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LETTER

Environmental variability and the evolution of the glucocorticoid receptor (*Nr3c1*) in African starlings

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Abstract

One of the primary ways that organisms cope with environmental change is through regulation of the hypothalamo–pituitary–adrenal (HPA) axis, the neuroendocrine system that controls reactions to stress. Variation in genes regulating the HPA axis – particularly the glucocorticoid receptor – may facilitate adaptation to changing climatic conditions by altering expression. Here we examine signatures of selection on the glucocorticoid receptor gene (*Nr3c1*) in African starlings that inhabit a range of environments, including those with variable climatic conditions. To investigate potential adaptive mechanisms underlying the vertebrate stress response, we sequence the *Nr3c1* gene in 27 species of African starlings. Although we find some evidence of positive selection, substitution rate is negatively correlated with variance in precipitation. This suggests climatic cycling in sub-Saharan Africa may have resulted in lower substitution rates to maintain a successful coping strategy. When environmental conditions fluctuate rapidly, variation in the strength of purifying selection can explain evolutionary rate variation.

Keywords

Adaptation, canalisation, environmental variability, fluctuating selection, stress response.

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INTRODUCTION

Climatic conditions that vary greatly in time can result in fluctuating selective pressures (Bell 2010). In an environment with extreme fluctuation, there is no single fitness function that allows individuals to evolve towards a globally optimal phenotype (Sæther & Engen 2015). Instead, environmental stochasticity generates a constantly moving optimum where rapid changes in the direction of selection result in a dynamic adaptive landscape (Calsbeek *et al.* 2012). How organisms adapt to stochastic changes in their environment depends largely upon the degree of predictability of environmental variation that they experience (Botero *et al.* 2015). If environmental stimuli are relatively predictable, phenotypic plasticity may enable individuals to cope with changing environments (Charmantier *et al.* 2008; Auld *et al.* 2010; Gomez-Mestre & Jovani 2013). However, in the absence of reliable environmental cues, rapid fluctuations in the environment may select for fixed phenotypes (i.e., bet-hedging strategies) that maximise fitness across the mean environment by reducing temporal variance in fitness (Frank & Slatkin 1990; Simons 2009). Over longer periods of environmental change, a phenotype can adaptively track the environment as the mean population trait changes gradually and lags behind the change in environmental conditions (Chevin 2013; Botero *et al.* 2015). Although we are beginning to develop a predictive theory of when organisms should adopt one type of evolutionary response to environmental change over another (Botero *et al.* 2015; Tufto 2015), we still do not fully understand how these phenotypic responses are governed at the genetic level.

Climatic conditions in some environments may fluctuate too unpredictably to allow for phenotypic plasticity or too rapidly to be tracked adaptively. In highly unpredictable environments, traits subject to fluctuating selection may show either increased genetic variation or reduced polymorphism (i.e. genetic canalisation) (Kawecki 2000; Botero *et al.* 2015). If environmental fluctuations are periodic and intermediate in length, genetic variation underlying a coping strategy is predicted to be advantageous (Ellner & Hairston 1994; Svardal *et al.* 2011; Le Rouzic *et al.* 2013). However, rapidly fluctuating selective pressures can lead to stabilising selection that reduces genetic variation (Le Rouzic *et al.* 2013). A reduction in genetic variation could help protect species against environmental perturbations by decreasing phenotypic variance (Pélabon *et al.* 2010; Svardal *et al.* 2011; Hallsson & Björklund 2012; Le Rouzic *et al.* 2013; Shaw *et al.* 2014; Miehls *et al.* 2015). Frequent and rapid changes in environmental conditions may therefore result in genetic canalisation that reduces sensitivity to environmental change (Gibson & Wagner 2000; Kawecki 2000; Flatt 2005). Under fluctuating selection, genetic canalisation may reflect adaptive tracking, bet-hedging, or a combination of the two coping strategies. The degree of predictability and/or relative timescale of environmental variation determines which strategy or combination of strategies (uniform or bet-hedging) is employed (Botero *et al.* 2015). Thus, environmental variability may result in

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fluctuating selection on behavioural and physiological traits underlying these coping mechanisms. Regardless of how populations cope with environmental change, genetic canalisation minimises temporal variation in fitness by allowing organisms to maintain a single phenotypic response across all possible environments.

