# THE EFFECTS OF FOREST DEGRADATION ON DNA METHYLATION IN CENTRAL AFRICAN SONGBIRDS

By

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ABSTRACT

THE EFFECTS OF FOREST DEGRADATION ON DNA METHYLATION IN CENTRAL AFRICAN SONGBIRDS

Andrew Kendall Wiegardt

Environmentally-induced stress can initiate a molecular response through DNA methylation, which can alter gene expression, thereby serving as a mechanism allowing individuals to acclimate to a changing environment within their lifetime. In addition to DNA methylation, the production and release of corticosterone is a physiological mechanism by which birds can cope with acute environmental stressors. To assess how environmental stress impacted DNA methylation and corticosterone, I collected blood and feather samples from three understory avian species (*Alethe castanea*, *Bleda notatus* and *Pseudalethe poliocephala*), along a disturbance gradient in the lowland Guinean rainforest adjacent to the village of Oyala, Equatorial Guinea. I used two binomial mixed-effect models for each species to identify gene loci that were differentially methylated with respect to the amount of corticosterone deposited in a tail feather and distance to forest edge when captured. I identified 195, 126 and 246 sites differentially methylated by feather CORT and 296, 166 and 254 sites differentially methylated by distance to forest edge for *Alethe castanea*, *Bleda notatus* and *Pseudalethe poliocephala*, respectively. I also found a significant negative relationship between feather CORT and overall feather brightness indicating there may be trade-offs between coping with

environmental stressors and fitness. I found substantial variation in CORT and DNA methylation along the disturbance gradient, suggesting that environmentally-induced molecular and endocrinological responses are prevalent in degraded tropical forests.

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# INTRODUCTION

Environmental stressors, including biotic factors such as limited food availability, the presence of predators, infection with pathogenic organisms, or interactions with conspecifics, as well as abiotic factors such as temperature, water availability, and toxicants (Schulte 2014), can trigger a molecular response in organisms through epigenetic changes that can alter gene expression and potentially individual fitness (Bird 2007). Epigenetic changes can be adaptive by facilitating a rapid adaptation to a changing environment (Baerwald et al. 2016, Rubenstein et al. 2016) but can also result in maladaptive changes that negatively affect fitness (Le Luyer et al. 2017). Interestingly, at least some epigenetic changes may be heritable, influencing gene expression in subsequent generations (Berger et al. 2009). DNA methylation is one of the most studied epigenetic changes and occurs when an environmental stimulus causes a methyl-group to attach to a cytosine base that is followed by a guanine base, termed CpG (Bird 2007). Dense CpG sites are often grouped near genomic regions associated with gene regulation. Methylation of CpG sites can result in promotion, reduction, or silencing of gene expression (Bird 2007). DNA methylation has been shown to facilitate acclimation to a variety of environmental stressors (Baerwald et al. 2016, Lea et al. 2016, Rubenstein et al. 2016). For example, prebreeding rainfall was strongly related to nestling DNA methylation in *Lamprotornis superbus* (Superb Starlings) with lower DNA methylation of the glucocorticoid receptor gene in poorer-quality years with a drier prebreeding season (Rubenstein et al. 2016). The glucocorticoid receptor is the receptor to which

corticosterone and other stress hormones bind and functions to regulate genes related to development, metabolism, and immune response (Rubenstein et al. 2016). DNA methylation played a critical role in an individual's ability to acclimate to climatic variation in the East African savannah (Rubenstein et al. 2016). Le Luyer et al. (2017) found that the hatchery environment can cause drastically different DNA methylation compared to wild-reared *Oncorhynchus kisutch* (Coho Salmon), which may play a role in the reduction of fitness in hatchery-reared salmonids. Across diverse taxa, DNA methylation can aid in the rapid adaptation of organisms to changing environments (Verhulst et al. 2015, Weyrich et al. 2015), yet also can be associated with reductions in fitness (Le Luyer et al. 2017). As such, the degree to which an organism's gene expression is altered via DNA methylation may affect its capacity to acclimate to human-dominated landscapes, degraded habitats and a warming climate.

One physiological mechanism that allows birds to cope with environmental stressors is the production and release of glucocorticoids, a group of steroid hormones. The release of glucocorticoids alters certain behaviors and physiological processes that enable birds to cope with environment stress, such as lack of resources or high densities of predators (Sapolsky et al. 2000). One of the most studied glucocorticoids is corticosterone (CORT), a hormone associated with the physiological stress response that is released from the avian hypothalamic-pituitary-adrenal (HPA) axis which can help birds cope, in the short-term, with acute environmental stressors (Wingfield and Kitaysky 2002). However, chronically high levels of CORT are associated with negative physiological effects, such as reduced feather quality, leading to higher energetic costs of

flight and self-maintenance (Romero et al. 2005). Advances in endocrinological techniques allow quantification of CORT deposited in feathers, where the amount of feather CORT has been linked to environmental stress (Bortolotti et al. 2008, Fairhurst et al. 2011, Lattin et al. 2011).

Feather brightness has been associated with fitness in several avian species (Keyser and Hill 2000), however the relationship between feather CORT and feather brightness remains unclear. For example, Kennedy et al. (2013) found no relationship between feather hue and feather CORT, but a strong negative relationship between feather CORT and feather brightness of the red wing bar on *Agelaius phoeniceus* (Red-winged Blackbird). Contradictory to this finding, Fairhurst et al. (2014) found a strong relationship between feather CORT and the strength of carotenoid signals in adult male *Acanthis flammea* (Common Redpoll). The relationship between feather quality and feather CORT also appears unclear. Fairhurst et al. (2014) also found that the width of feather growth bars was positively correlated with feather CORT, but negatively correlated with the probability of having fault bars, indicating that *Acanthis flammea* with more CORT grew their feathers more quickly, which is typically associated with poorer quality feathers, but had less of likelihood of fault bars, typically associated with better quality feathers. Although a potentially powerful method of assessing history of stress, general patterns and relationships between feather CORT and CORT in blood and plasma are obscured due to variation among species as well as individuals (Fairhurst et al. 2013). Experimentally altering CORT levels can alter gene expression through DNA

methylation in *Mus musculus* (Laboratory Mouse, Lee et al. 2010), however this relationship has not been explored in wild birds.

One of the most ubiquitous forms of environmental stress birds face within human-dominated landscapes is habitat fragmentation and degradation. Logging and forest fragmentation have been shown to decrease certain avian species' abundance and productivity, but the molecular mechanisms associated with these changes has been relatively unstudied in tropical birds (Peh et al. 2006). Disturbed habitats often have more edges and less complex canopy structure relative to undisturbed primary forest leading to increased sunlight reaching the ground, which can result in understory desiccation during dry periods (Pohlman et al. 2007). This leads to variable microclimates and heightened primary productivity during wet periods and suppressed primary productivity during dry periods, which in turn produces unpredictable arthropod resources for forest bird communities in degraded forest (Kremen et al. 1993, Chazdon 2014, Peters et al. 2009). Disturbed areas often have higher densities of certain raptor species that prefer open canopies (Jullien and Thiollay 1996), which can cause birds residing in disturbed habitat to encounter chronic stressors throughout their lives compared to birds in undisturbed habitat (Lens et al. 1999). These chronic sources of disturbance affect bird diversity in dynamic ways and disproportionately harm those bird species most reliant on pristine forest habitats (Thiollay 1999). Nectivores, upper canopy frugivores, omnivores, and gap, edge and secondary growth specialists tend to increase after disturbance, whereas understory insectivores and mixed-species flock obligates are particularly vulnerable (Thiollay 1999, Powell et al. 2015, Wolfe et al. 2015a, Powell et al. 2016). Birds

sensitive to habitat degradation are well-represented in lowland Congolese forest throughout the central African country of Equatorial Guinea. Human population growth and rapid development drive habitat degradation in Equatorial Guinea. Vast expanses of pristine forest coupled with rapid development has created a disturbance gradient upon which lowland tropical birds are subjected to variation in environmental stress.

The first main objective of this project was to determine whether forest birds were affected, at the molecular level, by logging and forest fragmentation, by identifying gene sites that were differentially methylated in relation to distance to forest edge and their potential functional roles. The second main objective was to determine whether DNA methylation in forest birds was related to feather CORT, by identifying gene sites that were differentially methylated in relation to feather CORT. To further assess how environmental stress influences forest birds, an additional objective was to determine whether feather CORT was related to overall feather brightness and growth bar width.

