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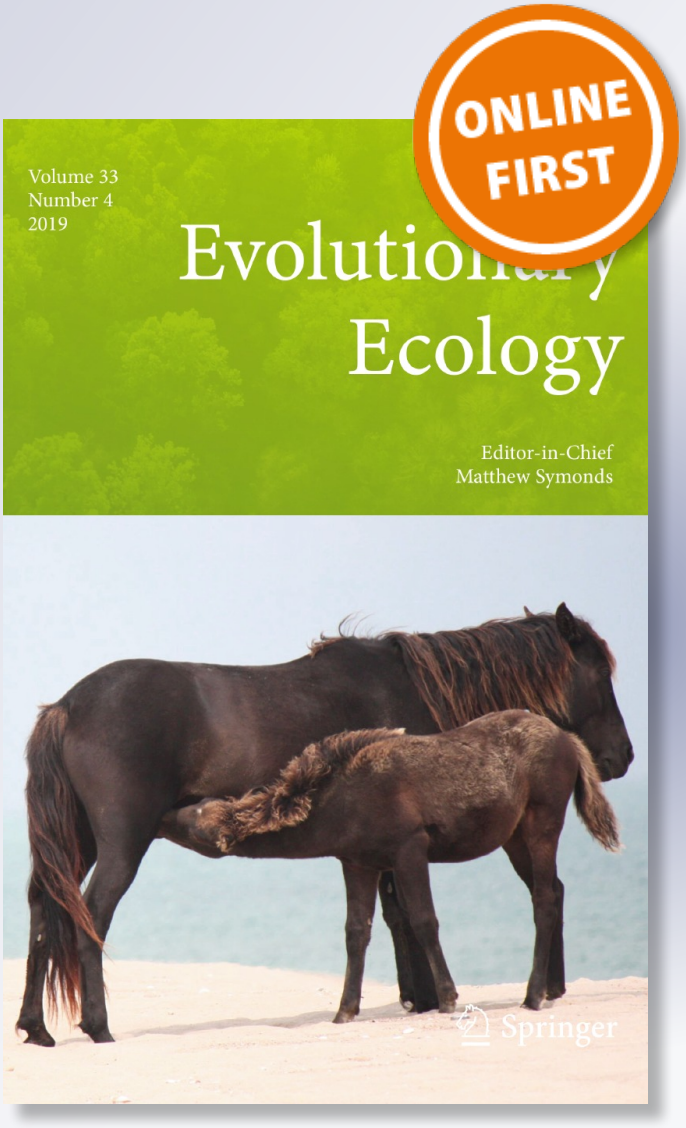

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ORIGINAL PAPER

Epigenomic changes in the túngara frog (*Physalaemus pustulosus*): possible effects of introduced fungal pathogen and urbanization

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Abstract
Amphibian populations are being threatened by human related activities including the spread of the fungal pathogen, *Batrachochytrium dendrobatidis* (Bd) and urbanization. With growing losses in global amphibian biodiversity, it is essential to document how amphibian populations are responding to rapid environmental changes. While most evolutionary processes, e.g. changes in allelic frequencies, may be too slow to allow adequate response to environmental changes, epigenetic modifications can rapidly translate environmental changes into adaptive phenotypic responses. Epigenetic modifications come in multiple, non-exclusive forms, the most notable being DNA methylation. Here we sought to examine variation in the frequency of DNA methylation among four túngara frog populations distributed across Gamboa, Panama; which vary in both their level of fungal presence/prevalence and urbanization. DNA samples were collected from amplexed (male–female) pairs and frequency of DNA methylation was analyzed using a methylation-sensitive amplified fragment length polymorphism protocol. We found significant variation in DNA methylation among populations, and correlations between Bd infection status and methylation patterns. Urbanization, however, had no influences on the frequency of DNA methylation. These data suggest epigenetic modifications are substantially flexible across fine-scale, environmental gradients and there appears to be possible biologically relevant links between DNA methylation and Bd infection status. Our results provide a basis for future work investigating the causal role epigenetics have in mediating phenotypic response to human-induced, environmental changes.

Keywords Túngara frog · *Batrachochytrium dendrobatidis* · Epigenetics · Urbanization · DNA methylation

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Introduction

The expansion of human activity has challenged organisms with ecological changes more rapid and larger in spatial scale than most other events in evolutionary history (Pereira et al. 2010). These rapid anthropogenic changes (including climate change, spread of exotic species/pathogens, urbanization, etc.) significantly impact both population dynamics (e.g. survival, reproduction, and dispersal) and global biodiversity (Palumbi 2001; Jackson and Sax 2010; Lankau et al. 2011). Some species exhibit flexibility with regard to environmental changes, which can lead to population expansion or promote invasion when introduced into new habitats (e.g. invasive species; Whitfield et al. 2002; Sih et al. 2010; Sih 2013; Asplen et al. 2015). For other species, these environmental changes pose significant challenges and can lead to formerly adaptive responses becoming maladaptive (e.g. evolutionary traps; Robertson et al. 2013). Further, many species may lack the ability to adaptively respond to these challenges and begin declining into extinction (Johnson et al. 2011).