One of the primary mechanisms that organisms use to cope physiologically with environmental change is modulation of the hypothalamo–pituitary–adrenal axis, the neuroendocrine system that controls reactions to stress. Biotic (e.g. predation pressure, social interactions) and abiotic stressors (e.g. climate) trigger a hormonal cascade in the brain that ultimately results in the release of glucocorticoids from the adrenal glands (Sapolsky *et al*. 2000; Creel *et al*. 2013; Wingfield 2013, 2014). The release of glucocorticoids can be adaptive in the short-term by rapidly altering behaviour and causing the mobilisation of energy reserves (Sapolsky *et al*. 2000), but elevated glucocorticoids can also be maladaptive over longer time periods by leading to changes in immune function, body composition, and behaviour that may ultimately result in death (Korte *et al*. 2005; Busch & Hayward 2009). As the downstream effector of the stress response, the glucocorticoid receptor can regulate an individual's stress responsiveness by binding to circulating glucocorticoids and up-regulating transcription (Sapolsky *et al*. 2000). The glucocorticoid receptor (*Nr3c1*) gene may thus be an ideal candidate to link physiological responses to cope with environmental change with the underlying adaptive genetic variation that facilitates these responses. Mutations in *Nr3c1* can change the transactivation potential or binding affinity of the receptor (Tung *et al*. 2011; Murani *et al*. 2012), shaping how *Nr3c1* interacts with other transcription factors to alter gene expression. *Nr3c1* expression, which can vary with environmental conditions (Liebl *et al*. 2013; Durairaj & Koilmani 2014), influences receptor density and thus stress responsiveness. Examining sequence variation in *Nr3c1* allows us to begin to explore the genetic architecture of phenotypic traits that enable vertebrates to cope with environmental stressors.

Here we test for signatures of natural selection on the *Nr3c1* glucocorticoid receptor gene in 27 ecologically diverse species of African starlings (Sturnidae). Starlings inhabit a variety of environments across Africa, ranging from arid deserts, to semi-arid savanna woodlands, to tropical and montane moist forests. These environments differ not only in their mean levels of precipitation, but also in their degree of within- and among-year variation in precipitation, with species that inhabit semi-arid savannas experiencing the most unpredictable climatic variation (Rubenstein & Lovette 2007). Although Africa has had a dynamic climatic history over the past three million years (Nicholson 1996), we assume that climates for which birds are adapted today are similar to those in which they evolved. To determine how environmental variability has influenced patterns of genetic variation in the starling *Nr3c1* gene, we examine phylogenetically controlled differences in substitution rate, or the ratio of non-synonymous to synonymous mutations (dN/dS), among species to identify signatures of positive (adaptive tracking) or negative (canalisation) selection. We hypothesise that a positive relationship between environmental variation and dN/dS would suggest adaptive tracking of environmental conditions and local adaptation, whereas a negative relationship between environmental variation and dN/dS would suggest genetic canalisation. In addition to looking for signatures of environmentally driven selection among species, we also examine differences in dN/dS among codon sites of the glucocorticoid receptor to determine whether adaptive divergence is associated with particular receptor functions. Ultimately, by studying the genetic architecture of stress responsiveness in wild vertebrates, we can begin to bridge the gap between proximate studies of molecular mechanisms and ultimate studies of fitness consequences in the wild (Romero 2004; Bonier *et al*. 2009; Breuner *et al*. 2013).

MATERIAL AND METHODS

Laboratory methods

DNA was extracted from vouchered museum tissues (Table S1), using a DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA). We designed primers for each exon (Table S2), using a superb starling (*Lamprotornis superbus*) transcriptome (Weinman *et al*. 2015). PCR reactions (10 μL) included 1 μL of genomic DNA (10–50 ng μL^{-1}), 0.8 μL 10× PCR buffer, 24–26 mM $MgCl_2$, 10 mM dNTPs, 0.015 μM of forward and reverse primer, and 0.5 U Jumpstart Taq polymerase (Sigma, St. Louis, MO, USA). Each PCR profile began within an initial denaturation at 95 °C for 3 min followed by two phases – phase 1 (touchdown phase) consisted of 15 cycles of three steps (95 °C for 30 s, 45 s at a starting temperature of 60–66 °C decreasing by 1 °C in each subsequent cycle, 72 °C for 60 s) and phase 2 (traditional phase) consisted of 25 cycles of three steps (95 °C for 30 s, 60 °C for 45 s, 72 °C for 60 s) – and a final extension at 72 °C for 5 min.

The PCR product was visualised on 2% agarose TAE gels to confirm amplification and product size. To remove dNTPs and single-stranded DNA remaining after PCR, we added 0.35 μL of Exonuclease I (USB) and Shrimp Alkaline Phosphatase (USB) to the remaining 7 μL PCR product and incubated at 37 °C for 30 min and then at 90 °C for 10 min. We conducted cycle sequencing reactions, using the amplification primers and a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA), purified sequencing product, using DyeEx 96 kits (Qiagen), and read sequences on an Applied Biosystems 3730 automated DNA sequencer. Sequences were aligned and checked manually in Geneious v8.0.2 (Biomatters, Auckland, New Zealand). Because Exon 3 failed to sequence and there was no sequence variation in Exons 2 or 4, concatenated alignments used in further analyses included only Exon 1 (the N-terminal domain) and Exons 5–8 (the ligand-binding domain) (Fig. 3a).

Tree building and inference

PAUP* v4.0b10 (Swofford 2003) and RAxML v7.2.8 (Stamatakis 2006) were used to build phylogenetic trees used in selection testing. In PAUP*, we conducted full heuristic searches using parsimony and likelihood methods. We assessed node support with 100 heuristic bootstrap replicates.

