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FROM THE COVER

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Heritability of interpack aggression in a wild pedigreed population of North American grey wolves

Bridgett M. vonHoldt[1] | Alexandra L. DeCandia[1] | Elizabeth Heppenheimer[1] | Ilana Janowitz-Koch[1] | Ruoyao Shi[2] | Hua Zhou[3] | Christopher A. German[3] | Kristin E. Brzeski[4] | Kira A. Cassidy[5] | Daniel R. Stahler[5] | Janet S. Sinsheimer[3,6]

[1]Ecology and Evolutionary Biology, Princeton University, Princeton, NJ, USA

[2]BioKnow Health Informatics Lab, College of Life Sciences, Jilin University, Changchun, China

[3]Department of Biostatistics, UCLA Fielding School of Public Health, University of California, Los Angeles, CA, USA

[4]College of Forest Resources and Environmental Science, Michigan Technological University, Houghton, MI, USA

[5]Yellowstone Center for Resources, National Park Service, Yellowstone National Park, WY, USA

[6]Department of Human Genetics and Computational Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA

Correspondence
Bridgett vonHoldt, Ecology and Evolutionary Biology, Princeton University, Princeton, NJ, USA.
Email: vonHoldt@princeton.edu

Janet S. Sinsheimer, Department of Biostatistics, UCLA Fielding School of Public Health, University of California, Los Angeles, CA, USA.
Email: jsinshei@ucla.edu

Present address
Ilana Janowitz-Koch, Hagerman Genetics Laboratory, Columbia River Inter-Tribal Fish Commission, Hagerman, ID, USA

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Abstract

Aggression is a quantitative trait deeply entwined with individual fitness. Mapping the genomic architecture underlying such traits is complicated by complex inheritance patterns, social structure, pedigree information and gene pleiotropy. Here, we leveraged the pedigree of a reintroduced population of grey wolves (*Canis lupus*) in Yellowstone National Park, Wyoming, USA, to examine the heritability of and the genetic variation associated with aggression. Since their reintroduction, many ecological and behavioural aspects have been documented, providing unmatched records of aggressive behaviour across multiple generations of a wild population of wolves. Using a linear mixed model, a robust genetic relationship matrix, 12,288 single nucleotide polymorphisms (SNPs) and 111 wolves, we estimated the SNP-based heritability of aggression to be 37% and an additional 14% of the phenotypic variation explained by shared environmental exposures. We identified 598 SNP genotypes from 425 grey wolves to resolve a consensus pedigree that was included in a heritability analysis of 141 individuals with SNP genotype, metadata and aggression data. The pedigree-based heritability estimate for aggression is 14%, and an additional 16% of the phenotypic variation was explained by shared environmental exposures. We find strong effects of breeding status and relative pack size on aggression. Through an integrative approach, these results provide a framework for understanding the genetic architecture of a complex trait that influences individual fitness, with linkages to reproduction, in a social carnivore. Along with a few other studies, we show here the incredible utility of a pedigreed natural population for dissecting a complex, fitness-related behavioural trait.

KEYWORDS

aggression, behaviour, canid, heritability, RAD-seq

vonHoldt and DeCandia authors contributed equally.

1 | INTRODUCTION

Aggressive behaviour across species is correlated with two central aspects of fitness, namely fecundity and reproductive success (Réale, Reader, Sol, McDougall, & Dingemanse, 2007; Wolf, Doorn, Leimar, & Weissin, 2007), shaped by the interaction of hormones, neurotransmitters, genetic variation and the environment (Nelson & Trainor, 2007). This quantitative and continuous trait is found to vary within natural populations (Brodkin, Goforth, Keene, Fossella, & Silver, 2002), yet little is known about the genetic components of aggression in natural populations (de Boer, Vegt, & Koolhaas, 2003). Quantifying the extent to which genetic variation contributes towards aggression can enhance our understanding of the evolutionary constraints on, or the plasticity of, this fitness-related behaviour (reviewed by Anholt & Mackay, 2012). Research on the molecular mechanisms of aggressive behaviour has historically focused on neurochemicals and their associated receptors, which are known to have a central role in regulating behaviours (Haller et al., 1998; Mandel et al., 1981; Takahashi & Miczek, 2014). Furthermore, domesticated species have been successfully used to discover some of the underlying molecular components of complex traits, particularly in dogs (e.g., aggression, Vage et al., 2010; Eo et al., 2013; Proskura et al., 2013; sociability, vonHoldt et al., 2017). Although these studies have provided insights into the genetic basis of complex behavioural traits, their interpretations are limited to systems that have been artificially modified and controlled. Here, we suggest an extension to study aggression in an extensively monitored grey wolf (*Canis lupus*) population in North America.

After six decades of extirpation, 41 grey wolves were reintroduced to Yellowstone National Park (YNP) in 1995 and 1996, and nearly every aspect of their recovery has been documented (e.g., life history traits, Stahler, MacNulty, Wayne, vonHoldt, & Smith, 2013; genetics, vonHoldt et al., 2008, 2010; pigmentation, Anderson et al., 2009). Furthermore, behavioural studies of wolf intraspecific aggression have been successful in northern YNP as wolves are highly visible in this region, which contains a high density of overlapping wolf territories and elk populations (Supporting Note). Such spatial overlap can result in higher intraspecific mortality rates (Cassidy, MacNulty, Stahler, Smith, & Mech, 2015; Cassidy, Mech, MacNulty, Stahler, & Smith, 2017; Cubaynes et al., 2014) and disease (Almberg, Mech, Smith, Sheldon, & Crabtree, 2009). Here, we harness the integrative power of these past studies, field observations, a quantified behavioural trait, pedigree information and newly collected genetic data to investigate the following facets of aggression.