Amphibians are a well-documented example of populations and species failing to adapt to changing environments. Staggering declines in amphibian populations have been documented globally and result from rapid anthropogenic environmental changes (Becker and Zamudio 2011; Hof et al. 2014; Johnson et al. 2011; Nori et al. 2015). Major identified threats to amphibian populations are chytridiomycosis, an infection by the fungal pathogen *Batrachochytrium dendrobatidis* (Bd); global climate change; and urbanization (reviewed in Hof et al. 2014). Individually, each environmental threat can impact amphibian population dynamics, but may also interact to exacerbate or dampen their negative effects on a population (Becker and Zamudio 2011). Bd is sensitive to temperature and humidity; often occurring in highland areas with cooler, more humid climate. The damaging effects of Bd thus can be exacerbated by shifts in ambient temperatures and precipitation brought on by climate change (James et al. 2015). Areas of high habitat-loss/fragmentation exhibit lower prevalence of Bd compared to undisturbed areas, likely due to reduced migration of infected individuals between populations and higher temperatures due to lack of canopy cover (Becker and Zamudio 2011). As such, amphibians in some areas may become increasingly susceptible to population decline due to interactions between these human-induced, environmental threats while other populations may be less susceptible (Kolby and Daszak 2016). Due to the growing threat of amphibian biodiversity loss it is increasingly important to detail whether and how amphibian populations are responding to these environmental perturbations.

Evolution may occur too slowly for a population to respond to rapid environmental changes, ultimately driving population decline or even species extinction (Kosch et al. 2016). Alterations to the epigenome, however, may rapidly translate environmental changes into adaptive phenotypic responses (reviewed in Bossdorf et al. 2008; Schrey et al. 2013; Burggren and Crews 2014; Liu et al. 2012; Ledon-Rettig et al. 2013; Smith et al. 2016), which could allow populations to persist until an appropriate evolutionary response is mounted (i.e. genetic assimilation; Robinson and Pfennig 2013). Epigenomic alterations act to alter gene expression without altering the underlying genetic sequence (Bird 2007) and take multiple, non-mutually exclusive forms, including methylation of cytosine residues (i.e. DNA methylation), histone protein modifications, and RNA interference (Weaver et al. 2004; Bossdorf et al. 2008; Herrera and Bazaga 2010, 2011; Smith et al. 2016). Epigenetic alterations can also be heritable (Johannes et al. 2009; Skinner 2014), making them potential targets upon which evolutionary processes may act. Of the numerous epigenetic modifications possible, DNA methylation is currently the most widely studied form (e.g.

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Weaver et al. 2004; Herrera and Bazaga 2010, 2011; Smith et al. 2016). Changes in DNA methylation occur at CpG sites along the genome and are regularly found within gene promoter regions. When these sites are methylated it commonly leads to a slowing or cessation of gene expression (Jaenisch and Bird 2003; Bender 2004; Bossdorf et al. 2008). Ultimately, alterations of DNA methylation and the resulting changes in gene expression may generate population-level phenotypic variation, which can be acted upon by natural selection, drive population adaptation and, possibly, speciation events (Smith et al. 2016). A point of contention, however, is that DNA methylation may also be driven by gene expression (i.e. reverse causation), may not necessarily constitute an epigenomic response to environmental changes, and, as such, any epigenomic-phenotypic relationship should be cautiously interpreted (reviewed in Greally 2018; Lappalainen and Greally 2017).

Population-level methylation studies have used methylation sensitive-amplified fragment length polymorphisms (MS-AFLPs) to generate epiallelic markers of non-model systems in the absence of genomic information (Liu et al. 2012; Perez-Figueroa 2013; Ellison et al. 2015; Smith et al. 2016; Hu and Barrett 2017). Variation in genome-wide, DNA methylation patterns have been documented among populations in several species (Liebl et al. 2013; Wenzel and Piertney 2014; Platt et al. 2015), even those which are geographically close enough in distance to allow gene flow (Smith et al. 2016). These studies have examined patterns and possible adaptive correlates of epigenetic variation. For example, alligator weed (*Alternanthera philoxeroides*) exposed to varying water levels exhibit changes in growth and morphology that correlate with significant changes in genome-wide, DNA methylation patterns (Gao et al. 2010). In the mangrove rivulus fish (*Kryptolebias marmoratus*), exposure to cooler temperatures during incubation induces temperature-determinant sex change (hermaphrodite to male) in genetically identical individuals and was attributed to significant, temperature-induced changes in genome-wide, DNA methylation patterns (Ellison et al. 2015). Further, a recent study in darter fish (*Etheostoma* sp.) found evidence that reproductive isolation, and ultimately speciation, is mediated first by population level alterations in genome-wide, DNA methylation patterns and followed by changes in allelic frequencies (Smith et al. 2016).

