1   **Habitat fragmentation in the Brazilian Atlantic Forest is associated with erosion of frog**

2   **immunogenetic diversity and increased fungal infections**

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14   **ABSTRACT**

15   Habitat fragmentation and infectious disease threaten amphibians globally, but little is

16   known about how these two threats interact. In this study, we examined the effects of Brazilian

17   Atlantic Forest habitat fragmentation on frog genetic diversity at an immune locus known to

18   affect disease susceptibility in amphibians, the MHC IIB locus. We used a custom high-

19   throughput assay to sequence the MHC IIB locus across six focal frog species in two regions of

20   the Atlantic Forest. We also used a molecular assay to quantify infections by the fungal pathogen

21   *Batrachochytrium dendrobatidis* (*Bd*). We found that habitat fragmentation is associated with

22   genetic erosion at the MHC IIB locus, and that this erosion is most severe in frog species

23   restricted to intact forests. Significant *Bd* infections were recovered only in one Atlantic Forest

24 region, potentially due to the relatively higher elevation. In this region, forest specialists showed

25 an increase in both *Bd* prevalence and loads in fragmented habitats. We also found that reduced

26 population-level MHC IIB diversity was associated with increased *Bd* infection risk. On the

27 individual-level, MHC IIB heterozygotes (by allelic genotype as well as supertype) exhibited a

28 reduced risk of *Bd* infection. Our results suggest that habitat fragmentation increases infection

29 susceptibility in amphibians, mediated at least in part through loss of immunogenetic diversity.

30 Our findings have implications for the conservation of fragmented populations in the face of

31 emerging infectious diseases.

32

33 **INTRODUCTION**

34   Amphibians are in decline worldwide due to anthropogenic stressors including habitat

35 modification and emerging infectious diseases (Stuart et al. 2007; Becker et al. 2010; Scheele et

36 al. 2019). The recent global rise in the amphibian disease chytridiomycosis caused by the

37 pathogen *Batrachochytrium dendrobatidis* (*Bd*) has raised questions about whether pathogen

38 virulence and/or amphibian susceptibility has recently increased. Given mounting evidence that

39 *Bd*'s presence predates known declines in several areas of the world (Rodriguez et al. 2014;

40 Talley et al. 2015; Carvalho et al. 2017) and that enzootic lineages of *Bd* continue to exhibit high

41 virulence in naïve hosts (Fu and Waldman 2019), increased host susceptibility seems a likely

42 explanation for the rise in disease outbreaks in many regions. One hypothesis is that the negative

43 impacts of widespread habitat modification have contributed to increased amphibian disease

44 susceptibility. Habitat modification including destruction and fragmentation negatively impacts

45 amphibians via several mechanisms. For example, loss of genetic diversity in fragmented

46 populations can reduce population-level fitness and resilience (Allentoft and O'Brien 2010) and

47   increase disease susceptibility (Pearman and Garner 2005). Collectively, the impacts of

48   increasingly modified habitats may have surpassed a threshold, tipping previously stable

49   populations to a point of increased susceptibility to disease and other stressors, and giving rise to

50   global increases in amphibian disease.

51   Habitat fragmentation can reduce genetic diversity in surviving wildlife populations

52   (Lesbarrères et al. 2002; Andersen et al. 2004; Johansson et al. 2007; Frankham et al. 2002) or

53   impact selection on immunogenes in the Major Histocompatibility Complex (MHC) that

54   contribute to fitness and immune function (Hernandez-Gomez et al. 2019; Gonzalez-Quevedo et

55   al. 2016; Belasen et al. 2019). The MHC gene family is composed of two classes, with Class II

56   genes primarily involved in the response to extracellular pathogens (Bevan 1987). In particular,

57   MHC Class IIB Exon 2 is associated with conformation of the peptide-binding regions of MHC

58   Class II molecules (Tong et al. 2006), which present pathogen-derived antigen peptides to

59   immune cells to stimulate the adaptive immune response (Bevan 1987; Richmond et al. 2009).

60   Previous studies have shown that MHC IIB genotype is associated with variability in amphibian

61   susceptibility to a variety of pathogens and parasites (Bataille et al. 2015; Savage and Zamudio

62   2011, 2016; Mulder et al. 2017; Savage et al. 2019; Hernández-Gómez et al. 2019; Belasen et al.

63   2019) and that MHC IIB heterozygosity confers elevated protection (*i.e.*, heterozygote

64   advantage) against *Bd* (Savage and Zamudio 2011).

65   Studies of the relationship between habitat fragmentation and MHC diversity have shown

66   mixed results. In a classic study of MHC diversity, Aguilar et al. (2004) showed that strong

67   balancing selection can maintain high MHC diversity even in the presence of genome-wide

68   genetic erosion (*i.e.*, genetic diversity loss) in historically fragmented vertebrate populations.

69   However, in some taxa, MHC diversity appears to be naturally low or to track neutral genetic

70   diversity; in these cases, demographic factors and genetic drift may outweigh selection (reviewed

71   in Radwan et al. 2009). For example, genetic erosion at MHC IIB was observed in frog

72   populations that had been fragmented and isolated for 12,000-20,000 years on land-bridge

73   islands (Belasen et al. 2019). It remains unclear whether more recent anthropogenic habitat

74   fragmentation has similarly eroded MHC IIB diversity in amphibians through inbreeding and

75   genetic drift or altered selection. In recently fragmented populations where inbreeding and strong

76   genetic drift are intense enough to outweigh balancing selection, genetic erosion may be

77   expected at MHC loci. This could increase susceptibility to infections on both the individual- and

78   population-level as a result of decreased heterozygosity and/or the loss of disease resistance-

79   associated rare alleles.

