Evolution and Conservation in a Changing World: Empirical and Conceptual Lessons from Bats, Salamanders, and Beyond

by

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To my family

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Abstract

Environmental change, particularly exceptionally rapid shifts triggered by humans, can be leveraged to understand factors underlying species survival outcomes and associated evolutionary processes. Such environmental shifts can trigger micro-evolutionary forces. For instance, increased mutation rates due to carcinogenic chemicals, increased inbreeding and genetic drift due to isolation of previously connected populations, and altered adaptation due to shifts in selective pressures.

In Chapters II and III, I explore the impacts of human-altered environments on one species of bat and salamander, respectively. In Chapter II, I ask whether little brown bats (*Myotis lucifugus*) suddenly faced with the disease white-nose syndrome (caused by an introduced fungal pathogen) are experiencing corresponding natural selection. I consider one population in Michigan, USA, which experienced losses of roughly 75% in the first few years following introduction of the disease. Survivors are apparently evolving in response to it, with evidence of some alleles undergoing natural selection (they are more common than would be expected among the surviving bats, as compared to bats that died from the disease). However, the survivors also apparently have reduced genetic diversity compared to the pre-disease population. In Chapter III, I consider whether coastal giant salamanders (*Dicamptodon tenebrosus*) have experienced negative genetic consequences due to habitat disturbance. Sampled throughout Oregon, USA included disturbed versus less-disturbed sites. Some populations exhibit genetic

signatures of potential conservation concern, including genetic bottlenecks and moderate levels of inbreeding. Many sites had experienced logging or fires, and sites were often separated by inhospitable landscapes, such as agricultural land. However, genetic signatures of concern were not clearly connected to any of the anthropogenic disturbances considered. This is likely, at least in part, because of the species' own mixed response to disturbance—populations have been documented to increase in abundance, at least temporarily, following logging events. Thus, our ability to detect genetic signatures is limited by both evolutionary timescales, but also by ecological ones.

Species' responses to anthropogenic change are likely to be at least partially conditioned on previous environmental changes they have experienced. These background changes, which are often cyclical, include regimes of glacial retreat and expansion as well as annual seasonal changes. Cold temperatures, in particular, can present a challenge to which species must adapt or succumb. The salamanders in Chapter III, for example, are relatively cold-susceptible, and bore the genetic legacy of the last glacial maximum—having been extirpated from much of their northern range by Pleistocene Glaciation, contemporary populations that had recolonized northern areas exhibit relatively reduced genetic diversity. The bats in Chapter II had winter hibernation cycles carefully calibrated to seasonal regimes, which became a problem when their hibernations were disrupted by the introduced pathogen. Given the potential importance of categorizing and quantifying species' cold-survival strategies in a comparable manner, in Chapter IV, I present a conceptual framework for doing so. I propose a framework with three clearly delimited axes of cold-survival and four tenants underlying relationships between axes. Species can use either 1) seasonal migration (cold avoidance), 2) torpor (cold tolerance), or 3) cold resistance, and cold-survival strategies i) are comprehensively encompassed within the framework, ii) can be used in conjunction, iii) are used to variable degrees, and iv) should vary (collectively) inversely and proportionally to one another to meet minimum survival thresholds (when comparing populations that are sympatric in the summer range).

Chapter I

Introduction

Both evolutionary and conservation outcomes are underlaid by the question of how species respond to and cope with environmental change. Research that addresses fundamental questions regarding the nature of evolutionary processes while also informing conservation efforts is therefore possible at the intersection of environmental change, conservation, and evolution. These three components each include interconnected factors (Fig. 1-1)—evolutionary responses shape species relationships to environmental regimes and can shift their conservation trajectories. The field of conservation is, at its base, about preventing species extinctions. Extinctions are foreshadowed by declining populations and shrinking distributions, and can accumulate into substantial levels of biodiversity loss (Fig. 1-2).

Changing environments and the people problem

Environmental changes can be divided, broadly, into anthropogenic (human-caused) and natural changes. These anthropogenic changes are in addition to the natural environmental changes that are ongoing in the background, such as seasonal changes and glaciation cycles. Natural changes are usually, at least partially, cyclical. Glaciation cycles, which occur over tens of thousands of years (Imbrie et al., 1992), and annual seasonal changes (especially winters, which are periodically freezing), can both be extremely challenging to species (Mann et al., 2019; Sherpa et al., 2022; Studd et al., 2021; Williams et al., 2015)—leading, for example, to species extinctions, range restrictions, and remarkable adaptations (e.g., hibernation and migration; Chapter IV; Auteri, 2022). These background environmental shifts can already be incredibly challenging to species. However anthropogenic changes can often occur in addition to these background changes, and at more rapid rates, potentially presenting insurmountable challenges. Examples of anthropogenic changes include factors like facilitating the spread of invasive species (Bellard et al., 2016), habitat destruction and fragmentation, and climate change associated with increased emissions of greenhouse gasses. Some species actually thrive in human-altered environments (Lowry et al., 2013; Mazza et al., 2020; Thomas, 2020), however many others do not, and these thriving species may contribute to conservation threats by displacing more sensitive species. Furthermore, species may be at first negatively affected by anthropogenic alterations, but may later be able to recover due to evolutionary rescue (Carlson et al., 2014; Catullo et al., 2019; Greenspoon and Spencer, 2021), or may also exhibit the reverse pattern—at first appearing relatively unaffected by artificial changes to the environment, but that later may experience severe declines (Trindade et al., 2020). In the wake of the overwhelming conservation challenges sparked by these environmental changes, many have begun to ask whether evolutionary forces might be able to mitigate these threats.

Relevance of evolution to conservation

Evolution is inherently linked to how species respond to environmental changes. Thus, it is fundamentally linked to conservation outcomes as well. In particular, natural selection can save species from extinction via adaptation (referred to as evolutionary rescue) (Carlson et al., 2014), but species can also experience negative outcomes such as inbreeding depression or low genetic diversity, potentially brought on by strong genetic drift occurring on conjunction with population declines (DeWoody et al., 2021).

Evolution, particularly evolutionary adaptation, is often thought of as slow. Thus, can evolution be fast enough to be relevant to conservation given the rapid rates of change in the Anthropocene? The idea that evolution is slow comes in part from historical context. After Darwin’s On the Origin of Species (Darwin, 1859), biologists became excited about evolution by natural selection, often taking on an adaptation-centric view of evolution. As our understanding of genetics increased, people often thought about natural selection as occurring in terms of a beneficial mutation happening, followed by subsequent natural selection favoring that allele. From this perspective, evolution would be expected to be very slow, because mutations are rare, especially good mutations. However, later biologists began to questioned this perspective (Barrett and Hoekstra, 2011; Gould, 1997; Pigliucci and Kaplan, 2000), drawing focus to the importance of neutral and nearly neutral genetic variation (Kimura, 1977, 1968; Ohta, 1992). A key to rapid evolution is preexisting, neutral, genetic variation, which may end up being adaptive later when the environment changes (when selective conditions change). This is important because it means the slow step—waiting for a beneficial mutation—is eliminated. There is potentially already plenty of genetic variation upon which changing selective forces can act.

Conservation can inform knowledge of evolutionary processes

Rates of anthropogenic changes tend to be faster that natural ones, which is why they can be so problematic from a conservation perspective. But all environmental changes can be inherently challenging to species and lead to conservation threats. Conservation is ultimately about preventing species extinctions. Some of the factors related to extinctions are population declines (decreases in abundance), range contraction (decrease in the extent of an area where a species persists), and biodiversity loss (decreases in the number of existing or extant species). In addition to being influenced by evolutionary processes, conservation outcomes relate to evolution, because such outcomes are, in a sense, metrics of species survival in the face of changing selective environments. Conservation questions, although sometimes considered only in the context of an applied discipline, can also presents tremendous opportunity to address theoretical questions in evolution and ecology. Evolution is, in essence, potentially sped up by rapid anthropogenic changes, so that we it is potentially more readily observable on a human-centric timeline.

Overarching research themes

The overarching focus of my work is when and how evolutionary forces can buffer, mitigate, or work in conjunction with the impacts of environmental changes. Chapter II (Auteri and Knowles, 2020) considers bats responding to an invasive species (a fungal pathogen. Chapter III (Auteri et al., 2022) considers the effects of anthropogenic and glacial environmental change on a species of salamander. Chapter IV (Auteri, 2022) considers how careful consideration of species' preexisting adaptations to seasonal changes might influence our ability to understand responses to past and forthcoming forms of environmental change.

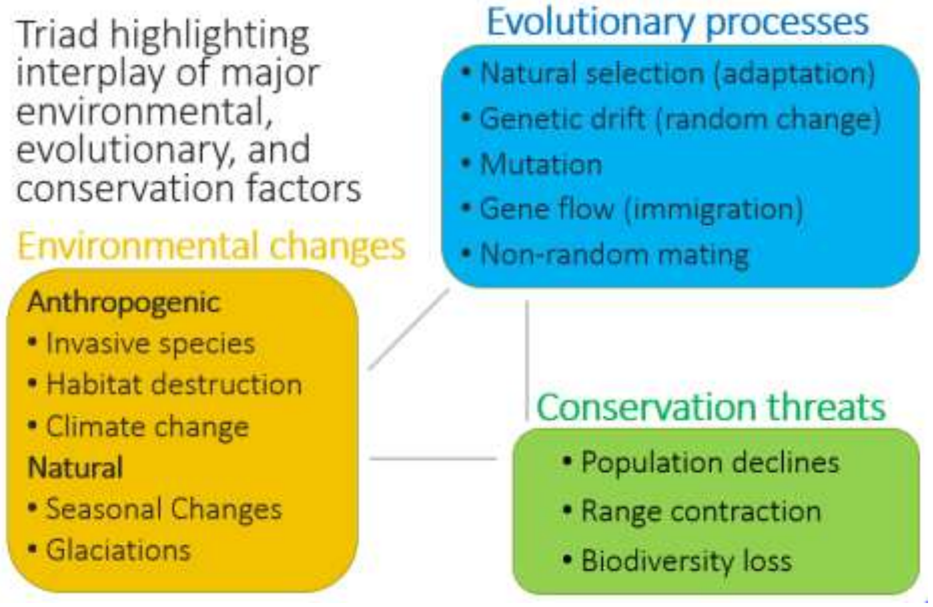

Fig. 1-1. Environmental changes, evolutionary processes, and conservation threats, including relevant subcomponents, many of which are touched upon in this dissertation. Evolution and conservation components are two aspects of species' responses to environmental change, but can also influence each other.

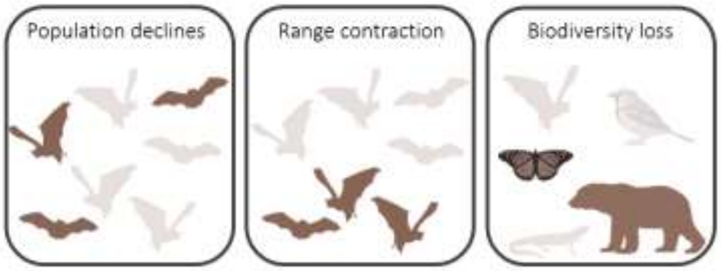

Fig. 1-2. Patterns of conservation concern—declines in population abundance (left), shrinkages in the extant range (center), and extirpations (or extinctions) of species (right).

Chapter II

Decimated Little Brown Bats Show Potential for Adaptive Change

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Abstract

The degree to which species can rapidly adapt is key to survival in the face of climatic and other anthropogenic changes. For little brown bats (*Myotis lucifugus*), whose populations have experienced declines of over 90% because of the introduced fungal pathogen that causes white-nose syndrome (WNS), survival of the species may ultimately depend upon its capacity for adaptive change. Here, we present evidence of selectively driven change (adaptation), despite dramatic nonadaptive genomic shifts (genetic drift) associated with population declines. We compared the genetic makeups of wild survivors versus non-survivors of WNS, and found significant shifts in allele frequencies of genes associated with regulating arousal from hibernation (GABARB1), breakdown of fats (cGMP-PK1), and vocalizations (FOXP2). Changes at these genes are suggestive of evolutionary adaptation, given that WNS causes bats to arouse

with unusual frequency from hibernation, contributing to premature depletion of fat reserves. However, whether these putatively adaptive shifts in allele frequencies translate into sufficient increases in survival for the species to rebound in the face of WNS is unknown.

Introduction

Events that kill large portions of populations, including naturally and anthropogenically induced disasters, increasingly threaten biodiversity (Dobson et al., 1997; Johnson et al., 2017). Invasive species are a major trigger of these declines (Clavero and García-Berthou, 2005), including invasive pathogens, against which native species can experience high mortality due to a lack of co-evolutionary defenses (Anderson and May, 1986; Cunningham et al., 2003; Parker and Gilbert, 2004). Introduced fungal pathogens can be particularly dangerous—they can frequently survive in the environment for extended periods, affect a relatively broad range of hosts, and can be highly virulent (Fisher et al., 2012), thereby driving mass-mortalities of native species (e.g. amphibian chytrid(Berger et al., 1998), snake fungal disease (Lorch et al., 2016), sea fan aspergillosis (Kim and Harvell, 2004), and others (Hansen et al., 2005; Holdich et al., 2009; Tompkins et al., 2003) as well as threatening agricultural crops (Institute of Medicine (US) Forum on Microbial Threats, 2011; Pennisi, 2010) (e.g. rice blast disease (Khush and Jena, 2009) and Fusarium wilt in bananas(Stokstad, 2019).

Although host mortalities may have little impact on fungal pathogens, the pathogens can exert incredibly strong selective pressures on their host populations (Castro and Bolker, 2005). A pressing conservation question is whether host populations can evolve resistance or tolerance during such epidemics—a necessary first step towards preventing extinction. Strong selective pressures might theoretically lead to an evolutionary rescue effect if host populations adapt

(Gomulkiewicz and Holt, 1995). However, acute events that kill off most members of a species also reduce the genetic diversity upon which natural selection can act, thereby limiting the capacity for adaptive change (Hoffmann and Sgrò, 2011).

