Integrative and Comparative Biology

Integrative and Comparative Biology, volume 58, number 4, pp. 763–776
doi:10.1093/icb/icy086

Society for Integrative and Comparative Biology

SYMPOSIUM

Species-Specific Means and Within-Species Variance in Glucocorticoid Hormones and Speciation Rates in Birds

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From the symposium "Understanding the Evolution of Endocrine System Variation through Large-scale Comparative Analyses" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2018 at San Francisco, California.

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Synopsis At macroevolutionary scales, stress physiology may have consequences for species diversification and subspecies richness. Populations that exploit new resources or undergo range expansion should cope with new environmental challenges, which could favor higher mean stress responses. Within-species variation in the stress response may also play a role in mediating the speciation process: in species with broad variation, there will always be some individuals that can tolerate an unpredictable environment, whereas in species with narrow variation there will be fewer individuals that are able to thrive in a new ecological niche. We tested for the evolutionary relationship between stress response, speciation rate, and subspecies richness in birds by relying on the HormoneBase repository, from which we calculated within- and among-species variation in baseline (BL) and stress-induced (SI) corticosterone levels. To estimate speciation rates, we applied Bayesian analysis of macroevolutionary mixtures that can account for variation in diversification rate among clades and through time. Contrary to our predictions, lineages with higher diversification rates were not characterized by higher BL or SI levels of corticosterone either at the tips or at the deeper nodes of the phylogeny. We also found no association between mean hormone levels and subspecies richness. Within-species variance in corticosterone levels showed close to zero repeatability, thus it is highly unlikely that this is a species-specific trait that influences diversification rates. These results imply that stress physiology may play a minor, if any, role in determining speciation rates in birds.

Advance Access publication July 13, 2018

Introduction

Range expansion, whereby spreading populations exploit new resources, reduce intraspecific competition, and fill empty niches, is a chief component of the speciation process (Price 2007). Expanding populations may establish larger population sizes and broader distribution ranges, all of which increases the probability of population divergence leading to new species and subspecies to emerge (Gaston 2003). Niche segregation within a species' distribution can also mediate the speciation process, as adaptations to specific environmental conditions within a population can lead to the formation of reproductive barriers therein (Nosil 2012). Range expansion and/or resource polymorphism can be achieved via the evolution of morphological, behavioral, and life history characters (Smith and Skulason 1996). These key innovations allow organisms to cooperate with the environment in novel ways (Losos and Mahler 2010) and serve as important engines of speciation (Sanderson and Donoghue 1994; Maddison et al. 2007; Hoorn et al. 2010). Focusing on birds that are one of the most species-rich vertebrate classes (Gill and Donsker 2018), here we propose that stress physiology may be involved in the speciation process with glucocorticoid hormones mediating ecological adaptation and diversification (see also Lexer and Fay 2005; Wingfield et al. 2015).

Corticosterone in most vertebrates is the main glucocorticoid that is involved in the physiological stress response (Romero 2002; Wingfield and Sapolsky 2003). At low levels, baseline (BL) concentrations govern organismal functions related to metabolic demands during the course of routine activities by orchestrating energy intake, storage, and mobilization (Landys et al. 2006). At higher circulating levels, stress-induced (SI) corticosterone triggers survival-supporting processes in response to unpredictable and highly challenging stimuli (Wingfield et al. 1998). When exploiting novel environments, pioneers often encounter suboptimal energetic conditions, higher allostatic load, and an increased number of confrontations with predators, competing species, and/or novel parasites (Wingfield et al. 2015). Therefore, the founders of new populations will be among those individuals that have the physiological capacity to deal with such environmental challenges. As a result, the fraction of the ancestral population with elevated corticosterone concentrations will more likely contribute to range expansion and subsequently experience genetic differentiation and diversification. Consequently, if new species emerge and radiate these will be characterized by mean corticosterone levels that are higher than that of the population of origin. Over evolutionary timescales then, the hormonal profile of the colonizing populations establishes the physiological basis of the exploitation of a new environmental opportunity, which will serve as a prerequisite for key innovation and speciation. This reasoning leads to the hypothesis about a positive evolutionary relationship between glucocorticoid concentrations and diversification rate.

Several case studies of birds suggest that physiological adaptation to less favorable conditions during range expansion is facilitated by elevated corticosterone levels. Studies on house sparrows, *Passer domesticus*, revealed that populations in recently colonized areas exhibited more active exploratory behavior and higher SI corticosterone responses than birds in the founder populations (Liebl and Martin 2012; Martin et al. 2017). Populations at the northern extremes of the breeding range exhibit higher SI corticosterone responses than do central populations of snow buntings, *Plectrophenax nivalis*, and lapland longspurs, *Calcarius lapponicus* (Walker et al. 2015). In the white-crowned sparrow, *Zonotrichia leucophrys*, populations breeding at high altitudes or at the northern limits of the species distribution exhibit higher levels of both BL and SI corticosterone (Addis et al. 2011; Krause et al. 2016). Furthermore, in several species, corticosterone levels from more recently established urban sites were higher than concentrations from rural sites, implying that during the urbanization process expanding populations also face greater environmental challenges that have either physiological consequences or underpinnings (e.g., Fokidis et al. 2009; Bonier 2012; Grunst et al. 2014; also see Partecke et al. 2006; Atwell et al. 2012). This line of evidence shows that many species may leverage variation in corticosterone levels to expand their range, and populations exploiting novel resources can be characterized by individuals with a higher corticosterone profile. Furthermore, at the among-species level, SI corticosterone levels positively correlate with latitude (Bokony et al. 2009; Jessop et al. 2013), implying that evolutionary responses to the demands of the environment do not only affect between-population but also between-species variance of mean hormone levels.