80   The majority of our knowledge about the relationships between habitat fragmentation and

81   amphibian disease susceptibility comes from studies on *Bd*, though the hypothesis that habitat

82   modification increases disease susceptibility has not been well-supported in these studies. In a

83   meta-analysis, Becker and Zamudio (2011) found that *Bd* prevalence was higher in populations

84   living in pristine (*i.e.*, unfragmented) forested habitats around the world. A logical explanation

85   for this pattern is that *Bd* is a psychrophilic and aquatic fungus, meaning that *Bd* grows optimally

86   in the cooler and wetter environments found in pristine forests (Puschendorf et al. 2009).

87   Nonetheless, *Bd* distribution often does not match habitat suitability model predictions (James et

88   al. 2015). In addition, the majority of studies supporting a negative relationship between *Bd*

89   prevalence and habitat fragmentation focus on individual host species that are locally abundant

90   habitat generalists (Becker and Zamudio 2011; Puschendorf et al. 2009; Kriger et al. 2007).

91   These generalist species may exhibit recalcitrance to both abiotic and biotic stressors (i.e., both

92   fragmentation and *Bd*). In contrast, species that are sensitive to environmental changes or those

93 with specialist ecologies may experience stronger negative effects due to habitat fragmentation

94 (reviewed in Harrison and Bruna 2012). Thus, it is important to consider a diversity of species to

95 fully understand the impacts of habitat fragmentation on disease susceptibility in diverse tropical

96 systems.

97   In this study, we examined the effects of recent habitat fragmentation on MHC IIB

98 diversity and infection prevalence in frogs of Brazil's extensively fragmented Atlantic Forest. To

99 examine the range of effects on immunogenetics and infections in diverse tropical frogs, we

100 sampled six endemic Atlantic Forest frog species including forest specialists and habitat

101 generalists. These populations were previously genotyped at neutral loci using a reduced-

102 representation library approach (ddRAD; Belasen et al. *unpubl.*). We collected tissue samples

103 and skin swabs from our focal species in fragmented and continuous forested habitats in two

104 sampling regions to quantify immunogenetic diversity at the MHC IIB locus and assess *Bd*

105 infection prevalence and load. We tested the following questions: i) How is MHC IIB diversity

106 and allelic composition affected by fragmentation in habitat specialists vs. generalists? ii) Does

107 fragmentation increase infection susceptibility across a range of species ecologies? and iii) Does

108 MHC IIB diversity and/or genotype determine infection susceptibility?

109

110 **MATERIALS AND METHODS**

111 *Study system and sample collection*

112   Brazil's Atlantic Forest (BAF) is one of the most heavily fragmented tropical ecosystems

113 in the world. More than 500 years ago the Atlantic Forest stretched 1.2 million km^2 across the

114 eastern coast of South America. Anthropogenic deforestation and fragmentation have now

115 reduced BAF to ~13% of this original area (Ribeiro et al. 2009). The remaining forest is

116   distributed among tens of thousands of small isolated patches, more than 80% of which are less

117   than 50 hectares in area. Despite this extensive fragmentation, BAF remains one of the most

118   biodiverse regions in the world, and contains 5% of all vertebrate species described on Earth and

119   60% of Brazil's threatened animals. Amphibian diversity is particularly high, with ~660

120   described species, more than half of which are endemic to the region (Haddad et al. 2013).

121   For this study, two regions in BAF were sampled that contained large tracts of continuous

122   forest as well as small ~100 year old isolated forest fragments within cattle pasture matrix:

123   northeastern São Paulo state (SP) and southeastern Bahia state (BA; Fig. 1). In São Paulo, forest

124   fragments were sampled in the municipality of São Luiz do Paraitinga (23°09'S 45°15'W, 840 m

125   asl). A section of the same original forest that has been preserved adjacent to a protected area

126   (Núcleo Santa Virginia, Parque Estadual da Serra do Mar; 23°25'S, 45°11'W, 620 m asl,

127   ~17,000 ha total area of natural forest) was sampled ~30 km from the fragmented area. Similarly,

128   in Bahia, forest fragments were sampled in the municipality of Igrapiúna (13° 50' S, 39° 13' W,

129   237m asl). A continuous forested site was sampled within the Reserva Ecológica Michelin (13°

130   50' S, 39° 14' W, 137m asl, ~1,800 ha total area of natural forest) ~2 km from the fragmented

131   area.

132   To examine how immunogenetics is impacted by habitat fragmentation, genetic samples

133   were collected from six focal species sampled from fragmented and continuous habitats in the

134   two regions, including habitat specialists (those that live and reproduce only in forested areas)

135   and generalists (those that disperse through, live, and reproduce in a variety of habitats including

136   intensively managed agricultural matrix). Four of the focal species were sampled in São Paulo,

137   and included two forest specialists (Hylidae: *Aplastodiscus leucopygius* and Brachycephalidae:

138   *Ischnocnema henselii*) and two habitat generalists (Hylidae: *Dendropsophus minutus* and *Boana*

139 *polytaenia*). The remaining two focal species were sampled in Bahia, including one forest

140 specialist (Hylidae: *Boana semilineata*) and one habitat generalist (Hylidae: *Dendropsophus*

141 *branneri*). Skin swab samples were collected from all focal species to detect *Bd* infections. To

142 increase power to detect differences in *Bd* prevalence and load across habitat types and species

143 ecologies, swab samples were also collected from two additional species from the same sampling

144 sites in São Paulo (Bufonidae: *Rhinella icterica*; Hylidae: *Scinax fuscovarius*; both habitat

145 generalists).

