Hybridization between closely related songbirds is related to human habitat disturbance

Running Title: Chickadee disturbance-mediated hybridization

Authors: Kathryn C. Grabenstein [a], Ken A. Otter [b], Theresa M. Burg [c], Scott A. Taylor [a]

ORCiDs
Kathryn C. Grabenstein: 0000-0003-4725-3008
Ken A. Otter: 0000-0003-2331-7900
Theresa M. Burg: 0000-0001-5096-3479
Scott A. Taylor: 0000-0001-9580-9125

[a] *Department of Ecology and Evolutionary Biology, University of Colorado Boulder, 1900 Pleasant St, Boulder, CO, U.S.A.*

[b] *Natural Resources and Environmental Studies, University of Northern British Columbia, 3333 University Way, Prince George BC, V2N 4Z9, Canada*

[c] *Department of Biological Sciences, University of Lethbridge, 4401 University Dr. W, Lethbridge, AB, T1K 3M4, Canada*

Correspondence: K. C. Grabenstein, Department of Ecology and Evolutionary Biology, University of Colorado Boulder, Boulder, CO 80309 U.S.A.
E-mail address: Kathryn.Grabenstein@colorado.edu (K. C. Grabenstein).
Telephone: (303) 735-5227

Abstract

Human habitat disturbances can promote hybridization between closely related, but typically reproductively isolated, species. We explored whether human habitat disturbances are related to hybridization between two closely related songbirds, black-capped and mountain chickadees, using both genomic and citizen science datasets. First, we genotyped 409 individuals from across both species' ranges using reduced-representation genome sequencing and compared measures of genetic admixture to a composite measure of human landscape disturbance. Then, using eBird observations, we compared human landscape disturbance values for sites where phenotypically diagnosed hybrids were observed to locations where either parental species was observed to determine whether hybrid chickadees are reported in more disturbed areas. We found that hybridization between black-capped and mountain chickadees positively correlates with human habitat disturbances. From genomic data, we found that 1) hybrid index significantly increased with habitat disturbance, 2) more hybrids were sampled in disturbed habitats, 3) mean hybrid indexes were higher in disturbed habitats versus wild habitats, and 4) hybrids were detected in habitats with significantly higher disturbance values than parentals. Using eBird data, we found that both hybrid and black-capped chickadees were significantly more disturbance-associated than mountain chickadees. Surprisingly, we found that nearly every black-capped chickadee we sampled contained some proportion of hybrid ancestry, while we detected very few mountain chickadee backcrosses. Our results highlight that hybridization between black-capped and mountain chickadees is widespread, but initial hybridization is rare (few F1s were detected). We

conclude that human habitat disturbances can erode pre-zygotic reproductive barriers between chickadees and that post-zygotic isolation is incomplete. Understanding what becomes of recently hybridizing species following large-scale habitat disturbances is a new, but pressing, consideration for successfully preserving genetic biodiversity in a rapidly changing world.

Keywords: chickadees, anthropogenic change, reproductive isolation, species barriers, hybridization, habitat disturbances

Introduction

Humans are a dominant force on earth as they continue to transform landscapes by reducing, homogenizing, and fragmenting habitats (Bürgi, Hersperger, & Schneeberger, 2005; Haddad et al., 2015; Harden et al., 2014). While changes in species' distributions and abundances in human altered habitats are well-documented (Devictor, Julliard, & Jiguet, 2008; Williams et al., 2010), a growing body of literature implicates human habitat disturbances in driving hybridization between naturally co-occurring, reproductively isolated species. Hybridization is the interbreeding of closely related species to produce mixed-ancestry offspring (Harrison, 1990) and has a variety of evolutionary outcomes, which can have positive or negative consequences for biodiversity (Gompert & Buerkle, 2016). In some cases, hybridization may decrease population viability and persistence if hybrid offspring are sterile or have reduced fitness compared to non-hybrids (Todesco et al., 2016), as is often the case (Abbott, Barton, & Good, 2016). If hybridization is common and hybrids are fertile, populations might experience genetic homogenization and a loss of rare genetic variants (Hasselman et al., 2014). Additionally, when hybrids are fertile and breed successfully, alleles can introgress between species (Taylor, Larson, & Harrison, 2015), which might be adaptive (Norris et al., 2015), and can increase the genetic potential of populations (Shafer et al., 2015; Whiteley, Fitzpatrick, Funk, & Tallmon, 2015). Regardless of the outcome, hybridization can have significant impacts on global biodiversity.

Increased hybridization and/or increased survival of hybrids in human modified habitats was first hypothesized by Anderson (1948). Advances in whole genome sequencing technologies have increased our ability to detect hybridization in wild populations of non-model organisms, especially for later generation hybrids which are often phenotypically indistinguishable from parental taxa (McFarlane & Pemberton, 2019). Using genomic tools, a growing body of work is

implicating human habitat disturbances in promoting hybridization (and/or increasing hybrid fitness) between naturally co-occurring, closely related, but reproductively isolated species for a wide variety of taxa (Grabenstein & Taylor, 2018). For our purposes, human habitat disturbance refers to direct, physical habitat alterations to the environment caused by humans, such as land clearing, water eutrophication, or noise pollution. This definition purposely excludes global climate change and the introduction of non-native species, both of which can increase hybridization and have been well-reviewed (Blois, Zarnetske, Fitzpatrick, & Finnegan, 2013; Moran & Alexander, 2014). While an increasing number of studies have detected hybrids following large-scale habitat disturbances in a wide variety of taxa (e.g., shrubs: Lamont et al., 2003, fishes: Huuskonen et al., 2017, birds: Carantón-Ayala et al., 2018, reviewed in Grabenstein & Taylor, 2018), comparatively few studies have intentionally been designed to explore the relationship between human habitat disturbances and hybridization of closely-related taxa (Ortego, Gugger, & Sork, 2016; Seehausen, Alphen, & Witte, 1997).