White-nose syndrome (WNS) is a disease affecting bats, which is caused by the invasive fungus *Pseudogymnoascus destructans* (Lorch et al., 2011). This highly destructive pathogen has decimated populations of bats, with 12 North American species currently affected (White-nose syndrome response team, n.d.), and some populations experiencing losses of 90–100% (Turner et al., 2011). The fungus was first inadvertently introduced to North America by humans in 2006 (in the northeastern U.S.) (Blehert et al., 2009), and is spreading across the continent, largely via infected bats (Wilder et al., 2015). The exact mechanism of death is not known, but bats apparently die from secondary physiological complications (e.g. depleted fat reserves) associated with too frequent arousals from hibernation (Reeder et al., 2012).

Here, we conduct a genome scan to test for evidence of evolutionary changes in little brown bats (*Myotis lucifugus*) in response to WNS. The recent expansion of the fungus into our study area in 2014 combined with the staggering impact of WNS on the local population (roughly 78%) (Kurta and Smith, 2018) provides an opportunity to study the initial evolutionary effects of this pathogen, which continues to spread throughout the continent. Eurasian bats within the genus *Myotis*—in the native range of the pathogen—tolerate fungal growths with no noticeable mortality (Breed et al., 2010; Martínková et al., 2010). In contrast, little brown bats were the most common bats in eastern North America prior to WNS, but due to population losses, the species has now been listed as endangered by the IUCN (Solaris, 2018) and the federal government of Canada (Government of Canada, 2014), with a similar decision by the U.S. government pending (U.S. Fish & Wildlife Service, 2016). Despite large observed declines, some individuals may have greater genetic-based tolerance or resistance to the disease, raising the potential for adaptive change in little brown bats via selective forces acting on standing genetic variation. However, dramatic population losses may confound the effectiveness of selection or purge potential adaptive variants via genetic drift. Information about these evolutionary processes can help inform the tempo and pace of management efforts for this species, by indicating which, if any, populations are adapting to the pathogen and what traits may be important for survival.

Results

In our tests for evolutionary changes in little brown bats, we compared the genetic makeup of "survivors" and "non-survivors" of the disease (see Fig. 2-1) in a genome-wide survey of 19,797 single nucleotide polymorphisms (SNPs) among 14,345 loci (140 bp segments) generated from a reduced representation library (ddRadSeq; Peterson et al., 2012). We detected the effects of stochastic, non-adaptive genomic changes in otherwise neutral portions of the genome (genetic drift) reflective of the large numbers that have died from WNS in this species. Nevertheless, we also identified genetic changes (based on F_{ST}-outlier analyses) that may have contributed to survival (as opposed to changes simply due to strong genetic drift), where the signature of selection can be detected by levels of genetic differentiation at a gene that exceeds background levels across the genome (Beaumont, 2005; Weir, 1996). See methods for more details.

Non-adaptive evolution associated with large number of deaths caused by WNS

To visualize the drift-induced changes that have occurred broadly across the genome, a PCA generated using the survivors, onto which the non-survivors were projected (Fig. 2-2), indicated the genomic makeup of survivors differs substantially from the non-survivors (which is robust to more stringent criteria for data filtering; Appendix A Fig. A-S1). Quantification of the rate of evolutionary change from an inferred common ancestor showed the rate of drift is an order of magnitude higher in survivors (mean $F = 0.04 \pm$ SE 0.0001) relative to non-survivors ($F = 0.006 \pm 0.0003$), using the F-model in STRUCTURE (Falush et al., 2003; Pritchard et al., 2000). This amount of drift-induced genetic change (Fig. 2-2) is especially striking given that these changes have accumulated over, at most, three years (with most of our samples separated by just one year; Table A-S1), in a species that can live for well over 20 years (Florko et al., 2017; Keen and Hitchcock, 1980) and in which females typically produce one pup per year (Kurta, 2008).

Selective divergence putatively driven by WNS

Quantification of locus-specific differentiation across the genome using F_{ST}-outlier analyses identified nine SNP alleles that are significantly more common among survivors than non-survivors across all three outlier detection methods (Table A-S2; for details on individual genotypes see Table A-S3). These nine variable sites were the only outliers identified using the AMOVA-corrected F_{ST} from STACKS (Fig. 2-3), and were also among the outliers recognized in the two other tests (see Figs. A-S2 and A-S3 in Appendix A). Analyses with and without four non-survivors that were collected several years prior to other samples (in 2014; Table A-S1; Appendix A) confirmed the robustness of these results to different collection dates (Figs. A-S2, A-S3, and A-S4; Appendix A).

Comparison of the nine top-candidate loci with the *M. lucifugus* reference genome (MYOLUC 2.0; Lindblad-Toh et al., 2011) indicates three mRNA-coding SNPs are located in introns of annotated genes (Table A-S2; Appendix A). These three genes are: the gamma-aminobutyric acid (GABA) receptor subunit beta-1 (GABRB1; Gene ID 102432079 in the reference genome), cyclic guanosine-3’,5’-monophosphate-dependent protein kinase 1 (cGMP-PK1; Gene ID 102431010), and the forkhead box P2 protein (FOXP2; Gene ID 102423801). Two other SNPs are close to annotated genes—one was near the previously identified cGMP-PK1 gene in our dataset (3,387 bp away), and the other was near phospholipase A2 group VII (PLA2G7; Gene ID 19253; 2,747 bp away). The remaining four SNPs are relatively distant from any area of the reference genome with known function (> 170,000 bp away on average).

Discussion

We studied the genetic differences between wild little brown bats that were survivors versus non-survivors of WNS, and found evidence that there is likely a genetic component to survivorship for individuals facing this disease. This apparent adaptation has occurred very quickly since the detected evolutionary changes took place after the WNS introduction in 2014, and survivors were sampled just a few years later.

The putative selectively driven genetic changes we identify (Fig. 2-3) have also occurred despite dramatic nonadaptive genomic shifts (genetic drift; Fig. 2-2) associated with population declines due to the disease. Together, this suggests that the putative adaptive changes have resulted from very strong selective forces acting on standing genetic variation. Such rapid evolutionary changes are not unprecedented. For example, populations of the steelhead trout (*Oncorhynchus mykiss*) introduced to the central USA from coastal areas show signs of adaptation to freshwater conditions, despite small founder populations (Willoughby et al., 2018). Likewise, extremely rapid phenotypic adaptation in Caribbean lizards followed a hurricane, with surviving lizards having larger toe pads which were presumably better at gripping surfaces during strong winds (Donihue et al., 2018).

The putatively adaptive SNPs among the surviving bats in our study are located within or in close proximity to four genes (cGMP-PK1, FOXP2, GABARB1, and PLA2G7), which when mapped to the annotated reference genome suggest different ways adaptive shifts might contribute to survival. GABARB1 is a receptor for the neurotransmitter GABA, which is a major neural inhibitor in the brains of vertebrates, and has also long been suspected to be involved in regulating hibernation (Nilsson and Lutz, 1993). In addition to GABA, these receptors are also sensitive to histamines (Saras et al., 2008), which similarly help regulate hibernation in mammals (Sallmen et al., 1999) and are released in response to tissue damage from WNS (Field et al., 2015). The importance of an individual's sensitivity to histamines is further hinted by PLA2G7, which regulates release of histamines from mast cells (Choi et al., 1989). Because arousals account for 80–90% of bats' energy budget during hibernation, genetic variation that contributes to even small changes in arousal frequencies could result in large differences in energy expenditures, making the difference between life and death (i.e., affecting susceptibility to WNS). We speculate that bats genetically predisposed to release fewer histamines, or be less prone to arousals induced by histamines, are better able to survive WNS through conservation of energy reserves.

Links between metabolic demands and survival are further suggested by cGMP-PK1, which was implicated by two significant SNPs in our dataset (one within the gene and one nearby). This gene is part of pathways involving cellular metabolism and breakdown of fat, and allelic variants have been linked to obesity in mammals (Dai et al., 2013; Jobgen et al., 2006), which might prove beneficial for WNS-infected bats facing premature depletion of winter fat reserves. In fact, a recent study documented a post-WNS phenotypic shift towards fatter bats of this species (Cheng et al., 2019). Although this may be due to a variety of potential mechanisms, including non-evolutionary ones (see discussion in Cheng et al., 2019), our findings suggest a genetic component to this shift.

In contrast to the SNPs linked to physiological mechanisms during winter hibernation, a SNP within FOXP2 suggests behavioral differences might confer a selective advantage. Specifically, FOXP2 is associated with vocalizations in other vertebrates, and echolocation in bats (Li et al., 2007). Because variation in calls is closely associated with the type of prey and habitat bats must navigate, echolocation is an important functional trait, and potentially adaptive shifts might be related to hunting proficiency, speed of developing foraging abilities in juvenile bats, or subtle differences in prey preferences. These could affect the type and amount of fat that bats store for hibernation. In addition to echolocations for hunting, bats also emit social calls. Sociality may influence the impact of the disease in this species (Langwig et al., 2012), and due to the importance of FOXP2 in communication, the gene has been linked to variations in social behavior in other species (Robinson et al., 2008; Shu et al., 2005; Wohlgemuth et al., 2014). A more detailed study is needed to test these hypotheses, and there are possibly alternative unknown functions of FOXP2 in bats. Interestingly, no individuals in our dataset were heterozygous for this SNP.

Although outlier analyses can contain false positives, potentially inferring selectively driven differentiation when there is none (Hoban et al., 2016), we think it is unlikely the mRNA-coding SNPs we detected are statistical artifacts. The four genes we identify had putatively adaptive alleles that were entirely absent from our non-survivors (with the exception of a single allele copy in one individual). With the much greater sampling of non-survivors (n=29) this difference is also not due to limited sampling (Fig. 2-1). An alternative consideration is that genetic drift, not selection, explains the elevated differentiation in what we identified as putatively adaptive alleles among the survivors. With inter-locus contrasts, the genome serves as the expected background for differentiation caused by drift (i.e., the expected variance in F_{ST}-values in this case; Fig. 2-3). However demographic processes can inflate the variance of the distribution of F_{ST}-values (e.g. population structure such as isolation by distance or expansion; reviewed in Hoban et al. (2006), potentially confounding the signals of selection and drift. Although we cannot rule out a role for non-selective processes, we note that annotation of the alleles suggests that selection is involved given that the functions are consistent with an adaptive response.

Whether the putative adaptive changes described here reflect host resistance or tolerance to the fungal pathogen has consequences for evolutionary and ecological pressures, as well as management strategies. While our study does not explicitly test whether bats survive WNS via resistance versus tolerance mechanisms, and the genomic approach we used only looked at a small portion of the genome, we found putative selection acting on non-immune genes, which suggests disease tolerance (Ayres and Schneider, 2008) may be important. Specifically, the alleles we identify could assist some bats in “holding out” until spring, when they leave sites in which growth of the pathogen is restricted to. While infected bats do exhibit an immune response to the fungus (Lilley et al., 2017), they likely ultimately die due to secondary physiological complications linked to starvation while hibernating (Cryan et al., 2010; Reeder et al., 2012). Such tolerance in little brown bats to WNS may be important for survival in both in intraspecific (Jonasson and Willis, 2011) and interspecific (Davy et al., 2017) contexts. However, others argue that resistance is the primary mechanism of survivorship (Langwig et al., 2017). Future work is needed to resolve this question.

Conclusions

What the outcome of the evolutionary change we report here might be and what it bodes for the future recovery of little brown bats is not clear—it is too soon to claim that the species will be “saved” via an evolutionary rescue effect. There have been dramatic population declines, and low population sizes inherently make species vulnerable to further perturbations. Furthermore, the disease has only been present in North America for thirteen years at the time of this publication, and with little brown bats surviving to more than 20 years old in the wild (Florko et al., 2017; Keen and Hitchcock, 1980) it will take time to determine whether surviving remnant populations have sufficient reproductive and recruitment levels to avoid extinction or extirpation. However, the functions of the genes we identify suggest that for this species, and possibly other bats effected by WNS, conservation of summer foraging habitat—not just winter hibernation sites—may promote population recovery, given that the selective advantages underlying shifts in FOXP2 would most likely manifest when bats are echolocating and hunting, and not in the hibernation sites where the bats are confronted with the fungus (Fig. 2-1). Other genes we identified are likely subject to strong selection during winter periods of infection, but could also be important year-round (cGMP-PK1, PLA2G7, and GABRB1), given their functions in cellular metabolism. With the limited representation of the genome, there may also be selective divergence in genes not studied here. Nevertheless, even without more extensive coverage of the genome, our work hints at the multifaceted nature of selection by identifying genes whose roles differ across habitats of highly seasonal environments, and are linked to both physiological and behavioral traits.

Materials and Methods

Study area

We chose northern Michigan, USA, for our study because it represents a reasonably isolated population of little brown bats (Fig. 2-1A); WNS is present throughout our study area, and was first detected there in early 2014. We sample non-survivors from hibernation sites during the winter and survivors during the summer (when they are no longer afflicted by the pathogen). However, because the species utilizes short distance seasonal migration (typically ≤ 500 km; Fleming and Eby, 2003), during warmer periods they do not roost in the same sites in which they hibernate, thus the relative geographic isolation is important for assuring that bats sampled during both seasons were from the same population. Winter hibernation sites are concentrated in the northwestern portion of our study area (hibernation sites are lacking in the central and southern Michigan), and primarily consist of abandoned iron and copper mines. As a consequence, bats in our area (Fig. 2-1A) are isolated from other populations by two factors: the Laurentian Great Lakes and the lack of suitable subterranean hibernation sites within migration range in central and southern Michigan. The seasonal sampling of bats is necessary because WNS non-survivors can only be documented in winter areas, and disease survivors can only be identified during summer.