# MATERIALS AND METHODS

## Field Methods

To examine effects of forest degradation on stress and its relationship with DNA methylation in forest-dwelling birds, I used mist-nets to capture landbirds in primary and second growth forest adjacent to the village of Oyala, Equatorial Guinea. The study area was comprised of both upland and seasonally-inundated lowland Guinean rainforest. The primary forest has only had very light selective logging in the 1980s and the secondary forest was commercially logged approximately 2 decades ago and has been regularly selectively harvested since. Additionally, the secondary forest plot was much closer to the city center being constructed (Figure 1). Due to the fragmented nature of the secondary forest there were two disconnected sections of secondary forest (Figure 1). With the help of Biodiversity Initiative, I operated mist-nets for 6 hours beginning at sunrise. There were 8 banding stations in primary forest plot and 7 in the secondary. We operated each banding station once in January 2016 then again in March 2016, where each station was comprised of six 12x2.5m mist-nets. In December 2016 to January 2017 and January 2018 we increased the number of mist-nets to 20 12x2.5m mist-nets for most stations where each station was run twice in successive days to maximize recaptures from previous banding attempts.

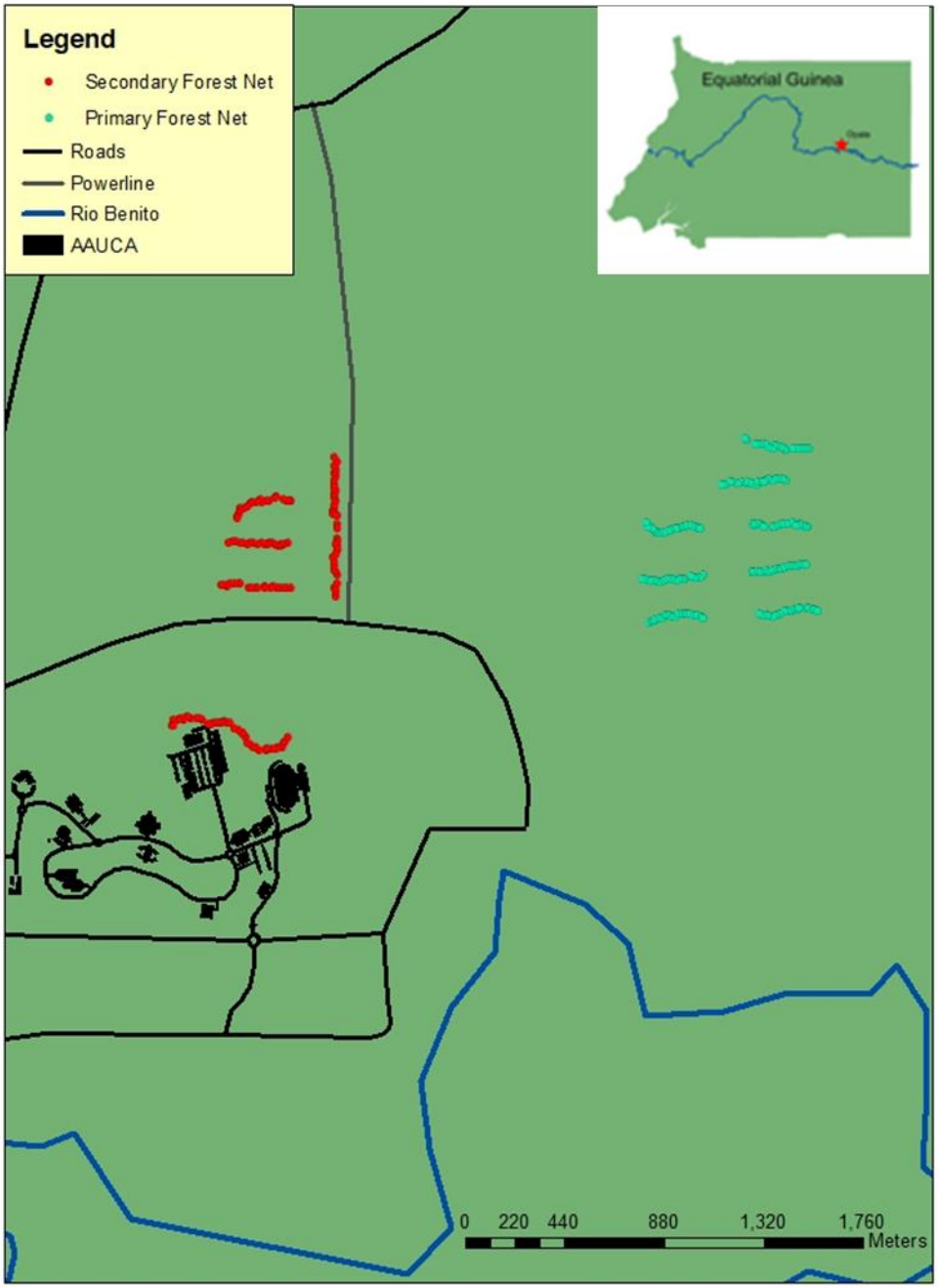


Figure 1. A map of the study area in Oyala, Equatorial Guinea in Central Africa, including net locations in primary forest (blue) and secondary forest (red), the Afro-American University of Central Africa and the roads surrounding (black), a large powerline that fragments the forest (gray) and the River Rio Benito (dark blue).

I chose three non-migratory study species that reside in both primary and secondary forest in Equatorial Guinea: Lesser Bristlebill (*Bleda notatus*), a generalist, and two understory insectivores, Fire-crested Alethe (*Alethe castanea*) and Brown-chested Alethe (*Pseudalethe poliocephala*). All three species will facultatively forage at ant-swarms, which is a guild that has been shown to be particularly susceptible to forest degradation (Peters et al. 2008, Wolfe et al. 2015b), however *Bleda notatus* can switch to frugivory when arthropod food resources are not plentiful. To maximize the likelihood that blood and feather samples represent habitats within my study plot, I selected study individuals which were recaptured on the same plot more than 7 months after initial capture (Table 1). We banded each captured bird with a unique aluminum band and collected blood, feces and a tail feather (outer right retrix). We collected blood from the brachial vein using 26.5-gauge needles then transferred using capillary tubes to 1.5 ml centrifuge tubes with 0.6 ml of Longmire Buffer (Longmire et al. 1988) and stored at room temperature until samples could be exported back to the United States where samples were stored at -80°C until use. We collected feathers in acid-free paper envelopes and stored at room temperature. I conducted all research under IACUC 17/18.W.06-A.

## Lab Methods

### DNA Methylation Library Preparation

I constructed DNA methylation libraries using a reduced representation bisulfate sequencing (RRBS) approach that preferentially sequences cytosines within CpG islands,

which are often found in gene regulatory regions (Meissner et al. 2005). First, DNA was extracted from blood samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen Cat No. 69504) and quantified using Qubit 2.0 fluorometer (ThermoFisher Scientific). Then, I digested 600ng of genomic DNA with a BsaWI enzyme (NEB Cat No. R0567S), which preferentially cleaves at 5'-WCCGGW-3' sites, where W denotes the nucleotide is an adenine or thymine. Given this cut site, BsaWI selects for these CpG islands, so whereas ultimately only ~2% of the genome was sequenced, it encompassed most methylated regions (Meissner et al. 2005). Enzyme digestion was verified using electrophoresis on 2% agarose gel. After digesting with BsaWI, AMPure magnetic beads were used to select DNA fragments of 200-350 base pairs (bp) in length using a DNA-to-AMPure bead ratio of 1:0.55 first then a ratio of 1:0.16. Before the bisulfite conversion, I included Lambda DNA (NEB Cat No. N3011S) in each sample to estimate bisulfite conversion rate for each sample. I used the Qiagen Epitect bisulfite conversion kit (Qiagen Cat No. 59104) to convert all unmethylated cytosines to uracils. I then amplified DNA using PCR with an initial denature of 95°C for 30 seconds, 15 cycles of 95°C for 15 seconds, 61°C for 30 seconds, and 68°C for 30 seconds, and a final extension period of and 68°C for 5 minutes. A final double AMPure bead clean and 80% ethanol wash was used to further remove any additional DNA fragments larger than 350 bp. The DNA to AMPure bead ratios for both final bead cleans were 1:1 for the first selection and 1:0.9 for the second. I uniquely indexed each sample with a unique combination of basepairs, pooled and sequenced using an Illumina HiSeq 2500 at Princeton University. During sequencing the

unmethylated cytosine which were converted to uracil were sequenced as thymine, thus, any cytosine sequenced represented a methylated site.

Sexing using PCR

I used polymerase chain reaction (PCR) methodologies with P8 (5'-CTCCCAAGGATGAGRAAYTG -3') and P2 (5'-TCTGCATCGCTAAATCCTTT -3') primers to determine sex of each bird from DNA extracted from blood samples following Jensen et al. (2003). I ran samples in a thermocycler with an initial denature period of 95°C for 30 seconds, 35 cycles of 95°C for 30 seconds, 52°C for 60 seconds, and 72°C for 45 seconds, and a final extension period of and 72°C for 5 minutes. These primers cleave sections of DNA of different lengths on the Z and W chromosomes. The section of the Z chromosome that was cleaved by the primer was approximately 320 bp and the section of the W chromosome was approximately 400 bp. I then dyed subsequent DNA fragments and assessed them using electrophoresis on a 2% agarose gel for 75 minutes which resulted in two distinct bands at 320 bp and 400 bp for females and a single band at 320 bp for males.