Most documented cases of disturbance-mediated hybridization appear to be *post hoc* explanations for observations of hybrids in habitats where they were previously undetected, rather than testing *a priori* expectations for the relationship between disturbance and hybridization. For example, Crego-Prieto et al. (2012) found an increase in hybrid flatfish following a major oil spill compared to hybrid numbers before the spill. Similarly, Lamb & Aviset (1986) detected hybridization between tree frogs in ponds where mowing had removed shoreline vegetation used for male vocalizations. These opportunistic, single site studies strongly implicate disturbances in driving hybridization and/or altering hybrid fitness landscapes such that hybrids survive long enough to be sampled but it is unclear how repeatable these patterns are across species ranges. One notable example of a range-wide pattern of disturbance-mediated

hybridization comes from California oaks where Ortego et al. (2016) found an increase in the rate of hybridization between two sister species relative to an increase in wildfire frequency across the entirety of their ranges. Establishing strong correlations between human habitat disturbances and hybridization based on robust *a priori* expectations is a critical next step before we can begin to explore the mechanisms by which disturbance erodes species barriers. Ultimately, understanding how rapid human landscape changes shift interspecific interactions will advance our understanding of how humans impact biodiversity at the genetic level.

We sought to explore whether there is a significant relationship between human habitat disturbances and hybridization between two closely related species of songbirds, black-capped (*Poecile atricapillus*) and mountain (*P. gambeli*) chickadees. Black-capped and mountain chickadees appear to hybridize primarily in human modified habitats based on 1) the distribution of purported hybrid chickadee sightings in eBird (Fig. 1c) and 2) microsatellite genetic studies examining chickadee admixture (Grava et al. 2012, Graham et al. 2021). However, explicit genomic investigations into the extent of hybridization in this system, as well as the context of hybridization, are lacking.

Black-capped and mountain chickadees are closely related songbirds, but not sister taxa, that are estimated to have diverged from a common ancestor over 2 million years ago (Harris, Carling, & Lovette, 2014) and exhibit strong genomic differentiation (average genome-wide F_{ST} = 0.34, Grabenstein et al., *in review*). Historically, hybridization in this system was considered to be rare (Howe, 1985; Hubbard, 1978; Martin & Martin, 1996), especially in comparison to the well-studied and geographically extensive hybrid zone between black-capped and Carolina chickadees (*P. carolinensis*) which extends across the entire band of these sister species' range overlap, from Kansas to New Jersey, USA (Reudink et al. 2007). Both black-capped and

mountain chickadees are common, widespread North American songbirds with substantial areas of range overlap throughout nearly all the Rocky Mountains (Fig. 1a). Where their ranges overlap, the two species occupy different, but often neighboring forest types and are effectively separated along elevational gradients (Hill & Lein, 1988). Mountain chickadees often occupy higher-elevation coniferous forests, while black-capped chickadees are found in lower-elevation mixed-wood forests; sympatry occurs at mid-elevation habitats in transitional forests. Both species co-occur and breed sympatrically in these transition zones (Colorado, USA: Grabenstein et al., *in review*; British Columbia, CA: Grava et al., 2012)

Hybridization between black-capped and mountain chickadees appears to occur in human modified habitats, such as cities or logged forests, based on eBird sightings of purported hybrids in and near urban centers (n = 271 from 1989-2021; eBird 2021; Fig. 1c) and two genetic studies using microsatellite markers (Graham et al., 2021; Grava et al., 2012). Outside of these two studies, hybridization between black-capped and mountain chickadees has been inferred from records of birds with intermediate plumage characteristics. The species have similar plumage patterns, with the main distinguishing characters being the white supercilium (i.e., eyebrow) of mountain chickadees and the buffy sides and white edging on the wings of black-capped chickadees (Feldmann et al. 2021; Fig. 1). Based on whole genome data from a confirmed F1 hybrid, F1 hybrids appear to have the buffy sides and white wing feather edging of black-capped chickadees paired with a thinner supercilium than typical for mountain chickadees (Grabenstein et al., *in review*; Fig. 1c). Chicks from the nest of the only known documented social pair between a black-capped and mountain chickadee (i.e., presumably all F1s) had this same intermediate phenotype (Martin and Martin, 1996). Thus, it is likely that all of the intermediate birds reported in eBird are F1 hybrids rather than backcrosses. Although quantification of

147 plumage traits in suspected F1 hybrids are lacking, additional whole genome data from 477 birds
148 indicates that after an F1 backcrosses, the offspring look either like black-capped chickadees
149 (most common) or mountain chickadees (less common) (Grabenstein et al., *in review*).
150 eBird is an expansive online global database of bird observations that can be used to
151 explore species' distributions and abundances (Sullivan et al., 2009). eBird users have reported
152 likely F1 hybrids between black-capped and mountain chickadees (based on intermediate
153 plumage) across western North America (Fig. 1c; eBird 2021) primarily in and near cities despite
154 both parental species being common and widespread with substantial range overlap. This mosaic
155 distribution of hybrid black-capped and mountain chickadees suggests that hybridization in this
156 system is context dependent (i.e., occurs non-uniformly when the two species co-occur).
157 To assess whether hybridization between black-capped and mountain chickadees
158 correlates with human habitat disturbance at the continental scale, we compared human habitat
159 disturbance metrics to chickadee hybridization using two complementary datasets (genomic &
160 phenotypic) to characterize hybridization. For our genomic dataset, we genotyped 409 black-
161 capped and mountain chickadees from across both species' ranges using reduced-representation
162 genome sequencing and compared measures of genomic admixture to metrics of human
163 landscape disturbance. Our ddRAD approach increases our ability to confidently detect hybrids
164 because a greater number of loci (> 400) are generated compared to previous studies using only a
165 handful of microsatellite markers. For the phenotypic dataset, we used eBird reports of likely F1
166 hybrid chickadees across North America (n = 271) to compare metrics of human habitat
167 disturbances for locations of reported hybrids to those of both parental species (n = 271 for both
168 species) to test whether phenotypic hybrids are reported in more disturbed areas. Here, we
169 document widespread hybridization between black-capped and mountain chickadees across their

range overlap (i.e., not a single site of hybridization) and find that hybridization positively correlates with human habitat disturbances.