Among canines, aggression can significantly impact fitness in individual interactions relating to territory defence, social dominance, predation events, and mate acquisition and thus reproduction (Maher & Lott, 2000). Wolves live in territorial, cooperative, kin-structured groups called packs that vary tremendously in structure, ranging from a single monogamous breeding pair to multiple mating pairs of subordinate ranks (Mech & Boitani, 2003; vonHoldt et al., 2008). Not surprisingly, aggressive territorial behaviour is prominent in group-living social mammals and is used as a means of territorial defence and dominance establishment (Maher & Lott, 2000). Levels of aggression are expected to influence reproductive success and probably evolve under balancing selection (Anholt & Mackay, 2012). Given the social hierarchy in wolf packs, breeding is cooperative, with rank and reproductive access often assumed to be correlated with aggression and are population density-dependent (Cubaynes et al., 2014; Sands & Creel, 2004) where successful interpack aggression leads to better access to resources such as territory, prey and pup-rearing space (Smith et al., 2015). These factors influence pack size, individual survival and fitness, which have important impacts on female reproductive success (Stahler et al., 2013). Wolves in YNP exhibit natural variation in aggressive interactions both within and between packs that is density-dependent and correlated with survival (Cassidy et al., 2015; Cubaynes et al., 2014). Sex-based differences in aggression are also known to occur in dogs, with males exhibiting higher aggression levels than females, and positively correlated with age in males but not in females (Proskura et al., 2013). Furthermore, aggression is also more likely to be exhibited among same-sex interactions (Eo et al., 2013). The relative numbers of wolves in each pack also has a strong effect on pack success and an individual's degree of aggression (Cassidy et al., 2015, 2017).

Previous research has found a clear association between aggression and neurotransmitter-related genes across taxa (Comai, Tau, & Gobbi, 2012; Pavlov, Chistiakov, & Chekhonin, 2012), including domestic dogs (Vage et al., 2010). In addition, a 3-bp mutation in the canine beta-defensin 103 (*CBD103*) gene is responsible for melanism and segregates as a Mendelian trait in grey wolves (Anderson et al., 2009). Although melanism is typically associated with increased aggression in a variety of taxa (Beziers, Ducrest, Simon, & Roulin, 2017; Ducrest, Keller, & Roulin, 2008; Roulin & Ducrest, 2011), Cassidy et al. (2015) found that grey-coated wolves were more aggressive than melanistic individuals during interpack conflict. The capacity for the *CBD103* gene to competitively bind other melanocortin receptors (Candille et al., 2007) that modulate aggressive behaviour (Ducrest et al., 2008) may decrease aggression in melanistic wolves. Support for this mechanism comes from evidence that black-coated dogs have lower aggression rates than nonmelanistic dogs (Amat, Manteca, Mariotti, Torre, & Fatjo, 2009; Houpt & Willis, 2001).

Here, we explore the inheritance and stability of aggression in a pedigreed grey wolf population. We hypothesize that: (i) individuals that have reproduced will exhibit increased levels of aggression, and (ii) due to familial aggregation, aggression will display a positive, narrow-sense heritability. We use a restriction site-associated (RAD) DNA marker sequencing approach to generate genome-wide single nucleotide polymorphism (SNP) genotypes to infer (or confirm) pedigree relationships (following vonHoldt et al., 2008). Using linear mixed models, we explore the relationship of interpack aggression (herein, aggression) with life history traits as

fixed effects and shared environment (i.e., natal packs) as a random effect to determine the degree of heritability of aggression. Our investigation provides both a perspective on the success of future gene mapping efforts to uncover possible universalities of genes and pathways, as well as further insights into the environmental or correlated factors that shape behaviours in a social canine.

2 | METHODS

2.1 | Sample collection and DNA extraction

Since their reintroduction, grey wolves are annually monitored in YNP. During winter captures using helicopter darting techniques following protocols approved by NPS (IACUC #IMR_YELL_Smith_wolves_2012), blood is collected in EDTA vacutainers, along with radiocollaring and morphometric data collection on age, sex and breeding status. YNP also collects tissue specimens from carcasses to ensure a high representation of individuals in the curated collections. We extracted high-molecular-weight genomic DNA from blood and tissue samples collected from YNP since 1995 until the present using the DNeasy Blood and Tissue Kit (Qiagen) or the BioSprint 96 DNA Blood Kit in conjunction with a KingFisher Flex Purification platform (Thermo Fisher Scientific) following the manufacturers' protocols. DNA was visualized on a 2% agarose gel with a 2-log DNA ladder (New England Biolabs) for degradation, quantified using either PicoGreen or Qubit 2.0 fluorometry, and standardized to a concentration of 5 ng/μl.

2.2 | Life history traits and behavioural data

We accessed the extensive collection of phenotype data for 205 individual wolves in YNP with at least one observation contributing to the individual aggression score (IAS), with higher IAS values indicating that an individual consistently displays higher levels of aggression (see Appendix S1). We limited our inclusion to individuals with at least three IAS and full covariate information, which resulted in 141 individuals. These individuals do not necessarily also have paired RAD marker sequencing (RAD-seq) genotype data. Cohorts based on date-of-birth and natal pack were utilized for family-based analyses in addition to a life history data table, documented for every individual in the study. Static life history data (annually documented) include sex, date of birth and death, lifespan, cause of death, breeding status, genetically confirmed parentage (vonHoldt et al., 2008), and coat colour (melanistic/grey) phenotypes. We used molecular methods to assign sex when field observations were unavailable (DeCandia, Gaughran, Caragiulo, & Amato, 2016). Individuals were considered "breeding" if they have genetically confirmed offspring at any point in their life, or otherwise as "nonreproductive."

