Genomic Comparison of Indigenous African and Northern European Chickens Reveals Putative Mechanisms of Stress Tolerance Related to Environmental Selection Pressure

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ABSTRACT Global climate change is increasing the magnitude of environmental stressors, such as temperature, pathogens, and drought, that limit the survivability and sustainability of livestock production. Poultry production and its expansion is dependent upon robust animals that are able to cope with stressors in multiple environments. Understanding the genetic strategies that indigenous, noncommercial breeds have evolved to survive in their environment could help to elucidate molecular mechanisms underlying biological traits of environmental adaptation. We examined poultry from diverse breeds and climates of Africa and Northern Europe for selection signatures that have allowed them to adapt to their indigenous environments. Selection signatures were studied using a combination of population genomic methods that employed FST, integrated haplotype score (iHS), and runs of homozygosity (ROH) procedures. All the analyses indicated differences in environment as a driver of selective pressure in both groups of populations. The analyses revealed unique differences in the genomic regions under selection pressure from the environment for each population. The African chickens showed stronger selection toward stress signaling and angiogenesis, while the Northern European chickens showed more selection pressure toward processes related to energy homeostasis. The results suggest that chromosomes 2 and 27 are the most diverged between populations and the most selected upon within the African (chromosome 27) and Northern European (chromosome 2) birds. Examination of the divergent populations has provided new insight into genes under possible selection related to tolerance of a population’s indigenous environment that may be baselines for examining the genomic contribution to tolerance adaptations.

KEYWORDS tolerance selective pressure genomic variation adaptation environment

The nature of specialized trait selection in livestock species within commercial operations, and in some instances experimental settings, has made them more sensitive to environmental extremes or rapid alterations in their environment (McMichael et al. 2007; Berry et al. 2011; Cisar et al. 2011; van der Most et al. 2011; Kantanen et al. 2015). This issue may be a by-product of artificial selection within very controlled production environments (Thornton 2010; Herrero and Thornton 2013; Lawrence and Wall 2014) and is particularly true for genetically elite livestock and poultry. This susceptibility to environmental extremes is a hindrance to expansion of the poultry industry into areas of the world where the environment and its stressors drastically differ from the environment under which selection was performed (Canario et al. 2013; Lawrence and Wall 2014; Rothschild and Plastow 2014). Multiple environmental stressors triggered by climate change phenomena, such as extreme weather, can generate multiple stressors that test the ability of commercial livestock to survive and produce in harsh environments (Thornton et al. 2009; Cisar et al. 2011). For
livestock, environmental stressors can prove insurmountable when genetic potential and feed resource allocation are not matched (van der Most et al. 2011). Future agriculture will take place in more areas where changes in climate have made the environment less amenable to commercial livestock that lack the genetic potential to adapt (McMichael et al. 2007; Thornton et al. 2009; Rothschild and Plastow 2014). In particular, for poultry, an inability to cope with rapid or extreme changes to their environment can be costly and limits their potential in developing countries (Lara and Rostagno 2013; Porto-Neto et al. 2014; Rothschild and Plastow 2014) where poultry are vital for economic and nutritional importance (Neumann et al. 2002). To gain a better understanding of how to mitigate the impacts of environmental stressors by genetic approaches, it is prudent to examine native livestock species that have evolved under a given environment. For example, these could be landrace or village chickens indigenous to environments where environmental stress is endemic or mimicked (Seebacher 2009; Zakrzewska et al. 2011; Mwacharo et al. 2013; Porto-Neto et al. 2014).

The current study utilized populations of noncommercial indigenous African and Northern European (Tixier-Boichard et al. 2011) chickens to identify signatures of selection centered on survival of the environmental stressors of their respective habitats. The ability of the environment to apply selection pressure has been observed in studies of other livestock species. In sheep and swine, researchers were able to show correlations between selection signals and the local environment of different sheep breeds that were related to the shaping of adaptive variations (Lv et al. 2014; Ramirez et al. 2015; Yang et al. 2016). Given the extreme differences between the climate, geography, and the animals of Africa and Northern Europe, the selective pressure enforced by the environment should be detectable in the genomic architecture of the two deviating groups of populations (Hoffmann and Hercus 2000; Lyimo et al. 2014). By surviving in environmental conditions that can be considered as stressors for multiple generations, chickens indigenous to harsh environments should have been under selective pressure to develop tolerance at a genomic level (Clarke 2003; Benestad 2005; Chen et al. 2009; Seebacher 2009; Nardone et al. 2010; Lawrence and Wall 2014; Porto-Neto et al. 2014). Our study used this contrast of bird populations and high-density genomic variation data to examine how pressure from environmental stress can lead to advantageous genomic adaptations that allow for survival in harsh environments.

