to 16 320 annotated genes, and N50 values between 2391 and 9231 kb (Supplementary File 2).

Estimation of Genome-Wide and Locus-Specific Differences in Expected Heterozygosity

Pooled sequencing produced ~142 Gb (1 401 074 154 total reads) of raw data (Supplementary File 2). Following quality control measures (i.e., trimming and culling), we retained 48.8 Gb (484 737 712 total reads), 50 Gb (500 622 232 total reads) and 38.8 Gb (380 011 982 total reads) of sequence data for golden eagles, imperials, and prairie falcons, respectively (Supplementary Files 3 and 4). We identified >267 000 SNPs in both nestlings and adults for each of the 3 species (golden eagles, imperial eagles, and prairie falcons; Supplementary File 5).

Genome-wide heterozygosity was lowest for imperial eagles and highest for prairie falcons (Figure 2; Supplementary File 5). Our resampling procedure (stratified across scaffolds) indicated that genome-wide expected heterozygosity did not differ between nestlings and adults in golden eagles or prairie falcons (i.e., the CI associated with the distribution of expected heterozygosity differences between adults and nestlings overlapped 0). The mean difference in genome-wide heterozygosity for golden eagles and prairie falcons was 0.0003 (95% CI = −0.0135, 0.0144) and −0.0027 (95% CI = −0.0097, 0.0043), respectively.

We found a small but statistically significant difference in genome-wide expected heterozygosity between nestlings and adults of imperial eagles (i.e., the CI associated with the distribution of expected heterozygosity differences between adults and nestlings did not overlap 0). We identified 267 109 SNPs with a minimum coverage of 15 in both nestlings and adults. To generate a mean difference in genome-wide heterozygosity between cohorts and CIs, a single SNP was subsampled from each of 1759 scaffolds before the mean difference in genome-wide heterozygosity was calculated. In addition, we applied a randomization process where we selected 80% of the scaffolds and repeated this process 1000 times to generate 95% CIs. Adults were 1.5–3.2% more heterozygous genome-wide than nestlings (i.e., mean difference of 0.0233, 95% CI = 0.0154, 0.0318; Figure 2).

To evaluate whether this result was an artifact of low coverage or differences in read depth between juvenile and adult pools, we repeated our analyses with reads downsampled to 22. Downsampling to a coverage of 22 reduces the number of SNPs considered from 267 109 to 56 823 (distributed on 784 scaffolds). To generate a mean difference in genome-wide heterozygosity between cohorts and CIs, a single SNP was subsampled from each of the 784 scaffolds before the mean difference in genome-wide heterozygosity was calculated. In addition, we applied a randomization process where we selected 80% of the scaffolds and repeated this process 1000 times to generate 95% CIs. We again found a small but statistically significant difference in genome-wide expected heterozygosity between imperial eagle cohorts (i.e., a mean difference of 0.0142, 95% CI = 0.0034, 0.0246).

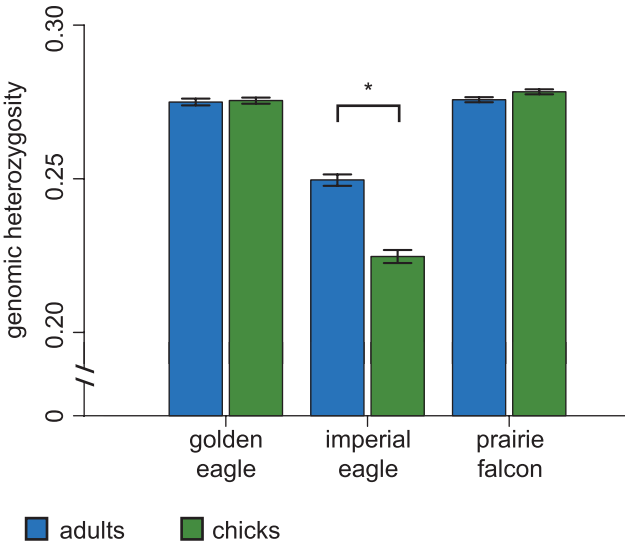

Figure 2. Average heterozygosity in golden eagle, imperial eagle, and prairie falcon nestlings and adults. We compared genome-wide estimates of expected heterozygosity in adults and nestlings using a resampling procedure that was stratified across scaffolds to reduce the effects of linkage on our estimates. For each species, we identified a random subset of 80% of all scaffolds, randomly selected a single SNP from each of these scaffolds, and calculated average heterozygosity. This process was repeated 1000 times to estimate a 95% CI around the mean.