In 1995, YNP embarked on a 16-year effort to document direct observations of interpack interactions in northern YNP among individually recognizable wolves (Cassidy et al., 2015, 2017) (Appendix S1). For all observations, the pack or group affiliation was recorded for each wolf. Interpack aggressive conflicts occurred when one wolf chased and physically displaced another wolf, with some cases in which not all interacting partners have been identified (Cassidy et al., 2015). In some cases, the aggressive conflict escalated to an attack that was defined by physical contact between individuals, some with a mortality outcome, where at least one wolf was killed or fatally wounded. Interpack aggression was summarized on an ordinal scale for each interaction per individual wolf, ranging from 1 (flee) to 10 (led a chase which resulted in a kill). Because a single individual score is not a good indication of underlying aggressive tendency, individual scores were then averaged by the total number of observations per individual (Appendix S1). To reduce the effects of viewer subjectivity and effects due to differences in wolf pack compositions, we required a minimum of three documented interpack interactions per individual for all subsequent analyses, with each individual average IAS derived from data collected across all documented interactions. We used these average IAS data to estimate the heritability of aggressive behaviour and explore genetic associations with aggressive interpack interactions. As a difference in pack size is a strong predictor of aggression (Cassidy et al., 2017), we also recorded the relative pack size at each interpack interaction and assigned every individual an average relative pack size by averaging over the documented interactions in which this individual was present. Note that if the average relative pack size for a wolf is greater than zero, then that wolf was on average in the larger of the two packs interacting, and if the average relative pack size is less than zero that wolf was on average in the smaller of the two packs interacting.

2.3 | RAD-seq and data processing

We prepared 75 ng of high-molecular-weight DNA from 589 samples representing 468 unique wolves for a modified RAD-seq protocol as described by Ali et al. (2016) (Table S1). Briefly, genomic DNA was digested with *sbf*I followed by ligation of a unique 8-bp barcoded biotinylated adapter to ultimately allow for pooled sequencing of 96 individuals. Pooled DNA was randomly sheared on a Covaris LE220 device to 400 bp, with subsequent enrichment for adapter ligated DNA fragments through a Dynabeads/M280 streptavidin bead assay (Thermo Fisher Scientific). We prepared each enriched genomic library using the NEBnext Ultra II DNA Library Prep Kit following the manufacturer's instructions for paired-end sequencing (2 × 150 nt) on a rapid flowcell of the Illumina HiSeq 2500 at Princeton University's Lewis-Sigler Institute for Integrative Genomics core facility. We conducted a size selection to retain genomic fragments of 300–400 bp using Agencourt AMPure XP magnetic beads.

After sequencing, both the forward and the reverse raw sequencing reads were aligned using a custom perl script (flip_trim_sbf1_150821.pl, see Data S1) to identify and then retain reads that contained the *sbf*1 cut site along with a barcode. We

demultiplexed pooled libraries using the *process_radtags* function and allowed two mismatches in STACKS version 1.42 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013). We discarded reads with > 2-bp barcode mismatches or quality scores below 90% within the sliding window (set to 15% of the read) and removed PCR (polymerase chain reaction) duplicates using default parameters in the CLONE _ FILTER program. We mapped all samples with > 500,000 reads to the reference dog CanFam3.1 genome assembly (Lindblad-Toh et al., 2005) using the paired-end mapping feature in STAMPY version 1.0.21 (Lunter & Goodson, 2011). We sorted and filtered mapped reads based on a minimum quality score (MAPQ> 96) and converted files to bam format in SAMTOOLS version 0.1.18 (Li et al., 2009). We then implemented the updated *gstacks* pipeline in STACKS version 2.2, due to its ability to confidently identify and genotype SNPs from low-coverage, paired-end data using the Marukilow model (Rochette, Rivera-Colón, & Catchen, 2019). This model implements a maximum-likelihood method that incorporates population-level genotype frequencies and error rates to assess the statistical likelihood of each polymorphic site and individual genotype call (Maruki & Lynch, 2017). When paired with the previous clone-filtering step, this model removes the need for subsequent coverage filtering, because a posteriori removal of statistically significant alleles may introduce more bias and allelic dropout than it corrects.

We implemented the *populations* module twice to remove duplicate samples and minimize missing data in the final SNP data set. We first included 485 high-quality samples that passed clone-filtering and set the --write_single_snp flag (which retains only the first SNP per locus) as the only filtering parameter. We used PLINK (Purcell et al., 2007) to assess missingness per sample, and removed duplicate samples with a higher percentage of missing loci and any sample with missingness > 20%. We then ran the *populations* module a second time with a reduced sample set containing 423 unique wolves and an additional filtering parameter that removed loci genotyped in fewer than 90% of individuals (–*r* .90). We conducted principal component analysis (PCA) of the genotype data using FLASHPCA (Abraham & Inouye, 2016) and the PCs used in subsequent analyses where appropriate.

2.4 | Parentage and pedigree construction

We conducted parentage testing to assign relationships using a multipronged approach that integrated past observational and parentage information from vonHoldt et al. (2008), which was previously constructed using 26 tetranucleotide microsatellite loci, with genome-wide SNP data obtained from RAD-seq methods both to update and to resolve challenging relationships. For SNP-based analyses, we applied strict data filtering parameters that are optimized for pedigree reconstruction (Huisman, 2017). We removed problematic individuals (e.g., putative monozygotic twins) and filtered the remaining wolves (n = 413) to retain SNPs that segregated two alleles (--biallelic-only --snps-only), had a minor allele frequency of 0.45 (--maf 0.45), were in Hardy–Weinberg equilibrium (--hwe 0.001), and exclude loci in statistical linkage disequilibrium (LD) using genotypic correlation (r < .2) as a proxy metric (--indep-pairwise 50 5 0.2) in PLINK (Purcell et al., 2007). We assessed the degree of missingness per sample to remove those with missing values higher than two standard deviations above the mean (referred to as the pruned data set).

To perform parentage analyses at a finer scale, we additionally created annual data sets for 1995–2018 and retained life history data regarding which individuals were reproductively mature (≥1 year old) as candidate parents with no a priori preferences for parentage testing based on pack affiliation, social rank or copulatory/mating behaviours. We filtered the data set to reflect annual mortality events and removed individuals with missingness higher than two standard deviations above the annual mean. For complicated families, we later included observations on reproductive access or the display of copulatory mating behaviours to manually resolve candidate parents.