MATERIALS AND METHODS

The techniques used to analyze the population data allowed for the examination of selective pressure from the environment. The analysis workflow was composed of a combination of allele frequency and haplotype-based (Voight et al. 2006; Qanbari et al. 2011, 2012, 2014; Gautier and Vitalis 2012) detection methods performed on data generated with the 580k SNP Affymetrix Axiom Genome-Wide Chicken Genotyping Array (Kranis et al. 2013). These methods have previously been successfully employed to find areas under selective pressure that were associated with known and novel traits of biological interest (Voight et al. 2006; Qanbari et al. 2012, 2014; Gholami et al. 2014; Gutierrez-Gil et al. 2015; Kim et al. 2016). To account for the stochastic nature of environmental stress, temperature was used as a proxy to represent a dominant stressor that would have threatened survival without tolerance (Benestad 2005; Gerken et al. 2006; Seebacher 2009; Thornton et al. 2009; Walter and Seebacher 2009; Cheviron et al. 2014; Porto-Neto et al. 2014; Valero et al. 2014; Zhao et al. 2014). Temperature at extremes that confer stress across species has been well characterized by previous researchers (Gerken et al. 2006; Sharifi et al. 2010; Ciscar et al. 2011; Lara and Rostagno 2013; Mwacharo et al. 2013; Cheviron et al. 2014; Li et al. 2014; Lyimo et al. 2014; Porto-Neto et al. 2014; Valero et al. 2014; Zhao et al. 2014; Napper et al. 2015; Van Goor et al. 2015; Wang et al. 2015; Wu et al. 2015), who have shown it to affect the immune system, growth, and reproduction. This proxy, along with information from the National Oceanic and Atmospheric Administration (National Oceanic and Atmospheric Administration) on 30-yr average temperature and precipitation for each country, and the principal component analysis (PCA) based on the genotypic data from the array, were used to code the birds as “case” for higher ambient temperatures (African) and “control” for lower ambient temperatures (Northern European) for downstream analysis (Supplemental Material, Table S1).

Animals

All animals (N = 718) were noncommercial indigenous breeds and ecotypes. All chickens except the Ugandan, Rwandan, and Kuroiler populations were provided by theSyn breed Chicken Diversity Panel (SCDP). The Ugandan, Rwandan, and Kuroiler data were taken from Fleming et al. (2016). The SCDP represents chicken breeds from: Africa (N = 375, Uganda, Rwanda, Kuroiler, Tanzania, Zimbabwe, Sudan, Egypt, Ethiopia, and Israel) and Northern Europe (N = 343, Germany, Iceland, Finland, Norway, and Poland). All birds were genotyped by using the Affymetrix 580k SNP chip and genomic DNA isolated from whole blood. Data of all birds sampled within a country were pooled to represent a region based on shared climate history (Table S1).

Quality control

PLink 1.9 (Chang et al. 2015) was used to carry out quality control and filtering of the single nucleotide variants (SNVs) genotype data. Quality control for the samples allowed inclusion based on passing of the following filters: ≥0.05 for minor allele frequency (MAF), ≥99% individual call rate, and ≥99% SNV call rate. The genotyping rate was 0.998 for all samples. Eighty-six variants were removed because they mapped to the same chromosome and position. Only autosomal variants that passed QC were included in the downstream analysis. Filtering on the above parameters reduced the number of informative SNVs from 580,961 to 483,812 and the number of individuals from 718 to 634. The majority of the loss in sample numbers was the result of poor genotype calls for those samples that were removed during filtering. The 634 birds were partitioned into 319 controls (European) and 315 cases (African) for downstream analysis of the remaining SNVs. The program Fastphase (Shect and Stephens 2006) was used for haplotype phasing.

PCA

The Plink 1.9 (Chang et al. 2015) PCA analysis module was used to identify covariate data to address the underlying subpopulations. The PCA was based on calculations of the variance-standardized relationship matrix of the genomic distances. The top three (N = 20) eigenvalues provided the clearest separation of the data and were used to construct the PCA plot.

FST (case/control) analyses

Fixation test (FST) analyses were conducted using PLink 1.9 (Purcell et al. 2007) to identify genomic regions of increasing genomic differentiation between the African and Northern European population samples. The populations were recoded into case/control categories using temperature as a proxy for environmental stressors. The case/control designation is based on a hypothesized natural selection for tolerance for higher ambient (African chickens) vs. lower ambient temperatures.
(Northern European chickens) as the case and control, respectively. Additionally, the –family argument was used in Plink 1.9 to account for possible stratification. Estimation of the genomic variation between the African and Northern European chicken populations was analyzed in Plink for FST. Overlapping windows were generated using in-house Perl and R scripts. Windows were 100 kb and the step size was set to 50 kb to examine data with a 50% overlap The FST results used for downstream analyses were based on the highest peaks (≥0.2). Peaks were measured by width of the base of the peak in order to determine regions for gene list creation. Differential allele frequency (AF) analysis was carried out by Plink to generate the MAF for each SNV by population and then used as a check of the FST data.

Selection signature analysis

Examination of the African and Northern European populations for signatures of selection was performed using the REHH package (Gautier and Vitalis 2012). Haplotypes were phased using FastPhase prior to signature of selection analysis. The REHH package was then used to calculate iHS to determine the existence of selection signatures within each population. Also, the standardized log-ratio of the integrated EHHIS (iES) between pairs of populations (Rsb) was used for a pairwise examination of the populations for SNVs that displayed the population-specific selective pressure differences. Both iHS and Rsb values were log transformed to normalize the data and calculated following the method established by Voight et al. (2006). Statistical significance of iHS data was determined by first converting the −log p-values generated by the REHH software package back into nonlog p-values. From here, a permutation test was applied to the nominal p-values using the R package Perm (Fay and Shaw 2010) to shuffle p-values and perform a permutation t-test to set the threshold for significance, as a means of setting the adjusted p-value without the use of multiple test corrections. The threshold for statistical significance was set using permutations based on using both 1000 and 5000 iterations to set the adjusted p-value. Based on the permutation test, the corrected p-value was set at ≤3.8 × 10−4. The adjusted p-value was then reduced to ≤1 × 10−4 as an additional stringency to help account for using temperature as a proxy phenotype for the case/control-based comparisons. The reduced p-value (≤1 × 10−4) corresponded to an iHS score ≥3.89 for a variant to be considered as showing evidence of selection. This iHS value was used for both populations as the cut-off for variants, and the genes annotated to them that were later used for downstream gene ontology analysis (GO). The nature of the iHS test is based on a normalization of data making it possible to view iHS scores as deviations from the mean (iHS normalized mean = 0) represented by extremes values outside three SDs. Statistically significant iHS variants that cluster within intragenic regions indicate a gene considered to be under strong selection. Other variants in the African and Northern European chickens considered as being under strong selective pressure and possible candidate genes were annotated within 2 Mb upstream or downstream of statistically significant variants. Statistical significance for the Rsb analysis was set at a Rsb value of ≥4 and an adjusted p-value ≤1 × 10−4. Gene lists for downstream analysis were compiled in the same manner as for the iHS data. Candidate genes were selected based upon variant clustering within regions.