Materials and Methods

Genomic population sampling

We sampled 196 phenotypic black-capped and 213 phenotypic mountain chickadees at 81 sites from across most of their contemporary North American distributions over ten years (2008-2018) during the May - August breeding season. We captured <10 birds at each site except one, which we accounted for in downstream analyses. Chickadees of both species were captured using either audio lures at mist-nets, baited Potter traps, or by hand at the nest. Birds were morphologically identified as either parental taxon using well-established field characters (i.e., the white supercilium diagnosed mountain chickadees and a black head and white wing bars were used to identify black-capped chickadees; Fig. 1). No phenotypically intermediate individuals were captured in any of the sampling bouts (i.e., intermediate phenotype individuals were not purposely excluded from this study but appear to be rare). The lack of phenotypically intermediate chickadees included in this study despite a broad geographic and temporal sampling scheme highlights that initial hybridization (i.e., the production of F1s) is rare, which is further supported by few records of intermediate birds prior to widely available eBird reports (Hubbard, 1978, Howe, 1985, Martin and Martin, 1996). Birds included in the genomic dataset were sampled in three separate sampling bouts for single-species population genetic studies (Adams & Burg, 2015; Bonderud et al., 2018; Grava et al., 2012). Because chickadees were haphazardly sampled to describe single-species population genetic structure, and not for calculating measures of hybrid ancestry, our sampling schematic is not biased towards overestimating hybridization by

193 focusing sampling on locations where we predicted hybridization is most likely to occur (a
194 prediction that we formed *after* the collection of the samples).
195 Birds were recorded as occurring either in sympatry or allopatry using current
196 distribution maps, eBird observations, and whether or not individuals of both species were
197 sighted and / or captured at a single site (Sullivan et al., 2009). If individuals from both species
198 were captured in a single location, we scored them as sympatric, regardless of distribution maps
199 or eBird data. This allowed for allopatry to occur within the range of overlap (i.e., at high
200 elevation sites where only mountain chickadees were sampled, or low elevation where only
201 black-capped chickadees were sampled). For each species, we included >10 individuals from
202 allopatric populations (mountain chickadees: n = 23 from California, USA; black-capped
203 chickadees: n = 11 Alaska, USA) to identify ancestry informative loci used to calculate hybrid
204 indexes (a measure of genomic admixture) and run simulations (to assign birds to genotypic
205 classes). Small blood samples (< 20 ul) were collected from the brachial vein and stored either as
206 whole blood in 2% lysis buffer, ethanol, or blood on filter paper stored in ethanol. We included
207 several pectoral tissue samples from the Smithsonian Museum and Berkeley Museum of
208 Vertebrate Zoology. Tissue samples were stored in ethanol. We recorded latitude and longitude
209 for capture location of all chickadees. All protocols were approved by University of Colorado
210 Boulder IACUC (2683) panel, UNBC ACUC (protocols 2004-07; A2008.0109.002; 2011.05;
211 2014.06 & 2017.01), and University of Lethbridge (protocols 1028 and 1504) animal care
212 committees and all methods in this study were performed in accordance with relevant guidelines,
213 permits, and regulations.
214
215 *Quantifying and extracting DNA*

Previous studies exploring hybridization between black-capped and mountain chickadees have relied on intermediate plumage or several microsatellite markers to diagnose hybrids. To examine hybridization between black-capped and mountain chickadees, we used a genomic approach to generate hybrid indexes for 409 chickadees from across both species' ranges (including areas of sympatry and allopatry) using reduced-representation sequencing. We extracted DNA from either whole blood or tissue samples using salt-precipitation (Miller, Dykes, & Polesky, 1988). Specifically, 40 µl of the blood sample or ~2 g of tissue was added to 200 µl of homogenizing solution (0.4 M NaCl, 10 mM Tris–HCl pH 8.0, and 2 mM EDTA pH 8.0), 20 µl of 20% SDS, and 10 µl of Proteinase K (20 mg/mL). We vortexed samples and digested at 56 °C overnight. To breakdown cell components and draw off DNA-associated proteins, we removed samples from the heat block, vortexed them, and added 150 µl of 6 M NaCl salt solution to each sample. We then vortexed samples for 30 seconds and centrifuged them for 30 min at 13300 rpm to spin down cell components. After centrifugation, we decanted the supernatant into a clean, labeled 1.5 ml tube and added 2 µl of Glycoblue™ (Thermo Fisher Scientific Waltham, MA) to co-precipitate and stain the DNA. To precipitate the DNA from the supernatant, we added 1000 µl of cold 100% ETOH and incubated the samples in -20 °C for 15 min. After incubating the samples, we centrifuged them for 30 min at 13300 rpm to spin down the precipitated DNA. We then decanted off the supernatant and added 1000 µl room temp 70% ETOH to wash the DNA and remove remaining salt. We repeated this wash step as needed until no visible salt remained around the DNA pellet. After washing the DNA, we air-dried the pellets for 10 min. Lastly, we resuspended the DNA pellet in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA at pH 8-9) and incubated at 37 °C for 15 min. Samples were incubated at 4 °C overnight

to fully dissolve the DNA pellet. We quantified DNA concentrations using a Qubit 3.0 fluorometer (Invitrogen; Carlsbad, CA).

Library preparation and genomic sequencing

To generate genomic sequence data, we used double-digest restriction site-associated DNA sequencing (ddRAD) following the protocol of Peterson, Weber, Kay, Fisher, & Hoekstra (2012) with modifications as described in Thrasher, Butcher, Campagna, Webster, & Lovette (2018). Because ddRAD digests DNA with two restriction enzymes, it is a cost-effective approach for generating genomic sequences for large sample sizes of non-model organisms. For each sample, we digested ~500 ng of DNA with the restriction enzymes *SbfI* and *MspI* (New England BioLabs, Ipswich, Massachusetts, USA). The ends of the digested DNA were ligated to P1 and P2 adaptors using T4 DNA Ligase (New England BioLabs). We ligated P1 adapters to 5' end of digested DNA with a *SbfI* compatible overhang and an inline barcode (5-7 bp long) to identify individual samples bioinformatically later in the analysis. We ligated P2 adaptors to the 3' end of the digested DNA with a *MspI* compatible overhang. We pooled samples with unique P1 barcodes into 22 different indexing groups after digestion/ligation. To remove enzymes and small DNA fragments, we purified DNA in each index group using 1.53 Agencourt AMPure XP beads (Beckman Coulter, Pasadena, California, USA). To ensure the same loci are recovered in all index groups, we size-selected fragments between 400 and 700 bp using Blue Pippin (Sage Science, Beverly, Massachusetts, USA). To add the full Illumina TruSeq primer sequences and unique indexing primers into each library, we performed a low cycle number PCR with Phusion High-Fidelity DNA Polymerase (New England BioLabs) with the following thermocycling profile: 98°C for 30 s followed by 11 cycles at 98°C for 5s, 60°C for 25s, and 72°C for 10s with

a final extension at 72°C for 5 min. We visualized amplified products on a 1% agarose gel and performed a final 0.73 AMPure cleanup to eliminate DNA fragments smaller than 200 bp. We visualized libraries on a fragment Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) to determine fragment size distribution. Finally, all 22 index groups were combined at equimolar ratios and sequenced on one Illumina NextSeq 500 lane (single-end, 150 bp) at the Cornell University Biotechnology Resource Center. Raw sequence data and associated metadata are available at the sequence read archive.