Corticosterone levels vary substantially within a species and a population, and such variance components, in addition to mean levels, could also facilitate range expansion and speciation. For instance, most of the studies included in the HormoneBase dataset report a substantial range for corticosterone levels

even for the same species analyzed under the same assay conditions (Vitousek et al. 2018). Within-species variance may reflect consistent differences among individuals, such that some individuals reliably employ a certain physiological strategy to cope with their environment, while others use another strategy (Bennett 1987; Williams 2008). Accordingly, some individuals will benefit under certain conditions, whereas others will thrive under other conditions, but no particular phenotype is unanimously favored by selection (Dingemanse et al. 2004). Consistent among-individual differences in a suite of physiological (and behavioral) traits can reflect the degree of individual specialization generating intrapopulation variation in resource use (Bolnick et al. 2003). Such intrapopular niche variation can facilitate rapid sympatric speciation through reproductive isolation among specialized individuals (Rosenzweig 1978). Heterogeneous populations are more likely to survive under unpredictable environmental conditions, resulting in more habitats explored, larger population sizes, and broader distribution ranges (Møller and Garamszegi 2012; Garamszegi and Møller 2017). As these success measures are often translated into higher speciation rates (Gaston 2003), we can hypothesize that within-species variance in corticosterone concentrations is also positively related to diversification rate.

Phenotypic variation in corticosterone levels may also reflect within-individual plasticity, whereby individuals flexibly adjust their hormone secretion according to the prevailing environmental challenges. Estimates of repeatability of corticosterone traits based on the repeated measurement of individuals revealed that within-individual variance is considerable in many species (review in: Taff et al. 2018), and that the evolutionary significance of phenotypic plasticity in the hormonal regulation of behavioral adaptation is underappreciated (Hau and Goymann 2015; Kilvitis et al. 2017). Given that theory suggests that phenotypic plasticity, in general, is an important driver of speciation (Price et al. 2003; Pfennig et al. 2010), it is reasonable to expect that the within-individual component of the within-species variance of corticosterone, in particular, also plays a role in this process.

The aim of this study was to test the hypotheses regarding the phylogenetic association between speciation rate and the mean and variance of corticosterone by using HormoneBase as the main data source for the hormonal traits (Vitousek et al. 2018) and a recent time-calibrated phylogenetic tree for all birds (Jetz et al. 2012). Birds are particularly ideal models, because there is remarkable variation in diversification rates along their phylogeny (Jetz et al. 2012); there is also considerable variation in mean corticosterone levels at the between-species level that suggests an important evolutionary role for the trait; birds occupy a broad range of habitats all over the world, implying that environmental constraints may have been important selective forces during their evolutionary history; and they are well-represented in HormoneBase, noting their important role in the study of endocrine function. For the phylogenetic modeling, we relied on the framework "Bayesian Analysis of Macroevolutionary Mixtures" (BAMM; Rabosky 2014) to determine the locations of shifts in the rate of lineage splitting on the avian phylogeny (rate shifts), and to derive species-specific estimates of inherited diversification rates. Accordingly, our strategy was to i) examine if, as a consequence of key innovations leading to the radiation of species, ancestral states of corticosterone levels are higher at the nodes of the tree where rate shifts occurred, and to (ii) test for the interspecific correlation between species-specific estimates of speciation and hormonal traits. We also explored whether the hypothesized mechanisms for the links between range expansion, resource partitioning, and stress physiology are manifested at a lower (within-species) hierarchical level. Therefore, we also aimed to investigate if, iii) corticosterone levels are associated with subspecies richness that reflects phenotypic polymorphism within species (Phillimore et al. 2007).

Methods

Hormone data

HormoneBase included 225 bird species, for which information on corticosterone could be obtained (Vitousek et al. 2018). Data for population-specific mean BL corticosterone (as given by the mean of individual measurements within a source study) were available for 167 species, of which 133 records also held information on the variability of measurements at the between-individual level. For the latter, we calculated the coefficient of variation (CV) based on the standard error, sample size, and mean to derive an estimate that is independent of the mean/variance relationship (Wright 1968). The sample sizes for SI corticosterone were: $n = 122$ (mean) and $n = 92$ (CV; the sample size for variance is smaller than for that of the mean because some studies did not report the appropriate data). In several species, we could obtain summary statistics for locally distinct populations, for different breeding seasons, years, and sexes. Based on these multiple

entries, we calculated the within-species repeatability of the focal hormonal traits by building a linear mixed model, in which we entered log-transformed corticosterone levels as the response; latitude, sex, and breeding stage as fixed predictors; and population ID and species ID as random factors. From this model that controlled for the potential confounders, we extracted the variance components to derive adjusted repeatability, R_{adj} (Nakagawa and Schielzeth 2010). This estimate was R_{adj} =0.441 (95% CI = 0.337–0.536) and $R_{adj} < 0.001$ (95% CI = 0.000–0.045) for BL corticosterone (mean and CV, respectively), and was $R_{adj} = 0.453$ (95% CI = 0.324–0.569) and $R_{adj} = 0.046$ (95% CI = 0.000–0.146) for SI corticosterone (mean and CV, respectively). Repeatabilities for mean corticosterone levels are, hence, statistically differentiable from zero, and have a magnitude that is considerable given what is generally observed for labile physiological and behavioral traits (Nespolo and Franco 2007; Bell et al. 2009; Wolak et al. 2012). On the other hand, repeatabilities for CV are close to zero. We therefore concluded that means of BL and SI corticosterone are species-specific traits, while across-sample variance within the same species in the CV of corticosterone levels is so high (even if we control for latitude, sex, and population effects) that it makes their use in comparative phylogenetic analyses difficult to interpret. For these reasons, we were unable to proceed with the tests of the predictions related to the variance of corticosterone, and we only report results associated with mean levels. We extracted species-specific estimates of mean corticosterone levels from the above models (as the respective random intercepts); thus, latitude, sex, breeding stage, and population effects were controlled in the rest of the study.