ROH analyses

ROH are areas of a genome that harbor long stretches of homozygous genotypes. These stretches represent regions of low heterozygosity and could signify genomic hitchhiking related to signatures of selection (Lencz et al. 2007). The ROH analyses were carried out in Plink 1.9 to examine the populations for the presence of unique ROH. ROH were calculated using a sliding window approach and defined by the following parameters within Plink: SNVs ≥100, length ≥1 kb, ≤3 heterozygous calls to account for the presence of genotyping errors and/or hitch-hiking events, and window-threshold ≤0.05; no missing calls were allowed and program defaults were used for all other parameters. Overlapping ROH were considered matching based on pair-wise allelic matching of 95% or greater concordance. The analysis used sliding windows to determine the genomic regions of overlap within each population (African/Northern European). Only the overlapping consensus ROH regions, as defined by Plink 1.9, of the ROH were used in downstream GO analysis. The ROH analysis included the 30-yr mean temperature data in the form of the case/control designations that were based on historical high and low ambient temperatures (National Oceanic and Atmospheric Administration), respectively, to represent the environment or “landscape” indigenous to each population grouping (African/Northern European). Regions were considered unique if the consensus regions belonged to only one population (African/Northern European). All consensus ROH matching the following criteria were labeled as “landscape ROH” (LROH): (a) ≥1 kb in length; (b) the region was consensus among all members (chickens) of a “pool” (i.e., overlapping ROH segments); (c) members of the pool represented ≥~5% of the total number of samples assigned to that temperature zone (low ambient/Northern European or High ambient/African); and (d) contained only birds from one temperature zone. Gene lists used for downstream analysis were based on genes located between the first SNV of the consensus ROH and the last SNV of the last consensus ROH of that chromosome. Analysis of overrepresented GO terms for functions, processes, and pathways were based on a 5 Mb window based on a start position of −2 Mb from the first SNV of the LROH and +2 Mb from the last SNV in the LROH.

GO enrichment analyses

Lists were created from genes annotated to statistically significant variants from each analysis and examined for overenriched processes and functions. The analysis approach used was termed “(w)HOL(e) ISTIC GO enrichment” and centered on the overlap of terms showing enrichment from multiple methods implemented in different software programs. The programs used consisted of GOfinder (Boyle et al. 2004), STITCH 4.0, STITCH 5.0 (both used due to version 5.0 being an update still in β) (Kuhn et al. 2014), DAVID (Huang et al. 2009; Jiao et al. 2012), g:Profiler (Reimand et al. 2011), and the Panther online database (accessed January 2016) (Ashburner et al. 2000; Gene Ontology Consortium 2015). The p-value was adjusted using the Benjamini–Hochberg method at FDR < 0.05 for iHS- and ROH-based gene lists and at FDR < 0.1 for Rsb gene lists for GO analysis. Adjusted p-values for the FST analysis were set using the Benjamini–Hochberg method (Benjamini and Hochberg 1995) at FDR < 0.05.

Analysis of environmental variables and genomic variance

The existence of an association between the variants under possible selection and the indigenous environment of the African and Northern European chickens was examined using the latent factor mixed model (lfmm) test (Frichot et al. 2013) performed using the R (Gentleman et al. 2004) software package LEA (Frichot et al. 2013, 2015; Frichot 2015). The software was used to generate a list of candidate loci associated with adaption to the given environmental factors, as well as the number of ancestral populations (K) for the African and Northern European chickens. The lfmm analysis was performed using the 30-yr averages for temperature and precipitation for each of the countries and was performed on each group (African/Northern European)
separately. The lfmm analysis was performed several times on each population before taking the median of the merged z-scores. The p-values were then adjusted using the computed genomic inflation factor ($\lambda$) for the median. The lambda values were chosen to be stringent and were 1 for the African and 1.4 for the Northern European populations. Significance was then based on a corrected FDR value set at $q \leq 0.1$ using the Benjamini–Hochberg multiple test correction method.

**Ethics statement**
Samples were taken from the chicken diversity gene bank at the Friedrich-Loeffer-Institut, which has been established for many years. New samples were collected in Germany in strict accordance to the German Animal Welfare regulations, and relevant notice was given to the authorities of Lower Saxony according to x8 of the German Animal Welfare Act. Breeders were asked for their agreement.

**Data availability**
The genotyping data and breed/ecotype identity of the samples analyzed for this study can be obtained upon request from Steffen Weigend (Steffen.Weigend@fli.de).