Quality control and filtering

To demultiplex chickadee samples, we used the process_radtags command in STACKS 2.41 Catchen, Hohenlohe, Bassham, Amores, & Cresko (2013). After demultiplexing, we trimmed and filtered sequence reads using a custom script. Specifically, we removed Illumina adapters provided in the TruSeq3-PE.fa file using TrimmomaticSE (Bolger, Lohse, & Usadel, 2014). First, we searched for seed matches allowing maximally one mismatch. Using a sliding window trimming approach, we scanned sequence reads from the 5' end in 4 bp windows and removed sequence reads when the average Phred quality score fell below 20. Finally, we dropped any reads shorter than 36 bp long. We used fastqc (Andrews, 2010) to calculate quality scores. After filtering, we aligned reads to a high-quality black-capped chickadee reference genome (Wagner, Curry, Chen, Lovette, & Taylor, 2020) using bwa mem (Li, 2013) and a custom script to create a sam file. We converted sam files to bam files using samtools (Li et al., 2009). Next, we used Picard-tools (Broad Institute, 2019) to mark duplicates and add/replace read groups. Lastly, we called variants based on a previously assembled black-capped chickadee reference genome (Wagner et al., 2020) with bcftools (Narasimhan et al., 2016) and the mpileup command

284 resulting in 517,699 unique loci. After calling variants, we filtered out single nucleotide
285 polymorphisms (SNPs) with a Phred Score below 30, loci with a minor allele frequency less than
286 0.01 and 50% missingness, and loci with a maximum depth of 10x and a minimum depth of 1x.
287 To ensure we were only using informative alleles, we used VCFtools (Danecek et al., 2011) to
288 calculate the fixation index (F_{ST}), a measure of population differentiation, per SNP. F_{ST} ranges
289 from 0 - 1, and values closer to 1 indicate fixed allelic differences between populations, or
290 species in this case, at a given locus. After calculating F_{ST} for each SNP, we filtered SNPs to
291 retain loci with $F_{ST} > 0.80$ (n = 443) to improve our estimation of population differentiation and
292 hybrid indexes. After filtering SNPs, we converted our variant call format (vcf) file to
293 STRUCTURE format using PGD Spider version 2.1.1.5 (Lischer & Excoffier, 2012) for
294 downstream analyses. This VCF table is available on data dryad.
295
296 *Examining population genetic structure*
297 First, we used the program STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) to
298 assess the number of genetic clusters in our genomic dataset on a thinned SNP dataset after
299 pruning SNPs in linkage and retaining only ancestry informative markers (n = 443 SNPs). We
300 ran STRUCTURE for K = 1, 2, & 3 and, using a delta K approach, confirmed that the best
301 supported number of clusters was K = 2 (Supplementary Fig.1). We similarly explored
302 population structure using a principal component analysis (PCA) of genomic variation between
303 black-capped and mountain chickadees (Supplementary Fig. 2) using the same SNP dataset (n =
304 443 SNPs).
305
306 *Identifying hybrids from genomic data*

To identify hybrids in our genomic dataset, we first calculated hybrid indexes and heterozygosity for all individuals using gghybrid (Bailey 2018) and a custom script (available on github), respectively, then we used NewHybrids 1.1 Beta 3 (E. C. Anderson & Thompson, 2002) following the approach of Shurtliff et al. (2014) to assign individuals to one of eight genotypic classes (up to two generations of backcrosses). First, we calculated hybrid indexes (HI) for all 409 individuals using the R package gghybrid (Bailey, 2018). Hybrid index ranges from 0 - 1 with 0 indicative of one parental population (here, black-capped chickadees) and 1 representing the other parental species (here, mountain chickadees). First generation hybrids (F1s) have a HI of ~0.50. gghybrid calculates HI based on the method of Buerkle (2005), and uses Bayesian Markov chain Monte Carlo to estimate what proportion of alleles originate from a predefined parental population. We assigned parental populations as allopatric black-capped chickadees from Alaska, USA (HI = 0) and allopatric mountain chickadees from California, USA (HI = 1). We used the esth function with a burn-in of 3,000 iterations and 10,000 total iterations (i.e., default settings) to estimate hybrid indexes for all birds. We did not use fixed loci (e.g., F_{ST} =1) for estimating hybrid indexes because our reduced-representation approach did not capture enough fixed alleles to inform HI estimation. We also followed the above approach of estimating hybrid indexes using loci with $F_{ST} > 0.65$ (n = 955), which yielded similar results to using loci with $F_{ST} > 0.80$ but had larger confidence intervals due to a greater amount of missing data. We report HIs and confidence intervals generated from loci with $F_{ST} > 0.80$ (Supplementary Table 1 & Supplementary Fig. 3). After generating hybrid indexes, we re-scaled hybrid index from 0 - 0.5 using the equation, g(x) = 0.5 - abs(x - 0.5) to facilitate downstream analyses so 0 = parental individuals of both species and 0.5 = F1 hybrids.

Next, we calculated heterozygosity for all individuals using 443 loci and compared heterozygosity to hybrid index for each individual. Parental genotypes are expected to have scaled hybrid indexes close to 0 and low heterozygosity. First generation hybrids (F1s) should have high heterozygosity (close to 1) and high scaled hybrid indexes (~ 0.5). In contrast, later generation hybrids (F2s and backcrosses), should have lower heterozygosity and intermediate scaled hybrid indexes.