We used the R package RELATED to calculate relatedness coefficients between each pair of wolves using both the 413 and 384 wolf data sets (Pew, Muir, Wang, & Frasier, 2015). Using the coancestry function, we implemented the dyadic likelihood estimator (dyadml = 1; Milligan, 2003) with allowance for inbreeding (allow.inbreeding = TRUE) on our parentage data sets. We selected this relatedness measure for its inbreeding allowance, computational efficiency and low error rate with SNP data sets, as prior simulations implemented in RELATED returned similar results for moment (Wang, 2002) and likelihood (e.g., dyadml) estimators (Milligan, 2003; Wang, 2011).

We next used the R package SEQUOIA to reconstruct pedigrees with our parentage-informative pruned data set, and separately with the annual data sets that contained individuals alive in each year (Huisman, 2017). This program implemented a heuristic hill-climbing algorithm to optimize the likelihood of unrelated, first-, second- and third-degree relationships in the data set. It subsequently assigned parent–offspring (PO) pairs, half-siblings sharing a "dummy" parent, and grandparents that sired unsampled "dummies" to build multigenerational pedigrees. We used default parameter settings with the estimated genotyping error rate (Err) relaxed to 1e-03 for each analysis. We then analysed the pruned data set a second time with Err set to 1×10^{-2} to enable additional PO assignments.

We merged results from RELATED, SEQUOIA and previous microsatellite analyses (vonHoldt et al., 2008) to create a consensus pedigree. We first assigned PO pairs when all three analyses were consistent in the identification of the same individual parent (our "gold standard of high support"). We next considered PO pairs that were supported by two of three analyses pairs: (a) RELATED and SEQUOIA relationship assignments inferred from RAD-seq SNP genotypes; or (b) RELATED inferences based on SNP genotypes and previous microsatellite data. When SEQUOIA and microsatellite assignments mismatched, we assigned the PO pair with the higher relatedness value based on SNP genotypes.

2.5 | Estimating the heritability of interpack aggression

The kinship for two wolves *i* and *j* was defined as the probability that a gene selected randomly from an autosomal locus originating in the genome of wolf *i* and a gene selected randomly at the same locus from the genome of wolf *j* are identical by descent (IBD) (Malécot, 1948). To estimate the kinship matrix needed for SNP-based heritability estimates, we further filtered the LD-pruned full SNP set to exclude loci with genotyping success rates less than 95%, significant deviations from Hardy–Weinberg equilibrium ($p < 1 \times 10^{-7}$), or minor allele frequency (MAF) <.05, and any individuals with more than 12.5% missing SNP data. We estimated the kinship matrix for the resulting wolves with genotype, covariate and phenotype data using a robust genetic relationship matrix (VanRaden, 2008; Wang, Sverdlov, & Thompson, 2017) as implemented in the SNPARRAYS package of OPENMENDEL (Zhou et al., 2019) using only the autosomal SNPs remaining after the above filtering. To address any differences that may be obtained from global kinship estimates derived from the genetic relatedness matrix (GRM) vs. those from the pedigree, we also estimated heritability using the theoretical kinship values using the pedigree structure and Jacquard's recurrence formulas (Emik & Terrill, 1949; Lange, 2002; Zhou et al., 2019).

We used an REML-based linear mixed model as implemented by the VC test routine (Zhou et al., 2017) of the OPENMENDEL package (Bauman et al., 2005; Lange et al., l1983; Lange et al., 2013; Lange, Westlake, & Spence, 1976; Zhou et al., 2019; Zhou, Hu, Qiao, Cho, & Zhou, 2016) to estimate both fixed and random effects. Our most general model is:

$$E(Y) = \beta^T X, V_{\text{pheno}} = 2v_A K + v_D D + v_M M + v_{\text{pack}} H + v_e I$$

In all analyses, the *X* matrix includes sex (male as the reference group), breeding status (nonreproductive as the reference group), coat colour (grey as the reference group) and average relative pack size as fixed effects; β denotes the corresponding vector of coefficients. The V_{pheno} matrix is composed of the additive genetic variance v_A, natal pack variance v_{pack}, independent environmental variance v_e, dominance genetic variance v_D and maternal effect variance v_M. These effects are treated as random. The design matrices were: (a) *I*, a matrix with 1 on the diagonal and 0 elsewhere; (b) *K*, the kinship matrix; (c) *D*, a matrix of probabilities of sharing two genes IBD; (d) *H*, a matrix of ones and zeros with 1 denoting wolves *i* and *j* from the same natal pack and 0 otherwise; and (e) *M*, a matrix of ones and zeros with 1 denoting wolves *i* and *j* as having the same mother and 0 otherwise. Heritability is defined as the fraction of phenotypic variation that is due to genetic effects. Typically, narrow-sense heritability (h^2) is estimated as the fraction of phenotypic variance due to the alleles acting independently. As an example, when dominance and pack are also included as random effects:

$$h^2 = \frac{v_A}{v_A + v_D + v_{\text{pack}} + v_e}$$

We calculated the fraction of phenotypic variance that is due to the natal pack. For the same example:

$$f_{\text{pack}} = \frac{v_{\text{pack}}}{v_A + v_D + v_{\text{pack}} + v_e}$$

We similarly used year of birth to define a common environment effect of birth year. As our goal was to assess the degree to which genetic effects may influence aggression, we used a stepwise approach to determine if any additional variance components significantly improved the model when additive genetic variance was also included. Similarly, to address the possibility of any residual population substructure, we tested whether the inclusion of the first three PCs as fixed effects improved the model fit when additive genetic variance was also included using the GRM as the estimate of kinship coefficient matrix.