Sampling of focal species

All sampled bats (Table A-S1) were categorized as either “survivors” or “non-survivors” of WNS. Survivors (*n*=9) were adult bats that had been born the previous year or earlier and thus had survived at least one hibernation period with the WNS pathogen (collected during summer s of 2016–2017, see Anthony (1988) for aging methodology). Most individuals which succumb to the disease are found within the subterranean sites that afflicted species of bats rely upon in winter, and in which the fungus thrives, however some infected bats leave hibernation sites prematurely in winter in search of food or water, but quickly die due to lack of available resources and sub-freezing temperatures. Correspondingly, most non-survivors we sampled were bats found dead in or near hibernation sites during winter (collected in early 2016; *n*=25; Fig. 2-1), although some tissue samples came from individuals with the pathogen that were euthanized during surveillance studies (i.e., they tested positive for the fungus; collected in early 2014; *n*=4). Note that comparing survivors to this more general group of non-survivors makes tests for loci under selection more conservative, in that some of the euthanized bats categorized as non-survivors may not have died from WNS naturally. However, if non-survivors actually carried adaptive alleles, this would not produce a bias (i.e., make it more likely) to detect putatively selected alleles – in fact it would make such detection more difficult. In addition, all analyses were repeated excluding the euthanized bats to confirm the robustness the results.

Samples for most non-survivors (*n*=23) were from bat carcasses found during winter either in or proximal to the caves or mines in which they were hibernating. Prior to the introduction of WNS, it was uncommon to find dead bats at hibernacula, whereas conspicuous numbers of dead individuals are found in and around these sites post-introduction of the disease

(Fig. 2-1B), and all sites were WNS-positive at the time of collection. The accidental inclusion of bats which had died due to other causes would make it more difficult to detect adaptation in our analyses. To reduce disturbance to hibernating bats, dead bats were collected in conjunction with routine surveys by the Michigan Department of Natural Resources (MDNR) and Eastern Michigan University. Four samples were contributed by the U.S. Geological Survey National Wildlife Health Center; these bats were found during hibernation with the fungus growing on them, but were euthanized (as discussed above). Lastly, two samples of non-survivors came from the MDNR Wildlife Disease Laboratory (see details below).

Among the survivors, collection methods varied (Table A-S1). Three survivors were captured during summer using mist-nets, and visual inspection confirmed evidence of recovering from WNS (i.e., the presence of healing wing lesions or scars). Tissue samples were collected via small biopsy punches (2 mm diameter, one punch for each wing, Premier Medical Products Company, Plymouth Meeting, Pennsylvania, USA), after which bats were immediately released. No individual was detained for longer than 30 minutes. Eight specimens were contributed by the MDNR Wildlife Disease Laboratory, which annually receives large numbers of bats for rabies testing after they are encountered by humans or pets (Auteri and Kurta, 2015). All individuals used in this study tested negative for the rabies virus. Six of these were considered survivors because they were submitted for testing in summer or fall; during the summer this species uses structures such as houses in addition to trees (Kurta, 2008) so there is no reason to believe that animals encountered by people during warmer periods were unhealthy. However, at the latitude of our study, little brown bats are not known to hibernate in buildings (Kurta, 2008). Consequently, any individual encountered by humans during sub-freezing periods is almost certainly on the cusp of dying from WNS. Individuals submitted to the MDNR Wildlife Disease laboratory in winter or early spring were therefore assigned to the non-survivor group (*n*=2 in this study).

DNA sequencing and data processing

DNA was extracted from membrane of wing tissue using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) and used to prepare a reduced representation genomic library for sequencing. Two restriction enzymes, *Eco*RI and *Mse*I, were used to digest extracted DNA (ddRadSeq; Peterson et al., 2012), to which barcodes (unique tags 10 base-pairs long) and adapters for Illumina sequencing were then ligated. Ligation and amplification were done via polymerase chain reaction (PCR), and 350 to 450 bp long fragments were size selected using Pippin Prep (Sage Science, Beverly, Massachusetts, USA). The library of 38 samples was sequenced in one HiSeq2500 lane (Illumina, San Diego, CA, USA), at the Centre for Applied Genomics (Toronto, Ontario, CA).

Genomic sequences were demultiplexed using the STACKS bioinformatics pipeline (Catchen et al., 2013; v. 2.1; specifically *process rad-tags*, *gstacks*, and *populations*), and processed in conjunction with supporting programs. The first step, *process radtags*, allowed up to one mismatch in the adapter sequence and two mismatches in the barcode, with rescue of RAD-Tags allowed. A sliding window of 15% of the read length was used for an initial exclusion of any reads with a *Phred* score (Ewing and Green, 1998) below 10 within the window (note additional filters of a minimum *Phred* score of 30 were applied in downstream processing, as discussed below). Of 102,419,857 initial sequences, *process radtags* removed 1,144,865 reads containing the adapter sequence, 18,775,218 reads with ambiguous barcodes, 156,274 low quality reads, and 2,495,192 reads with ambiguous RAD-Tags.

We then indexed a previously generated reference genome for the species, ftp://ftp.ncbi.nih.gov/genomes/Myotis_lucifugus (7x coverage; MYOLUC v. 2.0; Lindblad-Toh et al., 2011), and mapped our sequences to the genome using the Burrows-Wheeler Alignment Program (v. 7.17) indexing and MEM algorithms, respectively (Li, 2013; Li and Durbin, 2009). The resulting files were filtered (-F 0x804, -q 10, -m 100), converted to .bam files, and sorted using SAMtools (v. 1.8-27; Li, 2011; Li et al., 2009).

The reference-based method of *gstacks* (set to remove PCR duplicates) was run using the Marukilow model (Maruki and Lynch, 2017), minimum *Phred* (Ewing and Green, 1998) score of 30, and alpha thresholds (for mean and variance) of 0.05 for discovering single nucleotide polymorphisms (SNPs). This resulted in 59,888,201 BAM records and 581,607 loci (8% of reads were excluded because they were excessively soft-clipped, and 3% had insufficient mapping qualities to be included). All remaining loci were genotyped, with a mean per-sample coverage of 10.5x ± 7.1x, a mean of 138.5 bps per locus, and consistent phasing for 88.3% of diploid loci.

Populations was then run with default settings and the resulting loci were filtered with a custom script in R (v. 3.5.0; R Core Team, 2015) to remove loci and SNPs that may be artifacts of sequencing or alignment errors (Fig. A-S5; Appendix A) based on the number of SNPs per read position, resulting in exclusion of SNPs occurring in the last 2 bp of each read. Loci with unusually high levels of diversity were also removed from consideration (threshold θ >0.026), leaving 273,261 unique loci.

Using the list of vetted loci and SNPs, *populations* was then run again, retaining loci present in at least 56% of both survivors and non-survivors, ensuring a minimum sample size of at least six survivors; note the actual missing data was typically much lower (i.e., <15% in all but 7 individuals of survivors and non-survivors). This resulted in 40,963 loci (140 bp segments), of which were variable, containing 19,797 SNPs (our final SNPs), all of which had a minor allele frequency of > 0.01. Minor allele thresholds of 0.01 and 0.05 were evaluated for downstream analyses, and when warranted the higher threshold was used (noted below). Mean genotyped sites per locus was 142.41 bp (*SE* ± 0.02). Because some loci contained more than one SNP, the robustness of downstream analysis to inclusion of multiple versus a single SNP per 140-bp fragment was evaluated. Main findings did not differ, thus we present analyses based on multiple SNPs per locus in the main text (see Fig. A-S6 for results based on a single SNP per locus; Appendix A).

We also checked that the data were not biased due to different levels of genetic decomposition between the survivors and non-survivors by analyzing the Guanine-Cytosine (GC) content of each sample. Specifically, raw Illumina reads (immediately after *process radtags*) of survivors were compared with the non-survivors using BBMap (v. 38.01; Bushnell, 2014). The proportion of GC per individual per locus was averaged across all loci for each individual using a custom script in R. Mean GC content was 43% for survivors (*n*=9) and 42% (*n*=29) for non-survivors, which confirmed non-survivors were not biased towards higher GC because of decomposition.

In addition, the relatedness of sampled individuals was evaluated in two ways: with *related* (Pew et al., 2015) in R (R Core Team, 2015) and using Plink (Purcell et al., 2007). Due to program constraints in *related*, 250 loci were randomly selected to simulate 100 pairs of individuals in each of four categories: parent-offspring, full sibling, half sibling, and unrelated. Application of the Ritland estimator of relatedness (Ritland, 1996) to both the simulated and empirical dataset of 1,242 filtered SNPs (see Fig. A-S7 caption; Appendix A) indicated that none of the individuals in our dataset were related with the exception of two of the non-survivors, which may be half-siblings (Fig. A-S7; Appendix A). However, the Plink (Purcell et al., 2007) analysis of 6,237 SNPs (restricted to a single SNP per locus and minor allele frequency > 0.05, as per guidelines) indicated no related individuals within our dataset. We kept all individuals in downstream analyses, because the presence of a single pair of potential half siblings is not expected to influence estimates of allele frequencies or F_{ST}, and removal of putatively related individuals can actually increase the error (for more details, see Waples & Anderson 2017).

Lastly, to confirm that individuals from different sampling sites within the study area could be considered one population, we used STRUCTURE (V. 2.3.4; Falush et al., 2003) to evaluate if genome-wide differentiation indicated a single, panmictic population. We selected the ADMIXTURE model with ‘Allele frequencies correlated’ turned on and no prior information about sampling population and explored the best supported model, considering a range of genetic clusters (i.e., $k = 1$ to 5) with 10 repetitions for each k, for 500,000 Markov chain Monte Carlo iterations with a burn in of 50,000. Visual assessment was used to ascertain convergence by examining plots of FST, alpha, and likelihood versus iterations, and to check for consistency among the ten iterations. No evidence of genetic subdivision based on geographic sampling locality was detected (see Fig. A-S8; Appendix A).

Tests of genetic drift

Given the large numbers that have died from WNS in this species, genetic differentiation between survivors and non-survivors may result because some alleles, just by chance, will increase or decrease in frequency. These stochastic, non-adaptive genomic changes in otherwise neutral portions of the genome (genetic drift) can be particularly great when only a small proportion of the population survives, sometimes causing population bottlenecks. To visualize the drift-induced changes that occurred broadly across the genome, we conducted a principal components analysis (PCA) of the survivors, and projected the non-survivors onto the estimated PC axes, and the degree of drift was quantified using the F-model (Falush et al., 2003) in STRUCTURE (Falush et al., 2003).

The PCA was calculated for the survivors, onto which the non-survivors were projected (by applying the same scaling and centering used for survivors to the non-survivors; see Lipson et al. 2018). Generating a PCA in this manner is a method of visualizing differences when one group is a subset of the other (in terms of the proportion of variance), for example due to a series of founder events (Lipson et al., 2018).The PCA was performed in R (R Core Team, 2015), in conjunction with the packages *Adegenet* (v. 2.1.1; Jombart, 2008; and *Plyr* v. 1.8.4; Wickham, 2011) using the *prcomp* function. One survivor and four non-survivors were excluded from this analysis because of missing data (i.e., >50% missing loci), as were loci missing in >50% individuals (data were filtered using Plink v. 1.07; Purcell et al., 2007; see Table A-S1). After this, the actual missing data was <15% for all individuals except one survivor and one non-survivor, with just under 50% missing data. Missing data were then replaced with the per locus mean value across all individuals. Only genomic sites with a minor allele frequency of ≥ 0.05 that were variable in both survivors and non-survivors were considered, for a total of 11,462 SNPs. The PCA was repeated to confirm the robustness of the results to missing data threshold, this time using a minimum data threshold of 8.7% missing data per individual and 19% per locus (mean missing data was 1.9%), which resulted in 13,666 loci and 31 individuals being included.

We also directly estimated the amount of genetic drift between survivors and non-survivors in STRUCTURE (Pritchard et al., 2000) using the F-model (Falush et al., 2003; see also Harter *et al*. 2004). The *F*-model accounts for differences in population sizes, and has been used to quantify differences in drift between groups of contrasting sample sizes that are similar in proportion to our own (Harter et al., 2004). For our parameter of interest, *F*, we used a prior mean and SD of 0.10, which places similar probabilities on both large and small values of *F*. To implement this Bayesian approach, we preassigned individuals to one of the two groups (survivor or non-survivor), and used a burn-in of 50,000 followed by 500,000 reps. We fixed lambda at 1, and used a uniform prior from 1 to 10 for alpha, with a standard deviation of 0.025. Three iterations were run, with different random seeds for initiating the Markov Chains.

Tests of loci under selection

To identify genetic differences among individuals that might have contributed to their survival of WNS, we used F_{ST}-outlier analyses, where the signature of selection can be detected by considering the proportional split of allelic variants between groups relative to background levels across the genome (Beaumont, 2005; Weir, 1996). We identified candidate loci using three methods of outlier detection – identification of outliers via (i) the number of standard deviations from the mean using an AMOVA-corrected F_{ST} (Excoffier et al., 1992), (ii) by assessing confidence intervals from bootstrap permutation across loci, and (iii) measuring departure from a chi-squared distribution (detailed below). Variable sites which met all three requirements were regarded as candidate loci apparently undergoing positive selection. All tests of selection were conducted with and without the four non-survivors sampled in 2014 (collected prior to the other specimens), to confirm that the results were robust. Note that the low number of sampled survivors reflects the devastating impact of WNS on this species; despite the small sample size, it is not beyond a size in which SNPs under selection can be detected with F_{ST}-outlier analyses (Willing et al., 2012).