Corticosterone in Feathers

Dr. Christopher Tonra quantified feather CORT at Ohio State University using a methanol-based extraction, modified from Bortolotti et al. (2008), and a commercial ELISA kit (Corticosterone ELISA kit; Neogen Corporation, Ayr, UK) as validated by Carbajal et al. (2014). The protocol involved mincing samples and incubating them overnight in a methanol shaking water bath. Extracts were separated from solids using vacuum filtration, evaporated under nitrogen gas, then reconstituted in Neogen extraction

buffer and run through ELISA kit procedures. Assay recovery was assessed by adding 20μL of tritium-labeled CORT in each sample and using a scintillation counter, with a mean recovery rate was 0.93. Intra-assay variation based on duplicate samples was 3.5%, and inter-assay variation (n = 2) based on in-house standards was 6%.

Spectrometry and Ptilochronology

I used an Ocean Optics S2000 spectrometer, a PX-2 xenon pulse lamp, and a fiber-optic probe (Ocean Optics, Dunedin, Florida) to assess feather brightness across the wavelengths 320-700 nm, following Zirpoli et al. (2013). Rectrices were frozen for 48 hours to remove mites, thawed and completely dried before conducting spectrometry. I placed feathers on black paperboard and positioned the fiber-optic probe 90° to the feather and measured reflectance at 6 equidistant points between the dark bars on the outer vane (Zirpoli et al. 2013). I used program R (R Core Team 2018, version 3.4.3) and library *pavo* (Maia et al. 2017, version 1.3.1) to calculate feather brightness as the total amount of light reflected, relative to a white standard across the wavelengths 320-700nm.

I conducted ptilochronology following Grubb (1989), where each feather was placed on a Styrofoam board and light and dark growth bars were measured using calipers and small continental pins. I calculated standard deviation (mm) of growth bars (both the light and dark bars) to assess the relationship between variation in growth bars and feather CORT.

# Analytical Methods

## DNA Methylation

I aligned my DNA sequences from RRBS, to the *Taeniopygia guttata* genome (Zebra Finch, version 3.2.4) using BS-Seeker (Chen et al. 2010) with a mismatch setting of six to account for the distant relationship between my study species and *Taeniopygia guttata*. Then I determined the total count of methylated cytosines and the total number of reads at each locus sequenced. Loci with less than 10 reads were removed from the model. I used DNA sequences data from the RRBS to generate pairwise kinship coefficients for each individual of each species, using the software package *samtools* (Li et al. 2009, version 1.7) and the R package *GENESIS* (Conomos et al. 2018, version 2.8.1). Specifically, I used *samtools* to call single nucleotide polymorphs from the sequencing data and convert the sequencing data into variant call format. Then I used *GENESIS* to run a principal components analysis of related samples to ordinate my single nucleotide polymorphs and used the first two principle components to generate the pairwise kinship coefficients. I compiled the pairwise kinship coefficients into a genetic relatedness matrix, which I included in the model to address the confounding issue that DNA methylation may be more similar between closely related individuals.

To assess differential DNA methylation associated with residing in disturbed forest compared to primary forest, I created two binomial mixed-effect models, for each species, using program R (R Core Team 2018, version 3.4.3) and the package *MACAU2* (Lea et al. 2015, version 1.10). The two binomial mixed-effect models examined

differential methylation relative to feather CORT (pg/mm) and distance to edge (log-natural meters). Count of methylated reads was used as the response variable and a random-effect of the genetic relatedness matrix as well as read coverage for each site, sex, and conversion rate were used as a predictor variables. Significantly differentially methylated sites were determined when β-hat 95% confidence interval (CI) did not encompass zero. I ran the RRBS in 3 separate batches, which could lead to differential conversion rates, but after examining model output I decided to remove batch to improve model convergence, since there was little variation attributed to batch effects (Figure 2).

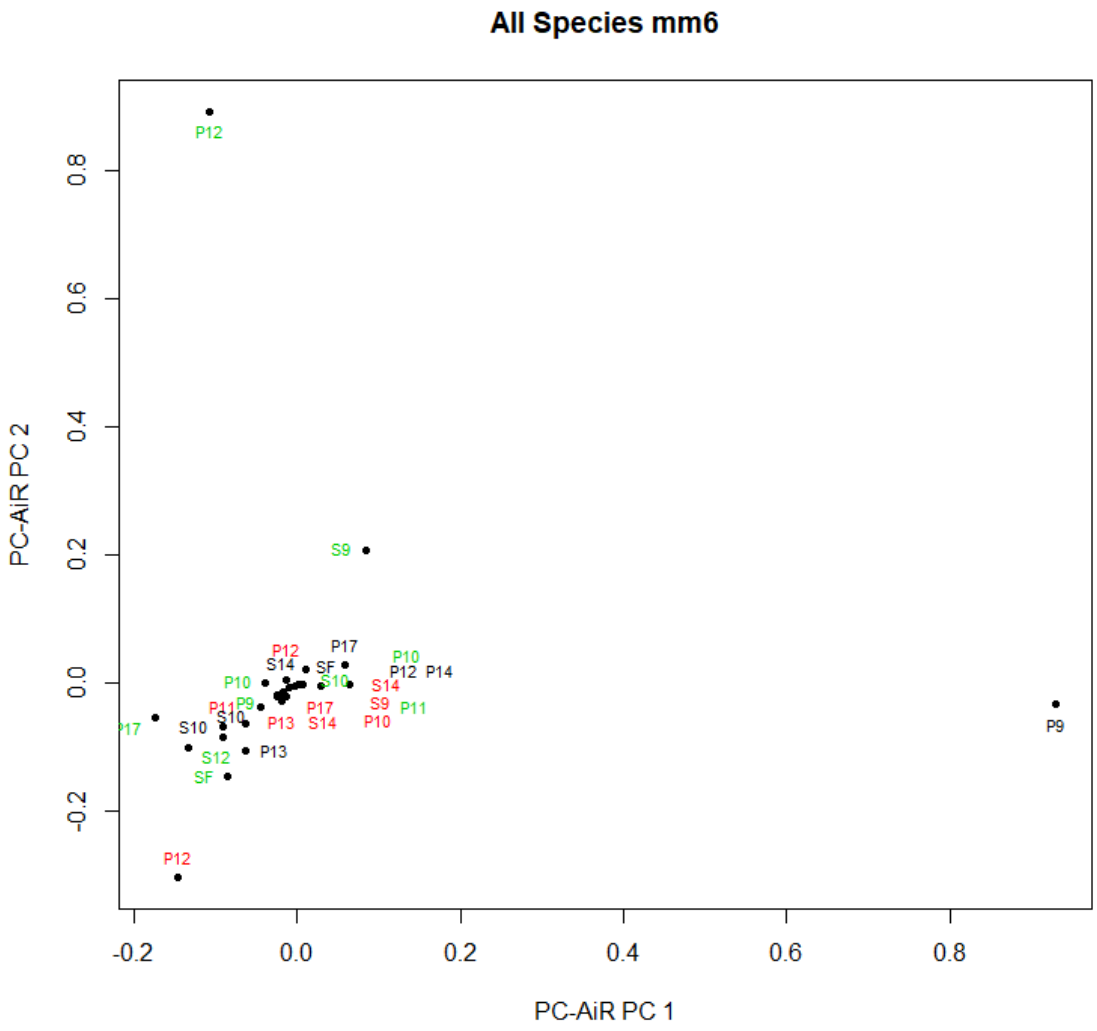


Figure 2. A PCA of related samples created using R package GENESIS (Conomos et al. 2018) with the first two principal components as the axes, with the banding station each bird was captured at colored by batch. Primary forest stations begin with P, secondary forest stations with S.

Once I determined significantly differentially methylated gene loci, I used the ENSEMBL BIOMART tool (Ensembl release 92) to download gene ontology terms for *Taeniopygia guttata* to define the functions of each of my differentially methylated gene loci. I examined the functions of my differentially methylated gene loci at three different levels to fully understand the role CORT and distance to edge play on DNA methylation. For the first and most fine-scale method, I used R (R Core Team 2018) and the libraries *clusterProfiler* (Yu et al. 2017, version 3.6.0), *DOSE* (Yu et al. 2017, version 3.4.0), *GO.db* (Carlson et al. 2018, version 3.5.0) and *qdapRegex* (Gray et al. 2017, version 0.7.2) to attach gene ontology terms and summarized their frequencies. The next and most broad scale method was to classify the gene ontology terms into three domains: biological process, cellular component, molecular function. For the last method of determining function, I used the Kyoto Encyclopedia of Genes and Genomes to determine the pathways that my differentially methylated genes are involved in. Visualizations were created using Arcmap 10.5 (ESRI 2017) and R (R Core Team 2018, version 3.4.3) libraries *ComplexHeatmap* (Gu 2017, version 1.17.1) and *Lattice* (Sarkar 2017, version 0.20-35).