2.6 | Pedigree-based genetic associations with interpack aggression

With the acknowledgement that this analysis is likely to be under-powered, we assessed genome-wide association of SNP variants with IAS. We filtered SNPs to retain sites with a maximum of 20% missing data per individual and MAF = 1%. We employed a linear mixed model (LMM) in GEMMA (Zhou & Stephens, 2014) and included a kinship relatedness matrix estimated for 391 individuals in RELATED (see above section for more details). We included IAS phenotypes for 121 individuals that had a minimum of three interpack aggression interactions observed, with individuals lacking such observational support excluded from the LMM analysis. We included sex, coat colour and breeding status as covariates in the LMM. We assessed the significance of the association using the likelihood ratio test (LRT) and inferred significance using an adjustment for multiple testing (B-Y modified; Benjamini & Yekutieli, 2001). We used an experiment-wide B-Y false discovery threshold of $\alpha = .01$. Our rationale is to acknowledge that the data set is expected to be under-powered and our goal was to minimize erroneous inference of genotype associations. All sites were catalogued with Ensembl's Variant Effect Predictor for their predicted impact (McLaren et al., 2016). We further conducted functional profiling using G:GOST in G:PROFILER to determine if outlier SNPs that were catalogued as genic were enriched in specific gene ontological categories using the Benjamini–Hochberg false discovery rate (FDR) of 0.05 (Benjamini & Hochberg, 1995; Raudvere et al., 2019). We searched only annotated genes and included all default data sources from ontology, biological pathways and regulatory motifs databases.

3 | RESULTS

3.1 | RAD-seq data processing and parentage analyses

We discovered 212,667 SNP variants in the 485 samples that passed initial clone filtering. After excluding duplicates, putative

monozygotic twins, and samples with low sequence coverage and low-quality data (n = 72), we retained 413 wolves with 120,327 SNPs for strict filtering and pedigree analysis. Our final parentage data sets consisted of 598 uncorrelated neutral SNPs with MAF > 45% (i.e., Hardy–Weinberg equilibrium p> .001). We assessed missingness per individual and removed an additional 29 wolves with missingness higher than two standard deviations above the mean, which produced two data sets of 598 SNPs for parentage analysis: (a) the full data set containing 413 wolves; and (b) the pruned data set containing 384 wolves. We additionally created annual data sets of wolves living in YNP between 1995 and 2018 to assign parentage within smaller subsets of wolves, where possible parents were restricted to individuals recorded to be alive in each year.

We conducted PCAs using 598 parentage-filtered SNPs across each of these data sets (full, pruned and annual), which revealed two components of the demographic history of wolves in YNP. First, PC1 reveals an axis that is polarized by the Nez Perce pack (low PC1 values), a pack that received translocated pups from the Sawtooth pack of northwestern Montana that represents a distinct genotype. These individuals eventually contributed to the genetic diversity of YNP wolves through gene flow. Second, PC2 differentiates the two source populations from which wolves were originally translocated (high PC2 values, 1995 relocation from Alberta; low PC2 values, 1996 relocation from British Columbia) (reviewed in vonHoldt et al., 2010) (Figures S1–S3).

3.2 | Pedigree construction and confirmation

We assigned SNP-based parentage results from SEQUOIA when also supported by concordant relatedness estimates across the full, pruned and annual data sets. In total, 505 PO assignments are supported: 264 PO pairs are supported by all SNP and microsatellite analyses ("gold standard of high support"); 140 PO pairs are supported by only SNP-based analyses; and 101 PO pairs are supported by only SNP-based relatedness estimates (parentage inference was not conclusive) and microsatellite analyses.

3.3 | LMM estimates for interpack aggression

After filtering, 111 wolves with full covariate information, at least three IAS values and 12,288 SNPs were used to calculate the robust GRM and to estimate the heritability of interpack aggression (Table 1). When additive genetic variance is included in the model, the first three principal components do not improve the model fit (Table 2). The best fitting model by Akaike's information criterion (AIC) included variance components for additive genetic effects, natal pack as a common environmental effect, and residual independent environment. The SNP-based narrow-sense heritability of aggression is h^2 = 0.369 and the proportion of the phenotypic variance explained by natal pack membership is f_{pack} = 0.134 (Table 3). The inclusion of dominance genetic effects, maternal effects and year of birth cohort effects did not improve the fit of a model that included additive genetic effects. We note that in our best fitting model, sex and coat colour are not significantly associated (p = .448 and .637), although their trends are in the previously observed direction where females tended towards lower IAS values than males, while melanistic wolves also tended towards lower IAS values than grey-coated wolves. Breeding status and average relative pack size are significantly associated ($p = 1.37 \times 10^{-4}$ and 7.93×10^{-8}) with breeding individuals having an IAS 0.713 higher than a nonbreeding individual, and a unit increase in average relative pack size increasing IAS by 0.111. Interpretation of these results is challenging because on a per interaction level the IAS value is ordinal, but clearly an individual that has reproduced will, on average, display a higher level of aggression relative to an equivalent nonbreeding individual. Similarly, average aggression levels are increased when individuals tend to travel in a larger pack, probably due to an advantage over any opponents in relatively smaller packs (e.g., Cassidy et al., 2015).