Phylogeny

Jetz et al. (2012, 2014) presented a time-calibrated maximum clade credibility tree for 9993 extant birds recognized as of 2012. Detailed description of the underlying methodology can be found in the supplements of the papers and on the website www.birdtree.org. Here we used the resolutions that were obtained based on the Hackett "backbone" tree (a phylogenetic hypothesis for 158 crown clade *sensu* Hackett et al. 2008). Furthermore, we only considered species that were added to "backbone" based on genetic data resulting in a fully resolved phylogeny for 6670 species (67% of extant species). Estimates of rate shifts and species-level lineage diversification rates are robust to alternative backbone topologies (e.g., Ericson et al. 2006) and the inclusion of the remaining species that can only be placed on the phylogeny based on taxonomic information and randomly sampled branching time (Jetz et al. 2012; Huang and Rabosky 2014).

For unbiased sampling, the list of species, for which we had information on corticosterone, should be randomly distributed along the phylogeny. To test this assumption, we compared tip speciation rate (see below) between species with and without hormone data. This comparison revealed no statistical difference between the two groups of birds (BL corticosterone: $t_{167.08} = -1.453$, $P = 0.148$; SI corticosterone: $t_{120.81} = -1.711$, $P = 0.090$), and verifies that data availability of the focal trait is not biased by the biological hypotheses at hand.

Number of subspecies

We counted the number of subspecies that have been described for each species based on the information provided in AVIBASE (http://avibase.bsc-eoc.org). To verify the reliability of this variable, we estimated subspecies richness for a subset of birds from another source (Cramp and Perrins 1977–2002). The independent sources provided highly correlated measures ($r = 0.945$, $P < 0.00001$, $N = 54$, log-transformed data), which justified the use of our estimate of subspecies richness in our interspecific framework.

Bayesian analysis of macroevolutionary mixtures

To estimate the rate of diversification along the nodes and branches of the avian phylogeny, we used the Bayesian approach implemented in the program BAMM (Rabosky et al. 2014, 2017). We chose BAMM because it rigorously separates the rate of lineage splitting from the ecological modulation of diversification (that also contributes to the species richness of a clade), which could potentially mask the effects of stress physiology on speciation rate (Rabosky 2009, 2010). In the BAMM framework, heterogeneous mixtures of dynamic processes (e.g., constant rate diversification, diversity-dependent speciation, key innovation, and rapid burst) are assumed to generate patterns of diversification within a time-calibrated phylogenetic tree. BAMM applies a reversible-jump Markov Chain Monte Carlo (MCMC) simulation to explore the universe of models that differ in the number of distinct macroevolutionary regimes, and identifies the number and locations of possible transitions on the tree that best explain lineage diversification (rate shifts). It assumes that shifts between regimes occur across

the branches of the tree under a compound Poisson process model, and that each regime can be described by an exponential time-varying process of speciation. However, it makes no *a priori* assumptions about the locations of regimes. As a main result, BAMM provides marginal distributions of speciation (and extinction) rates for every branch and node including the tips of the phylogenetic tree. To run BAMM the user is required to set up a number of parameters through a control file. We provide the parameter settings for this study in the Supplementary Material. The most important of these were the following: i) under the assumption of random taxon sampling, we set the global sampling probability (proportion of species sampled relative to the total number of species) to 67%; ii) the expected number of shifts (used to calculate the prior probability distribution on the number of rate shifts) was set to 10 as suggested for large trees; and iii) the number of generations was set to 100 million (of which we discarded the first 20 million as burn-in). Before interpreting BAMM outputs, we visually checked outputs (log-likelihoods and number of regimes) for convergence issues. To test the predictions of this study, we used the BAMM estimates of speciation rate at the tips of the phylogeny (for the species-level analyses), and the locations of rate shifts at the deeper nodes of the tree (for analyses focusing on the ancestral states). These latter localities were determined based on the maximum shift credibility configuration (the joint distribution of shift events that maximizes the marginal probability of the data).

There has been a recent theoretical discussion about the reliance of the BAMM protocol for estimating diversification rates and rate shifts (Moore et al. 2016; Rabosky et al. 2017; Meyer and Wiens 2018). This discussion provided conflicting results, and the current-state-of-the-art remains inconclusive for empiricists. To assess the robustness of our results, we compared BAMM estimates with that of alternative approaches by using the same phylogeny. On the one hand, we calculated species-level lineage diversification rate based on the equal split approach (Redding and Mooers 2006; Jetz et al. 2012). We compared this estimate with the one that was derived from the BAMM output as tip speciation rate—tip extinction rate. These two measures were highly correlated ($r = 0.682$, $P < 0.00001$, $n = 6670$), assuring us that our analyses at the species level are reliable.

We also determined the locations of rate shifts in the deeper nodes of the phylogeny by using MEDUSA (Alfaro et al. 2009), and examining the correspondence of these nodes to those that had been identified by BAMM as the maximum shift credibility configuration. The former approach detected 33 nodes where lineage splitting accelerated in relation to the background diversification rate, whereas the BAMM procedure obtained evidence for rate shift for 36 nodes. Across the two sets, 11 nodes were identical, 7 nodes were in direct ancestor-descendant relationship with each other, 4 nodes were separated by a single node, and 11 of them were linked through 5.63 intermediate nodes on average (the mean $\pm$ SE of number of nodes connecting two randomly selected nodes via the shortest path is 34.52 ± 1.19 on the phylogeny used). In a discrete shift framework, statistical support for a spike in evolutionary rates along a particular branch may signify biological reality, in which a minor set of evolutionary shifts is accumulated over several branches. Taking this inherent uncertainty into account, the concordance between the configuration of the approximate locations of rate shifts as given by the two approaches is relatively high. When we repeated our analyses on the level of ancestral nodes (see below) by using rate shifts as inferred from the MEDUSA output, the results we report below do not change qualitatively.