In our first approach, we used the AMOVA-corrected F_{ST} (Excoffier et al., 1992) calculated by *populations* in STACKS (Catchen et al., 2013). SNPs with an F_{ST}-value of greater than nine standard deviations from the mean (mean = 0.018 ± 1SD of 0.026) were considered outliers (similar to Willoughby *et al*. 2018). A threshold of five standard deviations is often used in detection of outlier SNPs under positive selection[42,86,87]. We increased our threshold of significance to nine standard deviations to reduce the potential for false-positives. In the second approach, confidence intervals (95% CI) were estimated using diversity (Keenan et al., 2013). Using the diffCalc function, Weir and Cockerham's F_{ST} (Weir and Cockerham, 1984) was calculated for all loci, with 1,000 bootstraps performed across loci. Only loci for which the lower limit of the CI remained five SD from the mean were considered outliers. In the third approach, outliers were identified with OutFLANK (Whitlock and Lotterhos, 2015), which estimates the expected neutral variation of F_{ST}-values under a chi-squared distribution. As per the developer guidelines (Whitlock and Lotterhos, 2015), we excluded loci with low expected heterozygosity (< 0.1), and visually adjusted the trim functions to best fit the observed distribution (LeftTrimFraction = 0.3 and RightTrimFraction = 0.05; Fig. A-S9; Appendix A). Significance was assessed using *qvalue* (Storey et al., 2015) in R (v. 2.12; R Core Team, 2015).

All results were visualized in R (R Core Team, 2015), often in conjunction with the package *ggplot2* (Wickham, 2009). A custom script was used to identify SNPs which were identified as candidate loci under all three methods, and putatively selected sites were then cross-referenced with the species' annotated reference genome (Lindblad-Toh et al., 2011) to infer possible phenotypic function (see Broad Institute, 2008; National Center for Biotechnology Information, 2015, for additional information on the reference genome and annotation). If the

SNP's position was not within a gene, the nearest annotated areas in each direction were identified.

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Data availability

Genomic data (raw reads) will be made available on GenBank (SRA accession PRJNA563655). All commands (STACKS, STRUCTURE) and scripts (PCA, F_{ST}) used for analyses are available on GitHub (https://github.com/giorgiaauteri/LittleBrownBats_WNSSurvivorsVsNonsurvivors).

Author contributions

GGA and LLK both conceived the study and contributed to the writing. GGA obtained samples, conducted laboratory work, performed statistical analyses (bioinformatics and downstream statistical work), and prepared figures, with LLK providing guidance and input at all stages. Both authors have reviewed the manuscript.

Additional information

The authors declare no competing interests.

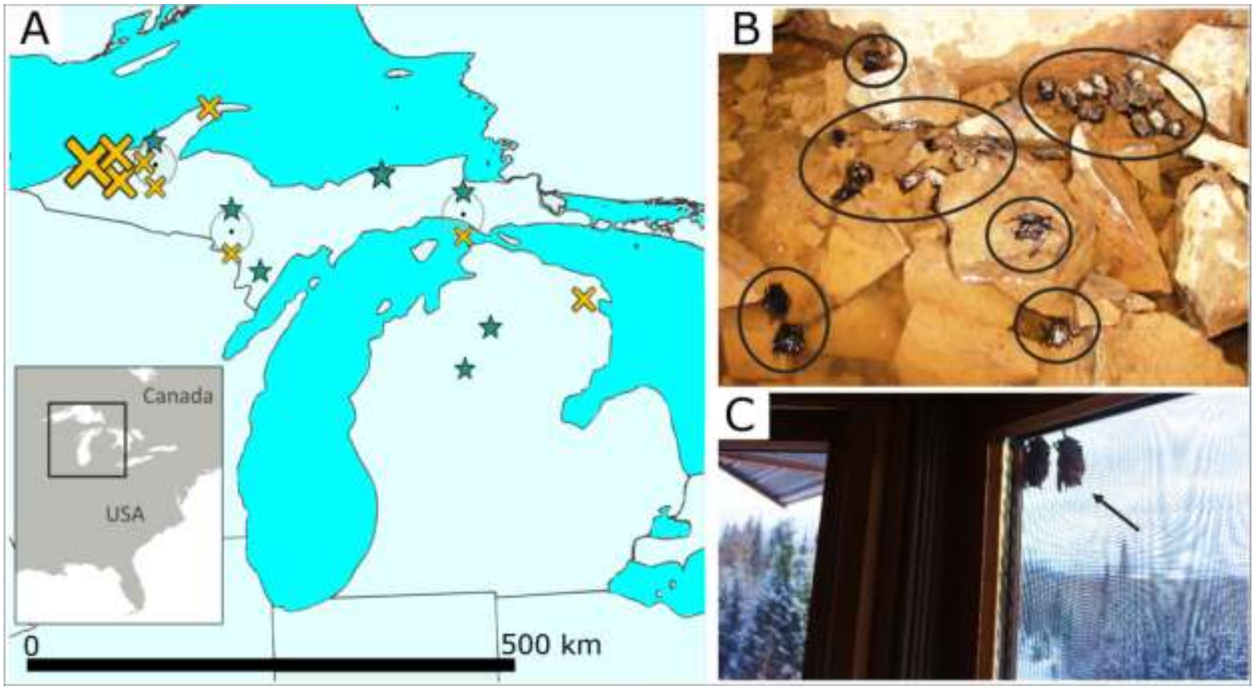

Fig. 2-1. Sampling locations of little brown bats. A, Sequenced survivors (*n*=9, marked by stars) and non-survivors (*n*=29, crosses), jittered around similar collection sites (black dots); the size of the symbol indicates relative differences in the number of samples per site (see Table A-S1 for details). Survivors undertake short-distance migrations away from hibernacula in spring, which is reflected in their scattered collection locations. Non-survivors are closely associated with underground hibernation sites, with most (**B**) collected within hibernacula (~26 carcasses marked by circles on the floor of a mine), although some (**C**) leave these sites prematurely, like these dead bats on the outer screen of a house < 1 km from a hibernaculum (note the snowy landscape). Photo credits A. Kurta (top) and C. Rockey (bottom).

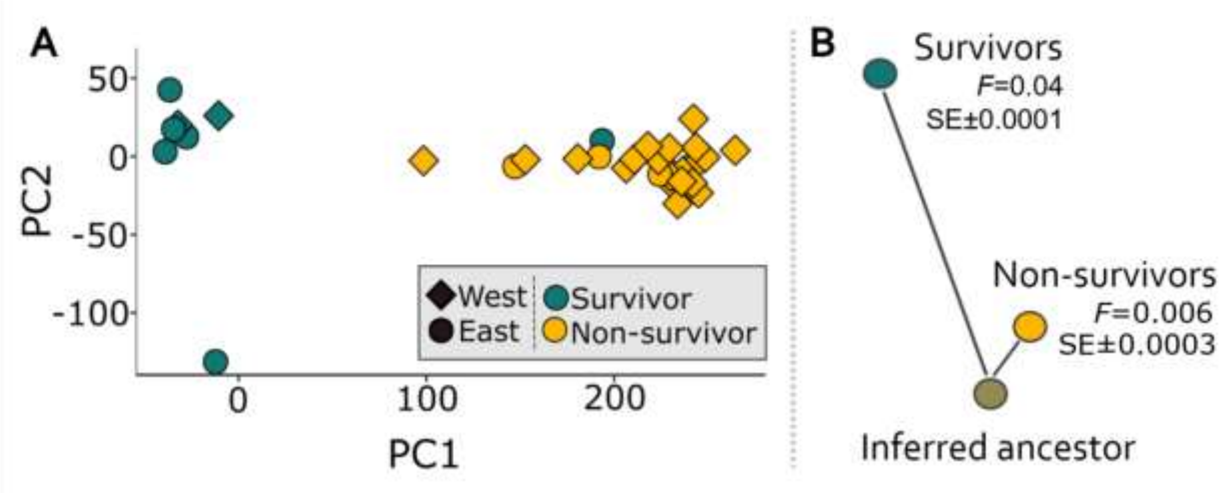

Fig. 2-2. Stochastic drift induced genetic change. A, PCA of survivors of WNS, with non-survivors projected onto the PC axes; PC1 explained 27% and 66% of the variance among survivors and non-survivors, respectively, and PC2 explained 13% and 6% of the variance. **B**, the estimated degree of genetic drift (*F*, as estimated in STRUCTURE[36,37]) is an order of magnitude greater for survivors compared to non-survivors, as illustrated by the contrasting branch lengths from an inferred common ancestor.

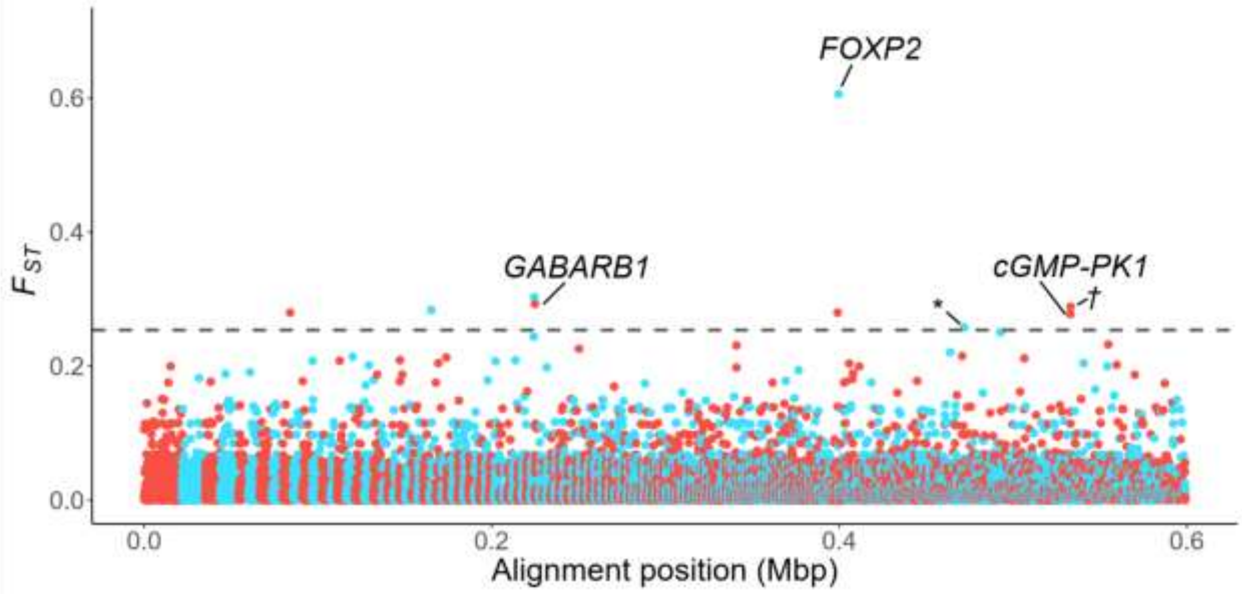

Fig. 2-3. Putative loci under positive selection. AMOVA-corrected F_{ST}-values of SNPs versus alignment position, highlighting the three genes that our SNPs map to, as well as an outlier SNP nearby to PLA2G7 (*), and the outlier SNP which is adjacent to CGMP-PK1 (†). The dashed line marks the significance threshold and alternating colors indicate different genomic scaffolds (1,214 in our dataset).

Chapter III

Landscape Connectivity among Coastal Giant Salamander (*Dicamptodon tenebrosus*) Populations Shows No Association with Land Use, Fire Frequency, or River Drainage but Exhibits Genetic Signatures of Potential Conservation Concern

Preamble: This chapter is undergoing minor revisions in the journal *PLOS ONE*. The citation is Auteri, G. G., Raquel Marchán-Rivadeneira, M., Olson, Deanna H., & Knowles, L. L. (2022). Connectivity in costal giant salamanders (*Dicamptodon tenebrosus*) shows no association with land use, fire frequency, or river drainage but exhibits genetic signatures of potential conservation concern. *PLOS ONE*. *In revision*.

Abstract

Determining the genetic consequences of both historical and contemporary events can clarify the effects of the environment on population connectivity and inform conservation decisions. Historical events (like glaciations) and contemporary factors (like logging) can disrupt gene flow between populations. This is especially true among species with specialized ecological requirements and low dispersal ability, like amphibians. We test for the genetic consequences of historical and contemporary disturbances in the coastal giant salamander (*Dicamptodon* *tenebrosus*) in the Pacific Northwest of the United States. We consider predictions based on the contemporary landscape (habitat connectivity, logging, forest fires, and topography), in addition to relatively ancient post-Pleistocene range expansion (following the last glacial retreat). To assess local versus larger-scale effects, we sampled 318 individuals across 23 sites, which were clustered in five distinct regions. Genetic variation was assessed using five microsatellite markers. We found evidence of (i) historical regional isolation, with decreased genetic diversity among more recently colonized northern sites, as well as (ii) high levels of inbreeding and loss of heterozygosity at local scales, despite relatively low population differentiation (F_{ST}) or strong evidence for population bottlenecks. Genetic diversity was not associated with contemporary disturbances (logging or fire), and there were not detectable effects on the genetic connectivity of populations based on intervening landscape features (habitat fragmentation and topography). However, lower genetic diversity in more northern compared to southern regions indicates a lag in recovery of genetic diversity following post-Pleistocene expansion. Additionally, some populations had evidence of having undergone a recent genetic bottleneck or had high inbreeding (F_{IS}) values. Lower genetic diversity in more northern sites means populations may be more vulnerable to future environmental changes, and managing for connectivity alone may not be sufficient given low motility. Evidence of recent reductions in sizes of some populations that isn't clearly linked to anthropogenic disturbances we examined suggests the type of changes that this species is sensitive to may not be well understood.