In RAxML, we used the GTR + G model and rapid bootstrapping. Selection tests were run using each topology to control for possible effects of the gene tree. We examined historical patterns of substitution in the glucocorticoid receptor with ancestral character-state reconstruction. Using the PAUP* gene tree, we inferred ancestral amino acids at each node, using unordered parsimony methods in Mesquite v3.03 (Maddison & Maddison 2015). Reconstructing substitutions that occurred in more than one place in the gene tree showed that these amino acid replacements did not appear to be associated with environmental factors. There were 33 variable codons in the N-terminal domain that required more than two steps to explain observed amino acid replacements, and these nonsynonymous substitutions were dispersed throughout the gene tree. Within the ligand-binding domain, we identified only two variable sites; both of these replacements were chemically inexpensive shifts between structurally similar amino acids, and thus would not be expected to change the structure of the receptor.

We tested for evidence of selection on *Nr3c1* using an alignment of 1272 bp with 97% coverage in 27 species of African starlings. To check for recombination in *Nr3c1*, we used the Genetic Algorithm for Recombination Detection (GARD) in Datamonkey (Kosakovsky Pond *et al.* 2006). We used three independent dN/dS methods to test for selection across 27 divergent species in independently evolving populations (Lovette and Rubenstein 2007): (1) Phylogenetic Analysis by Maximum Likelihood (PAML) (Yang 2007); (2) Datamonkey, a web-based server of HyPhy (Delport *et al.* 2010); and (3) TreeSAAP, which tests for selection on amino acid properties (Woolley *et al.* 2003). We also tested for selection on amino acid properties using TreeSAAP (Selection on Amino Acid Properties of phylogenetic trees). TreeSAAP quantifies the structural and biochemical properties of particular amino acid replacements and compares the resulting magnitude of change to an expected random distribution to identify sites with unique physiochemical properties that may be evidence of positive selection.

Testing for evidence of dN/dS skew

CodeML (PAML) was used to test for positive selection on specific amino acid sites based upon a series of three nested log-likelihood ratio tests that compare a null model that restricts dN/dS to an alternative model that allows an additional category of dN/dS (Yang 2007). First, we tested for rate heterogeneity between sites by comparing a model (M0) that allows no variation in dN/dS to a model (M3) that allows three discrete categories of dN/dS. Second, we tested for positive selection by comparing a neutral model (M1) with two categories of dN/dS to a model (M2) that includes an additional third category for sites with an estimated dN/dS > 1. Finally, we also tested for positive selection by comparing a flexible null model with beta categories (M7) to a model with beta + omega (M8). This M7–M8 test accounts for sites with dN/dS between 0 and 1 better than the M0–M3 test, since it estimates dN/dS using the flexible beta distribution. Each of these models was run with multiple starting values for omega (0.1, 0.4, 1) to control for potential effects of the starting dN/dS.

As a preliminary test for selection across sites, we applied five additional algorithms within HyPhy to our data: (1) single likelihood ancestor counting (SLAC) (Kosakovsky Pond *et al.* 2005); (2) fixed effects likelihood (FEL) (Kosakovsky Pond *et al.* 2005); (3) internal fixed effects likelihood (IFEL) (Kosakovsky Pond *et al.* 2005); (4) random effects likelihood (REL) (Kosakovsky Pond *et al.* 2005); and (5) fast unconstrained Bayesian approximation (FUBAR) (Murrell *et al.* 2013). These tests are often used to infer negative, or purifying, selection at each site if the number of synonymous substitutions (dS) is much greater than expected. Since the different algorithms vary in power, estimation of substitution rate, and the number of site classes of substitutions, applying all of them allowed us to compare results with varying statistical power and different assumptions (Kosakovsky Pond *et al.* 2005; Murrell *et al.* 2013).

Since dN/dS skew may result from site-specific and/or lineage-wide differential selection, alternative approaches that account for variation between branches may be more appropriate (Hughes 2007). Given these potential concerns, we further tested for differential negative selection in particular lineages rather than simply at specific sites. Mixed effects model of evolution (MEME) were developed to supersede other dN/dS approaches, as this branch-site model does not require specification of foreground branches that may be under different selective pressures compared to branches in the background. Specifically, MEME and the complementary branch-site REL (BREL) tests identify episodic diversifying selection using a modified branch-site random-effects likelihood approach that tests for all possible branches under selection (Murrell *et al.* 2012). To corroborate environmentally driven differences in dN/dS, we fit modified M3 models with fixed (null) and free (alternative) omega values in CodeML. We tested for differential selection across species using three comparisons where foreground lineages experienced (1) above-average mean precipitation, (2) above-average variance in precipitation, and (3) below-average predictability of precipitation. To verify that the results were not sensitive to how we classified foreground lineages, we ran additional tests that specified foreground lineages as those with precipitation measures that were one and two standard deviations above the mean. Results from these tests were qualitatively similar and therefore not reported.