Our general strategy to test our predictions was two-fold. First, we performed analyses at the species level, in which we related BAMM estimates for tip speciation rate (obtained based on the overall phylogeny for 6670 species) to mean corticosterone levels, as described by Huang and Rabosky (2014) for an analogous situation. Furthermore, we also investigated if subspecies richness is interspecifically associated with the hormonal traits. Accordingly, using a framework based on phylogenetic generalized least squares (PGLS; Symonds and Blomberg 2014), we built a regression model, in which mean corticosterone (log-transformed) was the response and speciation rate (log-transformed), or subspecies richness (log-transformed) was the focal predictor variable. We conditioned the covariance structure of model residuals based on the common ancestry of species as defined by the phylogeny that was trimmed to the species with data on corticosterone. We accounted for heterogeneity in data quality, and we weighted each species-specific entry by sample size (number of individuals; Garamszegi and Møller 2010). The model for SI corticosterone also included BL corticosterone to control for the dependence of the former on the latter. Furthermore, we entered body mass as a predictor to hold interspecific variation in the main aspects of life history constant, as it can mediate both speciation and stress physiology

(Phillimore et al. 2006; Bokony et al. 2009; Francis et al. 2018). Note that effects due to latitude, sex, breeding stage, and population ID were already corrected for when defining the focal variable from the raw data (see above). We performed these analyses for all species combined, and also aimed to do so separately for each family to reveal potential differences among these taxa with respect to the focal relationship. Due to the constraints of data availability (we proceeded with families with at least 10 measurements), we could repeat the within-family analyses for only one subset (Emberizidae).

Second, to assess whether patterns depend on the depth of phylogenetic resolution, we investigated if locations of rate shifts identified at the nodes of the tree coincided with high ancestral corticosterone levels, as could be predicted by bursts of innovations. We calculated ancestral state estimates of corticosterone relying on a maximum likelihood approach (Pagel 1997) for each node in the phylogeny. We investigated if these ancestral values were systematically higher for nodes that corresponded to the rate shift configuration of BAMM than for nodes not differing from the background diversification. Note that for this approach, we had to match the nodes of the large phylogeny that was used in the BAMM modeling with the nodes of the smaller phylogenies that were pruned to only the species with information on corticosterone (Figs. 3 and 4). To do so, we identified all species that descended from a node that corresponded to a rate shift on the large phylogeny, and retained only those that were also represented in the smaller phylogeny. Then we determined the most recent common ancestor for the subset of remaining species on the small phylogeny to identify the focal rate shift node. We repeated this procedure for each node in the maximum shift credibility configuration.

Results

BL corticosterone

Using species as the unit of analysis, there was no significant relationship between tip speciation rate and mean BL corticosterone levels when controlling for various confounds (Table 1 and Fig. 1a). This pattern was very similar when we restricted our analyses to a single family with sufficient sample size: the slope of the regression obtained separately for emberizid sparrows was not statistically different from zero ($t = -1.264$, $P = 0.219$). When we related subspecies richness to mean BL corticosterone levels, we also detected no significant association across all birds (Table 1 and Fig. 2a), or across emberizid species (Fig. 2a, $t = 1.229$, $P = 0.231$).

Table 1 The relationship among speciation rate, subspecies richness, and mean corticosterone levels

Model	Statistics		
	Coefficient (SE)	*t*	*P*
BL corticosterone λ^a=0.107			
Intercept	1.020 (0.111)	9.173	<0.001
Tip speciation rate	−0.078 (0.094)	−0.827	0.409
Body mass	−0.044 (0.029)	−1.507	0.134
BL corticosterone λ^a=0.125			
Intercept	1.066 (0.079)	13.547	<0.001
Subspecies richness	0.029 (0.038)	0.767	0.444
Body mass	−0.041 (0.030)	−1.389	0.167
SI corticosterone λ^a=0.144			
Intercept	1.222 (0.082)	14.968	<0.001
Tip speciation rate	0.022 (0.048)	0.458	0.648
Body mass	0.021 (0.020)	1.020	0.310
BL corticosterone	0.364 (0.047)	7.658	<0.001
SI corticosterone λ^a=0.200			
Intercept	1.201 (0.072)	16.770	<0.001
Subspecies richness	0.018 (0.022)	0.848	0.398
Body mass	0.021 (0.021)	1.008	0.316
BL corticosterone	0.352 (0.048)	7.344	<0.001

Notes: Outputs are from PGLS models, in which phylogeny, body mass (reflecting major life history strategies), and within-species sample size (reflecting heterogeneity in sampling effort) were held constant. Effects due to latitude, breeding stage, and sex were also controlled when estimating species-specific estimates of corticosterone traits.

[a]Phylogenetic signal in the model residuals (Symonds and Blomberg 2014).

We matched the nodes of the large phylogeny (including 6670 species) with the phylogeny of species with data on BL corticosterone (including 167 species), and identified those for which the BAMM provided evidence for rate shift (Fig. 3). When we compared the maximum likelihood estimates of ancestral states for BL corticosterone between nodes depicting rate shift with the others that correspond to the background diversification rate, there was no statistically significant difference between these ($t_{25.23} = -0.209$, $P = 0.836$; Fig. 3).