146 Frogs were individually captured at night using sterile plastic bags and transported to a

147 central field laboratory for sample collection. Following a ventral rinse with sterilized distilled

148 water, skin swab samples were taken according to a standard pathogen sampling protocol (Hyatt

149 et al. 2007) and either liver (lethal, post-euthanasia) or toe (non-lethal) tissue samples were taken

150 for immunogenetic analysis (IACUC protocols for humane animal care and use PRO00005605

151 and PRO00007691). Euthanized frogs were formalin-fixed and deposited as voucher specimens

152 in the Museu de Zoologia "prof. Adão José Cardoso" (ZUEC), Universidade Estadual de

153 Campinas, São Paulo (Appendix I), while non-lethally sampled frogs were released at the same

154 site they were captured. DNA was extracted using a DNeasy kit (Qiagen) using a modified

155 protocol for swab samples and the standard manufacturer's protocol for tissues.

156

157 *MHC IIB sequencing*

158 To sequence the MHC IIB immunogenetic locus, frog MHC primers were used to

159 amplify a 200-400 bp fragment of MHC IIB Exon 2. Tissue DNA extracts were amplified with

160 amphibian MHC IIB primers BCF6 and BobomSR (May and Beebee 2009). Prior to additional

161 library prep and sequencing, a subset of PCR products were cloned and sequenced using a TOPO

162   TA cloning kit (Invitrogen) and blue/white screening. Successful clones were sequenced and

163   compared against the NCBI GenBank database using blastx to confirm homology to amphibian

164   MHC IIB Exon 2.

165   Species-specific primers were developed for two species (*D. minutus* and *A. leucopygius*)

166   for which clean homologous sequences could not be consistently produced using BCF6 and

167   BobomSR, likely due to spurious amplification of paralogs. Primers were designed using a

168   genome walking approach (Clontech Universal Genome Walker Kit 2.0) to amplify the exon and

169   a portion of flanking intronic region to design new primers that would amplify orthologous loci

170   only. Tissue extracts were digested with four sets of restriction enzymes, then adapters were

171   ligated to cut ends of DNA strands. Nested MHC IIB Exon 2 primers were designed for each

172   species based on BCF6/BobomSR clone sequences. Two rounds of PCR were conducted with

173   nested gene-specific primers and nested adapter primers to amplify DNA fragments overlapping

174   MHC IIB Exon 2 along with flanking intron sequence. Final PCR products were then cloned and

175   Sanger sequenced to retrieve DNA sequences containing MHC IIB Exon 2 and flanking intronic

176   regions. These sequences were then used to design species-specific primers that would produce

177   orthologous amplicons.

178   Either BCF6/BobomSR or species-specific primers were modified with an attached

179   indexing primer overhang (Table S1). These were then used to PCR-amplify a 200-400 bp

180   fragment of the MHC IIB Exon 2 from each sample. After visualizing products on a 1% agarose

181   gel to confirm amplification, PCR products were diluted, and reduced-cycle PCR was used to

182   anneal each product to Nextera oligos containing Illumina flow cell adapters and a unique 10bp

183   index on each side. The resulting dual-indexed products were visualized on a 1% agarose gel

184   before being quantified on a Qubit fluorometer. Samples were then pooled using equimolar

185 volumes and purified using 1.8x AMPure magnetic beads. The pooled and purified library was

186 sequenced on the Illumina MiSeq platform (250 bp paired-end nano run) at the University of

187 Michigan Microbial Systems Molecular Biology Laboratory.

188

189 *MHC IIB genotyping and supertyping*

190 MHC IIB sequences were bioinformatically processed using the Mothur MiSeq pipeline

191 (Kozich et al. 2013). Briefly, MiSeq output data were split by frog species before paired reads

192 were assembled, quality-filtered to remove short or low-quality sequences, aligned to a reference

193 alignment of MHC IIB Exon 2 sequences from four frog species (downloaded from GenBank),

194 and clustered into $\geq$99% identical "OTUs" (operational taxonomic units) that represent putative

195 MHC IIB Exon 2 alleles. A threshold of 100 reads within a single individual was used to retain

196 and assign alleles to individual frogs. The most abundant sequence for a given OTU was

197 extracted as the allele sequence. Individuals with >2 alleles recovered (n = 12/114) were filtered

198 out of the dataset, as all target species are assumed to be diploid and a single orthologous locus

199 was being targeted. To confirm that the final set of haplotypes were orthologous, a PhyML tree

200 was constructed using the HKY85 model and 100 bootstraps in Seaview (vrs. 4.5.4; Gascuel

201 1997; Gouy et al. 2010).

202 To identify positively selected sites (PSS) and MHC IIB supertypes, allele sequences

203 were translated into amino acid sequences in MEGA (vrs. 7.0.26-mac). Sequences were aligned

204 with a previously published frog MHC IIB dataset (Bataille et al. 2015) to identify amino acid

205 residues hypothetically associated with peptide binding region (PBR) pocket conformation based

206 on analogous positions in human MHC class II alleles (antigen-binding groove pockets 4, 6, 7,

207 and 9; Bataille et al. 2015; Mulder et al. 2017; Brown et al. 1993; Tong et al. 2006). PSS in the

208 amino acid alignment were identified using a fixed effects likelihood model of site selection

209 implemented in Datamonkey 2.0 (Weaver et al. 2018; Pond and Frost 2005). Alleles were then

210 clustered into functional supertypes based on PSS amino acid physicochemical properties (amino

211 acid z-descriptors z1-z5; Sandberg et al. 1998) using a BIC-based k-means clustering algorithm

212 and discriminant analysis of principle components (DAPC) implemented in the R package

213 adegenet (Jombart et al. 2010).