Spectrometry and Ptilochronology

I created a single linear model to explore the relationship between feather CORT, brightness, and growth rate. Using the same 3 species and study individuals, I included feather CORT (pg/mm) as the response variable and species, total feather brightness, and the standard deviation of the growth bars as predictor variables. Residuals were examined following Faraway (2005) to test model assumptions.

# RESULTS

## DNA Methylation

The RRBS generated between 12369-124347 gene loci with more than 10 reads for each individual. The number of shared sites with greater than 10x coverage, was 5502 for *Alethe castanea* (n = 9), 3441 for *Bleda notatus* (n = 12) and 6223 for *Psuedalethe poliocephala* (n = 7). My bisulfite-conversion was efficient with a mean conversion rate of 95.1%. Some of the gene loci did not converge in the models due to lack of variation in methylation with respect to either CORT or distance to edge (Table 1). Additionally, there were several loci that didn't have annotated functions in the *Taeniopygia guttata* genome summarized in Table 1.

Table 1. Table summarizing the number of shared gene loci for *Alethe castanea* (n = 9), *Bleda notatus* (n = 12) and *Psuedalethe poliocephala* (n = 7), as well as the number of converged loci for two binomial mixed effect models, one with CORT (pg/mm) as the response and one with distance to edge (log natural meters) and the number of sites with annotated gene ontology functions from ENSEMBL genome browser.

| Species | Shared sites | Converged sites: CORT | Converged sites: Distance to edge | Annotated sites: CORT | Annotated sites: Distance to edge |
|---|---|---|---|---|---|
| *Alethe castanea* | 5502 | 3510 | 3498 | 1432 | 1388 |
| *Bleda notatus* | 3441 | 2594 | 2611 | 1032 | 1051 |
| *Psuedalethe poliocephala* | 6223 | 2562 | 2609 | 1057 | 1070 |

Overall, I found that many more gene loci annotated in the biological process domain were significantly differentially methylated than loci annotated in the cellular component or molecular function domains (Table 2). I found more gene loci in the molecular function domain that were differentially methylated with respect to distance to edge than CORT. I found a much greater proportion of differentially methylated sites in the intron of the gene compared to the exon or promoter regions (Table 3). The eight most common phrases included in multiple gene ontology functions are summarized in Figure 3. I determined the gene ontology terms of the top 50 most extreme beta estimates of the significantly differentially methylated sites from each model and found 13 GO terms shared among all three species for the CORT model, 11 for the distance model, and 9 in all three species in both models (Table 4).

Table 2. Number of significantly different shared gene loci, for each model, by gene ontology domain from ENSEMBL genome browser (Ensembl release 92). Model notation is the Latin name of each species and the predictor of interest (corticosterone and distance to edge).

| Model | Biological Process | Cellular Component | Molecular Function | Unknown |
|---|---|---|---|---|
| *A. castanea* CORT | 66 | 12 | 8 | 114 |
| *A. castanea* Distance to Edge | 78 | 7 | 14 | 197 |
| *B. notatus* CORT | 34 | 11 | 0 | 81 |
| *B. notatus* Distance to Edge | 45 | 8 | 3 | 110 |
| *P. poliocephala* CORT | 61 | 8 | 10 | 167 |
| *P. poliocephala* Distance to Edge | 70 | 8 | 12 | 164 |

Table 3. Total number of shared gene loci, with significant number of gene loci in parentheses, in the intron, exon, and promoter regions, for each model, from ENSEMBL genome browser (Ensembl release 92). Model notation is the Latin name of each species and the predictor of interest (corticosterone and distance to edge).

| Model | Exon | Intron | Promoter | Unknown |
|---|---|---|---|---|
| *A. castanea* CORT | 1090 (82) | 103 (6) | 239 (11) | 2078 (96) |
| *A. castanea* Distance to Edge | 1059 (89) | 97 (9) | 232 (24) | 1560 (174) |
| *B. notatus* CORT | 799 (44) | 57 (3) | 176 (12) | 1561 (67) |
| *B. notatus* Distance to Edge | 816 (58) | 56 (2) | 179 (12) | 1560 (94) |
| *P. poliocephala* CORT | 783 (80) | 78 (8) | 196 (9) | 1505 (149) |
| *P. poliocephala* Distance to Edge | 791 (82) | 78 (9) | 201 (15) | 1539 (148) |

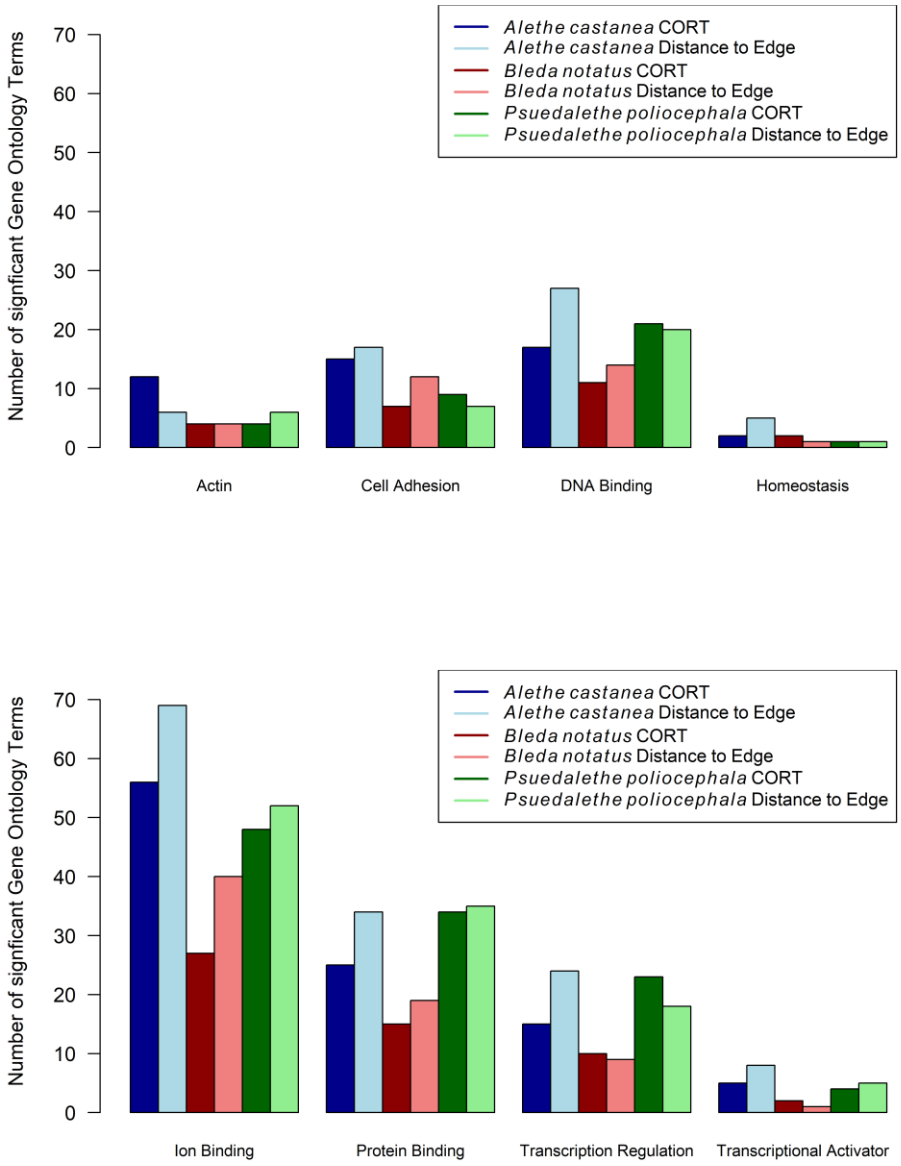


Figure 3. The number of significant differentially methylated gene ontology terms of eight different biological phrases, for *Alethe castanea* (blue), *Bleda notatus* (red) and *Psuedalethe poliocephala* (green) and the two different independent variables of interest: CORT (dark colors) and distance to edge (light colors).