Finally, we assigned birds to one of eight genotypic classes (up to two generations of backcrossing in either direction) using NewHybrids 1.1 Beta 3. First, we further filtered our SNP dataset to include ancestry informative loci and improve genotypic class assignment. Specifically, we filtered our SNP set to include loci with Fst > 0.90 and minor allele frequency in black-capped chickadees < 0.10 (n = 123) between our allopatric populations. Then we tested the power of this subset of SNPs to identify hybrids by using NewHybrids to simulate 184 known-hybrid individuals from a random subset of parental-type individuals (i.e., allopatric birds) and examined their probability of assignment to genotypic classes. We simulated 50 parental mountain chickadees, 44 black-capped chickadees, 15 F1s, 15 F2s, and then 15 of each direction of backcross up to 2 generations of backcrosses (i.e., 15 BCCH_Bx, 15 MOCH_Bx, 15 BCCH.2_Bx, & 15 MOCH.2_Bx). All individuals in this simulated dataset had high probability of assignment to genotypic classes in NewHybrids (p of $z > 0.95$; Supplementary Fig. 4) so we used the same subset of 123 SNPs to assign all unknown (i.e., real) chickadees in our dataset (n = 409) to the same eight genotypic classes using NewHybrids using a cut-off p of $z > 0.80$ (e.g., parental black-capped chickadee, parental mountain chickadee, F1, F2, and each direction of backcross up to two generations of backcrossing). Birds with p of $z < 0.80$ were not assigned to any genotypic class (n = 27) and were dropped from downstream analyses (Table 1). For birds

that were successfully assigned to genotypic classes, we further categorized these birds as either parentals or hybrids. Birds assigned to F1, F2, or any class of backcross were classified as hybrids. Birds assigned to parental classes were considered parentals.

Selecting phenotypic hybrids and parental chickadees from eBird

For our phenotypic dataset, we downloaded all observations of phenotypic black-capped / mountain chickadee hybrids (*Poecile gambeli x Poecile atricapillus*) from eBird from Jan 1989 – Dec 2021 (n = 751). Next, we filtered these observations by unique combinations of locality and date (month and year) to remove multiple sightings of the same bird. This yielded 271 unique observations of phenotypic hybrids from across North America (Fig. 4a). Then, we downloaded all observations of black-capped (n = 8,942,641) and mountain chickadees (n = 624,895) from the same date range as the hybrid dataset (1989-2021). For each parental taxon, we similarly filtered parental taxa observations to unique combinations of location and date to remove repeat sightings. For black-capped chickadees, we restricted observations to western North America (i.e., all observations west of 102° W) to match distributions of black-capped chickadee sightings to those of hybrids and mountain chickadees. We did not geographically restrict observations for mountain chickadees since their distribution is limited to western North America. Finally, we randomly sampled 271 observations for each parental taxa from these filtered subsets (Fig. 4a & Supplementary Fig. 5).

Calculating human habitat disturbance

To measure human landscape disturbances across the continental scale, we used the Global Human Influence Index (Geographic) v2 dataset (1995-2004; WCS and Columbia University

2005). This dataset is a map of anthropogenic impacts on the environment in geographic projection and is comprised of the Human Influence Index (HII) normalized by biome and realm. The HII is a measure of habitat disturbance produced as a global dataset of 1-kilometer grid cells, created from nine global data layers including human population pressure (population density), human land use and infrastructure (built-up areas, nighttime lights, land use/land cover), and human access (coastlines, roads, railroads, navigable rivers). Again, this metric purposely excludes global climate change, and is a holistic metric of human habitat disturbances, beyond land cover changes, to the physical environment. For both the genomic and phenotypic dataset, we plotted the capture location of individuals on the Global Human Influence Index map (Fig. 2a & Supplementary Fig. 5, respectively) and extracted Human Influence Index (HII) values for each chickadee sampling location using R v.4.2.0 (R Development Core Team 2018). The Human Influence Index (HII) ranges from 0 – 64 and creators of the dataset denote that HII < 10 indicate wild habitats, whereas HII > 10 are disturbed habitats (WCS and Columbia University 2005).

After calculating HII for each genotyped chickadee, we further classified chickadees based on their capture location as either occurring in wild habitats (HII ≤ 10) or disturbed habitats (HII > 10). While most of the genotyped chickadees were adults and therefore captured away from their natal nest, dispersal distances for both species of chickadees is small (< 2 km; Weise and Meyer, 1979, Pravosudov et al., 2003). Given that the resolution of the Global Human Influence Index is 1 km pixels, the calculated HII for each chickadee's sampling location is a reasonable proxy for the HII of their natal site.

Statistical Analyses

We explored the relationship between landscape disturbance and chickadee hybridization using both genomic and phenotypic datasets. For our genomic dataset, we first performed a generalized additive mixed model to explore the relationship between HI and HII as continuous, rather than categorical, variables. We then categorized both hybrid status and disturbance and used parametric statistical tests to compare 1) the proportion of hybrids found in disturbed versus wild habitats, 2) the mean hybrid index for chickadees sampled in wild versus disturbed habitats, and lastly, 3) the average disturbance metric (HII) of sampling location for hybrids compared to parentals. Finally, for our phenotypic dataset of hybrids, we used parametric tests to compare the mean disturbance metric (HII) of hybrid sampling locations to the sampling locations of both parental species.

For our genomic dataset, we used the package *mgcv* in R to construct a generalized additive mixed model with a Gaussian distribution to explore whether human influence index (HII) significantly predicts hybrid index (HI), while controlling for whether birds were sampled in *Sympatry*. We also included both *Year* and *Site* as random effects to control for non-independence both within sampling bouts and at single sites (i.e., to control for relatedness among individuals at the same site). Specifically, we constructed the following model:

Hybrid Index ~ s(Human Influence Index) * s(Sympatry) + (1|Year) + (1|Site)

Including both HII and whether birds were sampled in sympatry allows us to distinguish whether disturbance, independent of sympatry, is correlated with hybrid index, or whether birds only overlap in disturbed areas, and therefore can only hybridize in disturbed areas. We did not use a linear model to explore the relationship between hybrid index and human influence index

because we do not expect the relationship between hybrid index and human habitat influence to be linear. The ability of chickadees to survive and reproduce in heavily impacted habitats likely declines after some critical threshold, reducing the opportunity for hybridization to occur at maximum human influence index values (e.g., the center of an urban area).

Second, to test whether more hybrids (based on NewHybrids assignment) were detected in disturbed habitats versus wild habitats, we used a two-proportion, right-tailed Z-test with a Yates continuity correction. Then, we compared the average hybrid index for chickadees sampled in disturbed habitats versus chickadees sampled in wild habitats using Welch's two-sample t-test. To explore if parentals and hybrids cluster in landscapes with differing disturbance values, we tested whether the average disturbance value (HII) of sampling location for hybrids (based on NewHybrids assignment) was greater than parentals.

Finally, for our phenotypic dataset, we tested whether phenotypic hybrids from eBird are observed in more disturbed habitats using a one-way ANOVA to explore the effect of species (Hybrid v. *P. gambeli* v. *P. atricapillus*) on the HII of birds' sampling locations, followed by a Tukey HSD test. As a caveat, the eBird data reported here was collected over 10 years (2011-2021), however, our measure of habitat disturbance (HII) is a single aggregate from 1995-2004. While we expect HII to increase through time, we are unable to test this relationship directly. However, the single measure of HII used is still a reasonable proxy for comparing differences in disturbance association between the two chickadee species and their hybrids.