214

215 *Population genetic analyses*

216 To determine whether MHC haplotypes genetically clustered according to species

217 relatedness or local habitat, a haplotype network was constructed and visualized using the pegas

218 package in R (vrs. 3.5.1; R Team 2018; Paradis 2010). The network was constrained to four focal

219 species that represent congeneric species pairs: *D. minutus* and *B. polytaenia* from São Paulo,

220 and their congeners *D. branneri* and *B. semilineata* from Bahia.

221 To determine the impacts of fragmentation on MHC IIB diversity, summary statistics

222 were calculated in DnaSP (Librado and Rozas 2009). These included allelic diversity (N_A),

223 observed and expected heterozygosity (H_O and H_E), and nucleotide diversity (π). To determine

224 whether fragmentation was associated with significant reductions in immunogenetic diversity,

225 95% Confidence Intervals were calculated for mean H_E and mean π for each population. MHC

226 IIB genetic structure was evaluated among fragmented and continuous populations within each

227 species by calculating the fixation index (F_{ST}) in R. To compare MHC IIB diversity to neutral

228 genetic diversity, MHC IIB diversity summary statistics H_O, H_E, and π were treated as dependent

229 variables in separate general linear models that included the analogous summary statistic from a

230 reduced representation genomic library (ddRAD) constructed from the same samples (Belasen et

231   al., *unpubl*) as a fixed effect independent variable. Additional models that included habitat type

232   and species ecology (generalist vs. specialist) as factors were constructed using a stepwise

233   additive model building procedure. Adjusted R^2 values were used to select the best model for

234   each MHC IIB summary statistic. To test for signatures of selection across MHC IIB Exon 2, the

235   ratio of non-synonymous to synonymous sites (dN/dS) was calculated for each population and

236   the difference between dN and dS was statistically analyzed using z-tests in MEGA (vrs. 7.0.26-

237   mac).

238

239   *Detection and analysis of Bd infections*

240   Swabs were analyzed using a standard qPCR assay for *Bd* detection (Boyle et al. 2004).

241   Standard curves were produced using serial dilutions (10^6-10^0 zoospore equivalents, hereafter

242   ZE) of CLFT035, a *Bd*-GPL culture isolated from a Brazilian Atlantic forest tadpole. Samples

243   were run in duplicate to ensure accurate quantification, and only those containing ≥ 1 ZE were

244   considered positive for *Bd*.

245   *Bd* infection rates were compared across species ecologies (forest specialist vs. habitat

246   generalist) and habitat types (fragmented vs. continuous forest) using chi-square tests computed

247   in SPSS (vrs. 22). *Bd* loads were compared across species ecologies and habitats using a two-

248   way ANOVA after confirming that the data conformed to the assumptions of linear models. To

249   examine the relationship between genetic diversity and infections, general linear models were

250   constructed in R with *Bd* load as the dependent variable and additive stepwise combinations of

251   four explanatory variables: ddRAD or MHC IIB genetic diversity, species identity, species

252   ecology, and habitat type. Adjusted R^2 values were compared to select the best model for each

253   measure of genetic diversity. T-tests were used to determine whether *Bd* loads were associated

254   with MHC IIB heterozygosity on an individual-level. Chi-squared tests were used to compare *Bd*

255   infection rates between MHC IIB heterozygotes and homozygotes for both haplotype and

256   supertype.

257

258   **RESULTS**

259   *Immunogenetic diversity*

260   Across the six focal species, 72 unique haplotypes were recovered. Construction of a

261   haplotype network between congeneric species from São Paulo (SP) and Bahia (BA) showed that

262   haplotypes tend to cluster by genus rather than by sampling area or habitat type (continuous vs.

263   fragmented; Fig. 2). A single trans-specific haplotype was observed, and all remaining

264   haplotypes were only found in one species. While most haplotypes clustered within genera, the

265   trans-specific haplotype was shared between *D. branneri* and *B. semilineata* (both BA; haplotype

266   XL), and one *D. branneri*-specific haplotype (haplotype XLI) clustered within *Boana* (SP and

267   BA) haplotypes on the network. These results were corroborated by the larger dataset:

268   haplotypes predominantly clustered by species in the maximum likelihood tree (Fig. S1).

269   Five codon positions across the MHC IIB alignment showed signals of strong positive

270   selection (dN/dS > 10) and aligned with putative pocket residues of the PBR (Fig. S2). When

271   amino acid physicochemical properties from these five codon positions were evaluated, the 72

272   haplotypes condensed into seven unique MHC IIB supertypes that overlapped across regions and

273   species (Fig. S1; Fig. S3). Two supertypes were found only in a single species: ST1 was found

274   only in *D. branneri* (BA) and ST6 was found only in *D. minutus* (SP).

275   MHC IIB diversity was significantly lower in fragmented populations relative to

276   continuous populations according to non-overlapping 95% Confidence Intervals for expected

277   heterozygosity (H_E) in 5/6 focal species (Fig. 3A; Table S2). MHC IIB nucleotide diversity (π)

278   was also lower in the fragmented populations according to non-overlapping 95% Confidence

279   Intervals in all three specialist species and in the generalist *D. branneri* (BA; Fig. 3B, Table S2).

280   According to dN-dS z-tests, significant signatures of selection on MHC IIB were found

281   only in the São Paulo specialists *A. leucopygius* (positive selection in both populations) and *I.*

282   *henselii* (negative selection in the fragmented population and positive selection in the continuous

283   population; Table S2). No populations showed significant signatures of population bottlenecks

284   according to Tajima's D (Table S2).