Table 4. The gene ontology terms with the most extreme betas that were significantly differentially methylated with respect to CORT, distance to edge or both, in all three study species (*Alethe castanea*, *Bleda notatus*, *Psuedalethe poliocephala*). Complete lists of significantly differentially methylated gene ontology terms for each species can be found in Appendix A.

| CORT Model | Distance Model | Both Models |
|---|---|---|
| Cytoplasm | ATP binding | Cytoplasm |
| Cytosol | Cytoplasm | Cytosol |
| DNA binding | Cytosol | DNA binding |
| Identical protein binding | DNA binding | Integral component of membrane |
| Integral component of membrane | Integral component of membrane | Membrane |
| Membrane | Integral component of plasma membrane | Nucleoplasm |
| Nuclear speck | Membrane | Nucleus |
| Nucleoplasm | Nucleoplasm | Regulation of transcription, DNA-templated |
| Nucleus | Nucleus | Sequence-specific DNA binding |
| Regulation of transcription, DNA-templated | Regulation of transcription, DNA-templated | |
| Sequence-specific DNA binding | Sequence-specific DNA binding | |
| Transcription factor binding | | |
| Transferase activity | | |

For *A. castanea*, 195 of the 3510 converged gene loci were significantly differentially methylated with respect to CORT (Figure 4). Many of the gene loci are not involved in the 169 documented KEGG pathways documented for *Taeniopygia guttata*, but there were four of the significantly different loci function in the Adherens junction, basal transcription factors, oxidative-phosphorylation and ribosome pathways. 296 of the 3496 converged gene loci were significantly differentially methylated with respect to log-natural distance to edge (Figure 5) and 8 KEGG pathways were found to contain 9 significantly differentially methylated loci: ABC transporters, basal transcription factors, glycerophospholipid metabolism, glycerolipid metabolism, non-homologous end-joining, peroxisome, ribosome and spliceosome pathways. Two of the loci were involved in the ribosome pathway.

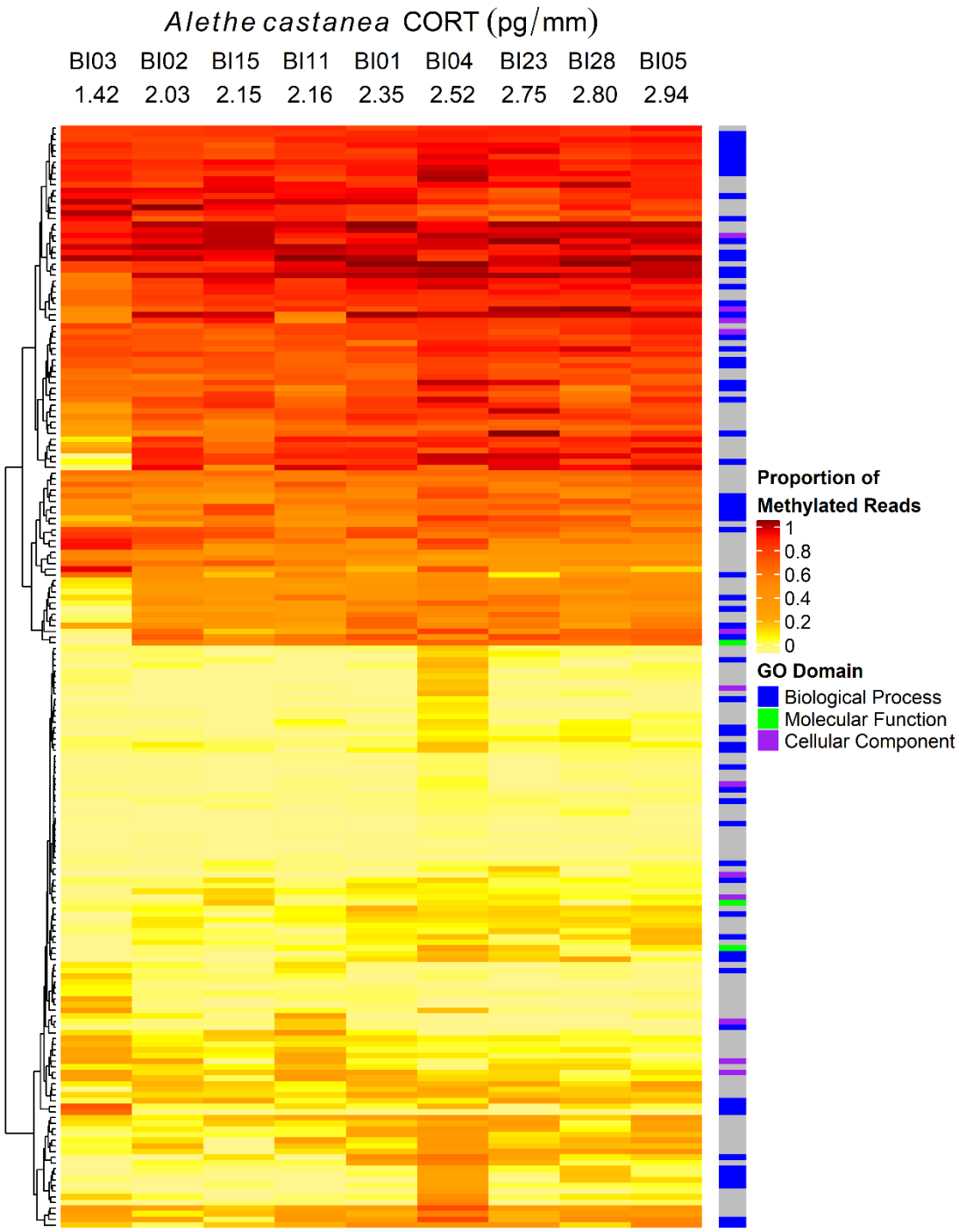


Figure 4. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to CORT for each individual of *Alethe castanea*. Feather CORT (pg/mm) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to CORT.

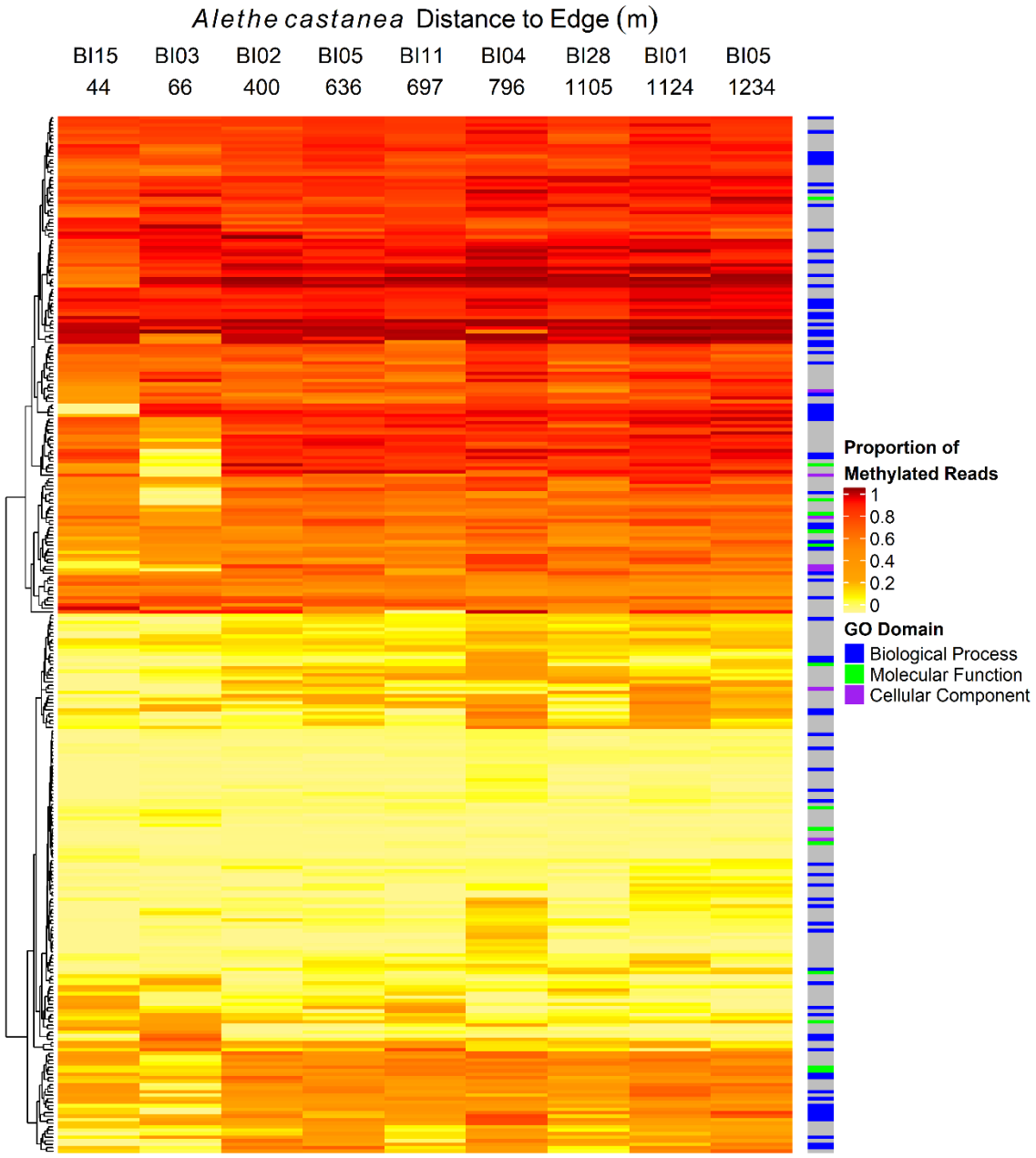


Figure 5. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to distance to edge, for each individual of *Alethe castanea*. Distance to edge(m) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to distance to edge.