## RESULTS

### PCA

PCA of the African and Northern European populations showed that the samples displayed several levels of clustering, which indicated the existence of subpopulations (Figure 1). The first level that showed distinct clusters grouped the samples by country of origin. The next level showed that countries clustered according to continent of origin, with all Northern European birds being grouped together and the Mediterranean and African birds clustering together. The distinct clustering of the PCA demonstrated that temperature profiles, land mass (continent), country, and genotype all separate the data in a similar fashion.

### $F_{ST}$ (case/control)

The sliding window analysis (Figure S1) indicated that chromosomes 4, 17, and 27 had the strongest $F_{ST}$ peaks ($F_{ST} > 0.2$) (Table 1). The peak on chromosome 4 was from 44 to 51 Mb, contained 131 genes, and was analyzed for GO term enrichment. Cross-referencing of multiple GO enrichment software (FDR ≤ 0.05) showed multiple statistically significant GO terms related to developmental pathways (epidermal growth factor receptor signaling pathway, GO:0007173), calcium signaling (ERBB signaling pathway, GO:0038127; biomineral tissue development, GO:0031214), and immune response (CXCR chemokine receptor binding, GO:0045236; cytokine activity, GO:0005125). The peak on chromosome 17 (Figure 2) covered an
area that included 14 genes under the 300 kb peak, possibly differentiated due to selection for the contrasted environments. A total of 19 genes, with the majority being feather keratin-like genes, fell within the peak area on chromosome 27 (Figure 2). This peak overlapped with a region of chromosome 27 under selective pressure based on the iHS results. Genes in this overlap region (1–1.4 Mb) were considered to be possible candidate genes, and included gap junction protein γ 1 (GJC1), myosin light chain kinase, smooth muscle-like (LOC428278), glial fibrillary acidic protein (GFAP), and phosphoinositide phospholipase C (PLCD3) (Table 2).

Selection signature analysis: iHS

**African population:** Examination of the iHS SNVs yielded 27 statistically significant SNVs, of which the top three iHS values were on chromosomes 25, 27, and 26, respectively (Figure 3). The genes that had statistically significant variants that clustered in their intronic regions were the highest iHS values considered to be under selective pressure. Chromosome 25 had the highest iHS score and encompassed keratin (LOC769139) and scale keratin-like (LOC769486) genes. The majority of the genes within the ±2 Mb window, which contained the variants on chromosome 25, were related to calcium signaling and feather and claw keratin. The strongest variant under selection on chromosome 27 was within an intron of polymerase I and transcript release factor (PTRF) and angiotensin I converting enzyme (peptidyl-dipeptidase A) 1 (ACE). Other statistically significant variants fell within introns of thyroid hormone receptor α (THRA). Within the ±2 Mb window were the stress related genes hypocretin (orexin) neuropeptide (HCRT), heat shock protein 25 (HSP25), and DnaJ (Hsp40) homolog, subfamily C, member 7 (DNAJC7). The variant under strongest selection on chromosome
26 fell within an intron of BCL2-antagonist/killer 1 (BAK1), while other statistically significant variants fell within exon 6 of adenosylhomocysteinase-like 1 (AHCYL1) (Table 3). The variant in the coding region of AHCYL1 caused a deleterious missense mutation that changed a serine (S) to arginine (R) (SIFT score = 0).

Northern European population: The chromosomal regions that had the highest significant iHS values for the Northern European chickens were on chromosomes 3, 2, and 20 (Figure 3). The Northern European chickens had less overall statistically significant SNVs and no statistically significant SNVs that clustered within any gene. Because of the limited number of variants under selection in the Northern European populations, significant markers falling on the same chromosome were also viewed within the ±2 Mb window. The highest iHS value on chromosome 3 (single SNV) was located in the intron of protein tyrosine phosphatase, nonreceptor type 14 (PTPN14); also under possible selection is protein kinase C ε (PRKCE). The single SNV of highest significance on chromosome 2 fell within an intron of spalt-like transcription factor 3 (SALL3). Another gene near this region of possible importance to the interaction of the Northern European chickens and their environment is the gene prolactin (PRL). On chromosome 20, one of the two variants under selective pressure fell within the intron of protein tyrosine phosphatase, receptor type, T (PTPRT). Genes of interest within the ±2 Mb window included DNAJ (Hsp40) homolog, subfamily C, member 5 (DNAJC5) and Bcl-2-like protein 1 (BCL2L1), along with multiple transglutaminase genes. Chromosome 20 had the highest gene density around significant SNV for the Northern European birds.

Selection signature analysis: Rsb: The results from the pairwise comparison (Rsb) of the African and Northern European chickens identified variants on chromosomes 2 and 27 as having the highest Rsb values (Figure 4). Chromosome 2 had the largest peak and number of

| Table 2 Candidate genes near peak for sliding mean Fst window for chromosome 27 |
|---------------------------------|-----------------------------------|--------------------------------------------------------------------------------|
| Gene Name                       | Function                          | Possible Relationship to Environmental Stress                                  |
| Gap junction protein, γ 1 (GJC1) | GO:00015700, vasculogenesis GO:0048738, cardiac muscle tissue development | Smooth muscle contraction, vasodilation (convection)                              |
| Myosin light chain kinase, smooth muscle-like: (LOC428278) | GO:0004683, calmodulin-dependent protein kinase activity | Ca²⁺, smooth muscle contraction, vasodilation/constriction                      |
| Glial fibrillary acidic protein (GFAP) | GO:1904714, regulation of chaperone-mediated autophagy | Heat shock protein activation                                                    |
| Phosphoinositide phospholipase C (PLCD3) | GO:0006629, lipid metabolic process; GO:0005509, calcium ion binding; GO:0001525 angiogenesis | Lipolysis and vasodilation (convection)                                         |

Candidate genes within ±2 Mb of the peak on chromosome 27. The genes in this region overlap with other regions of selection pressure seen in the iHS (integrated haplotype score) and Rsb (pairs of populations) analysis.