285   Relative to genetic differentiation (F_{ST}) across ddRAD loci, MHC IIB showed greater

286   genetic differentiation in three species (*A. leucopygius*, *D. minutus*, and *D. branneri*) and less

287   genetic differentiation in the remaining three species (*I. henselii*, *B. semilineata*, and *B.*

288   *polytaenia*; Fig. 3C). To determine the relationship between genetic diversity at the MHC IIB

289   locus compared with ddRAD markers in fragmented versus continuous populations, MHC IIB

290   diversity summary statistics H_E, H_O, and π were compared with summary statistics generated

291   from ddRAD data (genome-wide markers) from the same populations. A significant association

292   was only found for H_O, with a negative relationship between MHC IIB and ddRAD H_O across all

293   species and populations (SLR, β = -3.2, p < 0.05, R^2 = 0.37; Fig. S4).

294

295   *Incidence of Bd infections*

296   *Bd* infections were detected in all sites sampled in São Paulo. After running a subset of

297   samples (~50) collected from the lowland sampling area in Bahia we found ~5% prevalence of

298   *Bd* with positive samples showing very low loads (~1 ZE). As this is consistent with other

299   findings of very low *Bd* prevalence and loads from lowland areas in the Atlantic Forest

300   (Lambertini et al. 2021), and as loads <100 typically do not result in disease (Kinney et al. 2011),

301   we considered Bd to be functionally absent from the Bahia populations and restricted analyses of

302   Bd infections to São Paulo populations.

303   Within São Paulo, fragmented populations exhibited higher Bd infection rates relative to

304   continuous populations (30.8% mean Bd prevalence in fragmented populations compared with

305   9.5% in continuous populations; $X^2(1) = 10.783$, $p < 0.01$; Table S3). Specialists showed a trend

306   of higher Bd infection rates relative to generalists although this was not statistically significant

307   (43.3% mean Bd prevalence in specialists compared with 12.8% in generalists; $X^2(1) = 2.458$, $p >$

308   0.05; Fig. 4A). Bd infection loads tended to be higher in fragmented populations and in

309   specialists in both habitat types, although these trends were also non-significant (two-way

310   ANOVA, $p > 0.05$; Fig. 4B). While Bd load increased with fragmentation in specialists, loads

311   were similar across habitat types in generalists.

312   When infections were analyzed against genetic diversity within São Paulo, there was a

313   significant negative relationship between Bd prevalence and population-level MHC IIB diversity

314   for both measures of heterozygosity. The best models included habitat type (fragmented vs.

315   continuous) as an explanatory variable (MHC IIB H_E: $\beta = -84.61$, $p = 0.0311$, overall model $p =$

316   0.016, $R^2 = 0.8752$; MHC IIB H_O: $\beta = -52.64$, $p = 0.0307$, overall model $p = 0.026$, $R^2 = 0.8395$;

317   Fig. S4). On the individual level, MHC IIB heterozygotes were significantly less likely to be

318   infected with Bd ($X^2(1) = 9.5825$, $p < 0.01$; Fig. 4C). There were no significant relationships

319   between Bd prevalence and MHC IIB nucleotide diversity, or any measures of genetic diversity

320   generated from ddRAD markers (GLMs, $p > 0.05$). There was also no relationship between

321   individual-level MHC IIB heterozygosity and Bd load (t-test, $p > 0.05$).

322   Of the five supertypes that occurred across multiple species, ST2, ST4, and ST5 were

323   significantly associated with *Bd* infection status. Frogs showed a higher incidence of *Bd*

324   infections if they possessed these supertypes (ST2: $X^2(2) = 25.203$, p < 0.0001; ST4: $X^2(2) =$

325   7.0872, p = 0.07; ST5: $X^2(2) = 6.1613$, p < 0.05). Supertype heterozygotes were less likely to be

326   infected with *Bd* ($X^2(1) = 9.1077$, p < 0.01).

327

328   **Discussion**

329   *Habitat fragmentation is associated with erosion of immunogenetic diversity*

330   In this study, we built upon previous studies of amphibian immunogenetics and infection

331   risk to quantify the effects of landscape modification on MHC IIB diversity and infection

332   susceptibility across ecologically divergent host species. Overall, we found that habitat

333   fragmentation was associated with reduced MHC IIB diversity, with the most severe genetic

334   erosion in the forest specialists *A. leucopygius* and *I. henselii*. We also found that across all

335   species, MHC IIB diversity was inversely related to overall genetic diversity based on ddRAD

336   markers. Taken together with low Tajima's D values and MHC IIB F_{ST} values differing from

337   ddRAD marker F_{ST} values, this suggests that the loss of MHC IIB diversity may not exclusively

338   be due to genetic drift or inbreeding in fragmented populations. In half of our focal species,

339   MHC IIB genetic differentiation was lower than expected based on genome-wide genetic

340   differentiation, suggesting that selection may favor similar MHC IIB alleles in different

341   populations. This is corroborated by the MHC IIB haplotype network, which does not show

342   clustering according to population.

343   Trans-specific polymorphism (*i.e.*, the same haplotypes occurring in different species) is

344   thought to be common at MHC genes (Klein 1987), especially across species that encounter

345   similar pathogens. However, among the 72 MHC IIB haplotypes we recovered, we recovered

346   only one haplotype that was shared between focal species. This haplotype was found in both

347   species from Bahia, which implies that the local environment and/or local parasites (that were

348   not measured in this study) could be driving selection for this allele. At the supertype level,

349   however, there was evidence of trans-specific polymorphism, with 5/7 supertypes shared among

350   two or more focal species.