For *B. notatus*, 126 of the gene loci were significantly differentially methylated with respect to CORT (Figure 6). Eight of the significantly different loci were documented to function in the apoptosis, basal transcription factor, lysine degradation, RNA transport, ribosome and spliceosome pathways. Two of the significantly different loci were involved in the apoptosis and ribosome pathways. 166 gene loci were significantly differentially methylated with respect to log-natural distance to edge (Figure 7) and three of those loci were involved in the apoptosis pathway, three in the RNA transport pathway, two in the MAPK signaling pathway, and one in the endocytosis pathway.

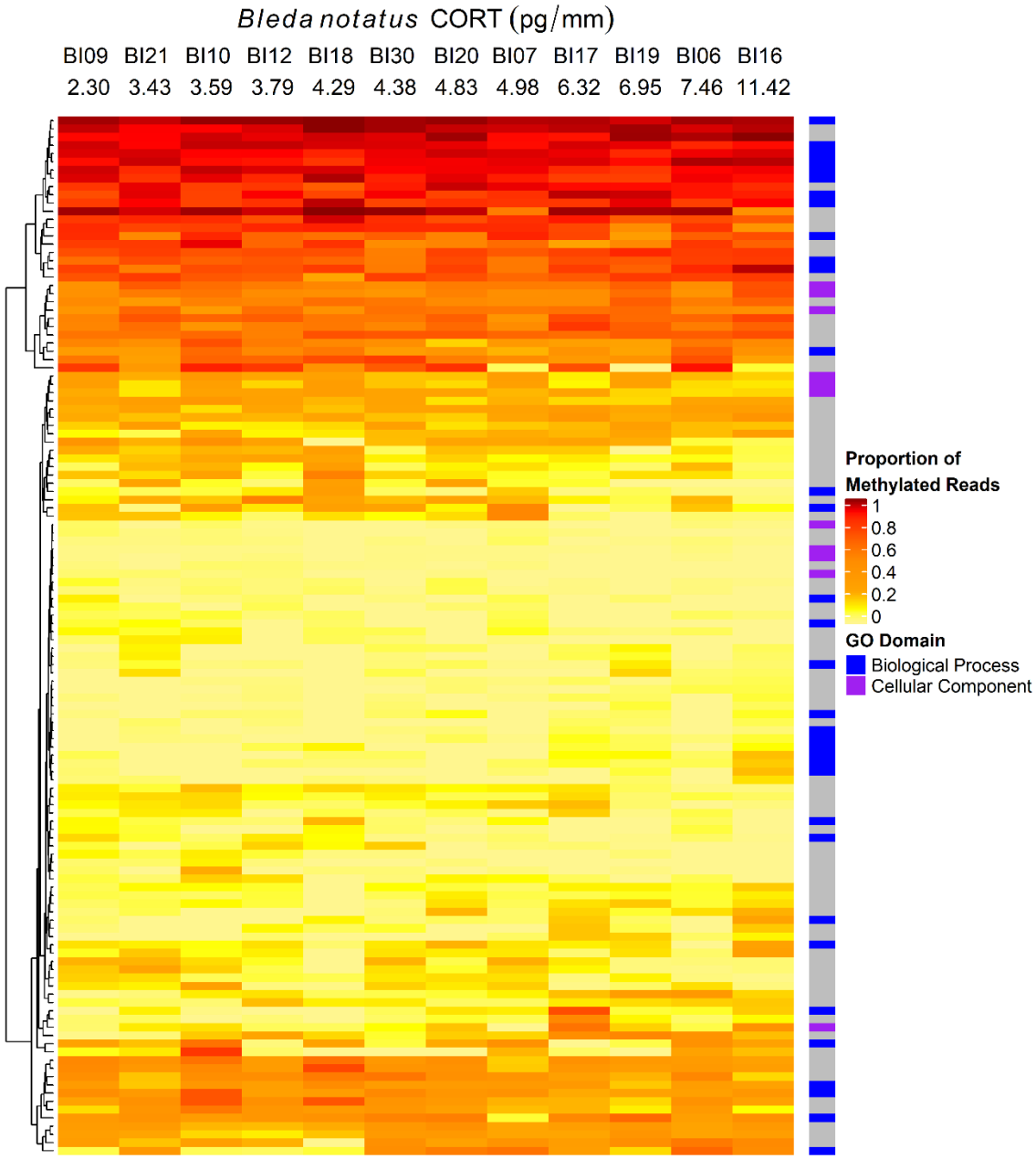


Figure 6. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to feather CORT, for each individual of *Bleda notatus*. Feather CORT (pg/mm) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to CORT.

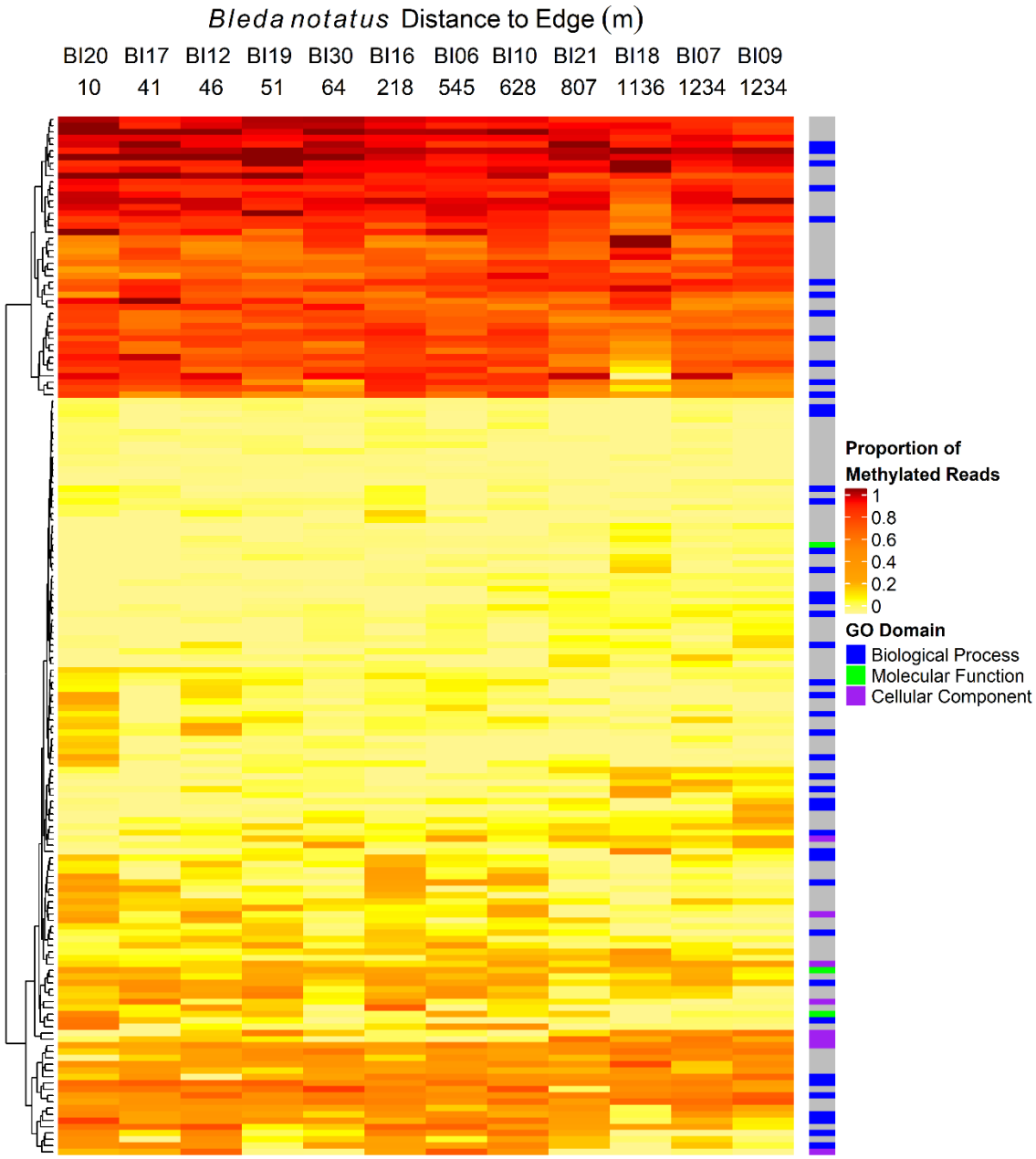


Figure 7. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to distance to edge, for each individual of *Bleda notatus*. Distance to edge (m) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to distance to edge.