Epigenetic modifications may be a key component underlying whether and to what extent amphibians and other sentinel species (i.e. fish and reptiles) are responding to human-induced, environmental changes, but research on this subject is significantly lacking (reviewed in Hammond et al. 2016). To the best of our knowledge no other study has examined possible links between epigenetic modifications and population responsiveness to human-induced, environmental changes in an amphibian system. In this study we sought to address this lack of information and examined individual and population level variation in genome-wide, DNA methylation patterns of an amphibian system, the túngara frog (*Physalaemus pustulosus*), utilizing the *MS-AFLP* procedure; a first in this well-studied system. The túngara frog has been extensively studied as part of sensory ecology, sexual selection, and population genetics research programs (e.g. Ryan 1985; Lampert et al. 2003; Gridi-Papp et al. 2006; Taylor and Ryan 2013; Halfwerk et al. 2014). Túngara frogs are frequently found in both pristine forests and urbanized landscapes and have come under threat from the southernly spread of Bd into South America within the past decade (Rodríguez-Brenes et al. 2016). Our aims were to document the frequency of genome-wide, DNA methylation within and among sites and examine whether DNA methylation patterns were correlated with differences in site characteristics (e.g. urbanization and/or Bd prevalence).

We examined variation in genome-wide, DNA methylation in túngara frogs from four distinct sites around Gamboa, Panama. Prior work has shown significant genetic differentiation among frogs at these sites, which are divided by a major barrier, the Chagras River/

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Panama Canal (Lampert et al. 2003). As such, we predict significant variation in overall DNA methylation among sites. Further, frogs within our study populations inhabit areas that vary in urbanization levels; ranging from relatively intact forest to completely urbanized environments. We thus predict that overall DNA methylation would vary dependent upon urbanization level. Lastly, two of our collection sites have been sampled for the presence of Bd as part of a separate study (Rodríguez-Brenes et al. 2016). Using these data, we examined differences in DNA methylation between infected and uninfected individuals within these sites. We predict that infected individuals would exhibit significant differences in overall DNA methylation relative to uninfected individuals.

Methods

Study sites and sample collections

We collected toe-clips from 120 frogs from four sites around Gamboa, Republic of Panama in June, 2013 (9.120, −79.703; Fig. 1). Three of the sites, Pipeline Road (PL; N = 29), Pre-Pipeline road (PP; N = 32), and Santa Cruz (SC; N = 30) are on the northwestern side of the Chagras River. The fourth site, Ocelot Pond (OP; N = 30), is located on the opposite, southeastern side of the Chagras. Two of the sites (OP and PL) consist of relatively intact

Fig. 1 Population map. A map detailing the location of each population sampled (Pipeline: 9 08′03″N, 79 43′12″W; Pre-Pipeline: 9 07′15″N, 79 42′53″W; Santa Cruz: 9 07′14′N, 79 42′12″W; Ocelot Pond: 9 06′04″, 79 41′05″W). Ocelot Pond to the South is separated from the remaining sites to the North by the Chagres River

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rainforest while the remaining two sites (PP and SC) are significantly more urbanized. PP is an unpaved road along a disturbed forest edge and is subject to periodic grading by heavy machinery. SC is urbanized; the frogs breed in a concrete water containment system or in standing water created by concrete curbs along street edges. Thus, we categorized these sites as undisturbed (PL and OP) and urbanized (PP and SC). The total area sampled covered a linear distance of approximately 10 km. As part of a separate study all focal individuals from OP and PL were swabbed and tested for the absence/presence of Bd using qPCR techniques (Rodríguez-Brenes et al. 2016). Rodríguez-Brenes et al. (2016) found that in June, 2013, seven (out of 30) individuals in OP and nine (out of 29) individuals in PL were infected with Bd. Having data confirming the presence/absence of BD infection, we were able to make comparisons of DNA methylation patterns among Bd infected and uninfected individuals within and among each location.

DNA extractions and MS-AFLP analysis

We extracted DNA (~5–50 ng/µl) from toe samples using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) following the manufacturer's protocols. MS-AFLP analyses followed Smith et al. (2016) except for DNA concentration since the toes yield lower concentrations. We divided all DNA samples into two, 20 µl aliquots and digested each aliquot using an MspI/EcoRI or HpaII/EcoRI enzyme combination, respectively. The EcoRI is a common enzyme used in AFLP analyses (rare cutter) while MspI and HpaII are isoschizmers which differentially cleave 5′-CCGG-3′ islands. MspI targets methylated or unmethylated internal cytosines but cannot when an external cytosine is methylated. HpaII can only cleave unmethylated (or hemi-) 5′-CCGG sequences. For MspI digestions, we combined 20 µl of DNA with 30 µl Master Mix [18.5 µl ultra-pure water, 10 µl Cutsmart buffer, 0.5 µl Msp1 enzyme, and 1.0 µl EcoR1 enzyme (Thermo Scientific, Massachusetts, USA)]. We incubated the samples in a Mastercycler Nexus Gradient (Eppendorf; Hamburg, Germany), with heat block disabled, at 37 °C for 2 h and then at 65 °C for 20 min to inactivate the enzymes. We ran the HpaII digestion in two stages. In stage one, we combined 20 µl of DNA with 30 µl Master Mix (24.5 µl ultra-pure water, 5 µl Cutsmart buffer, and 0.5 µl HpaII enzyme). We incubated the samples, with heat block disabled, for 1 h at 37 °C. In stage two, we removed the samples from incubation and added 0.5 µl EcoR1 enzyme and 6.25 µl Tango buffer to each sample. We then incubated the samples again, at 37 °C for 1 h and then at 65 °C for 20 min. We ligated adapters on both MspI and HpaII digested DNA samples, separately, by combining 20 µl digested DNA (either MspI or HpaII) with 10 µl Master Mix [5.75 µl ultra-pure water, 3.0 µl T4 DNA ligase buffer (Promega, Madison, WI, USA), 0.25 µl T4 DNA ligase, 0.5 µl EcoR1 adapter (Suppl. Table 1) and 0.5 µl MspI/HPAII adapter (Suppl. Table 1)]. The samples were incubated, with heat block disabled, at 22 °C for 1 h and then at 65 °C for 20 min to inactivate the enzymes. Afterwards, we added 30 µl ultra-pure water to the ligated samples (1:2 dilution). We performed Pre-Selective MS-AFLP reactions on both the MspI and HpaII ligated DNA samples, separately, by combining 2.0 µl ligated DNA (either MspI or HpaII) with 18.5 µl Master Mix [12.5 µl GoTaq (Promega), 3.0 µl EcoR1-A primer (Suppl. Table 1), and 3.0 µl MspI/HpaII-A primer (Suppl. Table 1)]. We transferred the reactions to the Mastercycler, ran the reactions at 95 °C for 2 min, then through 30 cycles—95 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min—and, 72 °C for 5 min. The pre-selective PCR product was diluted in a 2:20 dilution (2.0 µl Pre-Selective DNA with 20 µl ultra-pure water). As a quality check, we performed electrophoresis on all pre-select samples using 0.6% Agarose gels