351   The positively selected codons that we detected across the MHC IIB alignment are

352   corroborated by previous studies as sites that impact PBR pocket shape and thus pathogen

353   recognition (Bataille et al. 2015; Mulder et al. 2017). However, the diversity of haplotypes and

354   supertypes that we recovered are relatively lower than might be expected based on previous

355   studies. For example, Savage et al. (2016) recovered 84 alleles and 4 supertypes across 8

356   populations of a single species (128 individuals). In our study, we analyzed sequences from a

357   similar number of individuals (n=102) but included six focal species spanning two families and

358   four genera. It is somewhat surprising that only seven functional supertypes were recovered

359   across this level of species diversity, although this may be due to ascertainment bias (*i.e.*,

360   relatively small sample sizes within each species). As only a small number of previous studies

361   have identified MHC IIB supertypes in amphibians, it is unknown how many supertypes exist

362   across diverse amphibian species. It is possible that supertypes show a high degree of trans-

363   specific polymorphism if amphibians are subject to similar pathogens or other selective

364   pressures. Further comparative studies of the amphibian MHC IIB are needed to test this

365   hypothesis.

366

367   *Pathogen prevalence and load vary with elevation, habitat fragmentation, and immunogenetics*

368   *Bd* prevalence and loads were extremely low in the lowland Bahia sampling region.

369   However, *Bd* was detected in all São Paulo populations with the highest *Bd* prevalence and loads

370   in fragmented populations and in forest specialist species. On the population-level we observed

371   an inverse relationship between MHC IIB diversity and *Bd* prevalence and load, and on the

372   individual-level MHC IIB heterozygotes were less likely to be infected with *Bd*. This

373   corroborates previous studies in which MHC IIB heterozygotes showed lower *Bd* susceptibility

374   (Savage and Zamudio 2011, 2016). In another study, populations with higher heterozygosity

375   experienced higher *Bd* risk, potentially due to correlations between heterozygosity, dispersal, and

376   *Bd* transmission in more genetically diverse populations (Addis et al. 2015). Based on these latter

377   findings, we may expect to detect fewer *Bd* infections in fragmented populations, as these would

378   experience reduced transmission. However, in our study area, we did not observe evidence of

379   reduced *Bd* transmission, potentially as a result of generalist species transmitting *Bd* from

380   continuous habitats to isolated forest specialist populations. As habitat generalists show moderate

381   prevalence and relatively low *Bd* loads overall, these species could hypothetically serve as

382   tolerant pathogen carriers in this multi-host system.

383   MHC IIB supertypes ST2, ST4, and ST5 were associated with increased *Bd* infection

384   rates. Interestingly, two of these (ST4 and ST5) were rare in the Bahia sampling area, where *Bd*

385   likely poses little risk to amphibians due to extremely low prevalence and infection loads.

386   Although no supertypes were associated with protection against *Bd*, supertype heterozygotes

387   exhibited lower *Bd* infection risk. Previous studies did not find an MHC IIB supertype

388   heterozygote advantage against *Bd* despite heterozygote advantage at the allelic level (Savage

389   and Zamudio 2016). This may be due to higher concordance in functional complementarity

390   between allelic heterozygotes and supertype heterozygotes in our focal species, or due to the

391   larger sample size of supertypes in this study compared with previous studies.

392   It is possible that the associations we observed between infections and MHC IIB are due

393   to causal relationships between infection load and immune function, or to both factors being

394   independently associated with fragmentation. For example, MHC IIB selection dynamics may be

395   altered due to proximity to agriculture (Hernández-Gómez et al. 2019) rather than or in addition

396   to selection by parasites in the fragmented landscape. Likewise, parasite loads may be increased

397   by fragmentation due to physiological stress (Carey et al. 1999) rather than via impacts on

398   genetic diversity. Nonetheless, a growing number of studies support the role of MHC IIB in *Bd*

399   susceptibility. Both comparative genetic studies across diverse host species (Bataille et al. 2015)

400   and infection experiments (Savage and Zamudio 2011) have supported the mechanistic

401   relationship between MHC IIB genotype and *Bd* susceptibility. Future studies such as common

402   garden experiments would be valuable in distinguishing between the impacts of immunogenetics

403   versus stress in determining disease susceptibility in fragmented, immunogenetically eroded

404   populations.

405   Taken together, our results suggest that habitat fragmentation is associated with

406   decreased immunogenetic diversity and increased fungal infections in amphibians. First, we have

407   shown that immunogenetic diversity has eroded in fragmented populations. This has potentially

408   resulted from inbreeding and genetic drift, although the MHC IIB locus has likely undergone

409   selection as well according to our analyses. Second, we found that habitat fragmentation does not

410   reduce *Bd* incidence. We hypothesize that this generalist pathogen gains access to isolated forest

411   specialist host populations via high-dispersing tolerant habitat generalist hosts that may be

412   serving as disease carriers in this system. Our study expands knowledge of the amphibian MHC

413 IIB locus by demonstrating that habitat modification can affect diversity in this immunogenetic

414 region, which has implications for *Bd* infection susceptibility. Future studies of amphibian

415 genetic diversity and disease should consider the range of responses across the host community

416 to gain a holistic understanding of community-wide vulnerability in natural systems.

417

418 **Acknowledgments**

431

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633

634 **Data Availability Statement**

635   The data that support the findings of this study are openly available in Dryad at [to be added

636 upon acceptance] and GenBank [to be added upon acceptance].