For *P. poliocephala*, 246 of the gene loci were significantly differentially methylated with respect to CORT (Figure 8). Nine of the significantly different loci function in the Adherens junction, animal autophagy, cellular senescence, ferroptosis, galactose metabolism, lysine degradation, purine metabolism and spliceosome pathways. 254 were significantly differentially methylated with respect to log-natural distance to edge (Figure 9) and 8 KEGG pathways: adipocytokine signaling, animal autophagy, ferroptosis, galactose metabolism, neuroactive ligand-receptor interaction, purine metabolism, spliceosome, ubiquinone and other terpenoid-quinone biosynthesis.

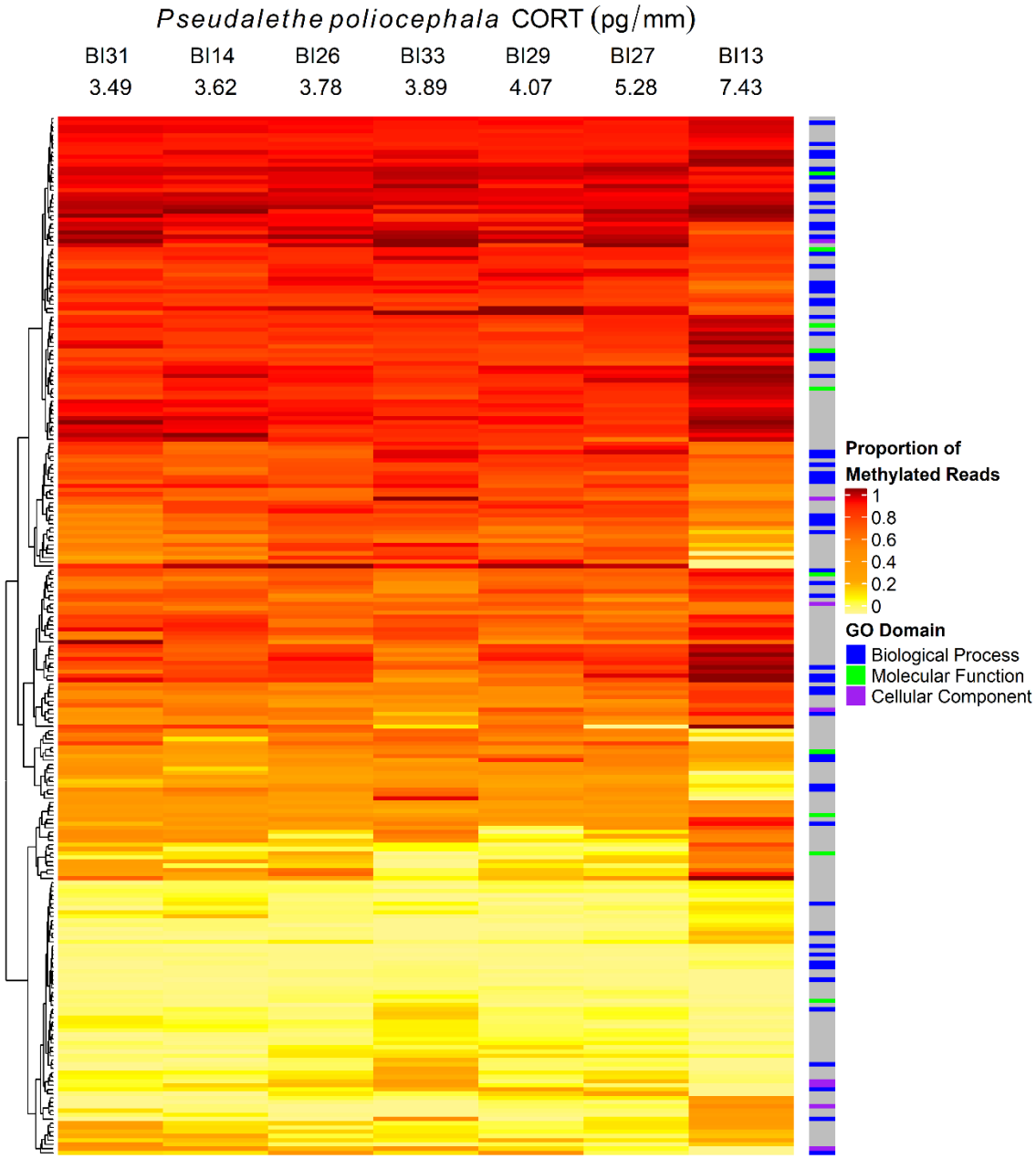


Figure 8. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to feather CORT, for each individual of *Pseudalethe poliocephala*. Feather CORT (pg/mm) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to CORT.

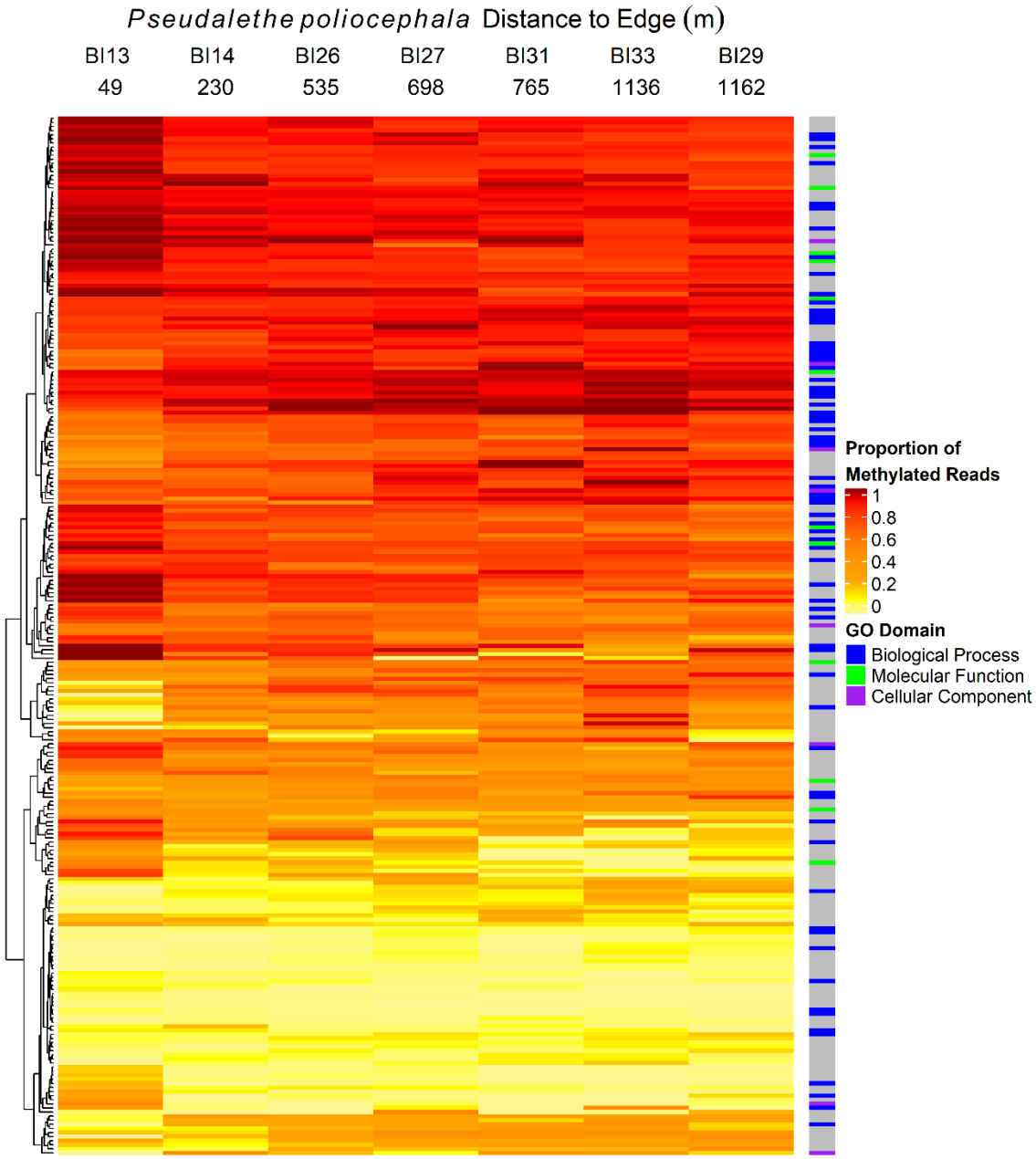


Figure 9. A heat map summarizing the proportion of methylated sites that were significantly differentially methylated with respect to distance to edge, for each individual of *Pseudalethe poliocephala*. Distance to edge (m) are below each individual's ID number. The rows are gene loci that have been clustered based on the how they are related to distance to edge.