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to confirm presence of Pre-Selective DNA. We performed four Selective MS-AFLP reactions on both the MspI and HpaII Pre-Selective DNA. The reaction volumes were 5.0 µl diluted Pre-Selective DNA (either MspI or HpaII) with 18.5 µl Master Mix [12.5 µl GoTaq and 6.0 µl EcoR1-ANN and MspI/HpaII-ANN selective primer combinations (3.0 µl of each primer; Suppl. Table 1)]. The selective PCR reactions were run with the following settings: 95 °C for 4 min, then through eight cycles [95 °C for 1 min, 65 °C for 1 min with − 1 °C per cycle (touchdown), and 72 °C for 1 min], and then 22 cycles (95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min). The selective PCR products were sent for fragment analysis at the Yale DNA Analysis Facility on Science Hill (New Haven, CT, USA) where the LIZ-500 size standard was added.

Methylation analysis

We performed fragment analysis using GeneMarker ver. 2.6.4 (SoftGenetic Company, State College, PA, USA). In GeneMarker we selected the pre-loaded "AFLP" package and added a custom size standard LIZ-500. Most of the AFLP package was left on the default settings, but we altered the following per Holland et al. (2008) suggestions: (1) allele call range to 75–350 bps, (2) peak detection threshold set to a minimum of 150 rfu (any peak below 150 rfu was considered noise), (3) global and local maximums set at 1%, and (4) shutter peak filter off and (5) Pass and Fail to 1.0. We visually inspected each sequence file and found a significant drop-off in fragments < 75 bp and > 350 bp. As such, we elected to use a call range between 75 and 350 bp. We performed separate analyses for each selective primer combination.

Epigenetic and genetic variance analysis

We performed our statistical analyses using "RStudio" version 1.1.463 (R Core Team 2014). Script for performing analyses in RStudio was provided by Wenzel and Piertney (2014).

Individual loci were coded based on the presence or absence of EcoRI-MspI and EcoRI-HpaII bands. We utilized the R package *msap* (Perez-Figueroa 2013) to classify all loci above or below the scoring-error threshold as either methylation-susceptible loci (MSL) or non-methylated loci (NML), respectively. We analyzed epigenetic variation based on banding patterns with polymorphic methylation states above a scoring-error threshold and analyzed genetic variation based on banding patterns with polymorphic methylation states below a scoring-error threshold (i.e. lacking a CpG motif); similar to AFLP analyses (Herrera and Bazaga 2010; Perez-Figueroa 2013; Wenzel and Piertney 2014).

We inferred population epigenetic and genetic structure with a discriminate analysis of principle component (DAPC) using the R package *adegenet* (Jombart et al. 2010; Jombart and Ahmed 2011). Similar to the popular STRUCTURE analysis (Evanno et al. 2005), DAPC estimates population structure but does so without prior knowledge of the underlying population genetic model and minimizes the influence of within-population variation. Pairwise comparisons between DAPC and STRUCTURE analysis using four standardized datasets found DAPC regularly outperformed STRUCTURE in correctly estimating population genetic structure (Jombart et al. 2010). We visualized epigenetic (MSL) and genetic (NML) differentiation among populations and between urbanization levels with Principal Coordinate Analyses (PCoA) utilizing the R package *msap*. We examined epigenetic and genetic differentiation among populations and between disturbance levels with a two-factor

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Analysis of Molecular Variance (AMOVA)—ran through 10^4 permutations to test for significance—using the R package *pegas* (Paradis 2010). We examined pairwise epigenetic and genetic differentiation using AMOVA-based differentiation statistics (*F Statistics;* F_{st}) among populations and urbanization levels. We accounted for type II error inflation from multiple testing by calculating false discovery rate (FDR) adjusted *p* values using the R package *fdrtool* (Strimmer 2008). Full details of the MS-AFLP procedure including detailed figures depicting the production and scoring of loci can be found in Perez-Figueroa (2013).