Using the consensus pedigree, 141 wolves with full covariate and at least three behavioural observations are included in the analysis (Table 1). These data, along with the theoretical kinships, were used to estimate the heritability of interpack aggression in a second LMM analysis. Again, we used a stepwise approach to determine the variance components that lead to the best fitting model by AIC, keeping in mind the hierarchical nature of some of the random effects (Table 2). The model including additive genetic variance provided a better fit than one with only the independent environmental effect (p = .01236). As with the GRM estimate of kinship, a model that included a dominance genetic effect along with an additive genetic effect or maternal effects was not the best fitting model. Similarly, the year of birth cohort effect explained only 2% of the total variance and was not included in the best fitting model. One major difference from the first analysis is that neither the additive genetic variance nor the natal pack variance is significantly greater than zero when the other effect is included in the model (p = .2273 and p = .0673,

TABLE 1 Summary statistics of individuals included in the linear mixed model analyses

Sample	Per cent males	Per cent melanism	Per cent reproductive	Mean IAS (*SD*)[a]	Mean relative pack size (*SD*; range)	Mean number of interactions (*SD*)[b]	Number of natal packs represented
GRM (n = 111)	47.7	47.7	37.8	4.50 (1.2)	5.87 (5.8; −8.4 to 22.2)	15.72 (14.5)	14
Pedigree (n = 141)	51.1	51.8	37.8	4.55 (1.2)	5.68 (6.1; −11.3 to 23.5)	15.56 (14.5)	17

Abbreviations: GRM, genetic relatedness matrix; IAS, individual aggression score; n, sample size; *SD*, standard deviation.

[a]The range of IAS was 1–7.

[b]The range for the number of interactions was 3–78.

TABLE 2 Log-likelihoods and Akaike's information criterion (AIC) for linear mixed models examined

Model	*df*	GRM log-likelihood	GRM AIC	Pedigree log-likelihood	Pedigree AIC
V_e	6	−153.9397	319.8794	−170.7956	353.5912
V_e; PCs	9	−152.5409	323.0818		
V_e, V_a	7	−151.6879	317.3758	−165.7513	345.5026
V_e, V_a; PCs	10	−151.0484	322.0968		
V_e, V_a, V_d	8	−150.7475	317.4950	−165.6432	347.2864
V_e, V_a, V_{mat}	8	−151.0995	318.1990	−165.9977	347.9954
V_e, V_a, V_{YOB}	8	−151.6714	319.3428	−165.3743	346.7486
V_e,V_a, V_{Pack}	8	−150.2500	316.5000	−164.6324	345.2648
V_e, V_{Pack}	7	−151.6069	317.2138	−164.9119	343.8238
V_e, V_a, V_{pack}; PCs	11	−149.4536	320.9072		

Note: All models include fixed effects of sex, breeding status, coat colour and average relative pack size. The fixed effects of the PCs were not included in the pedigree-based models because of extensive missing data.

Abbreviations: df, degrees of freedom; GRM, genetic relatedness matrix; PCs, the first three principal components; V_e, variance due to independent environmental effects; V_a, additive genetic variance; V_d, dominance genetic variance; V_{Pack}, variance due to natal pack treated as a random effect; V_{YOB}, variance due to year of birth cohort treated as a random effect; V_{mat}, variance due to shared maternal effect.

respectively). Indeed, when assessing model fits with AIC, the best fitting model included only the natal pack effect, with the next best model being the one with both additive genetic and natal pack effects (Table 2).

For comparison with the analysis using the robust GRM we consider this model further. The narrow-sense heritability of aggression, h^2, is 0.138 and the proportion of the phenotypic variance explained by natal pack membership, f_{pack}, is 0.160 (Table 3). As with the robust GRM, sex and coat colour are not significantly associated (p = .1386 and .8365), whereas breeding status and average relative pack size are significant ($p = 1.106 \times 10^{-6}$ and 2.141×10^{-12}). Breeding individuals are predicted to have a 0.8243 higher IAS value compared with nonbreeding individuals, and a unit increase in relative pack size is expected to increase IAS by 0.1124.

3.4 | Pedigree-based association

This data set included 391 wolves for 56,000 SNPs after filtering for 10% missing data per locus, an MAF of 1%, and excluding individuals with more than 20% missing data across all loci. Of these loci, 31,491 SNPs were informative for the model association. We restricted our association analysis to a total of 121 wolves that have a minimum of three behavioural observations and included sex, coat colour and breeding status as covariates. We identified 45 SNPs with alleles significantly associated with IAS (LRT, adjusted $p < 9.145 \times 10^{-4}$) (Table S3). Using Ensembl's Variant Effect Predictor, all 45 sites are catalogued as having a putatively "modifier" (noncoding variant) impact. Of these, only 17 are categorized as "genic" with associated genes (Table S4), two of which (*NOCT* and *EDC3*) belong to a single ontological category and passed the FDR (cytoplasmic mRNA processing body assembly, GO: 0033962, $p_{adj} = 4.112 \times 10^{-2}$). Despite our under-powered study, we do observe associations of genetic variation in the genes *MYO9A* and *TRAK1*. Although these gene functional categories do not surpass the FDR, their respective functions remain relevant and include involvement in neuronal growth and the regulation of endocytic trafficking of GABA-A receptors, respectively (Barel et al., 2017; O'Connor et al., 2016).

4 | DISCUSSION

To explore the life history, ecological and molecular factors associated with interpack aggression, we investigated behavioural and genetic data across a 16-year study of a pedigreed population of grey wolves. Overall, we found that aggression is heritable and subject to common environmental effects that are captured by natal pack. Aggression is predicted by breeding status, relative pack size and a small subset of functionally relevant genes. Our analyses suggest that aggression demonstrates moderate levels of narrow-sense heritability with additive genetic effects explaining 14%–37% of the variation in aggressive behaviour in grey wolves in YNP. The estimate of heritability based on a theoretical kinship matrix derived from the pedigree is 14%, which is substantially lower than the heritability estimate of 37% based on the correlation among the SNPs using the robust GRM. Both estimates are potentially biased, albeit in different directions. We further suggest that our variance models also are unlikely to fully capture the total evolutionary potential of aggression, and the role of indirect effects will probably add to our understanding of aggression in this wild pedigreed population of grey wolves (Alemu, Bijma, Moller, Janss, & Berg, 2014; Camerlink, Turner, Bijma, & Bolhuis, 2013).