SI corticosterone

We repeated the above set of analyses for SI corticosterone while including BL corticosterone in the models as an additional predictor. The general conclusions from these analyses were similar those for BL corticosterone, as we failed to demonstrate any phylogenetic relationship between the focal traits. In particular, SI corticosterone was not significantly

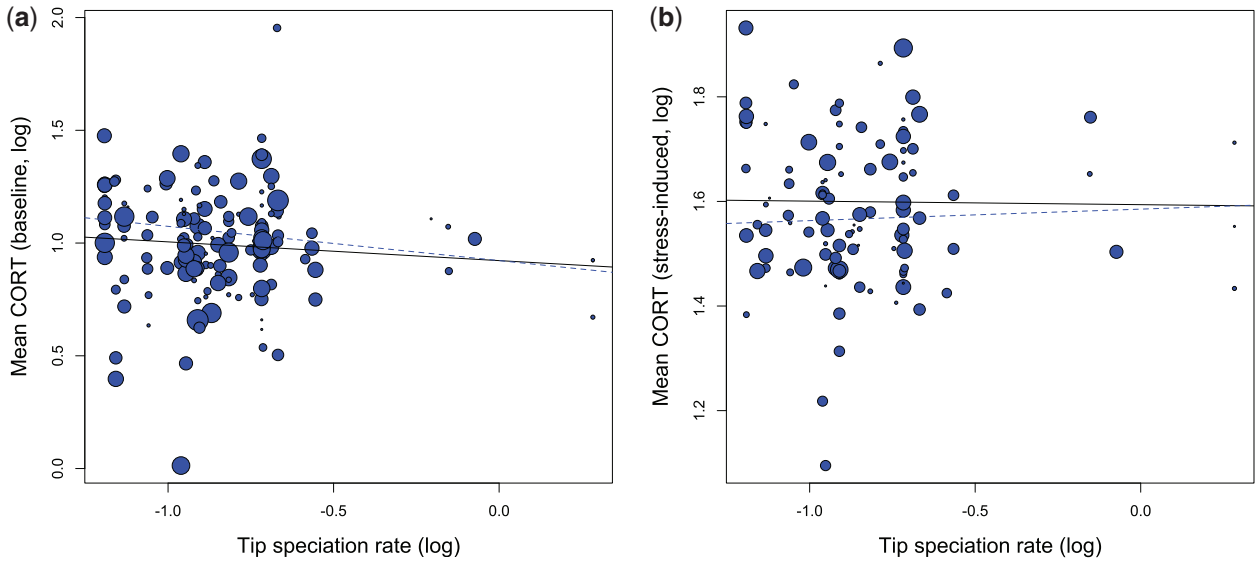

Fig. 1 Tip speciation rates and mean corticosterone in birds, for **a**) BL hormone and **b**) SI hormone levels. Dots are species-specific estimates of traits, with their size proportional to the underlying sample size. Solid black lines are the PGLS regression lines from the associated statistical model controlling for various confounds including all species available (Table 1). Dashed blue line corresponds to the PGLS regression for the family Emberizidae that included a sufficient number of species (BL corticosterone: $N = 26$; SI corticosterone: $N = 25$) to investigate family-specific effects.

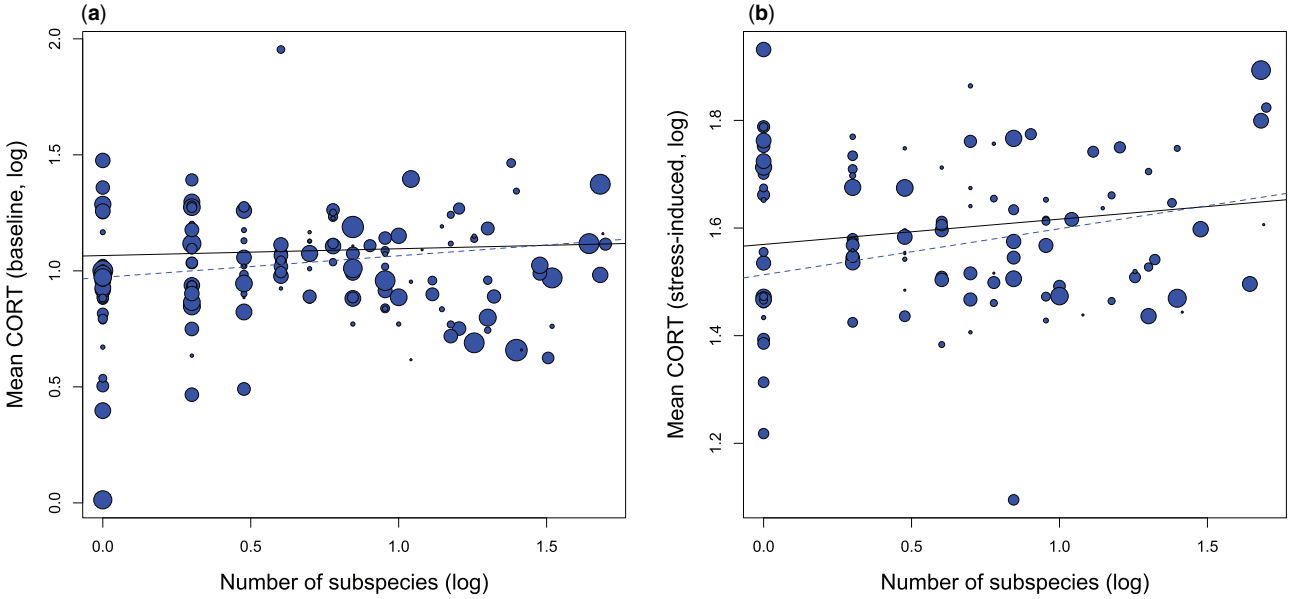

Fig. 2 Subspecies richness and mean corticosterone in birds, for **a**) BL hormone and **b**) SI hormone levels. Dots are species-specific estimates of traits with their size proportional to the underlying sample size. Solid black lines are the PGLS regression lines from the associated statistical model controlling for various confounds including all species available (Table 1). Dashed blue line corresponds to the PGLS regression for the family Emberizidae that included sufficient number of species (BL corticosterone: $N = 26$; SI corticosterone: $N = 25$) to investigate family-specific effects.

associated with tip speciation rate and subspecies richness, either when combining all birds (Table 1, solid lines on Figs. 1b and 2b) or when focusing on the Emberizidae (dashed lines on Figs. 1b and 2b, tip speciation rate: $t = 1.004$, $P = 0.327$; subspecies richness: $t = 1.113$, $P = 0.278$). Finally, ancestral state estimates of SI corticosterone did not differ statistically between nodes with and without strong evidence for rate shift ($t_{26.57} = 0.686$, $P = 0.499$; Fig. 4).