The number of outlier SNPs (those with absolute differences in expected heterozygosity >3 SDs from the mean) ranged from 356 in imperial eagles to 1282 in golden eagles (Supplementary File 5). Genome assembly quality and number of alignments (Supplementary File 5) may have influenced the number of outlier SNPs identified in each species. In golden eagles, the 1282 outlier SNPs were associated with 276 genes, whereas 66 outlier SNPs were associated with just 23 genes in imperial eagles and 171 outlier SNPs were associated with 61 genes in prairie falcons. We identified a number of significantly enriched gene ontology terms in golden eagle, imperial eagle, and prairie falcon genes associated with outlier SNPs (Supplementary File 6). Significantly enriched terms found in 2 species include regulation of embryonic development, regulation of cell migration, regulation of cell adhesion and telomere maintenance (in both golden eagles and prairie falcons) and presynaptic active zone, and structural constituent of cytoskeleton (in both imperial eagles and prairie falcons; Supplementary File 6). OrthoMCL identified 3 instances in which the same gene (i.e., ortholog) associated with an outlier SNP was found in 2 species. Orthologs of protein unc-13 homologue C (UNC13C) and AP-3 complex subunit delta-1 (AP3D1) were found

in the golden eagle and prairie falcon. Orthologs of ERC protein 2 (ERC2) were found in the imperial eagle and prairie falcon.

We identified 60 golden eagle genes with high average absolute differences in heterozygosity between nestlings and adults (i.e., 3 SDs from the z-transformed mean). We identified 9 and 16 such genes in imperial eagles and prairie falcons, respectively (Supplementary File 7). The OrthoMCL pipeline identified no genes common among these 3 data sets.

Additional Evidence of Selection

After mapping both imperial and golden eagle reads to the golden eagle genome (Supplementary File 8), we identified 225 481 SNPs polymorphic by sequence in both species. Of the 1282 golden eagle outlier SNPs, 173 were also polymorphic by sequence in imperial eagles. Genes associated with these SNPs are reported in Supplementary File 9.

We traversed 58 785 20-kb windows across the golden eagle genome and identified 157 with positive Tajima's *D* values that represented statistical outliers (i.e., greater than 3 SDs from the mean). We considered 11 791 100-kb windows and identified 40 outliers with positive Tajima's *D* values. In total, 65 unique genes overlapped with the 20-kb outlier windows, while 33 unique genes overlapped with the 100-kb windows. In both instances, however, some genes overlapped with 2 adjoining windows. Of the 1282 golden eagle outlier SNPs, 28 and 32 overlap with the 20- and 100-kb windows, respectively. These SNPs are associated with orthologs of 3 genes: CEP250-like (centrosome-associated protein) in a 20-kb window and KIAA0020 (minor histocompatibility antigen HLA-HA8) and NEO1 (neogenin 1) in 100-kb windows (Figure 3).

Discussion

Expected Allele Frequency Differences Between Cohorts

Our analyses illustrate that expected allele frequencies generated with pooled sequencing can provide evidence of viability selection. This evidence comes from the whole-genome estimates of heterozygosity (in imperial eagles) and from locus-specific differences (in all 3 species). These findings have implications for our understanding of how viability selection may work in natural populations, but it is important to consider how interpretation of our Pool-seq results differs from that of traditional studies. Previously described studies (e.g., Cohas et al. 2009; Lampila et al. 2011; Doyle et al. 2016) genotyped individuals separately, allowing for comparisons of observed heterozygosity between juveniles and adults. As pooled sequencing produces allele frequency estimates for entire groups and not individual genotypes, only estimates of expected heterozygosity are possible. Our models indicate that expected heterozygosity increases in postselection cohorts when initial allele frequencies are skewed and there is a survival disadvantage associated with homozygosity (i.e., heterozygote advantage). However, when initial allele frequencies are similar, increased survivorship of heterozygous individuals might go undetected by our method. We note that "directional selection" scenarios can also produce elevated expected heterozygosity in postselection cohorts, although not to the extent seen when mortality rates are elevated in both homozygote genotypes. Hence, by targeting loci with differences in expected heterozygosity between cohorts that represent outliers in the distribution (i.e., greater than 3 SDs from the z-transformed mean and with absolute differences in expected heterozygosity greater than 0.32), we target loci for which there is evidence of viability selection.