Introduction

Both contemporary and historical factors can contribute to the geographic distribution of genetic variation. These factors can increase the relative isolation of individuals, consequently reducing gene flow, population abundance, and genetic diversity. Considering genetic signatures can help link causes to consequences and guide conservation efforts by identifying populations with low genetic diversity that have recently experienced similar habitat disturbances. Genetic connectivity and diversity can be precursors for sensitivity to demographic changes, disease events, or future climatic changes (e.g., Dedrick and Baskett, 2018; Doi et al., 2010; Massatti and Knowles, 2020; Spielman et al., 2004). If gene flow becomes disrupted by contemporary changes to landscapes (Fahrig and Merriam, 1994; Spear and Storfer, 2008; Whitlock and Barton, 1997), mitigation might include restoring the focal habitats or the surrounding matrix in fragmented landscapes (e.g. Fahrig, 2001; Franklin, 1993; Franklin and Lindenmayer, 2009; Thomaz et al., 2016). Disruptions to gene flow may also have historical signatures, such as when populations become isolated as a consequence of shifting species distributions driven by glaciations (Knowles and Massatti, 2017; Marske et al., 2020; Slatkin and Excoffier, 2012). This historical isolation might impose similar conservation vulnerabilities as those from human-induced fragmentation. However, the management of these populations would call for different strategies (see Massatti et al., 2018; Winkler et al., 2018). As such, both historical and contemporary factors may need to be considered when developing management and conservation plans (see Kuzmin et al., 2009; Massatti et al., 2018), except when one factor is dominant (Mouret et al., 2011; Vandergast et al., 2007).

The degree to which contemporary and historical factors contribute to population isolation will vary across taxonomic and geographic scales. Historical and contemporary factors may be important for species that have persisted or expanded into areas that have undergone pronounced climatic shifts, such as previously glaciated areas. Species with limited dispersal and specialized habitat associations may be particularly susceptible to the negative consequences of reduced connectivity.

Amphibians are often reliant on spatially patchy and ephemeral habitats (e.g., seasonal pools for early development), and have high habitat specificity and strict ecophysiological requirements (Stebbins and Cohen, 1997; Zamudio and Wieczorek, 2007). Their dependency on multiple habitats means that disturbance events to any one of the habitats can have devastating effects on populations (e.g., Olson et al., 2007). Interconnectivity between habitats required for life-history functions (e.g., breeding, foraging, and overwintering; Pilliod et al., 2002; Pope et al., 2021; Semlitsch, 2008) is essential for population persistence (Olson and Burnett, 2013, 2009; Zamudio and Wieczorek, 2007). Over larger geographic scales, interconnectivity among populations may also be critical for population persistence. This is especially true when metapopulation dynamics are at play. For example, when species have been displaced to multiple refugia and must track shifting habitats across large geographic scales (Canestrelli and Nascetti, 2008; Slatkin and Excoffier, 2012; Svenning et al., 2008), or if metapopulation breed in isolated pools (Sjögren, 1991) but post-breeding life functions (such as foraging and dispersal) occur in differing habitats. To date, 41% of amphibian species are of conservation concern (International Union for the Conservation of Nature, 2021), and management efforts increasingly focus on facilitating species movements among required habitat types (within populations) and across geographic scales (among populations).

Here we test hypotheses about the effects of contemporary and historical factors on within- and among-population connectivity in the coastal giant salamander (*Dicamptodon tenebrosus*) from the Pacific Northwest (PNW) of the United States. The species breeds in naturally fragmented and disturbance-prone headwater habitats (Olson et al., 2007; Olson and

Burnett, 2013, 2009). Headwater stream-associated amphibians often use streams for breeding and larval development; but transform to terrestrial forms and use riparian and upland forest habitats for foraging, overwintering, and dispersal (e.g., Olson et al., 2007). The coastal giant salamander is associated with moist forests and has limited dispersal ability (Curtis and Taylor, 2004; Leonard, 1993; Nussbaum et al., 1983). Potential barriers and disturbances in the system could occur in either terrestrial or aquatic habitats. For example, headwaters are initiation points for downslope debris torrents (Burnett and Miller, 2007; Miller and Burnett, 2007), and terrestrial habitat in the PNW have been fragmented by timber harvest, agriculture, and rural community development (e.g., R. Davis et al., 2017; R. J. Davis et al., 2017; Miller and Burnett, 2007). Natural barriers to gene flow among amphibians may also be ridgelines (e.g., that define different stream basins) (Devitt et al., 2019), and unsuitable downstream aquatic habitats that are too warm, too large, and possibly include predators (e.g., Rundio and Olson, 2003, 2001). Moreover, the PNW has been subject to climatic changes associated with the Pleistocene glacial cycles. Multiple isolated refugia have been proposed within the PNW, including the mesic forests of the Coast Range and Cascade Range (Steele and Storfer, 2006).

Several studies have addressed the responses of PNW stream-associated salamanders to timber harvest practices. For example, In British Columbia, Canada, mobility of coastal giant salamanders was similar at sites where streamside riparian buffers were left intact during upland logging compared to fully forested sites, whereas logging these buffers significantly reduced salamanders’ movements (Johnston and Frid, 2002). Logging of buffers is also associated with decreased genetic diversity in the species (Curtis and Taylor, 2004). Maintaining buffers is therefore likely critical to mitigating both adverse effects on movements and abundance in this species. This is corroborated by the sensitivity of population connectivity in two other salamander species in the region (*Rhyacotriton* spp.) to land-cover type and the presence of roads (Emel et al., 2019). However, there are ecological differences between coastal giant salamanders and *Rhyacotriton* spp. hypothesize to result in varying vulnerabilities to disturbance (e.g., Mims et al., 2018; Olson and Burton, 2019). This makes it unclear if similar management strategies would be effective across species. Additionally, both *Rhyacotriton* spp. are rare relative to coastal giant salamanders, suggesting that their resilience to disturbance and gene flow among populations may differ with respect to contemporary and historical, isolating factors. With the unique assemblages of amphibians and arthropods associated with headwaters during reproduction and terrestrial dispersal in the PNW (Kluber et al., 2008; Olson and Weaver, 2007; Romanuk and Levings, 2003; Wahbe et al., 2004), understanding the species-specific factors affecting connectivity provides essential knowledge for maintaining a long-term stability in these communities.

We test hypotheses based on the population genetic variation of the coastal giant salamander to evaluate the potential effects of contemporary and historical factors on species vulnerability. We focus our sampling on the Oregon Coast Range within the PNW. By sampling at both regional and local (i.e., within-region) scales, we were able to test both historical (geographically broader) and contemporary (more localized) factors. Specifically, we explore genetic diversity and connectivity as related to land-use, logging, fire, as well across different river drainages.

Methods

Sampling

Tail or toe clips were collected from individual coastal giant salamanders across 23 headwater sites (designated 1–23) from five broadly separated regions (regions A–E; Fig 3-1) within the broader area of the central Oregon Coast Range. Sites were chosen to include either (i) streams in adjacent headwater drainages of distinct watersheds, such that freshwater habitat connectivity did not occur between them, or (ii) sites with interconnecting freshwater (tributary junctions downstream; Fig 3-1). Some regions included sampled streams in three distinct watersheds that met at their headwaters, providing three potential routes of terrestrial connectivity (i.e., “triads”; Olson and Burnett, 2013). Within a region, the distance between sites varied from 0.12 to 7.01 km (mean = 2.79), with six sites sampled in regions A and C, five sites in region B, four sites in region E, but only two sites in region D because of limited success in collecting animals. Region D was excluded from some analyses because of limited sampling (see details below). Tissues from a total of 318 individuals were collected, with between 4 and 29 individuals sampled per site (Table B-S1), occurring during July through September 2010. Animal handling and samples followed ethics guidelines approved by the University of Michigan’s University Committee on Use and Care of Animals (UCUCA; permit #10233) and were collected under Oregon Scientific Permit #014-10.

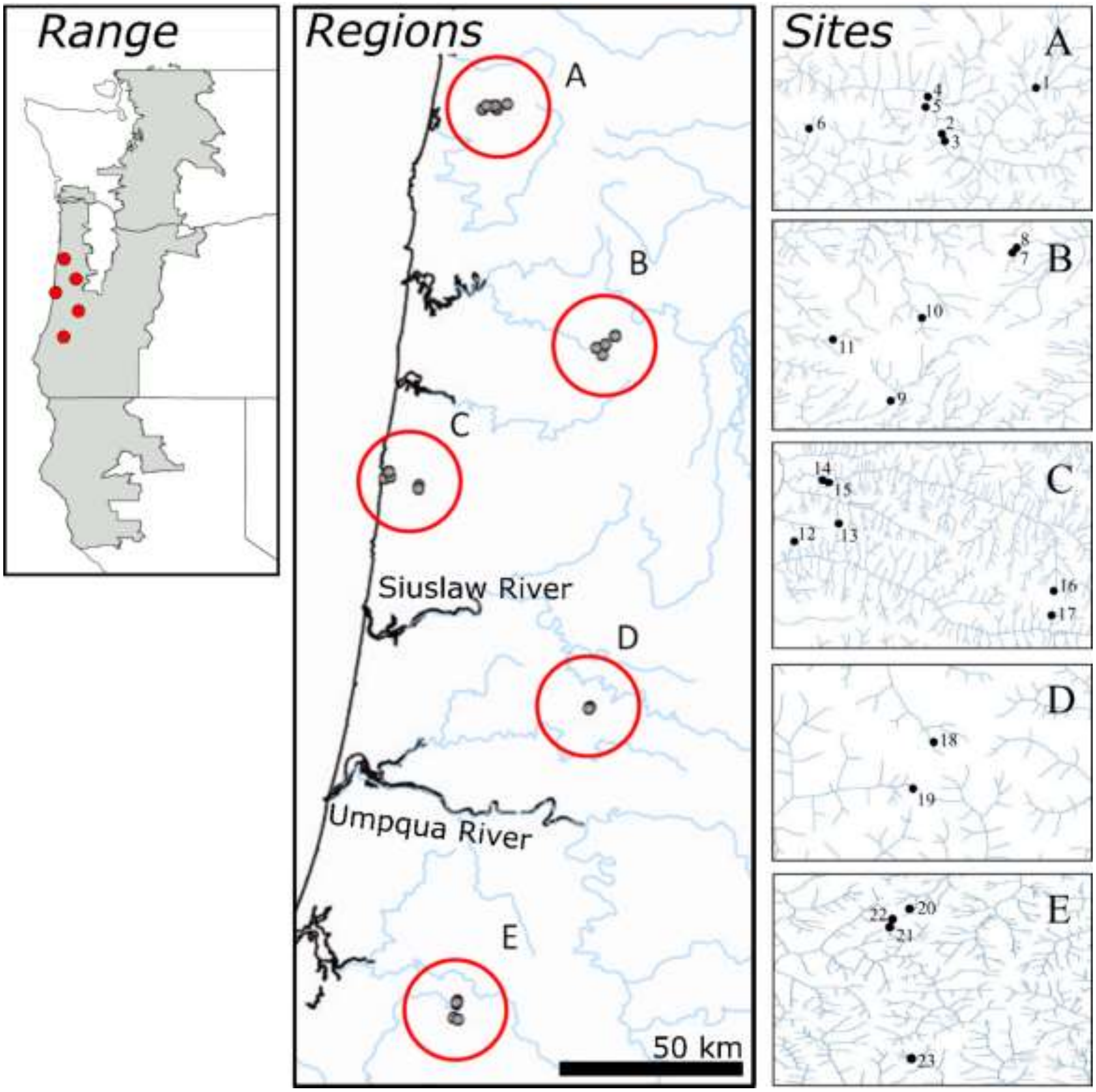

Fig 3-1. Sampling localities of salamanders. The geographic range of coastal giant salamanders(left panel) showing the five sampled regions (center panel: circles A–E) along the coast of Oregon, USA, and details of sampled sites within regions labeled from 1 to 23 (right panel; modified from Marchán-Rivadeneira, 2015). The left panel features species range information created using information from the IUCN Red List of Threatened Species (IUCN SSC Amphibian Specialist Group, 2015) and other maps feature waterways from United States Geological Survey (US Geological Survey, 2019).

DNA extraction and genotyping

Tissue samples were stored in separate vials of 75% ethanol (EtOH) upon collection. DNEasy Tissue kits (Qiagen, Inc., Germantown, MD, USA) were used for DNA extractions, following manufacturer guidelines. Individuals were genotyped for five microsatellite loci (Table B-S1) developed for this species using primers and protocols developed by Steele et al. (markers D6, D14, D17;) and Curtis and Taylor (Dte 11, Dte 14; Curtis and Taylor, 2000).

Quantification of molecular data

PCR products were genotyped, and alleles were manually scored using GeneMarker software (v. 1.97 SoftGenetics, State College, PA, USA). Potential genotyping errors, including null alleles, scoring errors, and allele dropout, were assessed using Micro-Checker (v. 2.3.3) (Oosterhout et al., 2004). Tests for significant deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD), as well as null allele frequency (r), were conducted for each locus using GenePop (v. 4.1; Raymond and Rousset, 1995); significant departures from HWE and LD were tested with 100 batches of 1000 iterations. Of the 318 individuals sampled, most (240) had data for all five markers, while 65 were missing data for one marker, 11 were missing data for 2, and two individuals only had information for one marker. Missing data for each marker ranged from 2% (Dte 11) to 13% (Dte 14), with other markers missing 3% (D6), 5% (D14), and 7% (D17). Allelic richness, observed heterozygosity (H_o), expected heterozygosity (H_e), and genetic differentiation between populations (F_{ST}) were calculated using Fstat (v. 2.9.3; Goudet, 2001).