As is often the case with wild populations, the pedigree is not exactly known and relatedness inaccuracies among even a few founders can bias heritability estimates towards zero (Wilson et al., 2010). In contrast, using SNP correlations to estimate the kinship among closely related individuals can lead to inflated heritability (Zaitlen et

TABLE 3 Coefficients for the best-fitting linear mixed models for the SNP-based analysis by Akaike's information criterion and corresponding model for pedigree-based analysis

Parameter	Mean	Sex	Coat colour	Breeding status	Relative pack size	v_a	v_{pack}	v_e	h^2	f_{pack}
Robust GRM-based estimates	3.749	−0.143	−0.092	0.713	0.111	0.383	0.139	0.517	0.369	0.134
SE	0.220	0.188	0.195	0.187	0.021	0.232	0.082	0.119	0.224	0.079
p-value		.448	.637	1.37×10^{-4}	7.93×10^{-8}	4.97×10^{-2}	4.49×10^{-2}			
Pedigree-based estimates	3.807	−0.175	−0.036	0.824	0.112	0.142	0.165	0.725	0.138	0.160
SE	0.223	0.166	0.173	0.169	0.016	0.190	0.110	0.163	0.158	0.107
p-value		.139	.837	1.11×10^{-6}	2.14×10^{-12}	.227	6.73×10^{-2}			

Abbreviations: f_{pack}, the proportion of the phenotypic variance explained by natal pack membership; v_a, additive genetic variance; v_e, variance due to independent environmental effects; v_{pack}, variance due to natal pack treated as a random effect.

al., 2013). We therefore suspect that the true estimate of IAS heritability is bounded by the pedigree and GRM-based estimates. Because we used the average effect of IAS estimated over a minimum of three interactions, we are not accounting for within-individual variability, which may inflate heritability estimates. As estimates of additive genetic variance can include other effects when the models are too simplistic, we explored models that might partially lead to apparent heritability. We found no evidence of maternal effects, dominance effects, residual population substructure or year of birth cohort effects, although our sample size is likely to be under-powered unless these effects are relatively large. We do find evidence suggesting a natal pack effect that explains 14%–16% of the total variation in IAS. This effect probably reflects shared exposure to environmental conditions and behavioural experiences as a group-living, territorial species, as well as potentially capturing a dominance effect as siblings are a major component of the natal pack membership.

Our heritability estimates for aggression in grey wolves are comparable to those reported for domestic animal temperament (~30%) (Chervet, Zöttle, Schürch, Taborsky, & Heg, 2011; Dingemanse, Both, Drent, Oers, & Noordwijk, 2002; Le Neindre, Boivin, & Boissy, 1996; Le Neindre et al., 1995; Lovedahl et al., 2005; Morris, Cullen, Kilgour, & Bremner, 1994; Pérez-Guisado, Lopoz-Rodríguez, & Muñoz-Serrano, 2006). Studies of PO trait correlations and applications of the animal model approach in wild populations have confirmed the low to moderate estimates of aggression (e.g., North American red squirrel h^2 = 0.08–0.12, Taylor et al., 2012; western bluebirds h^2 = 0.34, Duckworth & Kruuk, 2009; laboratory zebrafish h^2 = 0.36, Ariyomo, Carter, & Watt, 2013). Experimental simulations of territorial intrusions and regression modelling also estimated comparable estimates of heritability for aggressive behaviour in male great tits (h^2 = 0.260–0.266, Araya-Ajoy & Dingemanse, 2017). Furthermore, experimental breeding studies offer evidence that behavioural and complex traits have a heritable genetic component (Takahashi & Miczek, 2014). For example, the fear-selected line of silver foxes (*Vulpes vulpes*) exhibit a strong and heritable aggression response after generations of selection (Kukekova et al., 2011; Trut, 1980). A caveat of heritability estimates is that they are difficult to compare directly, as heritability models often vary in their components and depend on the amount of environmental variation. We included fixed effects in both analyses, which found that only breeding status and relative pack size were strongly significantly associated with aggression. Thus, our heritability analyses included sex and grey/melanism as covariates despite not being significant to allow better comparison to other studies of similar wolf populations.

Grey wolves rely upon aggression for acquisition and maintenance of both territories and mates. However, access to these resources central to individual fitness is often variable, may be density-dependent and may experience annual fluctuations. For example, by 2002, wolves in northern YNP had one of the highest densities ever recorded in North America at 98 wolves/1,000 km^2 (Paquet & Carbyn, 2003). Since 2008, it has stabilized to an average of about 39 wolves/1,000 km^2 (Smith et al., 2019). The Druid Peak