Discussion

The most important findings of this study were that i) the repeatability of the CV of corticosterone,

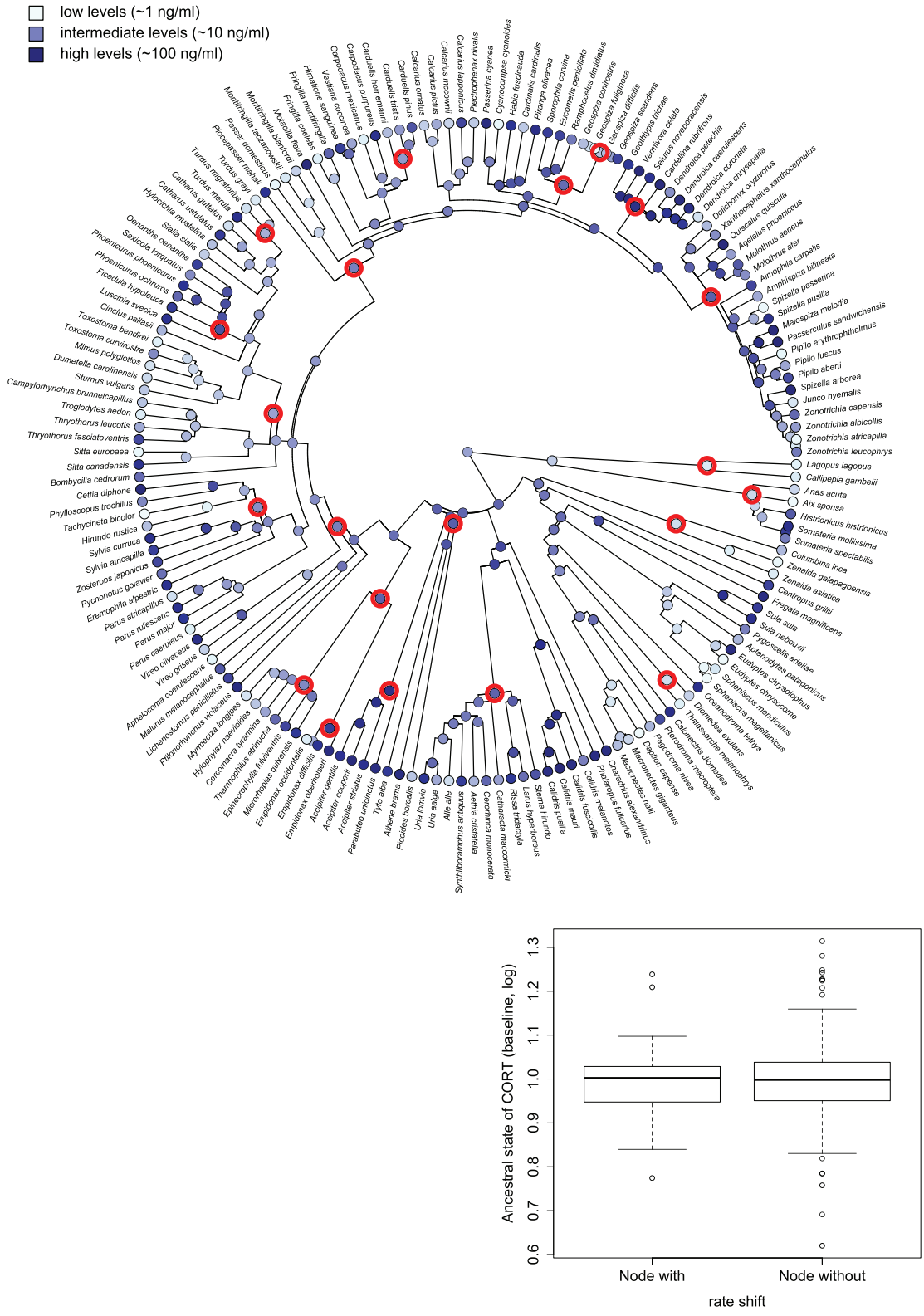

Fig. 3 Ancestral state reconstruction of BL corticosterone levels based on maximum likelihood approach. Highlighted nodes indicate nodes for which BAMM detected evidence of a rate shift. The boxplot shows the extreme of the lower whisker, the lower hinge, the median, the upper hinge, and the extreme of the upper whisker (dots are data points that lie beyond the extremes of the whiskers) separately for the two types of nodes.