Tests of genetic structure across space and drainages

Geographic structuring of genetic variation was evaluated in two complementary approaches: by principal component analysis (PCA) and a STRUCTURE analysis (v. 2.3.4; Hubisz et al., 2009; Pritchard et al., 2000). The PCA was performed in R (R Core Team, 2015) using RStudio (v. 1.1.453; RStudio Team, 2015) using the *prcomp* function and visualized using the package GGPLOT2 (Wickham, 2009). Missing values were replaced with the per-locus mean value. In the STRUCTURE analyses, *K*-values of 1 to 24 were run in the global analysis (i.e., all sample sites) without conditioning on geographic sites, using the admixture model with a 50,000 burn-in, followed by 500,000 repetitions. Ten independent runs were conducted for each *K*-value. We also conducted additional STRUCTURE analyses using *a priori* assignments. For these analyses, we conditioned by (a) population (K = 1–24), (b) drainage (K = 1–13), and (c) region (K = 1–6). The most likely *K*-value was estimated using STRUCTURE Harvester (Earl and vonHoldt, 2012), and assignment probabilities of individuals' ancestries were determined using CLUMPAK (Evanno et al., 2005; Kopelman et al., 2015).

We also evaluated isolation by distance (IBD) based on F_{ST} versus the minimum distances between sites from different drainages using RStudio (R Core Team, 2015; RStudio Team, 2015) and GGPLOT2 (Wickham, 2009). We evaluated the relative contribution of IBD within and among regions. The significance of IBD was evaluated for the entire dataset and the subsets of the data for each within-region comparison using Monte-Carlo simulations performed in the R package ADEGENET (Jombart, 2008) using 999 random replicates per test.

We examined the structuring of genetic variation as a function of watershed and drainage (B-S2 Table), controlling for the effects of geographic distance by analyzing the residuals of a linear model of between-site F_{ST} -values versus geographic distance, then testing whether these residuals were explained by boundaries between drainage basins. Specifically, differences among residuals based on drainage association (i.e., same versus different drainage membership) were analyzed using a Kruskal-Wallis test (Kruskal and Wallis, 1952) with a Bonferroni correction (Bland and Altman, 1995; Dunn, 1961) for multiple comparisons (i.e., an alpha-threshold of 0.01 for significance). These tests were carried out using multiple watershed categorizations to accommodate uncertainty regarding biologically relevant factors for *D. tenebrosus* specifically (explained below). The different categories for water catchments included: Hydrological Units (HUs) at three levels (HU 08, 10, and 12; U.S. Geological Survey, 2001), drainage basin (a continuous river system and its catchment area), and river systems separated by major waterways ("Level 1" streams; Esri, 2010). Larger waterways can act as barriers to headwater-associated amphibians due to increased currents, depths, temperatures, and predators (e.g., fish; Hecnar and M'Closkey, 1997; Pilliod and Peterson, 2001). However, adult coastal giant salamanders have been found in larger rivers (Welsh, 2005). Applying different watershed scales (i.e., HU 8, 10, 12, complete drainages, or watersheds separated by Level-1 streams), the number of different watersheds represented by our sampled sites are either 7, 9, 13, 8, or 16, respectively (see B-S2 Table).

Landscape effects on connectivity

The extent of different landcover classes was obtained from the National Land Cover Database (NLCD) from 2006 (Fry et al., 2011), given that the tissue samples were collected in 2010. We focused on terrestrial landcover types that are likely to impact this species. All sites at which we collected salamanders were within the evergreen forest (NLDC category 42), suggesting that the proportion of evergreen forest between sites would facilitate terrestrial movements (i.e., evergreen forest would be negatively correlated with genetic differentiation, as measured by F_{ST}). Conversely, habitat fragmentation and less desirable land cover types would increase genetic differentiation among sampling sites.

To quantify the landcover effects of areas separating sampled sites, we identified regions representing the shortest distances between each pair of sites in a region and created 80-m wide rectangles between the two sample sites. The width of rectangles (i.e., potential corridors) was estimated by quadrupling the maximum observed movement distance (of 20 m; Johnston and Frid, 2002) to hedge uncertainty in known mobility and edge effects. Each overlaid polygon was used to calculate the proportion and patchiness (Patch Cohesion Index) of the underlying landcover types using the Landscape Ecology plugin LECOS (v. 3.0; Jung, 2019) in QGIS (v. 3.6.1; QGIS Development Team, 2018). Although nine different landcover types occurred within the potential immigration corridors, we restricted downstream analyses to quantification based on two categories (Evergreen Forest and Shrub/Scrub), which were the two most common land-cover categories. To tests for the effects of land-cover, we fit a linear model to the F_{ST} -values and five different predictors: distance, proportion of evergreen forest, proportion of shrub/scrub, and patch cohesion of evergreen forest between sites, as well as whether sites were in the same watershed. We performed analyses in RStudio (R Core Team, 2015; RStudio Team, 2015). Only a single watershed membership was included in this model, as determined by population differentiation discussed in the previous section. The best model was identified using AIC score and a backward stepwise regression model (Akaike, 1974; Burnham and Anderson, 2002).

Influence of disturbance events on genetic diversity

We tested for the potential effects of disturbance events on genetic diversity using genetic bottleneck-tests within sites at each site using BOTTLENECK (1.2.02; Piry et al., 1999). This approach evaluates whether expected heterozygosity (H_e) is significantly higher than the equilibrium heterozygosity (H_{eq}). Significance was evaluated using a one-sided Wilcoxon sign rank test based on 1,000 iterations. Note that bottlenecks in this test are defined as severe population contractions resulting in a reduction of effective population size, not as a population contraction followed by an expansion. We used the developer's recommended settings for microsatellite data for our loci and individual sample size. Specifically, the settings were a two-phase mutation model with 95% of single mutations, a multi-step variance of 12, and the mode shift turned off. In addition, based on program guidelines and subsequent methodological studies (Peery et al., 2012; Piry et al., 1999), we restricted this analysis to sites for which at least 10 individuals had information from all five microsatellite loci (i.e. for a total of 14 sites, see B-S1 Table). We note that the tests may still have low power to detect bottlenecks; however, to ameliorate this we excluded sites with a limited sampling of individuals or loci.

We also tested for correlations between genetic diversity (average allelic richness) and whether a disturbance event had occurred in the last 70 years via Kruskal-Wallis tests (Kruskal and Wallis, 1952). We compiled information on whether logging or fires had occurred in the past 70 years at each site from historical records and databases of wildfires that occurred on federal lands (Bureau of Indian Affairs, Bureau of Land Management, Fish and Wildlife Service, National Park Service, and Forest Service, (U.S. Geological Survey, 2019); and historical satellite imagery on Google Maps, ending in 2016). We also noted whether trees had been maintained along the river during land-use disturbance events (no riparian forest buffer was assumed in the case of fires).

Results

All microsatellite loci were polymorphic. There were no consistent patterns of deviation from HWE, null alleles, or linkage disequilibrium across sites or across microsatellites, with the exception of the D6 locus (which was out of HWE). Consequently, all analyses were performed with and without the D6 locus, and because results were qualitatively similar, only analyses with all loci are presented here (see B-S1 Text for results based on analyses with D6 excluded).

Genetic diversity was generally high (globally across all alleles 18–40 alleles per site, mean = 28.6; see B-S1 and B-S3 Tables). Per locus and geographic sampled site, the number of alleles (NA) ranged from 2–15, expected heterozygosity (H_e) ranged from 0.07 to 1.00, and observed heterozygosity (H_o) ranged from 0.00 to 1.00. The F_{IS}-index per-population (averaged across loci) had a mean of 0.37 (SD ± 0.096) and ranged from 0.14 (site 11) to 0.57 (site 9; B-S3 Table). Per locus, F_{IS} ranged from -0.46 to 1.00 (B-S1 Table). Pairwise genetic differentiation between sampled sites ranged from an F_{ST} of 0.0 to 0.25.

Tests of genetic structure across space and drainages

The PCA showed overlap of some, but not all regions; individuals from the north (region A) did not overlap with individuals from the southern region (region E) on PC2. The northern region (A) also tended towards less genetic variation (i.e., occupied a smaller space in the PCA; Fig 3-2). STRUCTURE results generally supported regional differentiation, with a $K = 4$ as most probable (Fig 3-3), regardless of a-priori information used to condition assignments (i.e., conditioning on region, HU 8 watershed, or population; see B-S1 Fig; Appendix B). The one exception was for conditioning on sample site, which resulted in a most likely $K = 3$.

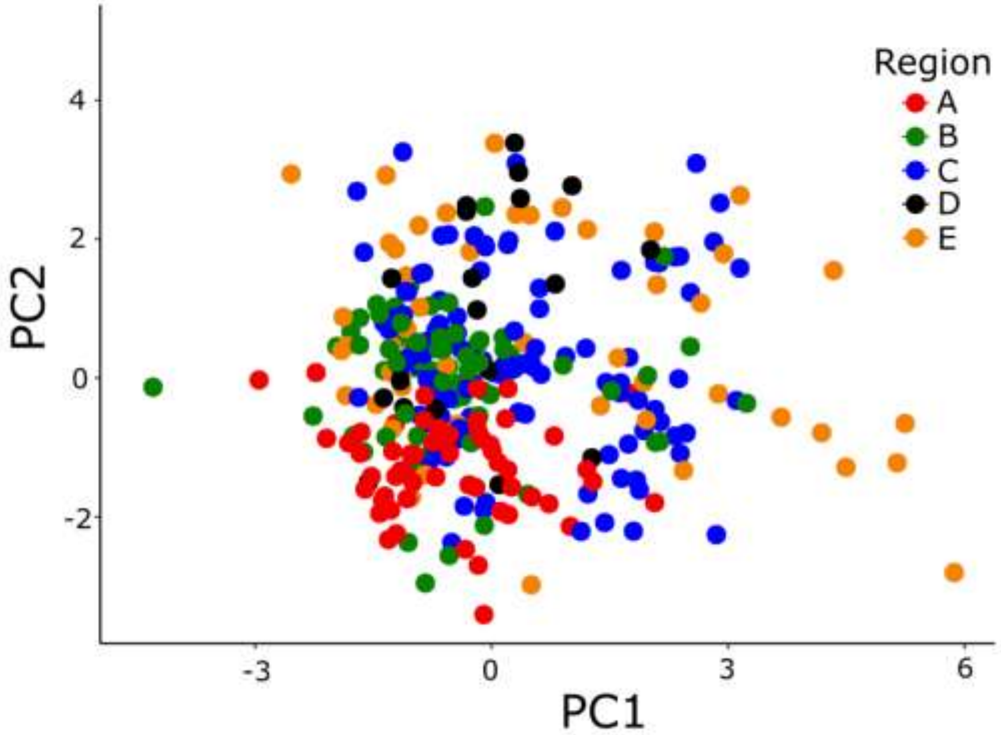

Fig 3-2. Principal Component Analysis of sampled individuals. The 318 individuals are color-coded by region (see Fig 3-1), where the first and second axes explained 21% and 18% of the variance, respectively.

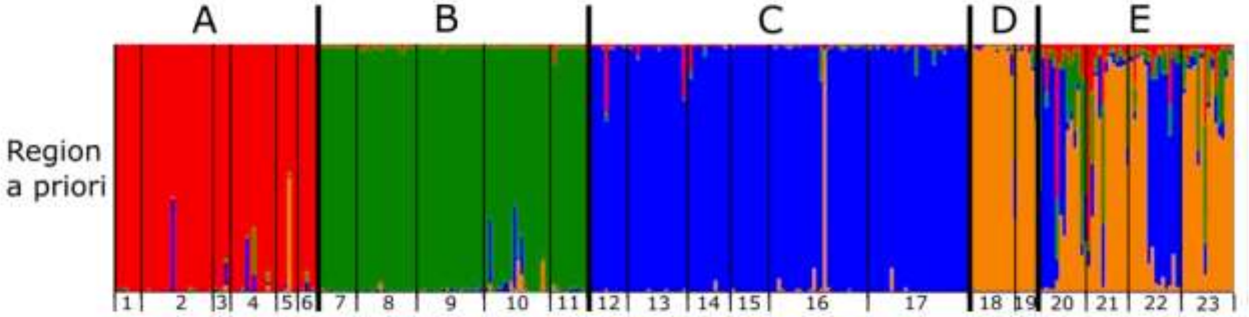

Fig 3-3. Plot of genetic STRUCTURE results. Individuals (vertical bars) are grouped by site (1–23) and region (A–E; separated by bold vertical lines; see Fig 3-1). Results are presented for the most likely number of genetic clusters, $K = 4$ when conditioning on region (see B-S2 Fig for runs with alternative priors; Appendix B).

IBD was significant across regions ($P \leq 0.001$), but not within regions ($P = 0.454$). Within regions, there is pronounced variation in the levels of genetic differentiation across sites (Fig 3-4). Within-regions, drainage basins did not generally contribute significantly to observed genetic differentiation among sites. However, one of the five water-catchment categories that we considered–specifically, watersheds separated by category 1 stream–was significant ($P = 0.044$) before, but not after Bonferroni correction for multiple comparisons.