Feather CORT, Spectrometry and Ptilochronology

Results from my linear model showed a negative relationship between feather CORT and the log natural overall brightness ($\beta$ = -2.587, $t$ = -2.263, 95% CI = -4.755, -0.236, Figure 10), but the relationship between feather CORT and the standard deviation of the growth bars was not significant ($\beta$ = 4.088, $t$ = 1.058, 95% CI = -3.240, 12.074).

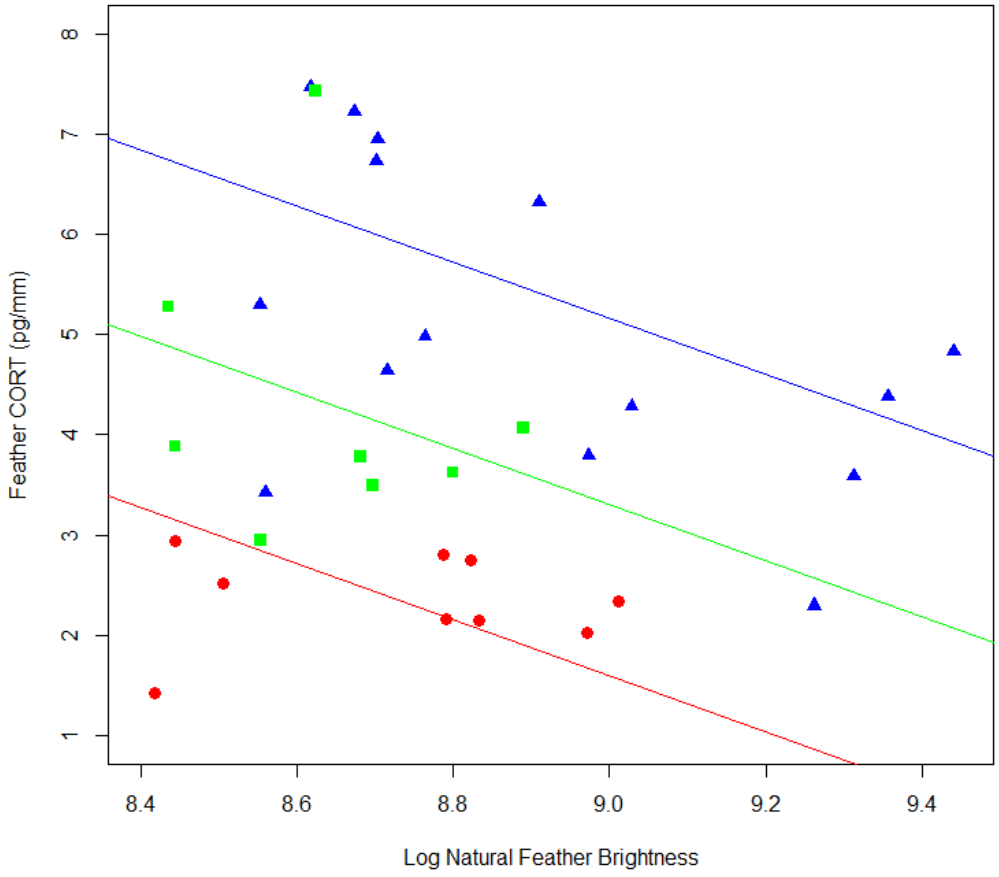


Figure 10. Plot of the relationship between feather CORT (pg/mm) and log natural feather brightness for three species of tropical forest birds *Alethe castanea* (red circles), *Bleda notatus* (blue triangles), and *Psuedalethe poliocephala* (green squares) with a linear regression line similarly colored. Linear regression equations are y= 26.75 -2.80x, y= 30.31 -2.80x, and y= 28.47 -2.80x for the three species, respectively.

# DISCUSSION

## DNA Methylation

I found that birds living closer to the forest edge had significantly different methylated sites than those residing in the interior of the forest. For some bird species, the long-term effects of forest degradation and logging have been shown to reduce abundance and fitness, and alter avian community composition, movement patterns, and distributions (Thiollay 1999, Dale et al. 2000, Laurance et al. 2004), but my results provide novel insight into the mechanisms which allow individuals to acclimate to changing environments. My study individuals appeared to be affected by the disturbance in the secondary forest, likely through long-term changes in salient aspects of the tropical forest habitat: microclimate, soil moisture, plant community and food resources. *Bleda notatus*, the most generalist of my study species, appears to have the most uniform proportion of methylated sites across individuals, compared to the other two more specialized insectivorous species. This conclusion consistent with Peters and Okalo (2009), who found that the most specialized understory insectivores were most susceptible to population declines due to forest fragmentation, but more generalist species were less vulnerable in the African tropics. My results support the idea that DNA methylation and consequent differential gene expression is part of an adaptive response to acute environmental stressors. However, there is likely a threshold when environmental

stress becomes chronic and stress responses to degraded landscapes manifest as reduced fitness, leading to changes in avian assemblages in human-dominated landscapes.

I found 5 KEGG pathways that contain gene loci, which were differentially methylated with respect to CORT, in two of the three study species: Adherens junction, basal transcription factors, lysine degradation, ribosome and spliceosome. These pathways function in protein synthesis, transcription and cell-cell communication, indicating that CORT may influence DNA methylation to alter gene expression in these pathways. Feather CORT appears to be strongly associated with DNA methylation in my study individuals, but more work is needed to disentangle the complex relationship between DNA methylation, feather CORT, and environmental stress. No doubt the timing of the environmental stress plays a large role in the variation between DNA methylation and CORT. These tail feathers were grown sometime during the 12 months before they were collected and took only a couple months to grow, so it possible that there is variation in CORT levels attributable to the months in which the feathers were grown. The amount of CORT deposited in the tail feather represents only a glimpse into the history of stress in the individual, whereas DNA methylation in the blood likely captures the history of environmental stress over a much larger time frame. Lee et al. (2010) found that exposure to chronic CORT reduced DNA methylation which altered gene expression in mice, however these results should be interpreted with caution because individuals were not wild and DNA methylation can be altered significantly over just one generation (Lea et al. 2016). Much of the literature on CORT focuses on manipulating CORT levels via supplemental feeding or implants, but such continued chronic exposure to CORT is

unlikely in a natural setting, therefore implications for wild birds are limited. Marra and Holbertson (1998) found that stress associated with lower quality habitat was related to physiological condition in *Setophaga ruticilla* (American Redstart) during the non-breeding season. Identifying natural thresholds at which acute stressors becomes chronic stress, will put researchers in a better position to understand species and community responses to disturbance.

It is possible that differential methylation is attributed solely to cell type heterogeneity, however since I was working with blood samples from non-model organisms isolating purified cell types was not an option. Since I collected blood from the brachial vein under the left wing from wild captured birds, I have no reason to suspect there would be differences in cell type proportions that would obscure the relationship between either CORT or distance to forest edge.

## Feather CORT, Spectrometry and Ptilochronology

My results, in association with other research, support the conclusion that feather CORT is negatively associated with feather brightness (Fairhurst et al. 2014). Feather brightness is related to fitness in several species (Vergara et al. 2015). Individuals with dull feathers often exhibit lower reproductive success (Keyser and Hill 2000), thereby suggesting a cascading effect of chronically high-levels of CORT on feather brightness resulting in diminished fitness. A review of the relationship between CORT and fitness by Bonier et al. (2009) found that overall there was a negative relationship between CORT and fitness, but at specific times in specific species CORT can be beneficial and

may actually increase fitness. In these instances when CORT is associated with an increase in fitness, CORT is likely facilitating acclimation to acute stressors. Rubenstein et al. (2016) found that DNA methylation was related to the likelihood of breeding in male and the likelihood of dispersal in female *Lamprotornis superbus*, which were assumed to be indices of fitness, however there is a lack of research on the relationship between DNA methylation and direct measures of fitness. Environmental degradation influences DNA methylation of regulatory regions associated with a stress response. This relationship may dynamically influence fitness in two ways: (1) help individuals cope with acute environmental stressors or (2) decreasing fitness due to chronic stressors resulting in reduced feather brightness.

## Conclusion

Measuring variation in a species' capacity to augment gene expression and acclimate to environmental stressors will help us identify those species most capable, and incapable, of persisting in disturbed habitats. Potentially these tools can provide insight into patterns of species loss, where species subject to extinction may have a lower threshold at which environmental stressors become chronic stress resulting in reduced fitness. Even though there is more genetic information available on model species, studies on non-model species of wild individuals are needed to fully understand the complex relationship between forest fragmentation, DNA methylation and CORT in wild birds, since DNA methylation is likely to be drastically different between wild and lab-raised individuals. Future studies relating direct measures of fitness to DNA methylation

will help identify levels at which environmental stressors diminish fitness which would shed light on how DNA methylation affects species on a population level over generations.

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