We elected to analyze the effects of Bd on individual-level epigenetic and genetic variances separately as we only had Bd data for two of our four populations. This subset of data only contained individuals with tested absence/presence of Bd. As above, we classified all loci above or below the scoring-error threshold as either methylation-susceptible loci (MSL) or non-methylated loci (NML), respectively, for this subset using the R package *msap*. We visualized epigenetic and genetic differentiation between infected and uninfected individuals with a Principal Coordinate Analysis (PCoA) using the R package *msap*. We examined epigenetic and genetic differentiation between Bd infected/uninfected individuals with a one-factor AMOVA—ran through 10^4 permutations to test for significance—using the R package *pegas*. We performed two additional one-factor AMOVA's in which we analyzed individuals from either population (OP or PL) separately. We opted to perform multiple, one-factor AMOVA's rather than a single two-factor AMOVA which included both absence/presence and population level effects because we lacked the degrees of freedom necessary to perform a two-factor AMOVA.

We examined reliability of all generated MSAP datasets (MSL and NML; see above) to identify epigenetic and genetic differences among our sampled populations with Intraclass Correlation Coefficient (ICC) tests using the R package *psych*. Following the recommendations of Koo and Li (2016), we calculated ICC estimates and their 95% confidence intervals based on mean rating (*k,3*), consistency, two-way mixed effects models. ICC estimates below 0.5 are considered "bad", between 0.5 and 0.75 are considered "moderate", between 0.75 and 0.9 are considered "good", and estimates above 0.9 are considered "excellent" (Koo and Li 2016). Reliability of our MSAP datasets can be found in Suppl. Table 3. All estimates of reliability across our datasets fell between 0.75 and 0.95, demonstrating good to excellent reliability of our generated datasets.

Results

Epigenetic and genetic variance among populations and urbanization levels

We identified a total of 764 loci (191 per primer combination) across all individuals; 555 of these loci were classified as MSL while the remaining 209 were classified as NML (Suppl. Table 2a). Further, we identified 548 MSL (95%) and 182 NML (81%) as being polymorphic across all individuals and utilized these loci in our analyses.

Our DAPC analysis detected significant differentiation in epigenetic, but not genetic, structure among the geographic populations (Fig. 2a, b). Among populations, our PCoA's explained between 3 and 9% of the total epigenetic and genetic variation; with all displaying substantial overlap (Suppl. Fig. 1a, b). Even so, our two-factor AMOVA revealed significant epigenetic, but not genetic, differentiation among populations (Table 1); that is, the frequency of DNA methylation significantly varied among populations. We found

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Table 1 Analyses of molecular variance (AMOVA) of populations and urbanization levels

	DF	SSD	MSD	Variance	Fixation index
Population and Urbanization					
MSL					
Among urbanization	1	150.93	150.93	0.0187 (0.015%)	$F_{ct}=0.0001$; $p=0.331$
Among populations	2	299.93	149.81	1.092 (0.93%)	**$F_{sc}=0.009$; $p=0.01$**
Within populations	117	13,666.27	116.81	116.81 (99.06%)	
Total	120	14,116.82	117.64		$F_{st}=0.009$
NML					
Among urbanization	1	37.07	37.07	0.359 (2.58%)	$F_{ct}=0.026$; $p=0.332$
Among populations	2	30.60	15.30	0.06 (0.43%)	$F_{sc}=0.004$; $p=0.164$
Within populations	117	1580.35	13.51	13.51 (96.99%)	
Total	120	1648.02	13.73		$F_{st}=0.03$

Results from two-factor AMOVA examining epigenetic (MSL) and genetic (NML) partitioning among populations and between urbanization levels. F-statistics were used to estimate the proportion of genetic variability found among populations (F_{st}), among populations within groups (F_{sc}) and among groups (F_{ct}). Significant analyses ($p<0.05$) are underlined and bolded

that pairwise epigenetic differentiation (F_{st}) ranged from 0.002 to 0.017 among populations and we retained three, significant pairwise comparisons after FDR correction (OP-PL, $F_{st}=0.017$, $p=0.006$; OP-SC, $F_{st}=0.013$, $p=0.001$; PL-PP, $F_{st}=0.017$, $p=0.002$). All other pairwise comparisons (N = 3) were non-significant ($p>0.05$). Between urbanization levels, our PCoA's also explained between 3 and 9% of the total epigenetic and genetic variation; with all displaying substantial overlap (Suppl. Fig. 1c, d). We did not detect any significant epigenetic or genetic differentiation between urbanization levels (Table 1).

Epigenetic and genetic variance between Bd infected/uninfected individuals

For these analyses we only used individuals from OP (N = 30; 7 infected, 23 uninfected) and PL (N = 29; 9 infected, 20 uninfected) populations as they were the only ones tested for Bd. Across Bd tested individuals we classified 520 loci as MSL and the remaining 244 loci as NML (Suppl. Table 2b) out of the initial 764 loci. We identified 499 MSL (96%) and 188 NML (77%) as being polymorphic across all individuals.