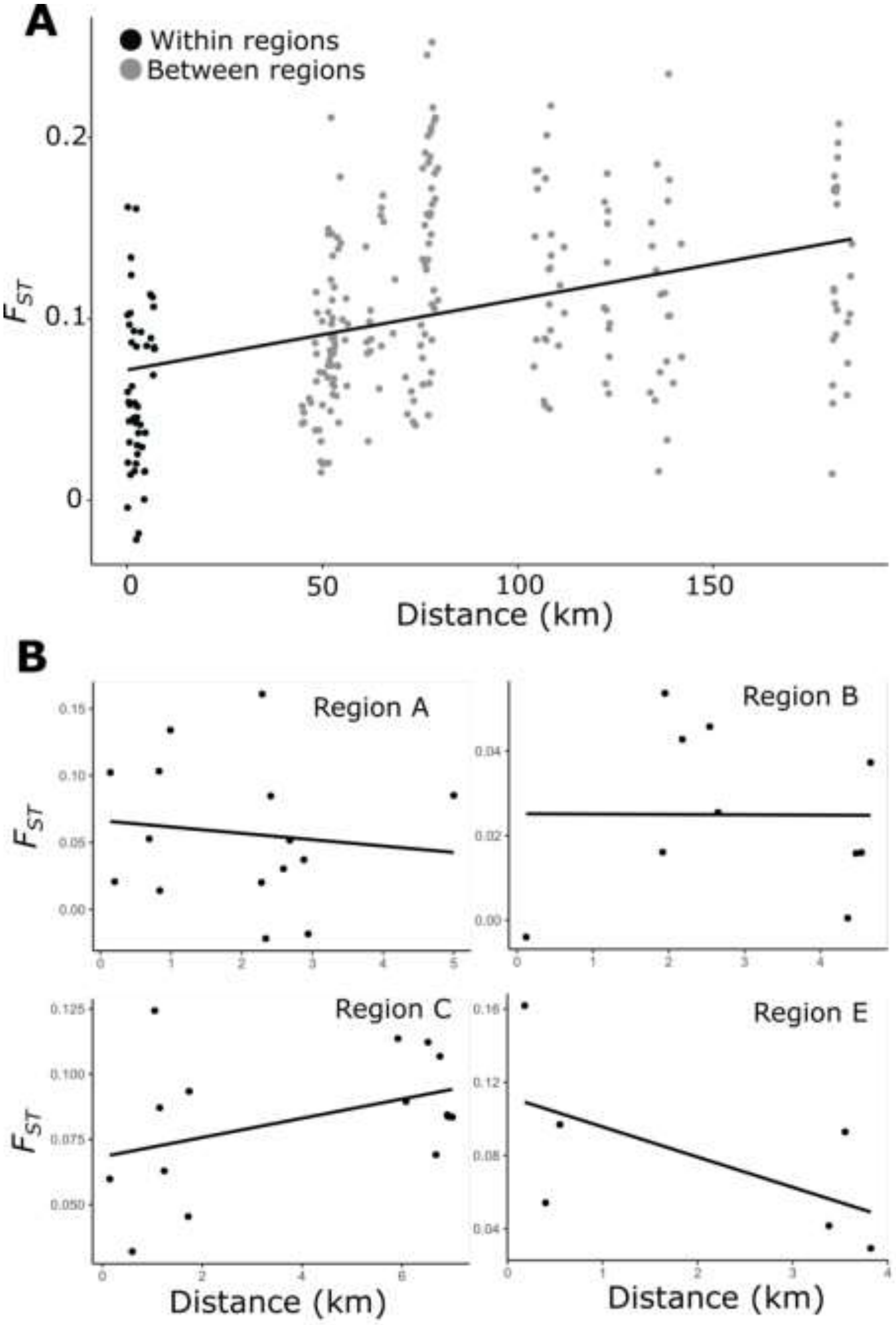

Fig 3-4. Isolation by distance plots. Segregation of allele frequencies (measured by F_{ST}) is shown between pairs of sites versus geographic distance. A. All site pairs in this study, with comparisons within a region shown in black and comparisons between regions in grey. B. Between-region site comparisons by region (region D is omitted since only two sites were sampled).

Landscape effects on connectivity

Although F_{ST}-values between sites within the same region were highly variable (and were sometimes of similar magnitude to F_{ST}-values between regions), our analysis did not identify a significant predictor of this differentiation. Of the five predictor variables, the best fit model included only one predictor–the proportion of shrub/scrub area between sampled locations (P = 0.055).

Influence of disturbance events on genetic diversity

Both types of disturbance–fire and logging–were common in the study area. Eleven sites had a recent history of fire, and eight sites had a recent history of logging. Five sites had been both burned and logged, and only one logged site had forested buffers left along the waterway.

Evidence of recent bottlenecks was apparent in four sampled sites: sites 2, 12, 20, and 23 (P = 0.031, 0.047, 0.016, and 0.016, respectively; B-S3 Table). However, according to the metrics we considered, only half (two) of these bottlenecked sites had a recent (≤ 70 years) history of disturbance–one had evidence of both fire and logging, and the other only had evidence of fire.

There was also no detected relationship between average allelic richness and recent disturbance events linked to logging and fires, or regarding the preservation of riparian buffers along streams (all P-values for Kruskal-Wallis tests > 0.1; B-S2 Fig; Appendix B).

Discussion

Species with restrictive movements that are sensitive to environmental changes and occupy disturbance-prone habitat fragments are likely to face contemporary environmental changes in conjunction with lower regional genetic diversity among more recently (re)colonized sites, which may compound the effects of future hazards. Our genetic analyses of coastal giant salamanders show evidence of lower genetic diversity among northern regions (Fig. 3-2) that is likely the legacy of historical disturbance associated with Pleistocene climate change. In addition, we found evidence of moderate-to-high inbreeding and losses of heterozygosity in local populations throughout the study area, despite apparent within-region connectivity and a lack of strong evidence for genetic bottlenecks.

Although impacts on genetic variation are more readily apparent from historical versus recent disturbances, this does not indicate hardiness to contemporary disturbance. It may be that sensitivity in this species is more context-dependent, that there has not been sufficient time for disturbances to leave a genetic signature, that signals of historical disturbance have overpowered those of more recent disturbances, that our results are statistically false negatives, or some combination of these. Indeed, the first reason is perhaps the primary driver for the lack of strong signature of particular disturbance regimes (e.g., logging and fire frequency; Fig B-S2; Appendix B) in our data. While studies of this species have shown individual mobility and abundance can be detrimentally affected by logging (Cole et al., 1997; Corn and Bruce Bury, 1989; Curtis and Taylor, 2004; Johnston and Frid, 2002), populations may experience upticks after logging (before the presumed) onset of population declines (Murphy and Hall, 1981). This is speculated to be a temporary effect of increased solar input, which increases stream productivity for a time. Additionally, populations of coastal giant salamanders occupying steeper streams have been shown to be less affected by logging (Corn and Bruce Bury, 1989; Murphy and Hall, 1981), presumably because there is less associated siltation. However, logging of steeper terrain is also more likely to result in longer-term ecological problems, such as increased erosion, which would likely negatively impact both headwater and lower elevation habitats. Thus, the unclear genetic consequences of anthropogenic change in our data are perhaps unsurprising, and, in conjunction with the lower genetic diversity in the northern region of our study site given historical restrictions in gene flow, potentially warrant future conservation concern. In addition, the species' relatively long generation time (10-15 years; Committee on the Status of Endangered Wildlife in Canada, 2014) may contribute to the relative lag in some genetic signatures (e.g., inbreeding).

Indicators regarding population stability

In our study, local genetic differentiation of coastal giant salamanders (within regional clusters; Fig 3-4B) was sometimes as high as differentiation of sites between regions (Fig 3-4A). However, this local genetic differentiation does not correspond with geographic distance, disturbance factor (e.g., fire and logging records), or drainage membership (Fig 3-4B). Many explanations might apply to any one of these observations, but there is no obvious overarching explanation when these three factors (distance, disturbance, drainage membership) are considered together. Especially given that none of the F_{ST}-values are high in an absolute sense, the genetic differences within regional clusters may or may not reflect the deterministic effects of some unmeasured environmental factors or random genetic changes.

Consideration of additional results–namely, lack of strong evidence for population bottlenecks (B-S3 Table; Appendix B), but moderate levels of inbreeding and loss of heterozygosity (B-S1 and B-S3 Tables; Appendix B)–would seem at odds with the moderate levels of gene flow suggested by the low F_{ST}-values (Fig 3-4). Any explanation for patterns of genetic differentiation within regions (i.e., connectivity as determined by low F_{ST}-values, but highly variable F_{ST}-values among sites within regions, with a lack of correspondence with fire or logging regimes) must also be compatible with the lack of strong evidence for population bottlenecks, moderate levels of inbreeding, and loss of heterozygosity (i.e., lower observed levels of heterozygosity than expected; B-S1 and B-S3 Tables; Appendix B).

When findings are considered jointly, these results are consistent with a general lack of population persistence, and specifically, locally fluctuating population sizes. Such fluctuations would drive down the effective population sizes, which could explain the inbreeding and loss of heterozygosity. Fluctuating population sizes, in particular, could also lead to the highly variable F_{ST}-values observed within regions (Fig 3-4B) that do not correspond with disturbance factors (i.e., any statistical association may be masked by fluctuations; B-S3 Table). Irrespective of the debate that might be leveled at this specific interpretation, dispersal among sites, as indicated by relatively low F_{ST}-values, may not be sufficient to compensate demographically for potential population vulnerability arising from inbreeding and loss of heterozygosity. The temporal mosaic of disturbance events (even within contemporary environmental changes), expounded upon above, may also contribute to unclear signals.

Implications for conservation under historical and contemporary disturbance

Whereas other studies of PNW salamanders have shown that genetic structure is affected by habitat disturbance associated with logging (e.g., coastal giant salamanders; Curtis and Taylor, 2004) or land-cover type and roads (e.g., *Rhyacotriton* spp.; Emel et al., 2019), we found genetic differentiation was not explained by any factors we tested (type of disturbance events, land cover type, distance, or watersheds/drainages; B-S3 Table). We caution that the lack of a significant predictor pertains only to our dataset (e.g., with more dense sampling at a local scale, such effects might emerge; Emel et al., 2019). The difference between this study and previous ones are not necessarily contradictory. Our work incorporates both regional and local components not found in past studies. Moreover, our sampling spans a range of heterogeneous conditions within a region to test for more “global” explanatory factors for local genetic structure (i.e., shared effects across each individual region, such as the importance of drainage or watershed membership). Past studies have suggested that the rarity of *Rhyacotriton* spp. in contrast to coastal giant salamanders (Rundio and Olson, 2001) may indicate differing sensitivities to disturbance.

Similarly, it would be a mistake to make management decisions based on the presumption that gene flow would mitigate any negative impacts of disturbance (e.g., fire and logging). In fact, the high genetic differentiation within a region (compared with between regions: Fig 3-4A) speaks to demographic events capable of causing drift-induced genetic differences, inbreeding and loss of heterozygosity (B-S1 Table). Moreover, if potential fluctuating population sizes (as described above) obscure effects of local landscape factors, the data does not speak to the lack of importance of the environment on population connectivity, but only that the assessed environmental influences may not be detectable in the genetic data. Since the species breeds in naturally fragmented and disturbance-prone small-stream habitats (Olson et al., 2007; Olson and Burnett, 2013, 2009) and transforms to terrestrial forms that inhabit riparian and upland forests for foraging, overwintering, and dispersal, then disturbance events to any one of the habitats either spatially or temporally may affect local population persistence (e.g., Olson et al., 2007). Also, it is possible that there may be genetic signatures in these salamanders of past disturbances, such as older fires or debris flows unrelated to recent fire and logging. In any event, our data suggests that because of detected bottlenecks and high inbreeding (F_{IS}) levels, coastal giant salamanders may be vulnerable to genetic losses from unspecified causes.

It is possible that a higher dispersal capacity contributes to the low regional genetic differentiation of coastal giant salamanders and a pattern of isolation by distance (although by itself, this wouldn't explain the lower genetic diversity in northern sites; Fig 3-2, B-S1 Table). Species reliant upon naturally fragmented habitats and subject to periodic disturbance can have correspondingly high dispersal proclivities that allow for recolonization and avoid permanent extirpations (Travis and Dytham, 1999). Thus, relatively high dispersal propensity in coastal giant salamander may be advantageous if habitats are naturally subject to periodic disturbance, as may be the case with headwater habitats due to landslides (Cover et al., 2010) and as has been suggested in other salamander species which rely on ephemeral ponds (Gill, 1978). However, this explanation (by itself) could be contradicted by the observed genetic structuring at the local geographic scale (i.e., within a region), due to its similarity to that observed at much larger geographic scales (Fig 3-4A).

Studies of other amphibians at similar geographic scales will be important for corroborating the patterns observed here, and will help evaluate our proposed interpretations. Such comparative studies will also be important for determining whether amphibian communities, and salamanders in particular, might be subject to the potentially damaging effects of inbreeding and reduced heterozygosity, despite different degrees of genetic structure (i.e., connectivity). If so, this would suggest that viewing genetic structure, or lack thereof, as a measure of greater resilience due to higher gene flow levels may potentially be misleading. While some species might be capable of higher dispersal and recolonization rates, if the local populations are nonetheless subject to fluctuations in size (resulting in inbreeding and reduced heterozygosity), such species are not necessarily less vulnerable than those where reduced gene flow creates a patchwork of small, isolated populations that also experience strong inbreeding and reduced heterozygosity (Lowe and Allendorf, 2010). Even though the coastal giant salamander has not been identified as a species of conservation concern, they exhibit levels of inbreeding and reduced heterozygosity that are odds with a robust species, possibly placing them in a precarious situation for dealing with future perturbations such as climate change and diseases (Mims et al., 2018). For salamanders and other relatively immobile species potentially still recovering from past glaciation events, management for enhanced connectivity (such as the development of habitat corridors that span political boundaries (Noss, 1991) may be key.

Data Accessibility

Data associated with this study are available on the University of Michigan's Deep Blue Data repository (https://deepblue.lib.umich.edu/data). Dataset title: Dataset of genetic (microsatellite) and associated habitat data of salamanders (coastal giant salamanders; *Dicamptodon tenebrosus*) in Oregon, USA (DOI: https://doi.org/10.7302/14hn-mb57).

Competing Interests

The authors have no conflicts of interest to declare.