Results

Black-capped and mountain chickadees exhibit strong population structure

We found that despite hybridizing throughout their ranges (Fig. 2b), black-capped and mountain chickadees exhibit distinct population structure. PC1 clearly separates black-capped chickadees from mountain chickadees (PC1 = 72.2%; Supplementary Fig. 2), and STRUCTURE results indicate strong population genetic differentiation between the two species (K =2; Supplementary Fig. 1).

Initial hybridization between black-capped and mountain chickadees is rare

Using 443 highly differentiated loci ($F_{ST} > 0.80$), we calculated hybrid indexes and heterozygosity for 409 chickadees. After scaling hybrid index from 0 – 0.5, hybrid index ranged from 0.0 to 0.47 for phenotypic black-capped chickadees (i.e., birds scored as black-capped chickadees in the hand) and 0.0 to 0.13 for phenotypic mountain chickadees (Fig. 3a). Heterozygosity ranged from 0.13 to 0.83 for phenotypic black-capped chickadees and 0.0 to 0.23 for phenotypic mountain chickadees (Fig. 3a). Using NewHybrids and a subset of 123 SNPs, we found that 43% of chickadees sampled had some proportion of hybrid ancestry: 160/375 sympatric chickadees were classified as one of the six hybrid genotypic classes (Table 1, Fig. 3b). For 53 of the 160 detected hybrids, we sampled parentals in the same sampling bout (i.e., in same year and site). We were able to classify nearly all birds to one of the eight genotypic classes (Table 1, Fig. 3b). We were unable to classify 27 birds to any genotypic class. All of the birds not meeting our cut off for assignment were scored phenotypically as black-capped chickadees, and all were split between parental black-capped chickadee and first/second generation backcrosses. Thus, it is likely these birds are later generation black-capped chickadee backcrosses, a pattern that is supported by whole genome data from a single sampling site (Grabenstein et al., *in review*). We detected two likely F1s (Fig. 3). Both of these birds were

466 males (one adult & one Hatch Year) and both had black-capped phenotypes (i.e., lacked a
467 supercilium), as identified by trained researchers in the field.
468
469 *Hybridization correlates with human habitat disturbances*
470 We explored whether human habitat disturbances correlated with chickadee hybridization using
471 a generalized additive model. We found that chickadee hybrid indexes were significantly higher
472 in more disturbed habitats: HI significantly increased with HII ($\beta = 0.0028$, SE = 0.00074, $P <<$
473 0.001). Neither *Sympatry* ($\beta = 0.10$, SE = 0.12, $P = 0.39$), *Sympatry*HII* ($\beta = -0.00017$, SE =
474 0.0032, $P = 0.96$), nor *Year* (HII: $\beta = 0.00031$, $P = 0.55$) significantly predicted hybrid index
475 (Fig. 2c). We did detect a significant effect of (1|Site) on hybrid index: (1|Site) (edf =
476 38.35, $P << 0.001$) because 81 sites were included in our analyses. We found that with an
477 increase of 1 HII, our model predicted an increase of 0.0028 in HI (i.e., a significant, but small in
478 magnitude, effect).
479 We used a two-sample, right-tailed Z-test to further explore this positive correlation. We
480 found that significantly more hybrids were sampled in disturbed habitats versus wild habitats
481 (HII $\leq$ 10 indicate wild habitats, HII > 10 are disturbed habitats; $\chi^2 = 20.91$, df = 1, p-value <
482 0.0001). We identified 98 hybrid chickadees in disturbed habitats out of 187 birds sampled
483 (98/187 = 52.4%) versus 62 hybrid chickadees out of 211 sampled birds in wild habitats (62/211
484 = 29.3%). Additionally, we found that the average hybrid index of birds in disturbed habitats (HI
485 = 0.17 ± 0.13; n = 187) was significantly higher than the average hybrid index of birds in wild
486 habitats (HI = 0.09 ± 0.12; n = 211; t = 5.17, df = 373.63, p-value < 0.0001; Fig. 2c).

Lastly, the average disturbance value (HII) for hybrids (hybrid HII = 21.01 ± 15.48; n = 160) was significantly higher compared to the average disturbance value (HII) for parentals (parental HII = 12.13 ± 9.26; n = 210) ($t = -6.41$, $df = 241.92$; p-value < 0.001).

Black-capped chickadees and hybrids are more disturbance-associated than mountain chickadees

From eBird, we found significant differences in habitat disturbance values for the report locations of black-capped, mountain, and hybrid chickadees (ANOVA: $F_{2,398} = 80.47$, $P < 0.0001$; Fig. 4b). Post hoc Tukey tests showed that disturbance values for locations of phenotypic hybrids (mean HII for hybrids = 38.53) were significantly higher than for mountain chickadee reports (mean HII *P. gambeli* = 24.28; $P < 0.001$), but not significantly different from black-capped chickadee reports (mean HII *P. atricapillus* = 39.54; $P = 0.38$). Similarly, we found that the mean disturbance values for black-capped chickadees were significantly higher than the mean disturbance values for mountain chickadee reports ($P < 0.001$).

Discussion

We found that black-capped and mountain chickadees hybridize across their range, and that hybridization is correlated with human habitat disturbances. Initial hybridization (i.e., production of F1s) is rare, and the F1s that are produced appear to predominantly backcross with black-capped chickadees, which produces cryptic later generation hybrids. Surprisingly, we found that nearly every black-capped chickadee we sampled contained some proportion of hybrid ancestry, indicating that post-zygotic isolation between black-capped and mountain chickadees is incomplete. Our results are concerning because we do not understand the long-term

consequences of hybridization for these songbirds. Understanding what becomes of recently hybridizing species following large-scale habitat disturbances is a new, but pressing, consideration for successfully preserving biodiversity in a rapidly changing world.