637

638 **Author Contributions**

639   AMB and TYJ conceived of the project; AMB, CGB, LFT, and TYJ performed the

640 fieldwork; AMB and RAC performed the labwork; AMB and KRA analyzed the data; AMB,

641 KRA, and CGB produced the figures; AMB wrote the paper; all authors contributed to reviewing
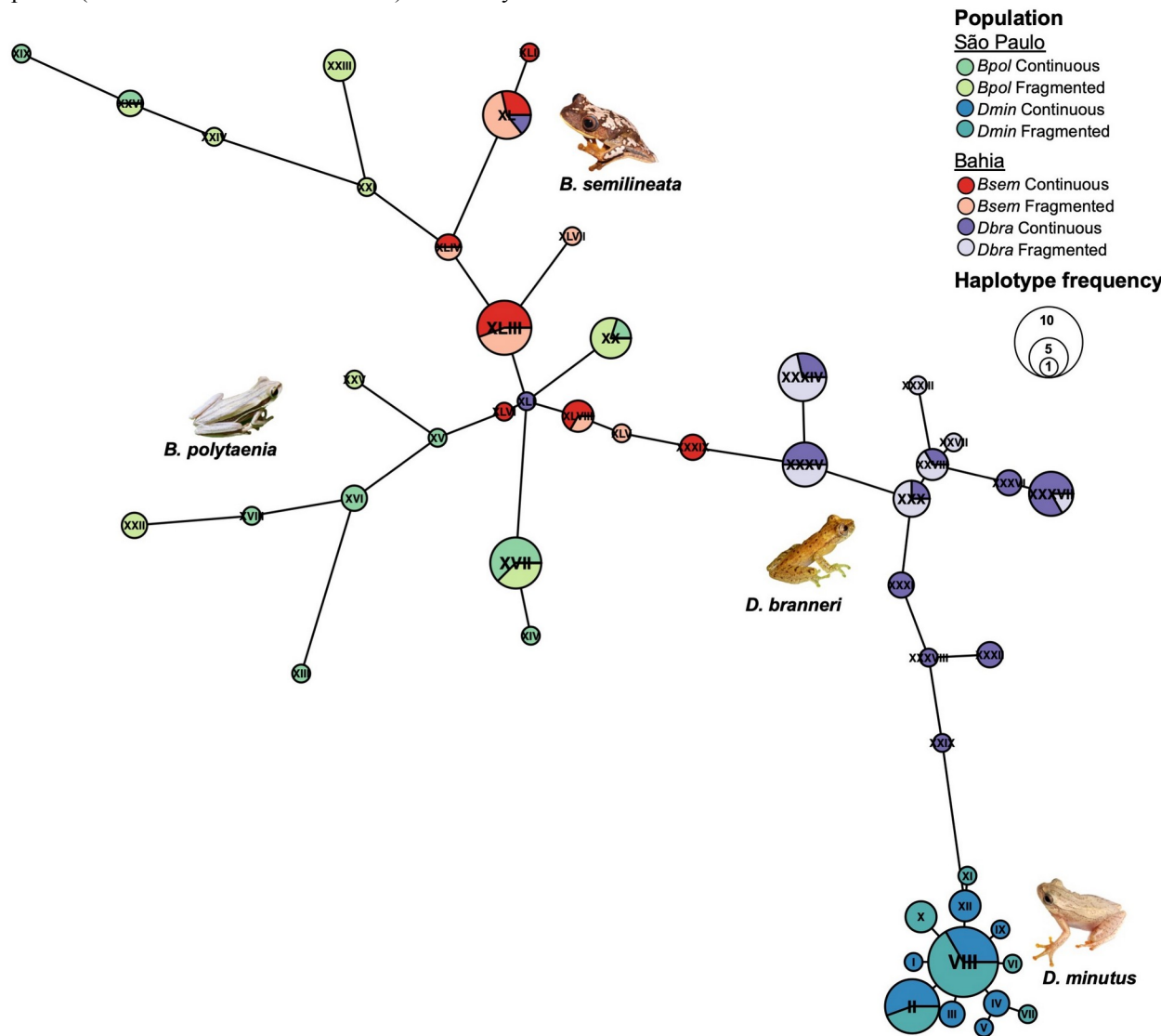
642 and revising the paper.