For Bd infected and uninfected individuals, our PCoA's explained between 5 and 13% of the total epigenetic and genetic variation; with all displaying substantial overlap (Suppl. Fig. 1e, f). Even so, our AMOVA revealed significant epigenetic differentiation between infected and uninfected individuals (Table 2); that is, the frequency of DNA methylation varied between Bd infection status. We also found significant genetic differentiation between Bd infected and uninfected individuals (Table 2), indicating that infected and uninfected individuals were genetically distinct from one another. When populations (OP and PL) were analyzed separately, we detected significant epigenetic differentiation between infected and uninfected individuals within each population (Table 2). Further, only individuals within the PL populations exhibited near significant genetic differentiation.

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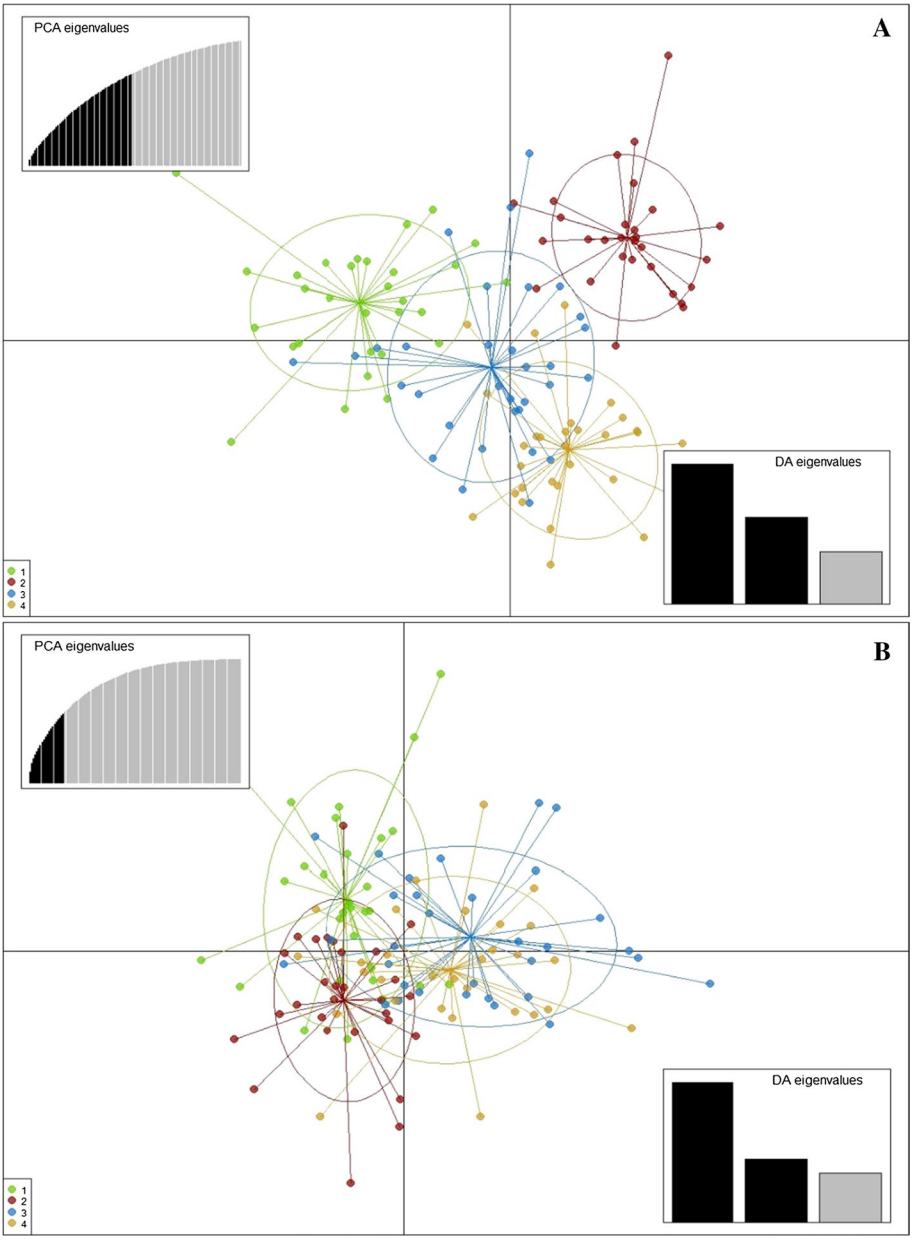

Fig. 2 Population genetic and epigenetic structure (DAPC). Estimates of population epigenetic (**a**) and genetic (**b**) structure from our Discriminate Analysis of Principal Components. Different colors and numbers represent our sampled populations. In order: 1 (green) = Ocelot, 2 (red) = Pipeline, 3 (blue) = Pre-Pipeline, and 4 (orange) = Santa Cruz. Lack of overlap between ellipses indicates significant differences in structure among populations. PCA and DA eigenvalue inlets display number of principle components and discriminant functions retained and used in each analysis. (Color figure online)

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Table 2 Analyses of molecular variance (AMOVA) of Bd infection status