We found a significant positive correlation between chickadee hybridization and human habitat disturbances. Importantly, we controlled for sympatry in this analysis and found no significant relationship between either sympatry and hybrid index, or disturbance*sympatry and hybrid index, highlighting that overlap between the two species is not restricted to disturbed habitats. Similarly, after categorizing birds as hybrid or parental and their sampling locations as either wild or disturbed, we found that birds in disturbed habitats had a higher mean HI compared to birds from wild habitats. Interestingly, this pattern does not appear to be driven by a few, rare F1s with high HIs sampled in disturbed habitats. Instead, we found more later-generation hybrids (with smaller HIs) in disturbed habitats. Further, from our genomic dataset, we found that hybrids were sampled in significantly more disturbed areas than parentals. From our analysis of eBird data, we found that mountain chickadees are reported in significantly less disturbed habitats than both hybrids and black-capped chickadees. Given that chickadees are resident songbirds with small dispersal distances (< 2 km), it is likely that both hybrids and black-capped chickadees are sired (and then reside) in disturbed habitats, rather than dispersing into them from neighboring rural areas. Together, these complementary datasets suggest that hybridization either occurs more readily in urban areas, hybrids are better able to survive in disturbed areas, or both. The possibility that human disturbance facilitates black-capped chickadee population expansion and increased interactions with mountains chickadees is intriguing and will be further investigated.

We identified 160 chickadees as hybrids out of 375 sympatric chickadees. The majority of hybrids identified were black-capped chickadee backcrosses: either first-generation backcrosses (n = 43) or second-generation backcrosses (n = 109), with comparatively few mountain chickadee backcrosses (second-generation mountain chickadee backcrosses: n = 6), F1s (n = 2) or F2s (n = 1) detected. This highlights that while production of F1s is rare, hybrids are able to survive and reproduce, at least to some degree. F1s also appear to predominantly backcross with black-capped chickadees rather than mountain chickadees. Whether this pattern is due to differential population sizes in urban areas (i.e., larger populations of urban black-capped chickadees), F1 preference for black-capped chickadees, or due to genetic incompatibilities that produce lethal combinations when F1s backcross with mountain chickadees remains unclear. Interestingly, 129/271 of the unique eBird hybrid sightings recorded hybrid chickadees associating with only black-capped chickadees (eBird 2021). In contrast, there were 22 records of eBird-reported hybrids observed with only mountain chickadees. In 69 observations, hybrids were reported in mixed species flocks with both black-capped and mountain chickadees, and in 51 instances, hybrids were not reported with either species. This pattern of phenotypic hybrids associating with black-capped chickadees matches our genetic data, which indicate repeated, and generally unidirectional, backcrossing between F1s and black-capped chickadees.

Historically, hybridization between black-capped and mountain chickadees has been considered rare but this is likely because hybrids beyond the F1 generation cannot be identified using phenotype alone. Surprisingly, our data suggest that many sympatric black-capped chickadees in disturbed habitats contain some proportion of hybrid ancestry though they lack intermediate phenotypes. Genotyped hybrid individuals were classified as black-capped or mountain chickadees rather than hybrids using phenotype by trained researchers in the field (i.e.,

555 hybrids were not recognized as hybrids in the hand). For example, both possible F1s from this
556 study were not recognized as hybrids in the hand. Despite many continental records of
557 phenotypically intermediate individuals (n = 271; eBird 2021), we captured no birds with
558 intermediate phenotype and therefore included none in our genomic analyses. While this
559 potentially leads us to underestimate the extent of hybridization in this system, it is more likely
560 that our lack of phenotypically intermediate individuals highlights the rarity of the production of
561 F1 hybrids. This makes our finding of extensive hybrid ancestry in black-capped chickadees
562 more surprising: despite rare initial hybridization there are lasting signatures of admixture in
563 many sympatric chickadee populations. Hybridization between black-capped and mountain
564 chickadees is likely much more common than previously thought, especially if most later
565 generation hybrids are cryptic and do not have intermediate phenotypes, as our data suggests.
566 Ultimately, assessments of the directionality of hybridization, frequency distribution of various
567 hybrid classes, as well as the genetic architecture of hybrid phenotypes are lacking and should be
568 a focus for future work.

569 While robust quantifications of the relative strength of pre- and post-zygotic isolating
570 barriers between black-capped and mountain chickadees are lacking, the two species appear to
571 have multiple pre-zygotic reproductive isolating barriers, including ecological differentiation in
572 physical, behavioral, and temporal isolation. In sympatry, the two species occupy different, but
573 often neighboring, habitats and are effectively separated along elevational gradients. In areas of
574 sympatry compared to allopatry, mountain chickadees, the subordinate species, appear to have
575 evolved character displacement. Mountain chickadees in sympatry with more dominant black-
576 capped chickadees have shifted their song frequency and chorusing behavior (Grava et al., 2013;
577 Lohr, 2008) compared to mountain chickadees in allopatry and breed later in areas of sympatry

578 compared to neighboring sympatric black-capped chickadees (Freshwater, Ghalambor, & Martin,
579 2014). It is these prezygotic barriers that appear to break down in human modified habitats.
580 Nearly every black-capped chickadee we sampled had genomic evidence of
581 hybridization, indicating incomplete post-zygotic isolation between the black-capped and
582 mountain chickadees. Given that black-capped and mountain chickadees are not sister taxa, this
583 is somewhat surprising; however, similar patterns have been found in other systems (e.g.,
584 Kuhlwilm et al., 2016). The degree of genetic differentiation between the two species is
585 significant (genome-wide $F_{ST} = 0.34$) and it is probable that genetic incompatibilities reduce
586 hybrid fitness (e.g., Price and Bouvier, 2002) such that hybrids do not persist well in the
587 population or that certain crosses are sterile or inviable. Previous work has documented reduced
588 body condition (calculated using scaled morphology) for sympatric black-capped and mountain
589 chickadees, thought to be driven by cryptic, low fitness hybrids (Grabenstein et al., 2022).
590 Specific intrinsic incompatibilities between black-capped and mountain chickadees have not yet
591 been investigated, but may include breakdowns in metabolic and cognitive function as is the case
592 for other hybridizing chickadees (Taylor et al., 2014; Wagner et al., 2020). Indeed, fatty acid
593 synthesis pathways differ significantly between black-capped and mountain chickadees (S.
594 Taylor, unpublished data). Ultimately, understanding the long-term outcomes of recent, and
595 potentially novel, hybridization due to human habitat disturbances is an outstanding question and
596 should be prioritized given the rapid rate of global change.
597 Disturbance-mediated hybridization is being documented at an increasing frequency;
598 however, little is known about how human habitat disturbances drive hybridization and what
599 becomes of hybridizing populations in the long term. While experimental explorations are
600 lacking, human habitat disturbances appear to promote hybridization by three potential, non-