Testing for associations between dN/dS and environmental variability

To determine whether the observed differences in dN/dS among lineages correlated with environmental factors, we used phylogenetic generalised least squares regression in the R package '*ape*' (Paradis *et al.* 2004) with an Ornstein–Uhlenbeck model of correlation structure. Using yn00 in PAML, we calculated dN/dS for each lineage as follows (Yang & Nielsen 2000). We first reconstructed the ancestral sequence of *Nr3c1* at the most basal node including all African starlings, and then extracted the dN/dS estimate that compared each species to the ancestral African starling. We calculated dN/dS from pairwise comparisons with the putative ancestral glucocorticoid receptor in African starlings for two reasons: (1) this

approach removes phylogenetic constraint by collapsing the tree into a star phylogeny with the ancestral sequence at the base of the tree; and (2) variation in *Nr3c1* across African starlings was so low that dN/dS estimates between terminals and the most recent node were equal to zero in the majority of comparisons. Since this approach to estimating dN/dS averages the shared ancestry, we also calculated dN/dS for each terminal by measuring lineage-specific dN/dS as the average of all pairwise dN/dS estimates between that lineage and all other starling lineages as calculated in yn00 (Yang 2007). Since subsequent tests were insensitive to the dN/dS estimation method, results are shown using only the ancestry-corrected dN/dS estimates.

To characterise environmental variation in this system, we measured precipitation across the entire range for each species (Table S4). We used previously published climatic data (Botero *et al*. 2014) where mean, variance, and predictability were calculated using monthly global maps (0.5 × 0.5 degree cells) for the period of 1901–2009 from the CRU-TS 3.1 Climate Database, where these data were available for 25 species of African starling (which includes all species sequenced except *Lamprotornis iris* and *Lamprotornis albicapillus,* for which range-wide data was not available). Predictability was calculated using Colwell's P, which accounts for both intensity and seasonality of periodic phenomena such as the annual patterns of monsoon-driven rains in sub-Saharan Africa. We then modeled the relationship between phylogenetically controlled dN/dS and all three measures of precipitation (mean, variance, and predictability) for 25 species. Because these environmental measures are related, we tested for autocorrelation among the variables: the variance inflation factor (VIF) was 1.39 for the final model fit, which used both variance and predictability of precipitation as predictors. Although mean and variance in precipitation are correlated (VIF = 5.93), mean precipitation does not predict dN/dS variation.

Testing for other sources of variation in dN/dS

Interspecific variation in dN/dS could reflect differences in effective population size or life history. Since only one sample was sequenced in the majority of species in this study, we cannot directly calculate effective population size and instead used range area as a proxy. Range area was calculated as the breeding distribution listed in the 2010 IUCN Red List of Threatened Species v2010.4 (www.iucnredlist.org; downloaded 17 April 2016). We tested whether range area predicted dN/dS to determine if reductions in population size drive the observed increases in substitution rate. Since generation time could also vary among species and influence dN/dS variation, we tested whether body size (as a proxy of generation time) predicts dN/dS.

Variation in dN/dS could also be age-dependent, such that older lineages have different dN/dS than younger lineages. Since synonymous substitutions (dS) accumulate at a faster rate than nonsynonymous substitutions (dN), lower dN/dS may simply result from systematically higher dS rather than purifying selection against dN. If dN/dS does not evolve in a clock-like manner, then differences in dN/dS could reflect systematic variation in evolutionary rate among codon sites. Tests of clock-like evolution using Bayesian relaxed clock methods in BEAST resulted in very low effective sample sizes for the posterior distribution, and thus these results are not presented. However, a correlation between dN/dS and dS would also suggest that accumulation of dS and not purifying selection against dN over time drives variation in dN/dS. As a final test of age-dependent variation in dN/dS, we tested for an association between terminal branch length and variance in rainfall, as bias in the overall number of substitutions across species could drive observed patterns of dN/dS.

Finally, since the observed variation in dN/dS could reflect selection across the genome and thus may not be specific to *Nr3c1*, we tested whether the cytochrome C oxidase subunit 1 (COI) gene showed the same patterns of dN/dS variation as the patterns observed in *Nr3c1* across African starlings using PAML and HyPhy as described above. We also calculated dN/dS in COI and used the PGLS methods described above to test whether precipitation influences COI evolution.