	DF	SSD	MSD	Variance	Fixation index
Chytrid infected/uninfected					
MSL					
Among individuals	1	183.10	183.10	3.27 (2.77%)	**$F_{st} = 0.029$; $p = 0.0009$**
Within individuals	57	6111	107.2	107.2 (97.23%)	
Total	58	6294	108.5		
NML					
Among individuals	1	24.18	24.18	0.35 (1.96%)	**$F_{st} = 0.022$; $p = 0.03$**
Within individuals	57	910.10	15.97	15.97 (98.04%)	
Total	58	934.2	16.1		
Ocelot pond only					
MSL					
Among individuals	1	130.7	130.7	3.29 (3.33%)	**$F_{st} = 0.03$; $p = 0.02$**
Within individuals	28	2671	95.4	95.4 (96.67%)	
Total	29	2802	96.61		
NML					
Among individuals	1	21.96	21.96	0.24 (1.22%)	$F_{st} = 0.012$; $p = 0.21$
Within individuals	28	544	19.43	19.43 (98.77%)	
Total	29	565.9	19.51		
Pipeline only					
MSL					
Among individuals	1	154	154	4.61 (4.54%)	**$F_{st} = 0.05$; $p = 0.002$**
Within individuals	27	2614	96.83	96.83 (95.46%)	
Total	28	2768	98.87		
NML					
Among individuals	1	28.52	28.52	0.63 (2.95%)	$F_{st} = 0.03$; $p = 0.055$
Within individuals	27	558.8	20.70	20.70 (97.05%)	
Total	28	587.3	20.98		

Results from one-factor AMOVA examining epigenetic (MSL) and genetic (NML) partitioning among ***Bd*** infection status across all individuals sampled. Separate AMOVA's also were performed on each sampled population. F-statistics were used to estimate the proportion of genetic variability found among individuals (F_{st}). Significant analyses ($p < 0.05$) are underlined and bolded. Analyses approaching significance ($0.06 > p > 0.05$) are underlined

Discussion

Amphibian populations are exhibiting significant susceptibility (i.e. population decline) to anthropogenic, environmental changes (Becker and Zamudio 2011; Hof et al. 2014; Nori et al. 2015; Kosch et al. 2016). Epigenetic variation, e.g. DNA methylation, may be one avenue for amphibian populations to respond and persist in the face of these rapid environmental changes (Bossdorf et al. 2008; Schrey et al. 2013; Burggren and Crews 2014). Here we assessed variation in DNA methylation among four populations of túngara frogs. We were particularly interested in whether DNA methylation varied among our focal populations, and whether this variation may be correlated with anthropogenic influences (i.e. urbanization and Bd introduction). In line with our hypotheses we found significant

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differences among sites in overall DNA methylation and epigenetic structure. We also discovered significant differences in overall DNA methylation among individuals dependent upon Bd infection status; a trend which held across sites. Interestingly, we found that urbanization had no significant impact on overall DNA methylation. Overall, it seems that anthropogenic disturbance levels had seemingly little impact on túngara epigenetic structure, while Bd infection status exhibited a significant statistical pattern in epigenetic structure, although we do not understand the biological relevance of this pattern at this time.

It has been well established that epigenetic structure can exhibit substantial variation across environmental gradients and among populations (Liebl et al. 2013; Wenzel and Piertney 2014; Platt et al. 2015; Foust et al. 2016) and can do so in the absence of genetic variation (Smith et al. 2016). It has been hypothesized that this fine-scale, epigenetic variance can promote range expansion into novel environments (Liebl et al. 2013), rapid response to environmental changes (Platt et al. 2015) and may even facilitate speciation events (Smith et al. 2016). Further, epigenetic variation has been documented in urban vs rural species of Darwin's finches (McNew et al. 2017). Prior work performed on our study populations had discovered significant genetic differentiation between populations on opposite sides of the Chagras River in Panama (Lampert et al. 2003). It was suspected that the Chagras River was impeding gene flow between Northern and Southern populations of túngara frogs. Based on this work, we hypothesized that there would be significant differentiation in DNA methylation between populations, specifically between the OP site and the PL, PP, and SC sites as it is separated from these sites by the Chagres River. Indeed, we found that each site had its own, unique epigenetic structure, that overall DNA methylation of frogs from OP was significantly different than PL and SC, but not PP. We also found that for PL and PP, there also was significant differentiation in overall DNA methylation frequency. Based on our findings we would suspect that reduced gene flow across the Chagras River is, in part, generating the observed differentiation. However, we did not detect significant genetic differentiation among our sites. Previously, Lampert et al. (2003) did find significant genetic differentiation between populations separated by the Chagras River but it was noted that populations closest to the south eastern side of the Chagras were not genetically distinct from those on the northeastern side, suggesting some level of gene flow, whether by land bridge or human activity. Our findings thus corroborate Lampert et al. (2003) original findings. Epigenetic modification is considerably flexible in response to differing environmental conditions (Weaver et al. 2004; Bossdorf et al. 2008; Schrey et al. 2013; Burggren and Crews 2014; Liu et al. 2012; Smith et al. 2016). Our findings that populations with likely gene flow among them still exhibited significant differentiation in DNA methylation suggest that fine-scale environmental differences may generate epigenetic changes, superseding any genetic changes.