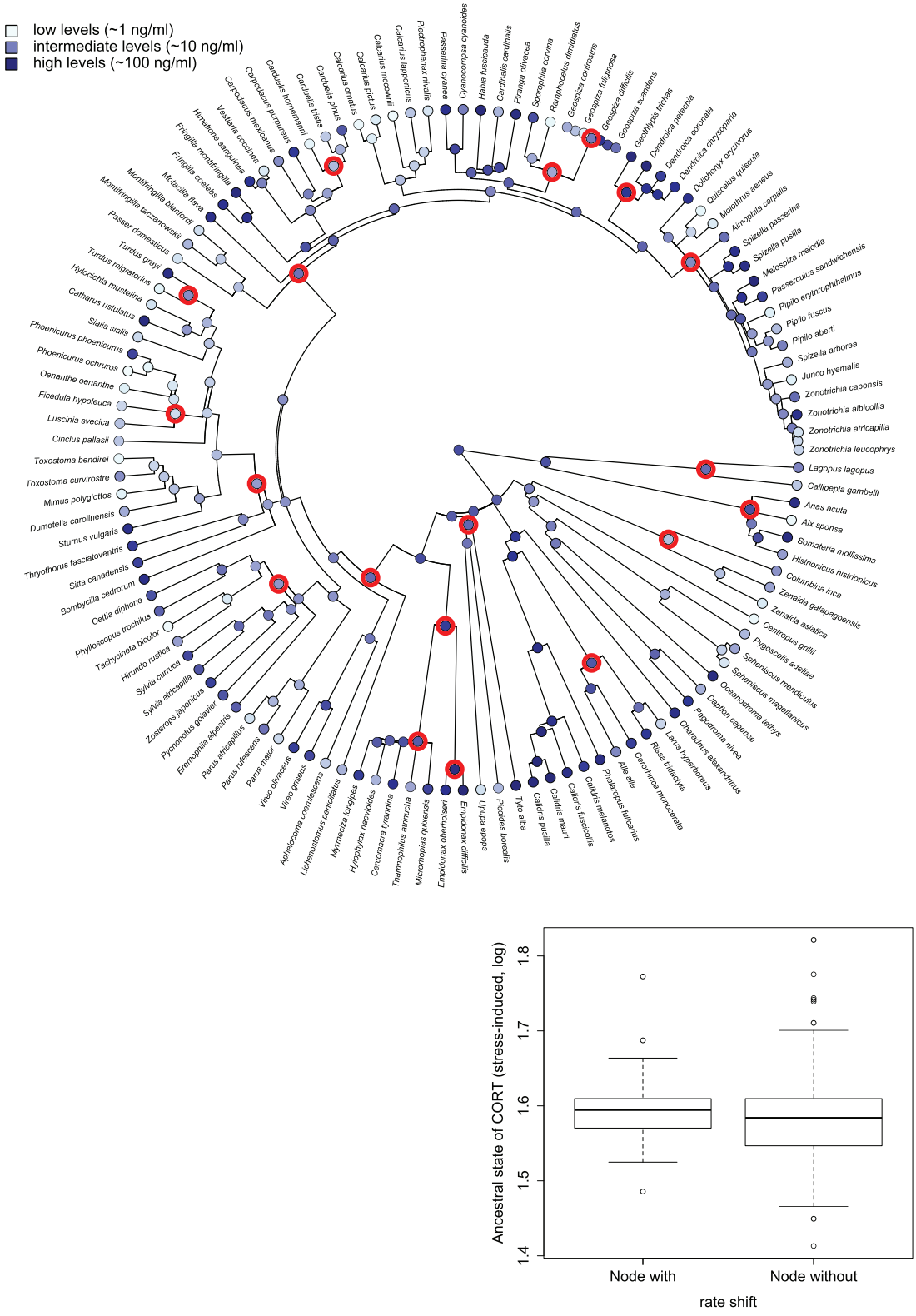

Fig. 4 Ancestral state reconstruction of SI corticosterone levels based on maximum likelihood approach. Highlighted nodes indicate nodes for which BAMM detected evidence of a rate shift. The boxplot shows the extreme of the lower whisker, the lower hinge, the median, the upper hinge, and the extreme of the upper whisker (dots are data points that lie beyond the extremes of the whiskers) separately for the two types of nodes.

which we used to reflect the within-population variance of the hormonal traits, was close to zero, precluding further analyses at the between-species level; ii) across all birds considered, species-specific means of BL and SI corticosterone were unrelated to tip speciation rate and the number of subspecies; iii) the latter two estimates of diversification rate did not predict the means of the hormonal traits even within a well-sampled family of birds (Emberizidae); and iv) ancestral state reconstruction of corticosterone did not reveal evidence for higher hormone levels at the nodes of the phylogeny with shift in diversification rate. Therefore, our results, in general, suggest that the measures of stress physiology included in these analyses play a minor, if any, role in determining speciation rates in birds. In this taxon, the evolution of circulating levels of corticosterone and the diversification of species might have occurred in an independent fashion, or the underlying evolutionary relationship is so weak that it remained unidentified at the resolution we could achieve in this study. Below, we discuss several factors that could contribute to the failure of detecting statistical support for the investigated hypotheses.

First, low statistical power cannot be excluded, as we could use empirical data on only a small proportion of extant species (i.e., representing 2.5% and 1.8% of species, for BL and SI corticosterone, respectively, on the resolved avian tree), which could have generated type II errors. Huang and Rabosky (2014) presented a simulation study in which they show that, depending on scenarios considered for the variance in speciation rate, effect sizes in the range of $r = 0.08$–0.28 coincide with acceptable statistical power (~80%) for a tip-level analysis that includes several hundred species. Given that our sample size was remarkably smaller than that of the simulation, and that an effect size $r = 0.1$ can have evolutionary importance (Møller and Jennions 2002), we can infer that currently available data preclude firm conclusions about weak but biologically relevant effects. Note that a large amount of missing data relative to the whole phylogeny not only has implications for the analyses at the tip level, but also for the estimation of ancestral states, which can be associated with increased rates of errors (Schultz et al. 1996). Furthermore, when the nodes of the complete phylogeny are matched with the nodes of the smaller tree that is pruned to only the species with empirical data, several internal nodes collapse, thus undermining our efforts to reveal a link between the locations of rate shifts and key innovations.

Second, it is also plausible that we did not account for some evolutionary factors that confound the focal relationship. Diversification rates in birds can be mediated by, for instance, cognition (Nicolakakis et al. 2003), color polymorphism (Hugall and Stuart-Fox 2012), feather ornamentation (Møller and Cuervo 1998), dispersal ability (Phillimore et al. 2006; Claramunt et al. 2012), or morphological adaptation (Ricklefs 2012; Price et al. 2014). If our sample is non-random with respect to these traits, this may blur the focal relationship. Our analyses accounted for body mass (reflecting key aspects of physical constraints and life history strategies), latitude, sex, and breeding stage effects, but obviously we were unable to consider all potential confounding variables in a phylogenetic context. Furthermore, speciation may be primarily shaped by the biogeography of species and not by their phenotypic attributes (Wiens and Donoghue 2004), which could undermine the potential relationship between a species-specific physiological trait and diversification.