RESULTS

In African starlings, lineage-specific variation in *Nr3c1* is associated with environmental variability (Fig. 1). After controlling for phylogeny, we found that dN/dS is negatively correlated with variance in precipitation across each species' range ($t = -2.56$, $P = 0.018$, $R^2 = 0.16$). This relationship suggests that birds experiencing greater variation in annual precipitation have lower dN/dS than those experiencing less variation in annual precipitation. In support of this pattern, lineage-specific dN/dS varies substantially among species (mean ± SD = 0.36 ± 0.13; range = 0.16–0.62; as estimated

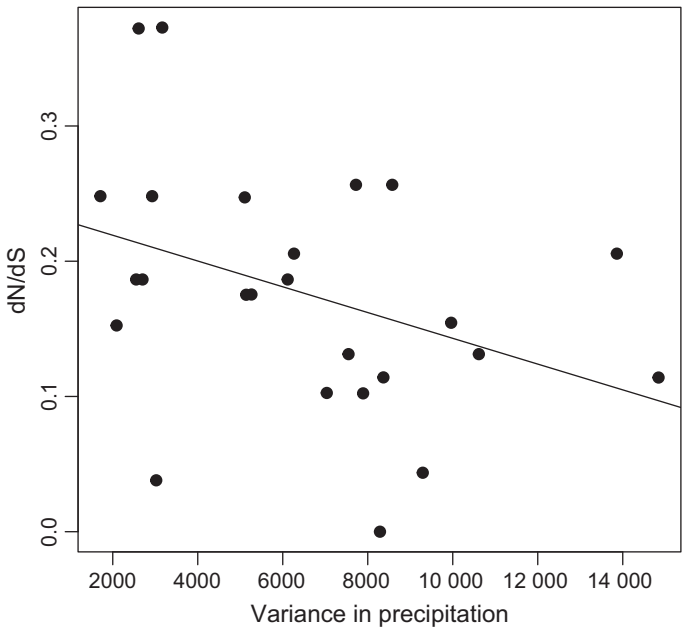

Figure 1 Relationship between variance in precipitation across the ranges of 25 species of African starlings and dN/dS values from a phylogenetic least squares regression. Variance in precipitation is negatively related to dN/dS, such that species in more variable environments show lower dN/dS than those in less variable environments.

in CodeML) (Fig. 2, Table S4). Models that allowed variation in dN/dS in *Nr3c1* between species (i.e. a free omega) fit significantly better than models that constrained dN/dS to a single measure across species (Table S5). Furthermore, branch-site tests that specified which lineages are expected to be under selection did detect evidence of lineage-specific diversification in *Nr3c1*, when species that experience high variance and low predictability in precipitation are set as the foreground lineages expected to be under selection (Table S5). These branch-site tests confirmed that environmental conditions – especially variability in those conditions – are associated with patterns of genetic variation in *Nr3c1*.

These patterns appear to be driven by selection and not neutral variation in dN/dS in *Nr3c1*. Although we cannot rule out a historical bottleneck without a direct measure of effective population size, range area does not predict dN/dS ($t = -1.10$, $P = 0.28$, $R^2 = 0.01$), suggesting that observed variation in dN/dS is not a result of genetic drift due to variation in population size among species. Generation time also does not appear to drive differences in dN/dS, since body size is not associated with dN/dS ($t = 0.05$, $P = 0.97$, $R^2 = -0.04$). Although we cannot reject the hypothesis that variation in dN/dS is age-dependent, dN/dS variation does not appear to reflect differences in evolutionary rates among codon sites alone: the number of synonymous substitutions (dS) does not explain variation in dN/dS ($t = -1.40$, $P = 0.17$, $R^2 = 0.04$), and thus accumulation of dS is not likely to be the primary driver of differences in dN/dS. However, terminal branch length is weakly correlated with variance in rainfall ($t = 2.14$, $P = 0.04$, $R^2 = 0.15$), which means that time since speciation could explain at least some of the observed variation in dN/dS. Together, these results suggest that systematic variation in dN/dS based upon lineage age is not the only explanation for the observed patterns of dN/dS variation. Furthermore, these patterns of dN/dS variation appear to be limited to *Nr3c1*. We find no evidence of selection or lineage-specific diversification in COI across African starlings (Table S3), and dN/dS in COI is not related to variance in rainfall ($t = -0.074$, $P = 0.86$, $R^2 = -0.078$). Thus, the relationship between environmental variability and substitution rate seems to reflect selection on *Nr3c1* and not neutral variation.

Although dN/dS varies between species of African starlings, the global dN/dS across sites is relatively low (0.22), as estimated by a model that restricts dN/dS to a single rate category. The overall mean genetic distance for all 27 species is 1.5%, with only 95 variable nucleotide positions across the 1272 bp alignment. Despite this low level of differentiation, topologies of the *Nr3c1* gene tree are congruent among tree-building algorithms, and these trees are generally consistent with the previously published phylogeny based on mitochondrial DNA and nuclear markers for both the African clade (Lovette & Rubenstein 2007) and the entire Sturnidae (Lovette *et al.* 2008). In *Nr3c1*, more than 93% of all amino acid sites have an estimated dN/dS ≤ 1 in four independent calculations (Fig. 3c) showing that non-synonymous substitutions in *Nr3c1* are rare. Furthermore, amino acid replacements are observed in only 35 of 424 sites in *Nr3c1*. No substitutions significantly alter the physiochemical properties of any site as