mutually exclusive mechanisms: (1) bringing formerly isolated (ecologically and/or temporally) species together by reducing habitat structure and/or by altering phenology, (2) impeding the ability of naturally co-existing species to discriminate between conspecifics and heterospecifics by visual, chemical, and acoustic interference, and/or (3) creating novel environments with reduced selection against hybrids such that they survive and are detected in populations (Grabenstein & Taylor, 2018). We hypothesize that human-mediated disturbances bring formerly ecologically isolated black-capped and mountain chickadees together in artificially extended transitional forest habitat. In most western urban areas, where hybrids appear to be produced and persist, humans have cultivated an artificial mosaic of native and non-native deciduous trees alongside native conifers (Ma et al., 2020). Based on disturbance values from eBird reports, we found that black-capped chickadees appear to be more human-associated than mountain chickadees. It is possible that large urban populations of black-capped chickadees that rely on planted deciduous trees in locations that would otherwise be unsuitable for black-capped chickadees (e.g., the high plains of Colorado) might promote hybridization by increasing contact between black-capped and mountain chickadees at range edges. We found that hybrids reported in eBird are found in highly disturbed habitats, but that these disturbance values do not significantly differ from black-capped chickadees, suggesting that hybridization is likely occurring in disturbed habitats since dispersal distance for both chickadee species is limited. The fact that both hybrid and black-capped chickadees are found in more disturbed habitats than mountain chickadees, implicates urban areas as playing a role in hybridization, potentially by artificially increasing black-capped populations in urban areas, reducing selection against hybrids via supplemental feeding (i.e., urban feeders), or a combination of both mechanisms.

Conclusions

We found that black-capped and mountain chickadees hybridize across their range and that hybridization in this system is significantly correlated with human habitat disturbance. The majority of published studies have quantified human-mediated hybridization at small spatial scales, often in a single city or field site, and have suggested disturbance as a post-hoc explanation for increased and/or novel hybridization. We cannot predict what becomes of hybridizing species following human habitat disturbances or if the most likely long-term outcomes (e.g., adaptive introgression, species collapse, stable hybrid zone) differ from those in more classic hybrid zones. Regardless, hybridization because of human habitat disturbances will impact biodiversity and population persistence with accelerating global change by either reducing genetic diversity or increasing adaptive potential. Understanding the causes and consequences of disturbance-mediated hybridization is of utmost importance for conserving biodiversity in a rapidly changing world.

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Tables

Table 1. Summary of each genotypic class and their associated genomic metrics. Genotypic classes are: parental black-capped chickadee (BCCH), parental mountain chickadee (MOCH), first generation hybrid (F1), second generation hybrid (F2), first generation black-capped chickadee backcrosses (BCCH_BX), first generation mountain chickadee backcross (MOCH_BX), second generation black-capped chickadee backcross (BCCH.2_BX), & second generation mountain chickadee backcross (MOCH.2_BX).

HYBRID CLASS	N	MEAN HYBRID INDEX ± SD (UNSCALED)	MEAN HETEROZYGOSITY ± SD
BCCH	5	0.10 ± 0.02	0.22 ± 0.03
MOCH	205	0.98 ± 0.02	0.03 ± 0.03
F1	2	0.48 ± 0.06	0.79 ± 0.05
F2	1	0.44	0.63
BCCH_BX	43	0.29 ± 0.04	0.56 ± 0.07
MOCH_BX	0	NA	NA
BCCH.2_BX	109	0.18 ± 0.04	0.33 ± 0.07
MOCH.2_BX	6	0.90 ± 0.04	0.21 ± 0.03
UNASSIGNABLE	27	0.26 ± 0.11	0.37 ± 0.12

Figure Legends

Figure 1. **Geographic context of study.** (a) Range distribution of black-capped (pink) and mountain chickadees (blue), highlighting substantial area of range overlap (purple). Map created by Daniel Jackson. (b) Sampling schematic of black-capped (pink) and mountain chickadees (blue) included in the study. (c) Locations of all eBird sightings of reported hybrids (purple) from 1989-2021. Hybrids are classified by intermediate plumage (photo inset C; eBird): namely, buffy sides and white wing bars of black-capped chickadees (middle left) paired with smaller-than typical white eyebrow of mountain chickadees (bottom left). Chickadee illustrations by Jessica French. Map lines delineate study areas and do not necessarily depict accepted national boundaries.

Figure 2. **Human habitat disturbance and chickadee hybridization are positively correlated.** (a) Map of Human Influence Index (0-64) with sampling locations of chickadees (black crosses). (b) Map of all sampled chickadees colored by hybrid indexes from 0 (white) to 0.5 (dark purple). Insert (upper right) shows only hybrids. (c) Chickadee hybrid index significantly increases with human influence index. Points represent individual chickadees. Green dashed line denotes 'wild' habitat cut-off (HII = 10). Blue Trendline with shaded 95% confidence interval show prediction from generalized additive model. (d) More hybrids sampled in disturbed habitats. Mean of hybrid index are significantly higher in disturbed habitats (orange) compared to wild habitats (green).

Figure 3. **Many, late-generation black-capped chickadees identified.** (a) Heterozygosity plotted against hybrid index for black-capped (pink) and mountain chickadees (blue). Allopatric populations for black-capped chickadees shown in dark pink. Allopatric mountain chickadees shown in dark blue. (b) Assignment probabilities for chickadees to genotypic classes: parental black-capped chickadee (pink), parental mountain chickadee (blue), first generation hybrids (purple), second generation hybrids (dark purple), first generation mountain chickadee backcrosses (medium blue), first generation black-capped chickadee backcrosses (medium pink), second generation mountain chickadee backcrosses (dark blue) & second generation black-capped chickadee backcrosses (dark pink). * indicates allopatric populations.

Figure 4. **Hybrid and black-capped chickadees are more disturbance-associated than mountain chickadees**. (a) Maps of eBird report locations for subset of black-capped chickadees (top, pink), all unique phenotypic hybrids reported to eBird (middle, purple), and subset of mountain chickadees (bottom, blue). (b) Mean disturbance values for reports of both phenotypic hybrids from ebird (purple; mean Hybrid HII = 38.53) and black-capped chickadees (mean *P. atricapillus* HII = 39.54*;* pink) were significantly higher than for mountain chickadee reports (mean *P. gambeli* HII = 24.28; blue). No significant difference between hybrid and black-capped chickadee HII. Dashed green line (HII = 10) indicates wild (below line) v. disturbed (above line) habitat.