Figures
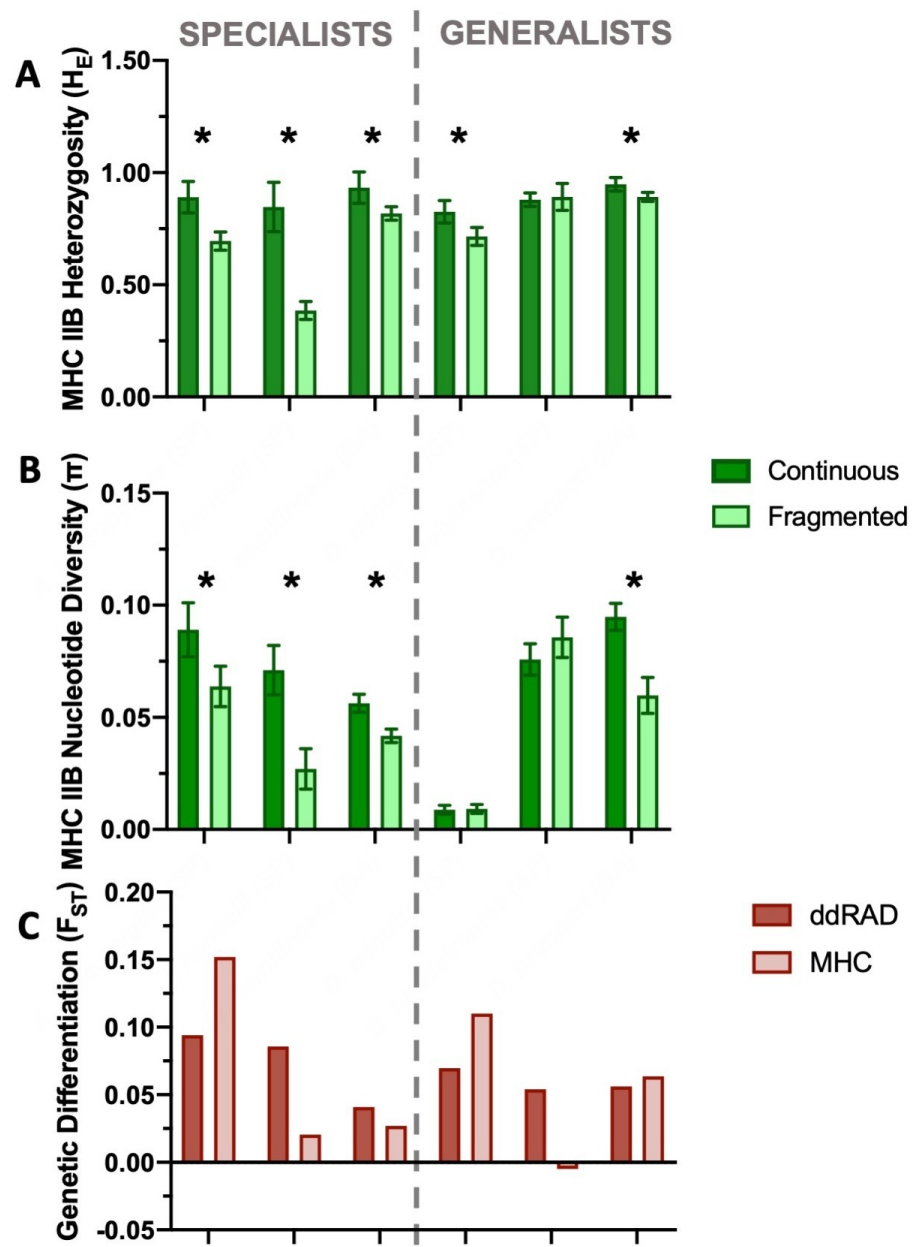
Figure 1. Sampling locations. Preserved continuous forests (CO1 and CO2) are denoted with white circles, and forest fragments (FR1-FR4) are denoted with red triangles. See Table S2 for sample sizes and species associated with each site.
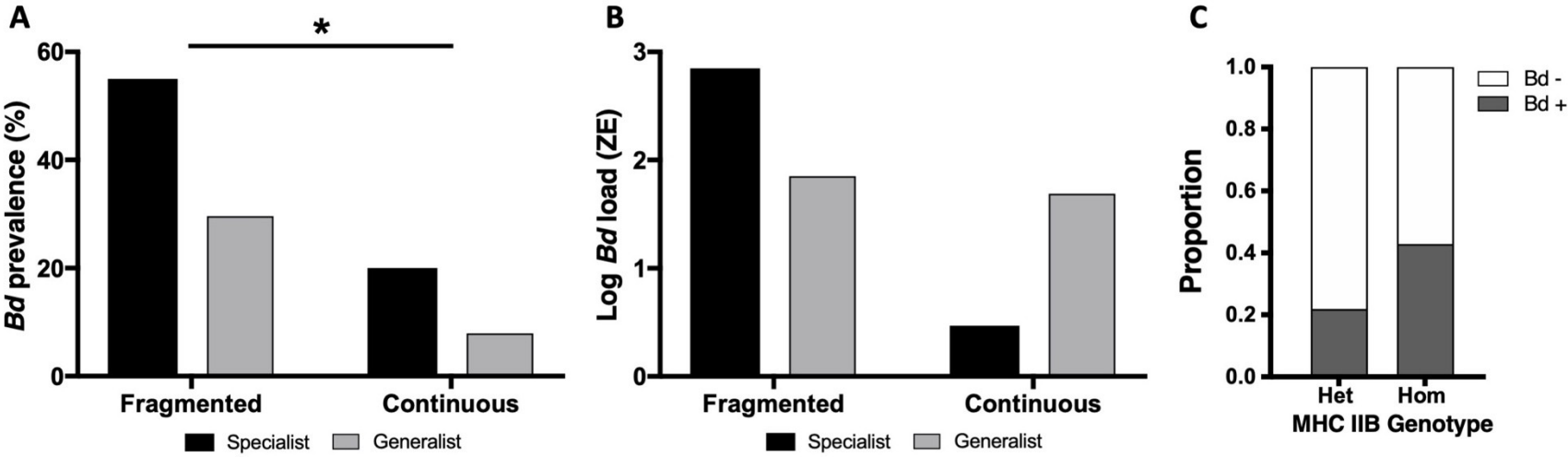
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Figure 3. MHC IIB summary statistics across all focal species. Sampling region (SP = São Paulo, BA = Bahia) is specified in parentheses after each species' name. **(A, B)** MHC IIB immunogenetic diversity erodes in fragmented populations as measured by both expected heterozygosity (A) and nucleotide diversity (B). Dark green bars represent populations from continuous forests and light green bars represent populations from fragmented forests. Asterisks represent a significant difference according 95% Confidence Intervals shown by error bars. **(C)** Genetic differentiation (fixation index, F_{ST}) at MHC IIB vs. ddRAD markers. ddRAD F_{ST} mean values are shown by dark red bars with 95% CI error bars and MHC IIB F_{ST} values are shown by light red bars.

665   and B include data from six São Paulo amphibian species, while C only includes data from the four species that were genotyped for MHC IIB (see text for details).
666   **(A) Bd prevalence in São Paulo was significantly higher in fragmented than continuous** habitats. Prevalence tended to be higher in specialists in both habitat ty
667   es. Asterisk indicates significant difference across   habitat types. (B) Bd infection loads tende   d to increase in fragmented habitats in specialists and d
668   id not change between habitat t
669