Figure 3 Manhattan plots for the African and Northern European chicken populations. The most extreme iHS peaks were on chromosomes 25, 26, and 27 for the African birds and chromosomes 2, 3, and 20 for Northern European chickens. Plots indicate that selective pressure on both populations is contrasted with selection toward different genomic regions that is likely the result of the difference in habitats. The blue line is the average, black dotted line is cut off at three SD of the mean iHS (≈ 0), red line shows iHS regions above 3. SD Arrows show highest iHS peaks used for downstream analysis. iHS, integrated haplotype score.
### Table 3 Genes within 2 Mb of statistically significant ($\leq 1 \times 10^{-4}$) iHS variants for African and Northern European chickens

| Chr | Location (n/Distance) | Gene | Function/Process |
|-----|-----------------------|------|-----------------|
| Africa 25 | Intron (3) | LOC769139 (keratin) | Intermediate filament (claw, feather, and scale) |
| 27 | Intron (3) | PTRF (Human) (polymerase I and transcript release factor) | Lipid metabolism, insulin-regulated gene expression |
| 27 | Intron (3) | THRA (thyroid hormone receptor, a) | Neuroactive ligand-receptor interaction pathway response to cold (GO:0009409) |
| 27 | Intron (2) | ACE [angiotensin I converting enzyme (peptide)-dipeptidase A 1] | Increased vasocstruction (UniProtKB - Q10751) Response to cold (GO:0009409) |
| 27 | Upstream (65 kb) | HCRT [hypocretin (orexin) neuropeptide] | Feeding behavior (GO:0007631) |
| 27 | Upstream (87 kb) | HSP25 (heat shock protein 25) | Response to heat |
| 27 | Upstream (165 kb) | DNAJC7 [DnaJ (Hsp40) homolog, subfamily C, member 7] | Heat shock protein binding |
| 26 | Intron (7) | BAK1 (BCL2-antagonist/killer 1) | Apoptosis (chicken), heat-shock protein chaperone (human) |
| Europe 3 | Exon 6-missense (1)* | AHCYL1* (adenosylhomocysteinase-like 1) | Oxidation-reduction process (GO:0055114) |
| 3 | Intron (1) | PTPN14 (protein tyrosine phosphatase, nonreceptor type 14) | Lymphangigenesis (GO:0001946), Dephosphorylation (GO:0016311) |
| 3 | Downstream (423 kb) | PRKCE (protein kinase C, ) | Positive regulation of insulin secretion (GO:0032024), cellular response to hypoxia (GO:0071456) |
| 2 | Intron (3) | SALL3 [Sal-like 3 (Drosophila)] | Limb morphogenesis, smoothened signaling pathway |
| 2 | Downstream (1.6 Mb) | PRL (Prolactin) | Thermal stress in cattle, egg production |
| 20 | Intron (1) | PTPRT (Protein tyrosine phosphatase, receptor type, T) | Dephosphorylation (GO:0016311) |
| 20 | Upstream (1.1 Mb) | DNAJC5 [DnaJ (Hsp40) homolog, subfamily C, member 5 (DNAJC5)] | Negative regulation of neuron apoptotic process (GO:0043524) |

Genes in the table were considered to be under strong selection due to the presence of statistically significant variants falling in intragenic regions. The list genes reads like a list of stress tolerance genes, complete with heat-shock proteins, characterized as key responders to cellular stress events. Chr, chromosome. a, SNV fell within exon.

Statistically significant SNVs between the two groupings. Genes of interest that contained statistically significant variants on chromosome 2 included: IKAROS family zinc finger 1 (Ikaros) (IKZF1), von Willebrand factor C domain containing 2 (VWC2), TBC1 domain family, member 5 (TBC1D5), and interferon regulatory factor 4 (IRF4). The significant variants on chromosome 27 fell within the introns of acid-sensing ion channel 2 (ASIC2) and in the intron and 3’-UTR of Golgi SNAP receptor complex member 2 (GOSR2) (data not shown). Many of the genes highlighted were used to perform a GO analysis to examine which processes and functions showed strong selection pressure differences between the African and Northern European chickens (Table 4).

**GO analysis of selection signature results (iHS/Rsb)**

GO analysis was conducted on the gene lists generated from significant SNVs from the iHS analysis of each grouping and the pairwise comparison of the two. In the African chickens, processes and pathways emerged related to cell death (apoptotic process, GO:0006915), immune function (response to interferon-γ, GO:0034341), and homeostasis (homeostatic process, GO:0042592). The cadherin signaling pathway (P00012) was the only significant pathway in the African chickens (Table S2). In the Northern European chickens, significant molecular functions and biological processes were mostly related to cell adhesion (GO:0007155) and angiogenesis (P00005) (Table S2). Regions of interest used for analysis of overenriched GO terms from the Rsb results for chromosome 2 fell within windows of two separate regions, located from ~31 to 36 Mb and ~79 to 83.7 Mb. The window on chromosome 27 was from ~1 to 3 Mb. Data from the Rsb analysis was statistically significant (FDR < 0.1) for biological processes involved in digestive tract mesoderm development (GO:0007502), angiogenesis (GO:0001525), pattern specification process (GO:0007389), skeletal system development (GO:0001501), and mucin type O-Glycan biosynthesis (KEGG:00512) on chromosome 2. Overenriched pathways and processes on chromosome 27 included frizzled binding (GO:0005109), NF-kB-inducing kinase activity (GO:0004704), melanogenesis (KEGG:04916), and regulation of systemic arterial blood pressure by aortic arch baroreceptor (GO:003026) (Table S2).