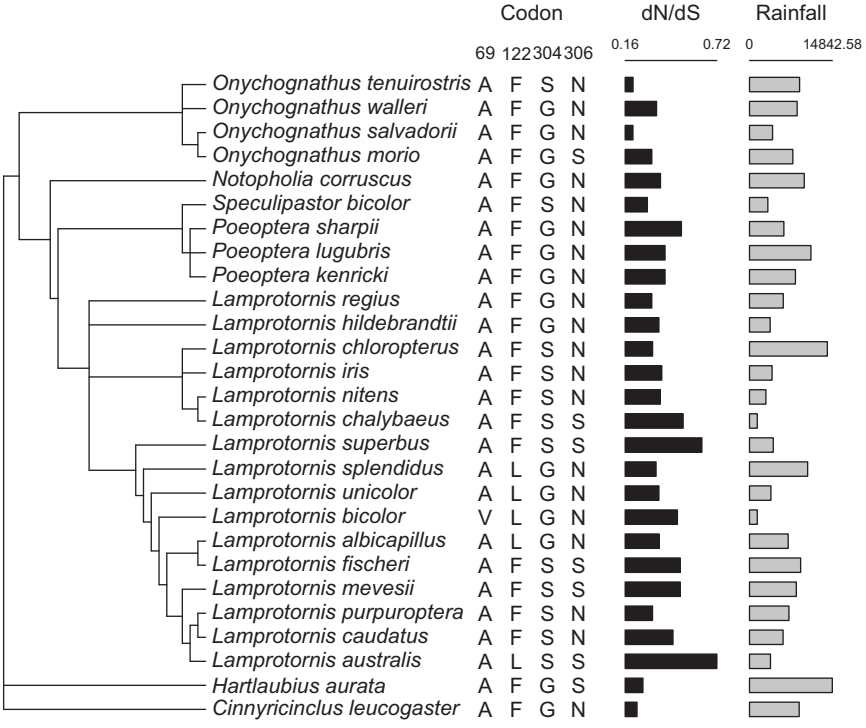

Figure 2 Maximum parsimony gene tree of African starlings showing dN/dS in *Nr3c1*, rainfall (variance in precipitation ranging from 0 to 14 842.58 cm year^{-1}), and variable codons under selection of each species. Codon 69 is under negative selection, whereas Codons 122, 304 and 306 are under positive selection. Letters under each codon indicate different amino acids (e.g. A indicates alanine, V valine, F phenylalanine, L leucine, G glycine, S serine and N asparagine).