The introduction and spread of *Batrachochytrium dendrobatidis* has had devastating effects on amphibian populations and global amphibian biodiversity (reviewed in Hof et al. 2014; Becker and Zamudio 2011; James et al. 2015). Since the late 1980s, Bd has been spreading through the highlands of Central America (Pounds and Crump 1994; Pounds et al. 1997; Phillips and Puschendorf 2013) and has a documented entry into Panama as early as 1997 (Berger et al. 1998). The population of túngara frogs that we studied in the Gamboa area did not have a documented case of Bd until 2010 and the prevalence of the disease has been rising steadily within the area since (Rodríguez-Brenes et al. 2016). Even with the introduction of Bd into the area, declines in the túngara frog populations have not been observed. It is suspected that individuals within these populations may be more tolerant of infection and could be a potential reservoir of Bd (Kosch et al. 2016; Rodríguez-Brenes et al. 2016). A growing body of research

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suggests that epigenetic modification due to early-life environmental exposure may mediate an individual's susceptibility to disease (Jirtle and Skinner 2007; Vaiserman 2015; Nilsson and Skinner 2015). Here we assayed the possible relationship between Bd infection status and epigenetic patterning and found significant differences in overall DNA methylation patterns between infected and uninfected individuals. This significant trend still held even when each population was examined individually; although not as strong compared to when all individuals were examined as a single pool. Our findings support our initial expectation and suggest a possible link between epigenetic mechanisms and infection with Bd. We make this claim cautiously as: (1) our study was not designed to determine the causal relationship between epigenetic responses and BD infection and (2) the possibility of reverse causation (Lappalainen and Greally 2017; Greally 2018) confounding our results. Further investigation is necessary to determine if there is a causal relationship between epigenetic modification and Bd infection; that is, does infection elicit an epigenetic response and is this related to tolerance, or does an epigenetic alteration earlier in life make an individual more or less prone to infection (Jirtle and Skinner 2007; Vaiserman 2015; Nilsson and Skinner 2014)?

We also found significant genetic differences between infected and uninfected individuals. However, when examining each population separately, this trend only held in the PL population and only approached significance ($0.06 > p > 0.05$). A recent study in another lowland species, the leopard frogs (*Lithobates yavapaiensis*), discovered that susceptibility, tolerance, immunological response, and physiological consequences (e.g. decreased growth) differed among Bd infected individuals (Savage et al. 2016). The authors hypothesized a gene-by-environment interaction between the host, Bd, and abiotic factors were driving these individual-level differences. Further, Kosch et al. (2016) found that a high-altitude population of túngara frogs, i.e. more vulnerable to Bd, which has been in contact with Bd for longer, had higher frequency of an MHC allele related with tolerance to Bd. In túngara frogs from Gamboa, i.e. lowland populations less vulnerable to Bd, which has less time in contact with Bd, there is a lower frequency of the relevant MHC allele. It was hypothesized that there might have been directional selection in the highlands for frogs to be more tolerant to Bd than their lowland counterparts. Our findings, in part, support these initial hypotheses and suggest an evolutionary response (e.g. change in allele frequency) to Bd introduction within these populations.

Urbanization can generate significant genetic and epigenetic differentiation among populations (Herrera and Bazaga 2016) and is one of three major factors contributing to amphibian population decline globally (Hof et al. 2014). In Panama, túngara frogs are quite adaptable and can be found in habitats ranging from near pristine to completely urban. As such, we hypothesized that this urbanization would have a significant influence on overall DNA methylation. However, our results were contrary to our hypothesis. We found little evidence that urbanization has any influence on DNA methylation among our focal populations. While we did see significant differences in DNA methylation patterns among sites, this trend does not appear to be driven by variance in urbanization among sites. One possibility for this is that another as-of-yet determined environmental factor, Bd infection perhaps, is generating variation in DNA methylation within rural and urbanized sites at high enough levels that it masks the effects of urbanization of DNA methylation frequency. Alternatively, urbanization may be less of a stressor for these túngara frog populations than we had originally predicted. A recent study has suggested population fitness might be higher in disturbed compared to undisturbed populations as urbanized sites may contain less stressful conditions (e.g. predator risk) relative to undisturbed sites (Halfwerk et al. 2019). Our findings along with this new research

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lead us to hypothesize that individuals within these urbanized sites are under less stressful conditions and thus may not produce or require an adaptive epigenetic response.

Our work examined epigenetic variation across multiple scales, among populations and across multiple environmental variables, urbanization and Bd introduction, in an attempt to understand whether and how túngara frog populations are responding to rapid, environmental changes. We have shown that epigenetic structure is variable among populations and correlates with Bd infection status but not urbanization. These findings have left us with several important questions. What environmental factors generated the observed variation in DNA methylation among populations? Why did urbanization have no appreciable effect on DNA methylation? Is there a causal relationship between Bd infection and epigenetic variance? Lastly, how does variation in DNA methylation impact the persistence of túngara frog populations in the face of rapid environmental change? We hope that our findings encourage future research endeavors to investigate the causal links between epigenetic modifications and human-induced, environmental perturbations.

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Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interests.

Ethical standards Handling and toe clipping were performed in accordance with The American Society of Ichthyology and Herpetologists' "Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research." All experiments were conducted under Salisbury University's Institutional Care and Use Committee (IACUC Protocol # SU 0036).

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