**ROH (landscape consensus ROH)**

Analysis of all ($N = 634$) chickens in the study yielded a total of 4167 overlapping pools of ROH. Analysis of the overlapping consensus regions revealed that the Northern European chickens harbored 22 pools of LROH that were unique to the Northern European birds.
The unique ROH were located on chromosomes 1, 2, and 4. Chromosome 2 had the largest number of ROH pools and individuals. Genes within the consensus ROH were used to carry out GO and pathway analysis for chromosomes 1 and 2 as there were no genes in the region on chromosome 4. The single region on chromosome 1 contained only eight genes, of which calcium channel, voltage-dependent, L type, α 1C subunit (CACNA1C), interleukin 17 receptor A (IL17RA), and BCL2-like 13 (apoptosis facilitator) (BCL2L13) appeared to be biologically relevant. The statistically significant processes related to the consensus LROH (Table 5) for chromosomes 1 and 2 included multiple hits related to glycolysis, neural signaling, and protein inhibition and recycling. There were known QTL for body morphological traits and intramuscular fat that were almost completely within ROH on chromosome 2 (Figure S1 and S2).

Environmental factor association analysis (lfmm)
The Iffm analysis of the African and Northern European chicken populations returned results on the diversity of the population structures (K) and listed statistically significant SNV loci associated with possible adaptation to the environmental factors of temperature and precipitation. The African chicken population exhibited a heavily admixed population structure with a K = 9 (out of 23 breeds/ecotypes from sampling) (Figure S6), and had candidate loci lists that totaled 2489 for temperature and 1961 for precipitation. The Northern European chicken population was only slightly stratified with a K = 19 (out of 22 breeds from sampling) (Figure S6) and produced a candidate loci list of 9738 for temperature and 7676 for precipitation. Some of the candidate loci discovered to be associated with selective pressure to adapt to the environment overlapped with genomic positions from the iHS, FST, Rsb, and ROH analyses. In the African chicken population, this included chromosomes 25, 26, and 27 from the iHS analysis (Table S2). In the Northern European chickens, candidate loci overlapped with iHS regions on chromosomes 2, 3, and 20 (Table S2). The lfmm analysis supported the previous results for candidate loci near genes of interest under selective pressure.

DISCUSSION
The stochastic nature of the connection between genotype, stress, environment, selective pressure, and adaptive phenotype makes it difficult to attribute changes in any of these phenomena to any single stressor or genetic phenomenon. Although, it is possible that different populations or breeds may converge around an adaptive advantage through the process of random drift, it is unlikely that random drift would leave identifiable “footprints” across multiple breeds due to its randomness. To identify and examine the “footprints” and the effects that contrasted environments have on the genomic architecture, historical temperature profiles of the countries were used as proxies for environment to account for potential differences in thermal stress. By using thermal stress as proxy for environmental stress, we can examine multiple issues related to environmental stress in livestock and view the data by genomic and physiological processes related to environmental stress tolerance in general, and thermal stress tolerance specifically. The biological pathways and network functions based upon the genomic variation of the chickens that address these may offer the information needed to select for environmental stress tolerance of multiple climates (Seebacher 2009; Walter and Seebacher 2009; Valero et al. 2014). Despite this, many of the processes and genes found to be under selection are part of a general stress (Zakrzewska et al. 2011) response meant to provide adaptability to multiple stressors in the animal’s environment, which may or may not be quantifiable or captured by temperature as a proxy (Clarke 2003; Canario et al. 2013; Newman et al. 2013; Zhao et al. 2014; Napper et al. 2015; Nguyen et al. 2015; H. Y. Sun et al. 2015; L. Sun et al. 2015; Van Goor et al. 2015). PCA was able to show us that the samples could be split into two distinct groupings based upon divergence in the populations by both genotype and environment. The

Figure 4 Manhattan plot for the African and Northern European chickens showing the pairwise comparison of selection pressure. Peaks on chromosomes 2 and 27 were analyzed for selection pressure and were shown to be the two chromosomes on which the populations were the most diverged. The data from the FST analysis supports the results on chromosome 27 as being under differential selection between the populations. Rsb, pairs of populations. black dotted line is cut off at three SD of the mean iHS (=0) red line shows iHS regions above 3 SD.
overlap between these two categories seems to indicate that each group of chickens (African/Northern European) has evolved genetic functions related to survival of their specific, indigenous environments that should present as distinct differences in their genomic architecture. The PCA results allowed the analysis to be performed as a case/control study, with temperature as a proxy phenotype, to allow the results to be viewed as genomic regions under possible selection for environmental stress tolerance differing by population and related to each population’s indigenous environment. The examination of the African and Northern European chicken populations utilized multiple methods that showed overlap in the genomic regions carrying selection signals. The overlap between methods presented chromosomes 2 and 27 as being highly differentiated between the two groups, and under high selective pressure within each group (Northern Europe and Africa), respectively.