Third, another important complication may arise from receptor-mediated mechanisms. Altering the concentrations of circulating corticosterone in response to environmental fluctuation is only one component of the neuroendocrine and molecular cascade that is modulated by the stress system. The physiological action of the hormone is actually realized via a large number of processes involving different precursor molecules, receptors, metabolizing enzymes, co-factors, binding proteins, etc. (Breuner and Orchinik 2002). Accordingly, if strong selection operates on any of these components, this fact would mask the effect of peripheral corticosterone levels to integrate detectable evolutionary responses, such as speciation (Adkins-Regan 2008). In other words, changes in receptor-mediated mechanisms should make between-species comparisons meaningless, because a certain corticosterone level in one species may have completely different effects in other species operating with different receptor density. However, this would imply that comparative studies using species-specific estimates of corticosterone levels could deliver null results only, which is not the case (Bokony et al. 2009; Hau et al. 2010; Lendvai et al. 2013; Brischoux et al. 2015; Pap et al. 2015; Casagrande et al. 2018).

Fourth, within-individual plasticity may preclude genetic differentiation via genotype-by-environment interactions, which can hamper the speciation process (Kitano et al. 2014). When considerable within-individual variance in corticosterone levels exists, it permits individuals to plastically adjust their hormone levels according to the prevailing ecological conditions, leading to a reaction norm with non-zero elevation along a continuous environmental

gradient (Lendvai et al. 2014; Taff et al. 2018). Therefore, the higher hormone levels that are detected in populations in more recently colonized areas (e.g., Addis et al. 2011; Liebl and Martin 2012; Walker et al. 2015; Krause et al. 2016; Martin et al. 2017) may be a result of the fact that individuals in these areas are sampled from different parts of their reaction norms but do not necessarily diverge in their mean phenotype from members of the ancestral population. The fact that some common garden studies report tendencies that are in conflict with the general expectation of higher corticosterone level in colonizing populations (Partecke et al. 2006; Atwell et al. 2012) may reflect the importance of individual plasticity. When plastic physiological responses to the environmental stimuli are available to pioneer individuals, range expansion and/or resource polymorphism will not alter corticosterone concentrations at the population level. Estimates of within-individual repeatability of corticosterone indicate that there is a high potential for phenotypic plasticity to be involved in ecological adaptation processes (Hau and Goymann 2015; Kilvitis et al. 2017; Taff et al. 2018). The moderate within-species repeatability and the weak phylogenetic signal we found for mean corticosterone traits in this study may also be a consequence of the plasticity of stress physiology at the within-individual level.

Fifth, one can postulate that we were unable to detect strong relationships between patterns of diversification and corticosterone levels, because the proposed mechanisms are operating at a finer scale than what we covered in our analyses. Even if populations that exploit new resources or expand breeding ranges adapt, in general, to the new environmental situations by elevating corticosterone levels, such short-term responses may be dissolved over the phylogenetic time scale. Maintaining consistently high corticosterone levels is costly, as it can impair immune response (e.g., Stier et al. 2009), reproductive success (e.g., Crossin et al. 2013), survival (e.g., Merkling et al. 2014), as well as growth efficiency and cognitive abilities (e.g., Kitaysky et al. 2003). Therefore, an initial increase in hormone levels in the pioneering populations may be followed by the relaxation of corticosterone activity during the long-term adaptation process, which will ultimately result in the disappearance of the interspecific correlations of species-specific mean trait. Note that in a companion study (L. B. Martin et al., submitted for publication), there was no general evidence for corticosterone levels being different between the edge and the core of the geographic area of species, indicating that mechanisms related to range expansion remain undetectable at ecological time scales as well.

Sixth, the homeostatic system to which corticosterone belongs, the hypothalamic–pituitary–adrenal axis (HPA), might be so conserved evolutionarily in birds that it is unlikely to mediate speciation within this particular taxon. The HPA axis evolved in the ancestor of all jawed vertebrates, and the descendent major tetrapod groups share the genetic infrastructure of the system (Uchoa et al. 2014). For example, glucocorticoid receptors that play a central role in the regulation of the HPA's activity diverged from the ancestors of mineralocorticoid receptors about 450 million years ago (Bridgham et al. 2006; Carroll et al. 2011). This was an early evolutionary step in vertebrates, through which the intrinsic function and specificity of the glucocorticoid receptors were irreversibly acquired as a key innovation long before birds diversified (Bridgham et al. 2009). If stress-mediated homeostasis, in general, largely depends on the integrity of the entire HPA axis (Nader et al. 2010; Nicolaides et al. 2015), this trait could have enhanced the divergence of all bony vertebrates. As a consequence, a study focusing on a lineage that is fixed for the ancestral HPA may be unlikely to capture a phylogenetic association between stress responsiveness and speciation. Accordingly, it might be that there is no relationship between corticosterone levels and diversification rate, which would support the null hypothesis of this study.

Acknowledgments

We thank the Company of Biologists, the SICB DCE, DAB, DCPB, and DEE divisions that sponsored our participation in the SICB 2018 meeting. We are grateful to J. Wingfield for generously providing unpublished data to HormoneBase.

Funding

This work was supported by funds from the Spanish Ministry of Economy and Competitiveness (CGL2015-70639-P) and the National Research, the Hungarian Development and Innovation Office (K115970) [to L.Z.G.].

Supplementary data

Supplementary data are available at *ICB* online.

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