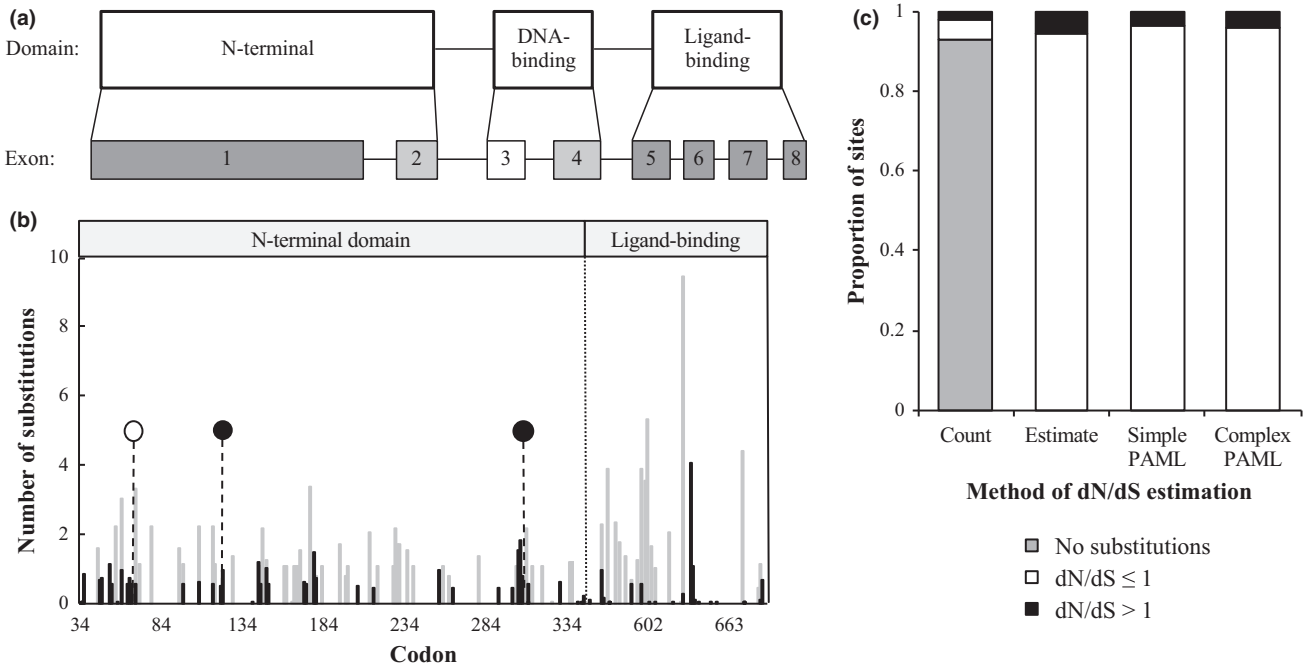

Figure 3 Sequence variation in *Nr3c1* is relatively low across African starlings. (a) Structure of avian *Nr3c1* glucocorticoid receptor gene. Darker grey exons showed variation between species, while exons shaded in light grey showed no substitutions. Exon 3 was not sequenced. (b) Estimates of dN/dS across sites in *Nr3c1* using single likelihood ancestor counting (SLAC). Non-synonymous substitutions (dN) are indicated by black bars and synonymous substitutions (dN) by grey bars. Sites under positive selection are indicated by black circles, and sites under negative selection by white circles. (c) Proportion of sites in each category of dN/dS out of all 424 sites in *Nr3c1*. The count method uses single likelihood ancestor counting (SLAC), while estimate uses the random effects likelihood (REL) method. Simple PAML uses Model 2, which constrains dN/dS to three categories, and complex PAML uses Model 8, which does not constrain dN/dS and instead assigns categories based on the gamma distribution.

estimated by TreeSAAP, and most of these replacements occur in only a single species.

We found that dN/dS varies significantly among sites ($P < 0.001$ for all M0–M3 comparisons; Table S3) and, as in other vertebrates, most of the variation in *Nr3c1* is found in the N-terminal domain (Fig. 3a,b) (Stolte *et al.* 2006). Models of dN/dS estimated that < 5% of the 424 codons in *Nr3c1* have an estimated dN/dS > 1 (Fig. 3c), and only three of these sites are under positive selection. All of the codons under positive selection are in the variable N-terminal domain and show dN/dS > 1 (BPP > 95; Table 1). Amino acid replacements at these selected sites may alter receptor function. Despite the low levels of variation across *Nr3c1*, we identified some sites under positive selection but several codons under purifying selection. Fifteen of the 424 codons in *Nr3c1* are inferred to be under negative selection with dN/dS < 1 by three or more algorithms in HyPhy (Table 1). Ten of these sites are in the N-terminal domain, and the remaining five sites are in the ligand-binding domain. Thus, there is strong evidence for negative, or purifying, selection on specific codons, even within variable regions of *Nr3c1*.

DISCUSSION

Substitution rate in *Nr3c1* in African starlings is associated with environmental variation, but we observe lower dN/dS in species that live in more variable environments (Fig. 1). This relationship suggests that increasing environmental variability leads to stronger purifying selection and may promote genetic canalisation of *Nr3c1*. In support of this prediction, we also find that (1) most codons do not vary between species (Fig. 3c) and (2) many of these sites are under strong negative selection (Table 1). Overall, there are few nonsynonymous substitutions in the glucocorticoid receptor across 27 species of birds that are ecologically diverse (Fig. 3c); dN/dS in *Nr3c1* is higher in species that inhabit less variable environments. Variation in the strength of purifying selection can explain the patterns of dN/dS variation that we observed, suggesting that lineages experiencing variance in precipitation have experienced selection to constrain substitutions in *Nr3c1* as a result of fluctuating environmental conditions.

In sub-Saharan Africa, annual variation in rainfall is a key environmental stressor that influences social behaviour, reproductive life history, and physiology in African starlings (Rubenstein 2007a,b, 2016; Rubenstein *et al.* 2008). Rainfall has been highly variable since at least the Plio-Pleistocene (Verschuren *et al.* 2000; deMenocal 2004), and the open, arid grasslands and savannas now characteristic of the area have existed for 16 Mya (Jacobs 2004; Bobe 2006). Climatic cycling in this heterogeneous environment continued throughout the radiation of African starlings, which is thought to have occurred 12.9–17 Mya during the Miocene (Zuccon *et al.* 2006). These rapidly fluctuating environmental conditions likely influenced the molecular evolution of *Nr3c1*, as well as the behavioural and ecological strategies for coping with this environmental change (Rubenstein & Lovette 2007;

Table 1 Selection inference on *Nr3c1* in African starlings indicates that four variable codons in the N-terminal domain are under selection (+ indicates positive selection and − indicates negative selection). Column headers indicate statistical tests used, where values represent statistical support. The majority of these codons were found to be under selection by additional selection tests (not shown); see Material and methods for explanation. Variable codons (shown in bold) show amino acid replacements and thus vary among species

		Selection test	
Site		FUBAR*	BEB†
N-terminal domain			
57	−	0.94	0.01
69	−	**0.97**	**0.34**
79	−	0.95	0.01
122	+	**0.92**	**0.96**
147	−	0.96	0.01
176	−	0.98	0.00
213	−	0.96	0.00
229	−	0.97	0.00
230	−	0.61	0.00
231	−	0.89	0.00
304	+	**0.87**	**0.99**
306	+	**0.92**	**0.99**
Ligand-binding domain			
509	−	0.96	0.01
564	−	0.96	0.01
583	−	0.97	0.01
584	−	0.99	0.00
597	−	0.97	0.00
606	−	0.99	0.01

*Test within HyPhy.
†Test within PAML.