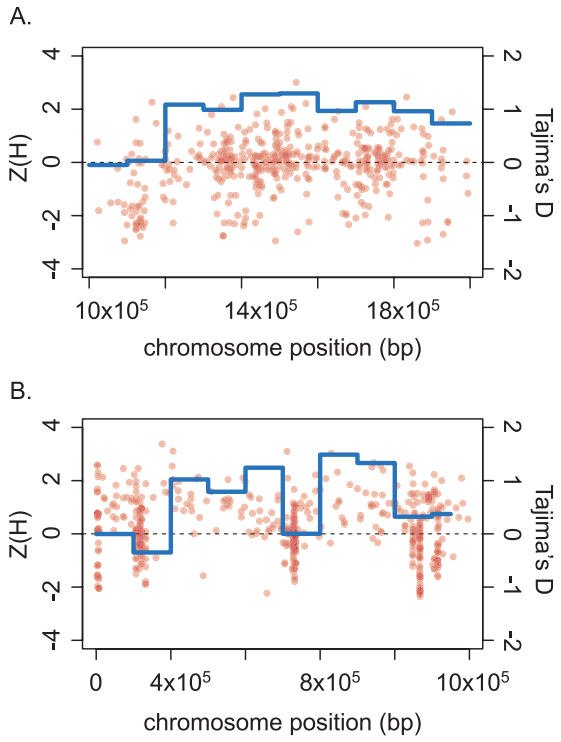

Figure 3. Absolute difference in H_E between adult and juvenile golden eagles at SNPs plotted across 1-Mb windows on scaffolds (**A**) 011950949 and (**B**) 011950872. The line indicates Tajima's *D* averaged over 100-kb windows. Each point indicates the z-transformed difference in expected heterozygosity between nestlings and adults at a given locus. Outlier SNP and Tajima's *D* windows are associated with an orthologs of KIAA0020 (minor histocompatibility antigen HLA-HA8) and NEO1 (neogenin 1) along scaffolds 011950949 (A) and 011950872 (B), respectively.

Genome-Wide Differences in Expected Heterozygosity

We found a small but statistically significant increase in genome-wide heterozygosity in adult imperial eagles relative to juveniles but did not detect this trend in the other 2 species. Reduced fitness may be associated with genome-wide heterozygosity if a population suffers from inbreeding depression (Hansson and Westerberg 2002; Balloux et al. 2004). More specifically, homozygous juveniles born into the population might suffer from more deleterious recessive alleles and have reduced chances of surviving to adulthood, resulting in increased genetic variability in the adult population relative to the juvenile population. However, previous work has not identified evidence of inbreeding or inbreeding depression in the Kostanay Olbast imperial eagle population. Rudnick et al. (2005) found that mated individuals were no more closely related than would be expected at random. Furthermore, there was no relationship between pair relatedness and clutch size (Rudnick et al. 2005).

It is worth noting, however, that the imperial eagle is threatened by illegal poisoning, electrocution, food shortages, habitat loss, and disturbance (Horvath 2009; Karyakin et al. 2009; Horváth et al. 2011; Karyakin 2011). The International Union for Conservation of Nature considers the imperial eagle "vulnerable" and estimates the global population size throughout Eurasia is between 2500 and 9999 individuals (http://www.birdlife.org; BirdLife International 2019). In contrast, the Partners in Flight Science committee estimates there are 130 000 golden eagles globally and 110 000 prairie falcons present throughout their range in western North America

(http://pif.birdconservancy.org; PopEstimates, 2019). A smaller census population size suggests a smaller effective population size (perhaps 11–15% the size of the census population size; Frankham 1995; Palstra and Ruzzante 2008). Over multiple generations, we would expect genetic drift to reduce heterozygosity and increase inbreeding in imperial eagles at a faster rate than in golden eagles and prairie falcons (Freeland et al. 2011; Frankham et al. 2015). Furthermore, if the associated genetic load is higher in imperial eagles, we might expect that viability selection would be more pronounced because deleterious mutations at any given locus may not be purged as effectively as in larger populations of golden eagles and prairie falcons. Our results suggest that decreased genetic variability in imperial eagles contributes to reduced fitness.