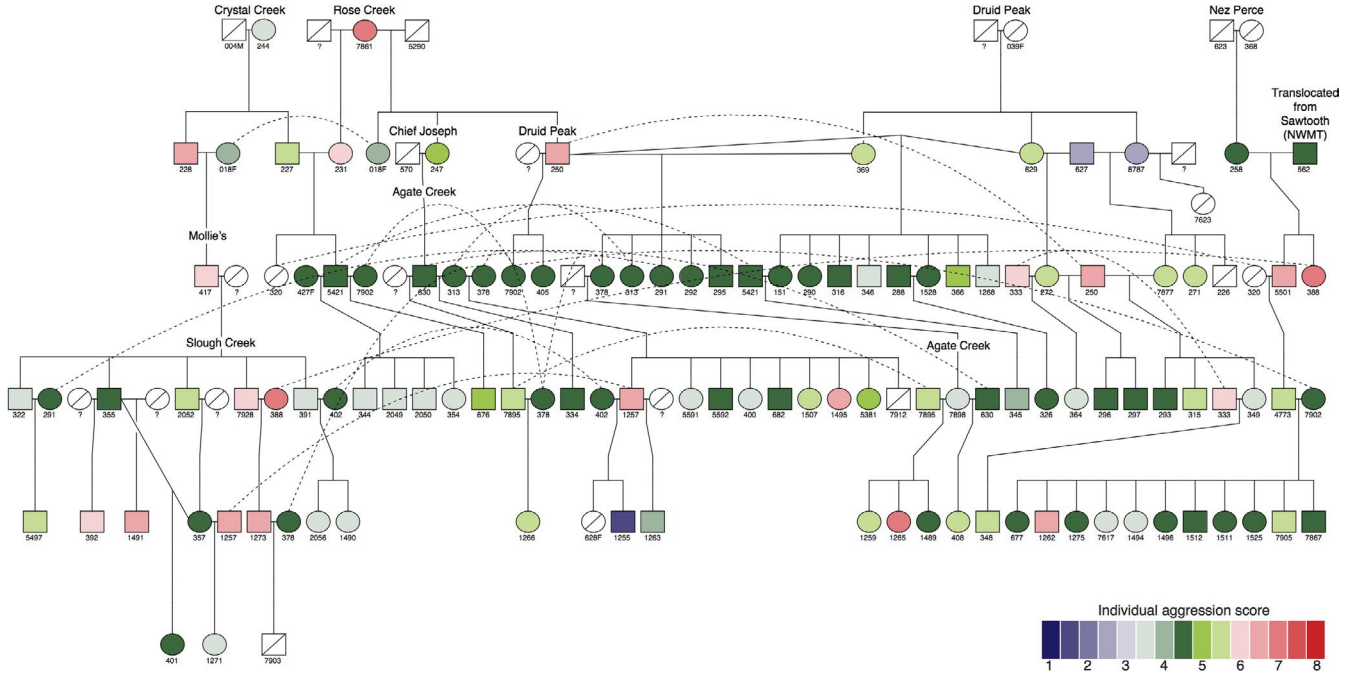

FIGURE 1 An example pedigree for a subset of Yellowstone National Park wolves with SNP genotypes and symbols (male, squares; female, circles) shaded to represent their individual aggression score (IAS) level. This pedigree is of the Druid Peak, Slough Creek and Agate Creek packs. Symbols with a diagonal line indicate the lack of data for the aggression behavioural phenotype. Dashed lines indicate where an individual was involved in parentage events across disparate sections of the pedigree. Pack names are indicated at the place in the pedigree when the pack was established. NWMT, northwest Montana

(1996–2009), Slough Creek (2003–2008) and Agate Creek (2002–2012) packs maintained territories concurrently in the northern range for >6 years and each pack was observed intensively for thousands of hours by biologists. The genetic relationship composition within and between packs was annually augmented due to changing memberships at the pack level. The Slough Creek and Agate Creek packs were formed by females dispersing from Druid Peak and joining males from other packs (Figure 1). All three packs alternated in being the largest and most dominant pack in the area. Interpack aggressive conflicts were common, with at least 14 mortalities documented. All three packs eventually disintegrated after a significant loss of a key member during an aggressive conflict with neighbouring packs.

Similarly, Cubaynes et al. (2014) showed that wolf survival was dependent upon wolf density. Intraspecific aggressive behaviour is presumably expected to serve as the primary mechanism for resource acquisition and defence, although aggression may also be influenced by group size and composition (Cassidy et al., 2015, 2017), or modulated at the individual level relative to their environment or social composition. The maintenance of the aggression trait is likely to be under stabilizing selection, where strongly or weakly aggressive behaviours are likely to lower individual fitness through decreased access to critical resources or mortality, respectively. Consequently, plasticity in aggression may be constrained by underlying molecular mechanisms (e.g., epigenetic gene regulation), which may have resulted in the evolution of evident genetic polymorphisms for aggressive behaviour in this species.

We explored the genetic association of aggression in grey wolves and it is unclear whether these genetic variants play a direct role in regulating gene expression. We found evidence suggesting that changes in neuronal growth and the GABA-A receptors may influence aggression levels, with the latter playing a well-established role in aggression (Bannai et al., 2009; Miyakawa et al., 2003; Takahashi & Miczek, 2014) and similar findings recently reported in heritable aggression in dogs (MacLean, Snyder-Mackler, vonHoldt, & Serpell, 2019).

Taken together, our results suggest that aggression is influenced by heritable genetic variation. The long-term pedigree, combined with robust behavioural observations, provides an unprecedented opportunity to integrate trait and genome-wide molecular data to discover associations with a complex, fitness-related trait in a natural population of a social canine. This study provides a new foundation that can support future studies that aim to expand upon explicit evaluations of individual-level fitness, ecological models, and explorations of natural selection in a pedigreed natural population.

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AUTHOR CONTRIBUTIONS

B.M.V. and I.J-K. designed the study; K.A.C. and D.R.S. contributed behavioural data from Yellowstone National Park grey wolves; E.H., I.J-K. and A.L.D. prepared the DNA for RAD-sequencing, A.L.D. and E.H. processed the RAD-seq data; A.L.D. conducted pedigree and parentage analyses; B.M.V., A.L.D., G.A.S., R.J. and J.S.S. analysed the data; G.A.S., R.J., J.S.S. and H.Z. wrote software subroutines to analyse the data. All authors contributed to writing the manuscript.

DATA AVAILABILITY STATEMENT

Mapped bam files for these 413 wolves are available on NCBI's public Sequence Read Archive (PRJNA577957). Additional metadata for each individual wolf can be found in Table S1.

ORCID

Bridgett M. vonHoldt https://orcid.org/0000-0001-6908-1687
Alexandra L. DeCandia https://orcid.org/0000-0001-8485-5556
Elizabeth Heppenheimer https://orcid.org/0000-0002-9164-3436
Ilana Janowitz-Koch https://orcid.org/0000-0003-3594-3108
Ruoyao Shi https://orcid.org/0000-0001-8957-1712
Kristin E. Brzeski https://orcid.org/0000-0002-5104-3719
Kira A. Cassidy https://orcid.org/0000-0003-4857-1059
Daniel R. Stahler https://orcid.org/0000-0002-8740-6075
Janet S. Sinsheimer https://orcid.org/0000-0001-9540-5956

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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