Selection signatures unique to the divergence of the African chickens

The results on chromosome 27 may be the most biologically relevant results from the iHS analysis, due to their overlap with results from the FST and Rsb analyses that indicate there is selective pressure in the region of ~1–4 Mb of chromosome 27 within the African (high ambient temperature) populations. Within this area is the gene THRA, which has homologs with functions related to environmental stress (response to cold GO:0009409) and muscle function in the heart (regulation of heart contraction GO:0008016). The area of chromosome 27 overlapped by the FST, iHS, and Rsb results also holds the genes HSP25 and DNAJC7, both of which are directly related to stress tolerance responses (Jenuth 2000; UniProt Consortium 2015). The presence of selective pressure where these genes are located could be indicative of a region involved in environmental stress responses in chicken. The gene HSP25 has been shown to be upregulated in indigenous Taiwanese chickens (hot climate) (Wang et al. 2015) under acute heat stress, which supports the idea of selection toward this gene in warmer habitats. The gene ASIC2, under selective pressure on chromosome 27, also supports selection toward conductance for heat tolerance, but also helps regulate pH balance, which could indicate homeostatic functioning. The strongest evidence of selective pressure on chromosome 27 being related to thermal tolerance, by more than just proxy, is demonstrated by the selection around the ACE gene involved in vasculogenesis (Jenuth 2000). Van Goor et al. (2015) reported that both the ACE gene and a previously unreported QTL for body temperature (BT28-20) under heat stress mapped to the same region(s) identified in the current study as being under selective pressure in the African population. The QTL region found by Van Goor et al. (2015) on chromosome 27, and the areas under selection in the African chickens, overlap around several possible candidate genes in addition to ACE. The results from both studies appear to point to a region of chromosome 27 that is involved in heat tolerance, and this region may have been selected for a thermal advantage in Africa’s ancient chickens and persisted through the continued domestication and improvement of African poultry breeds and ecotypes. The lack of selective pressure in this region among the Northern European chickens that were analyzed may suggest that the region is tied to some functional advantage that is useful for hotter environments. Analysis of the GO enrichment for chromosome 27 based on the iHS and Rsb results also indicated a heat tolerance region located in the vicinity of ~1–3 Mb on chromosome 27. The terms homeostatic process (GO:0042592), regulation of systemic arterial blood pressure by aortic arch baroreceptor (GO:0003026), frizzled binding (GO:004704), and melanogenesis (KEGG:04916) were all overenriched for the region under selection on chromosome 27 (Table S2). Studies have shown that genes involved in these processes are differentially regulated under experimentally generated heat stress (Gerken et al. 2006; Li et al. 2014; H. Y. Sun et al. 2015; L. Sun et al. 2015).

Selection signatures unique to the divergence of the Northern European chickens

The regions under the strongest selection pressure in the Northern European birds were located on chromosomes 3, 2, and 20 (Table 3). Chromosomes 3 and 20 both have genes that function in dephosphorylation and thermal stress and may indicate selection toward thermogenesis through energy uncoupling as a means of generating body heat in order to achieve cold tolerance in Northern European chickens (Seebacher 2009; Newman et al. 2013; Zhao et al. 2014; Napper et al. 2015). Another gene of biological interest on chromosome 3 from the Northern European iHS analysis is PRKCE. The gene has functions related to insulin secretion and response to low oxygen environments in chickens (Jenuth 2000; UniProt Consortium 2015; Herrero et al. 2016; Kersey et al. 2016). In humans, PRKCE is considered a stress response gene involved in multiple biological processes related to insulin secretion and response to low oxygen environments in chickens (Jenuth 2000; UniProt Consortium 2015; Herrero et al. 2016; Kersey et al. 2016).
response gene involved in cardiac tissue (Barnett et al. 2007); however, its impact is less defined in chickens. The selection signatures in the Northern European birds are possibly related to stress and/or some advantage in sugar oxidation that increases thermogenesis. Selection toward PRKCE also supports the hypotheses from previous work that suggest that kinases, such as PRKCA, were involved in the response to the environment in poultry. Studies have demonstrated that both PRKCA and PRKCE can have opposite effects on NADPH usage (F. Chen et al. 2014), a process that seems to be under unique selection in the Northern European chickens and echoed in the LROH GO enrichment results for chromosome 1 (Table 3). However, the major overlap in genomic regions that showed evidence of selection signatures identified by multiple methods (iHS, Rsb, and LROH) occurred on chromosome 2. The variants under selective pressure on chromosome 2 from the iHS (Table 3) analysis intersect with the LROH (Figure S3 and Figure S4) and seem to signify selective pressure around the PRL gene. In relation to environment, PRL has been shown to have variants that can cause tolerance phenotypes to heat or cold stress in cattle that affect hair morphology (Littlejohn et al. 2014). Likewise, variation in the PRL gene in turkeys and the PRLR receptor in chickens has been shown to affect feathering (Smyth 1954; Elferink et al. 2014). The variants associated with the PRL gene alleles based on the iHS data (Figure S5) proved to be no identifiable signaling networks in the Northern European birds based on the genes annotated to the iHS. It is possible that the Northern European chickens are more dependent on the pentose phosphate pathway than the glycolysis pathway. It is also possible that the selective pressure differences in energy metabolism pathways seen in the Northern European birds have been influenced over time by different nutrient availability. Taken together, the region on chromosome 2 could be related to energy metabolism, inhibition of protein digestion, and fatty acid oxidation (X. Y. Chen et al. 2014; Nguyen et al. 2015) as naturally occurring tolerance mechanisms in indigenous Northern European chickens. Considering that these results are unique to birds from the Northern European environment, the discovery of these LROH may point to a selection signature for adaptation to temperate or cold conditions.