Our conclusions are dependent on accurate allele frequency estimates. Studies such as Gautier et al. (2013) and Schlötterer et al. (2014) have indicated that pooling 40–100 individuals and sequencing to coverage of 100X will result in allele frequency estimates more accurate than when estimated with whole-genome sequencing of barcoded individuals. Although our approach did not meet these benchmarks, our downstream analyses (i.e., resampling procedure stratified across scaffolds) indicate that our primary conclusion that genome-wide heterozygosity differs amongst juvenile and adult cohorts is statistically and biologically significant. Furthermore, our approach allows us to target imperial eagles and other species with small effective population sizes for subsequent studies of viability selection.

Locus-specific differences in expected heterozygosity

We identified over 2000 outlier SNPs with extreme differences in heterozygosity between juvenile and adult cohorts in golden eagles, imperial eagles, and prairie falcons. We subsequently identified genes associated with outlier SNPs that 1) also represented polymorphisms by sequence in golden and imperial eagles or 2) were present in 20- or 100-kb windows with positive Tajima's *D* values. We recognize that it is difficult to detect evidence of balancing selection (Kreitman and Di Rienzo 2004; Asthana et al. 2005; Fijarczyk and Babik 2015) and that not all of these outlier SNPs represent "true positives" (Pavlidis et al. 2012). However, these approaches allow us to leverage the whole-genome, comparative nature of our analyses of 3 raptor species representing 2 widely divergent clades to identify outlier loci that may be of particular interest in future studies of viability selection.

Many convincing examples of balancing selection center on immune genes (Andre et al. 2008; Hedrick 2012; Martin and Vinkler 2015). Genetic variability can underlie heterogeneity in immune function, allowing hosts to counteract varied and rapidly evolving pathogens. Several outlier SNPs serve as interesting targets for future studies of genetic variability and immune function, including those associated with members of the toll-like receptor family (e.g., TMED7, Liaunardy-Jopeace and Gay 2014; Liaunardy-Jopeace et al. 2015; CD180, Divanovic et al. 2005; Divanovic et al. 2007) and genes implicated in antioxidant defense (e.g., SEPP1, Saito et al. 1999; Foster et al. 2006; Papp et al. 2007). An outlier SNP that also represents a polymorphism by sequence is associated with a solute carrier gene SLC4A8. Andre et al. (2008) and Macmanes and Eisen (2014) found evidence of balancing selection on solute carrier genes in mammals. The membrane proteins encoded by these genes may control pathogen movement into cells and mediate the response to infection (Andre et al. 2008).

Several studies have identified olfactory receptor (OR) genes to be under balancing selection in mammals (Gilad et al. 2000; Alonso et al. 2008; Zhao et al. 2013), although similar evidence has not previously been identified in avian species (but see Zhan et al. 2013 for evidence of rapid functional evolution of olfaction genes in falcons). ORs interact with odiferous molecules and trigger the neuronal responses associated with "smell." Although, historically, olfactory ability was thought to be underdeveloped in avian species, recent work has shown that birds use olfaction to identify appropriate nesting materials (Clark and Mason 1987; Petit et al. 2002), navigate (Papi et al. 1972; Nevitt et al. 2004; Abolaffio et al. 2018), forage (Grigg et al. 2017; Avilés and Amo 2018), and detect predators (Amo et al. 2008; Mahr and Hoi 2018). Several lines of evidence indicate a signal of selection on raptor OR genes, particularly in the golden eagle. First, the GO category "olfactory receptor activity" was overrepresented in genes associated with golden eagle outlier SNPs. Second, a golden eagle gene that is part of the OR51 family (OR51H1) was identified as a gene with significantly increased heterozygosity in adults relative to juveniles across 8+ SNPs. Finally, a golden eagle gene that is part of the OR14 gene family contains an outlier SNP that also represents a putative trans-species polymorphism in imperial eagles. Taken together, these results indicate a signal of increased heterozygosity at OR genes that in some cases (e.g., our OR14J1-like ortholog) may influence survival to adulthood. The ecological relevance of selection on OR genes for golden eagles is particularly relevant, as these birds often consume carrion (Kochert et al. 2002) and other scavengers use olfaction to find food (Grigg et al. 2017).