Rubenstein 2016). For example, a bird's responsiveness to environmental stressors influences the amount of time and resources it has to invest in reproduction, and birds that balance reproduction and stress responsiveness may be more fit than birds that are more sensitive to stressors. Year-to-year variation in the onset and duration of annual rains – which influence reproductive timing in African starlings (Rubenstein & Lovette 2007) – could thus result in selection to maintain a successful coping strategy (e.g. moderate tolerance of environmental stressors). Thus, environmental variability among years should select for maintenance of a successful strategy rather than adaptive tracking of rapid environmental change, which is consistent with the low levels of substitutions in *Nr3c1* observed in this study.

If variability in the environment exerts strong selective pressure on an animal's stress responsiveness, species that must cope with extreme variation in climate should show genetic canalisation of *Nr3c1*, which results from fewer non-synonymous substitutions and lower dN/dS. Environmental variability does appear to constrain genetic variation in *Nr3c1* in African starlings, such that species that live in more variable environments have lower dN/dS (Fig. 1). Amino acid replacements in *Nr3c1* may reduce fitness by disrupting receptor function (e.g. binding affinity or transactivation potential), whereas canalisation could help maintain the function of *Nr3c1*. Although functional studies of the glucocorticoid receptor are rare, substitutions in the ligand-binding domain have been shown to alter the sensitivity of the glucocorticoid receptor (Murani *et al.* 2012), suggesting that the amino acid replacements observed in our study could change glucocorticoid receptor function. We find that < 9% of codons in *Nr3c1* vary across sites (Fig. 3c), and that the global dN/dS is low (0.22), showing that non-synonymous substitutions are rare. Furthermore, this limited variation in *Nr3c1* seems to result from negative selection specific to *Nr3c1* and not simply genetic drift. Thus, in African starlings, the strength of purifying selection to maintain *Nr3c1* function appears to vary among lineages that experience different environmental conditions.

Given that the glucocorticoid receptor is the downstream effector that enables animals to respond to stressors, it is in some ways unsurprising that this gene is highly conserved, not only across vertebrates (Stolte *et al.* 2006) but also within a single family of birds. Comparative studies of other nuclear receptors suggest that this conservation is not uncommon (Katsu *et al.* 2010; Kostrouchova & Kostrouch 2015), but additional studies of species-level evolution of nuclear receptors are needed to understand the genetic mechanism of changes in hormone responsiveness. With rapidly changing climates, conservation of key modulators like the glucocorticoid receptor could allow for fine-tuning of stress responsiveness via more plastic mechanisms like the hormone ligand. Studies like ours that explore standing variation in genes underlying physiological responsiveness to environmental change will be critical for understanding how phenotypic plasticity influences the evolution of complex traits (Murren *et al.* 2015). Similarly, in a common garden experiment with *Melospiza* sparrows, Angelier *et al.* (2011) showed that two closely related subspecies differed in stress responsiveness even when raised in the same environmental conditions, suggesting that changes in glucocorticoid responsiveness are not completely plastic. Furthermore, pedigree analyses in swallows and quail suggested that there is some genetic component to variation in glucocorticoid responsiveness (Jenkins *et al.* 2014; Homberger *et al.* 2015). Many modulators – including hormone receptors, processing enzymes, binding globulins, and even other transcription factors – control how responsive an animal is to glucocorticoids. A combination of selection on plasticity and on genes underlying glucocorticoid production (e.g. the gene for corticotropin releasing hormone; Schulkin 2011) could facilitate observed differences in stress responsiveness.

Since environmental variability will continue to increase with global climate change (Easterling *et al.* 2000; Cai *et al.* 2014; Trenberth *et al.* 2015), understanding how species have adapted over evolutionary time to naturally unpredictable environments will be essential for predicting how other species might cope with anthropogenically driven increases in environmental uncertainty. We have shown that increased environmental variation can select for a slower rate of protein evolution in the avian glucocorticoid receptor, a pattern consistent with the idea of genetic canalisation. As environmental conditions continue to fluctuate from year to year, genetic canalisation may therefore result in greater fitness than adaptive tracking of these unpredictable changes in the environment. Since slower evolutionary rates in response to environmental variability could constrain adaptation, we suggest that examining a wider variety

of genetic mechanisms for adaptive strategies for coping with environmental change will help to clarify how organisms adapt to environmental variability.

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AUTHORSHIP

D.R.R. and N.R.H. conceived experiments and secured funding, N.R.H. performed experiments and analysed data, and N.R.H. and D.R.R. wrote the manuscript.

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