Conclusions

The data from the FST, LROH, iHS, and Rsb analyses all support the presence of strong selection pressure on regions of chromosomes 2 and 27. The genes and processes under selective pressure on chromosome 27 are unique to the African populations as there were no significant iHS scores for any chromosome 27 variants in the Northern European chicken populations sampled. The same proved true for chromosome 2 within the African populations. The Northern European chickens appear to be under less selective pressure than the African population, as evidenced by fewer variants reaching significance. The difference in the number of variants under selection could possibly reflect a less diverse or less harsh environment being encountered by the Northern

Response gene involved in cardiac tissue (Barnett et al. 2007); however, its impact is less defined in chickens. The selection signatures in the Northern European birds are possibly related to stress and/or some advantage in sugar oxidation that increases thermogenesis. Selection toward PRKCE also supports the hypotheses from previous work that suggest that kinases, such as PRKCA, were involved in the response to the environment in poultry. Studies have demonstrated that both PRKCA and PRKCE can have opposite effects on NADPH usage (F. Chen et al. 2014), a process that seems to be under unique selection in the Northern European chickens and echoed in the LROH GO enrichment results for chromosome 1 (Table 3). However, the major overlap in genomic regions that showed evidence of selection signatures identified by multiple methods (iHS, Rsb, and LROH) occurred on chromosome 2. The variants under selective pressure on chromosome 2 from the iHS (Table 3) analysis intersect with the LROH (Figure S3 and Figure S4) and seem to signify selective pressure around the PRL gene. In relation to environment, PRL has been shown to have variants that can cause tolerance phenotypes to heat or cold stress in cattle that affect hair morphology (Littlejohn et al. 2014). Likewise, variation in the PRL gene in turkeys and the PRLR receptor in chickens has been shown to affect feathering (Smyth 1954; Elferink et al. 2008) and may represent tolerance to the environment involving feather structure for improved evaporation, insulation, or filtration in the Northern European chickens. The terms negative regulation of ossification and negative regulation of nitric oxide mediated signal transduction (GO:0010751) are associated phenotypes with PRL that may putatively be involved in increased skeletal muscle development and skeletogenesis. Additionally, these GO terms have been observed in studies of cold tolerant birds (Walter and Seebacher 2009; Newman et al. 2013). Nitric oxide is capable of stimulating perfusive heat loss (Seebacher 2009), which Northern European chickens may have been selected against as an evolutionary adaptation. Also on chromosome 2 is evidence of population-specific selection on the SALL3 gene alleles based on the iHS analysis and on IKZF1, TBC1D5, and IRF4 alleles from the Rsb analysis. These genes are involved in processes that can be seen in experimental studies related to hypothermia and stress (Muhammad et al. 2008; Pinard-van der Laan et al. 2009; Seaman et al. 2009; Cheviron et al. 2014; Zhao et al. 2014). The variants annotated to these genes also fell within a selective sweep previously described in Qanbari et al. (2012), supplying more evidence of the existence of selection toward this area in natural populations.

When compared to the African population, the results of GO analysis of the LROH, iHS, and Rsb seem to indicate possible selection for metabolic processes that employ ion channel transport driven by sugar oxidation and NADPH+ as an alternative means of energy metabolism in response to the environment of Northern Europe (Table 3 and Table 5). Additionally, unlike the African iHS data (Figure S5), there proved to be no identifiable signaling networks in the Northern European birds based on the genes annotated to the iHS. It is possible that the Northern European chickens are more dependent on the pentose phosphate pathway than the glycolysis pathway. It is also possible that the selective pressure differences in energy metabolism pathways seen in the Northern European birds have been influenced over time by different nutrient availability. Taken together, the region on chromosome 2 could be related to energy metabolism, inhibition of protein digestion, and fatty acid oxidation (X. Y. Chen et al. 2014b; Nguyen et al. 2015) as naturally occurring tolerance mechanisms in indigenous Northern European chickens. Considering that these results are unique to birds from the Northern European environment, the discovery of these LROH may point to a selection signature for adaptation to temperate or cold conditions.

Conclusions

The data from the FST, LROH, iHS, and Rsb analyses all support the presence of strong selection pressure on regions of chromosomes 2 and 27. The genes and processes under selective pressure on chromosome 27 are unique to the African populations as there were no significant iHS scores for any chromosome 27 variants in the Northern European chicken populations sampled. The same proved true for chromosome 2 within the African populations. The Northern European chickens appear to be under less selective pressure than the African population, as evidenced by fewer variants reaching significance. The difference in the number of variants under selection could possibly reflect a less diverse or less harsh environment being encountered by the Northern
European birds due to their status as heritage birds. The differences in variant numbers may also possibly reflect less change in one environment vs. the other (high ambient vs. low ambient temperature) over time. It is possible that, when viewed as being entirely subject to their environment, the Northern European birds may have had some relaxation in environmental pressures of thermal stress under modern husbandry; however, the African chickens are mostly village chickens in a rural, scavenger/forage type of farming system allowing for more environmental exposure over more diverse environments (i.e., desert, mountain, jungle, or basin). The study supports the suggestion from experimental studies of chickens under heat stress that chromosome 27 is related to tolerance and survival under stressful environmental conditions. This result proved applicable in both experimental and natural populations encountering stress from high ambient temperatures. Additionally, the study also gives novel insights into unique genomic regions on chromosome 2 in the Northern European chickens that are possibly related to development and environment. Overall, the results indicate that environmental differences may have a part in selection for some portion of the genomic divergence seen in development and metabolic processes of these very distinct populations.

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