Lancet (1994) proposed that variability at OR genes would be positively correlated with the number of different odorant-binding sites associated with an individual's genome, potentially influencing survival through improved prey detection or navigation. Alternatively, multiple OR genes often cluster in close proximity to major histocompatibility complex (MHC) genes in both mammalian and avian species (Younger et al. 2001; Santos et al. 2010; Miller et al. 2014b; Miller and Taylor 2016) and the signal of balancing selection may stem from linkage disequilibrium between alleles at OR genes and those at MHC genes (Jahromi 2012). Balancing selection on MHC genes has been documented extensively (Hedrick 1998; Klein et al. 1998; Garrigan and Hedrick 2003; Zhang et al. 2018; Minias et al. 2019), and MHC variability is associated with avian resistance to pathogens (Sepil et al. 2013; Jones et al. 2015; Aguilar et al. 2016).

Both genes referenced above (i.e., members of the OR14 and OR51 families) are indeed located in close proximity to other members of their gene family (e.g., the OR14J1-like gene is located on the same scaffold as OR14C36 and OR14A16 orthologs), although no members of the MHC are found on the relevant scaffolds. Although 3 golden eagle genome assemblies are currently available from NCBI, none are resolved to the level of complete chromosomes. The imperial eagle and prairie falcon genomes are similarly fragmented. Techniques such as nanopore sequencing and genome mapping should ultimately result in chromosome-level assemblies, allowing us to better understand how selection maintains variability at OR and MHC genes.

Viability Selection and Heterozygote Advantage

Empirical evidence for viability selection due to heterozygote advantage has historically been derived from a small handful of molecular markers, starting with only 3 allozyme loci in wild oats (Clegg and Allard 1973). In the case of functional allozymes like the esterase loci used in wild oats, viability selection could be due to direct effects of the assayed genes. However, individuals heterozygous at any given locus are more likely to be heterozygous at other loci in the genome (Clegg and Allard 1973) and presumed local effects have been observed in surveys of a few noncoding microsatellite loci (e.g., Hufford and

Hamrick 2003; Forcada and Hoffman 2014). Recently, Doyle et al. (2016) extended the empirical data from less than a dozen markers to 162 SNPs in the golden eagle and found elevated levels of heterozygosity in adults relative to juveniles. Nearly half of those SNPs were associated with genes with critical biological roles in other avian species (e.g., bone morphogenesis or immune function), so viability selection was consistent with their presumed functions. Herein, we broadened the survey of genetic variants to genome-wide SNPs. We did not find genome-wide evidence of viability selection in golden eagles—perhaps in part because our search was not focused on nonneutral variation as in Doyle et al. (2016)—but we did find evidence of a genome-wide heterozygote advantage consistent with viability selection in the imperial eagle. A relatively smaller effective population size may prevent imperial eagles from purging deleterious mutations at any given locus as effectively as golden eagles and prairie falcons.

Forcada and Hoffman (2014) found that breeding female Antarctic fur seals (*Arctocephalus gazella*) were significantly more heterozygous (as measured with 9 microsatellites) than nonbreeding counterparts, and this was due to viability selection as opposed to mate choice. Similarly, Hoffman et al. (2014) used a large panel of anonymous SNPs to find strong heterozygosity-fitness correlations (parasite infection status) in harbor seals (*Phoca vitulina*). Thus, the genomic evidence for viability selection is not restricted to birds. Overall, our data and the studies cited herein (e.g., Forcada and Hoffman 2014; Hoffman et al. 2014) suggest that the effects of viability selection are probably magnified in species with smaller population sizes that harbor a significant genetic load.

Conclusions

Herein, we considered genome-wide and locus-specific differences in expected heterozygosity in golden eagles, imperial eagles, and prairie falcons. We note that genome-wide heterozygosity is significantly greater in the adult imperial eagle population than in the juvenile population. Notably, of the 3 species considered, imperial eagles have the smallest global population size. We argue that homozygosity in this species reduces the fitness of individuals born into the population. We have identified a subset of gene-associated SNPs that may be of interest for future studies that investigate how heterozygote advantage, frequency-dependent selection, and selection over spatial and temporal scales maintain variation at genes associated with immune function and olfaction. Our results demonstrate how a pooled-sequencing approach can be used to explore how genetic variability is maintained across species (within and between divergent phylogenetic clades) and between cohorts, providing targets for studies of viability and balancing selection in nonmodel species.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data availability

We have deposited the primary data underlying these analyses as follows:

- Sequence reads: NCBI SRA PRJNA556680
- Genome assemblies and annotations: Dryad doi:10.5061/dryad.j6p4nb3
- Custom scripts: https://github.com/jwillou/viability_eagles

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