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Chapter IV

A Conceptual Framework to Integrate Cold-Survival Strategies: Torpor, Resistance, and Seasonal Migration

Preamble: This is chapter has been accepted for publication in the journal *Biology Letters*. The citation for this chapter is Auteri, G. G. (2022). A conceptual framework to integrate cold-survival strategies: torpor, resistance, and seasonal migration. *Biology Letters*. (*Accepted*)

Abstract

Freezing temperatures are inherently challenging for life, which is water based. How species cope with these conditions fundamentally shapes ecological and evolutionary processes. Despite this, there is no comprehensive conceptual framework for cold-survival strategies—seasonal migration, cold resistance, and torpor. Here, I propose a framework with four components for conceptualizing and quantifying cold-survival strategies. Cold-survival strategies are 1) collectively encompassed by the proposed framework, and that this full breadth of strategies should be considered in focal species or systems (comprehensive consideration). These strategies also 2) exist on a spectrum, such that species can exhibit partial use of strategies, 3) are nonexclusive, such that some species use multiple strategies concurrently (combined use), and 4)

should collectively vary inversely and proportionally with one another when controlling for external environment (e.g., when considering species that occur in sympatry in their summer range), such that use of one strategy reduces, collectively, use of others (proportional use). This framework is relevant to understanding fundamental patterns and processes in evolution, ecology, physiology, and conservation biology.

1. Cold-survival: a fundamental question studied in separate pieces

How species cope with seasonal variation is fundamental to central themes in biology, including factors shaping biogeographical patterns (Bergmann, 1847; Humphries et al., 2002; Kimura, 2004) and species' responses to past (Lovegrove et al., 2014; Winger et al., 2019) and current (Geiser and Turbill, 2009; Williams et al., 2015) climate change. **Winter** (see Glossary) can be particularly challenging (Studd et al., 2021). Despite this, species' **cold-survival strategies**—**seasonal migration, cold resistance**, and **torpor** (including hibernation and dormancy)—are largely studied in isolation of one another. Separate treatment misses opportunities to identify broader patterns and mechanisms. For example, in periods of rapid environmental change, different cold-survival strategies could influence species responses. Here, I argue for viewing cold-survival strategies under an integrated framework.

An example of a missed opportunity—where lack of a unifying cold-survival framework has hindered our understanding of biological patterns—is seen in Bergmann's rule. This rule refers to the trend of larger body sizes at higher latitudes, which purportedly facilitates cold resistance due to lower surface-area-to-volume ratio (Bergmann, 1847). However, some animals unpredictably deviate from (Ashton et al., 2000; Ashton and Feldman, 2003; Freckleton et al., 2003; Meiri and Dayan, 2003), or even reverse (Ashton and Feldman, 2003), this pattern. Small mammals were expected to conform to the rule more strongly than large ones, because they are under stronger pressure for heat conservation, yet they conform *less* stringently (Ashton et al., 2000).

Discrepancies in Bergmann's rule are resolved if we consider that the focal metric (size) represents just one cold-survival strategy—resistance. A reassessment under an integrated framework recognizes that species can use seasonal migration or torpor as alternatives. This resolves the mystery of why some taxa "break" the rule. Small mammals do not follow the rule well (Ashton et al., 2000; Freckleton et al., 2003) because they often use torpor instead of resistance. Migratory birds also conform less strongly (Meiri and Dayan, 2003) because, like small mammals, they are using an alternative—migration. A reframing of Bergmann's rule under an integrated framework would be that species are bigger towards the poles, *to the degree which they utilize cold-resistance (as opposed to seasonal migration or torpor)*.

Beyond Bergmann's rule, this framework can be leveraged to answer questions involving capacities for colonizing high latitudes (Kimura, 2004), adaptive tradeoffs (e.g., brain size; Heldstab et al., 2018; Vincze, 2016), disease dynamics(Martinez-Bakker and Helm, 2015), niche partitioning (Fandos et al., 2020; Gómez et al., 2016; Landry-Cuerrier et al., 2008; Smeraldo et al., 2018), bioenergetics (Humphries et al., 2002), and how changes in seasonal regimes impact ecological networks (Williams et al., 2017). A broader consideration of cold-survival strategies has been called for or hinted at (Barclay et al., 2001; Williams et al., 2017; Wilsterman et al., 2021; Winger et al., 2019), but no formal framework exists.

2. A conceptual framework for cold-survival: integrating cold-resistance, torpor, and seasonal migration

Here, I present an integrated conceptual framework for cold-survival strategies in animals. I propose that 1) resistance, torpor, and seasonal migration should be considered collectively (*comprehensive consideration*), that 2) species can (and commonly do) use multiple strategies (*combined use*), and that 3) each of the three strategies exist on a spectrum, permitting *partial use*. If the first three are accounted for, 4) species should exhibit *proportional use,* where use of one strategy correspondingly decreases use of other strategies. Species are expected to employ strategies to the degree necessary to meet adaptive thresholds, thereby avoiding extinction (or extirpation). Adaptive thresholds are determined by environment and geography (e.g., local winter severity and distance to suitable habitats). The framework is summarized in Fig. 4-1 and expanded upon below.

(a) Comprehensive consideration of cold-survival strategies

The claim, that resistance, torpor, and migration are alternatives is often made in both general and scientific literature (e.g., Elischer, 2015; Robinson et al., 2009; Webber and McGuire, 2021; "Where do insects go in the winter?," n.d.; Williams et al., 2017, 2015). However, strategies are usually not formally evaluated in conjunction. While exclusive treatment is often appropriate, *chronically separate consideration eliminates the opportunity for discoveries that require comparisons across strategies*. The remedy is retaining a perspective of *comprehensive consideration*—each cold-survival strategy exists as one of three alternatives. Considering only a single strategy can lead to over-grouping non-focal strategies—comparing the focal strategy versus "the other" (e.g., migratory versus "non-migratory"). Catchall groups defined by what they are not, rather than what they are can lead to results that are tricky to interpret and indecipherable mechanisms. The same is true of only considering one or two strategies if all three are used.

The consequences of restricted perspectives are apparent when considering the claim that species using a particular strategy will be especially sensitive to anthropogenic climate change. Expected range shifts have been evaluated separately based on hibernation propensity (Humphries et al., 2002), tolerance (Crozier, 2003), and migratory tendency (Sparks et al., 2005), or lack thereof (Parmesan et al., 1999). Justifications that a given strategy might confer increased sensitivity to climate change are often made with anecdotal evidence using a narrative approach. Some claim that migratory species will be especially sensitive because of specialized habitat associations (Both et al., 2010) or potential mismatches in phenology (Newson et al., 2009; Robinson et al., 2009). However similar arguments can be made for species that hibernate, which have phenologies impacted by climate change (Lane et al., 2012) and depend on multiple habitats throughout the year (Goldberg and Conway, 2021). Cold-resistant species also have phenologies linked to dwindling fat reserves or food resources (Jagielski et al., 2021) and are closely tied to high-latitude habitats (Laidre et al., 2008) undergoing rapid change (Studd et al., 2021). There are even conflicting claims—for instance that migratory species might be *less* sensitive to climactic changes (e.g., Parmesan, 2019). Without formal comparisons between strategies, species-comparative work is artificially constrained.

(b) Combined use—strategies can be used in conjunction

Cold-survival strategies are often treated as alternatives despite not being mutually exclusive (i.e., species can utilize multiple axes of Fig. 4-1). Species are sometimes considered, for example, as either migrating *or* hibernating. In contrast, *combined use* occurs when multiple cold-survival strategies are used (either by individuals, populations, or species). For example, individual blue jays (*Cyanocitta cristata*) may migrate south, but most remain throughout winter (Nunneley, 1964).

Combined use may be the norm (especially if the full scope of resistance is considered, including endothermy; Porter and Kearney, 2009). Many high-latitude bats use both torpor and migration (Fleming and Eby, 2003). Even species from clades traditionally considered unequivocally migratory, resistant, or hibernatory may exhibit combined use. Swallows (*Hirundo*), hummingbirds (*Trochilidae*), and warblers (*Sylviidae*) at high latitudes migrate, but may also use torpor (e.g., Wojciechowski and Pinshow, 2009), and torpor-assisted migration is explicitly studied in birds (Carpenter and Hixon, 1988; Clerc and McGuire, 2021; Eberts et al., 2021; Wojciechowski and Pinshow, 2009) and bats (McGuire et al., 2014). There may even be required co-occurrence of strategies, especially when constraints (evolutionary, physiological, or developmental) prevent species from using a single strategy to the degree necessary to survive winter.

(c) Partial use of strategies to varying degrees

Each cold-survival strategy exists not as a binary, but on a spectrum. This allows for *partial use*—species may utilize strategies to varying degrees. There is continuous variation in migration distances, torpor depths, and degrees of resistance. Partial use (relative to a hypothetical maximum) may be tenable due to milder winters or combined use of strategies. Despite this, capacities for migration, adaptive heterothermy, and cold-resistance are commonly reduced to binaries (e.g., migratory versus nonmigratory) or categories (e.g., long- versus short-distance migration). Yet use of strategies is highly variable. For instance, “hibernating” black bears (*Ursus americanus*) only drop their body temperature by 2-8˚C (Tøien et al., 2011), versus more than 30˚C in many smaller mammals (Geiser, 2004).

Treating strategies as binary or categorical can be useful (e.g., Geiser, 2004), but quantification can increase resolution and statistical power. For example, migration distance, not just migratory status, has been shown to have fundamental impacts on species life history (Winger and Pegan, 2021). Birds that migrate longer distance spend significantly less time in their breeding areas and have lower reproductive output (Winger and Pegan, 2021). Quantifying the axes of cold-survival (Fig. 4-1) may require combining multiple metrics of a strategy’s components into a single index (Section 3, below).

(d) Proportional tradeoffs between strategies

An extension of partial and combined use is *proportional use*—oppositional relationships between strategies. Cold-survival strategies are fundamentally about surviving energy deficits imposed by reduced solar energy (freezing temperatures and subsequent reduced productivity, including food availability). Decreased use of one strategy should result in increased reliance (collectively) on others, when controlling for external energy (e.g., winter severity). Individuals may rely heavily on one strategy, or use multiple strategies to lesser degrees. For example, some amphibians undergo extreme torpor, withstanding freezing body temperatures (Storey, 1990), and would therefore not be expected to simultaneously rely heavily on migration or cold resistance.

Individual eastern chipmunks (*Tamias striatus*) use torpor to a lesser degree when food caches are abundant (shifting from torpor to resistance) (French, 2000; Humphries et al., 2003). The common green darner dragonfly (*Anux junius*) exhibits tradeoffs between migration and torpor, with more northern populations being exclusively migratory (May, 2013). Human-altered energetic environments (additional food or heated areas in winter) have contributed to reductions in migration (e.g., Gilbert et al., 2016; Kurta et al., 2018; and torpor, Beckmann and Berger, 2003).

Potential interspecific examples of proportional tradeoffs are suggestive but not formally evaluated. The Swainson's Thrush (*Catharus ustulatus*) and Hermit Thrush (*C. guttatus*) both breed in northern North America, but the former undertakes long-distance migrations to the neotropics (Winger and Pegan, 2021), while the latter undertakes shorter migrations to areas which periodically experience cold snaps. Similarly, temperate bats include species which have short-distance migrations, undertake deep hibernations, and are less cold resistant (*Myotis* spp.) whereas other species have longer migrations, reduced torpor use, and more resistance (e.g., larger bodies, more fur, and periodic winter foraging; *Lasionycteris* and *Lasiurus* spp.) (Fleming and Eby, 2003). Heterothermic capacity (quantitatively treated) is negatively related to presence/absence of migration (categorically treated) in mammals (Webber and McGuire, 2021), but approaches that are fully quantified and consider all three axes are ideal.

Interspecific relationships (such as in Fig. 4-1) should be observable when comparing species (or populations) that are sympatric in the summer range (similar winter severity and distance to clement areas). Species for which use of axes is collectively higher (exceeding minimum survival thresholds) likely do so because of increased adaptive payoffs (like migrants capitalizing on seasonally abundant resources).

3. Measuring the axes of cold-survival

Quantifying axes of cold-survival (Fig. 4-1) is challenging but possible given tools like GPS and temperature-sensitive skin tags (Bridge et al., 2011), thermal cameras (Sinclair et al., 2015; Tattersall, 2016), stable isotopes (Eerkes-Medrano et al., 2021), and relevant repositories (PanTHERIA: Jones et al., 2009 ; MoveBank : Wikelski et al., 2022). Measurements that reflect natural regimes are preferrable (Sinclair et al., 2015), and careful thought is needed as to what is being measured. “Cold tolerance” commonly refers to the ability to withstand low body temperatures in ectotherms (here, a component of torpor), but the ability to remain active (despite cold external temperatures) in mammals and birds (here, cold resistance). One or multiple measurements may be used to quantify each axis. Rigorous guidance is beyond the scope of this paper, but I present some direction.

Torpor — Measurements of torpor include duration, body temperature (potentially relative to ambient or normothermic body temperatures), and frequency of torpor bouts, among others (Geiser, 2021). Measuring body temperature is straightforward in insects (Sinclair et al., 2015) but less so in larger animals. Considerable differences can occur, for example, between brain versus rectal temperature. Arguably, skin temperature balances accuracy and accessibility.

Migration — Migration is most directly measured as distance typically traveled per year, but potentially relevant components include stopover sites and transportation mode. Distances between summer and winter ranges (e.g., using range or abundance maps, The Cornell Lab of

Ornithology, n.d.; Winger and Pegan, 2021; mark-recapture, e.g., Buskirk et al., 2009; or tracking studies, Baert et al., 2018) may be used. Temperature differentials in the overwintering climate (or microclimate), may be a suitable substitution for distance traveled (reflecting a cold-avoidance axis rather than migration per se).

Cold-resistance — Quantifying cold-resistance can be extremely challenging, potentially requiring careful tallying relative energy inputs versus losses across a variety of disparate substrategies. These include energy inputs through caloric intake (winter food sources or burning stored fat reserves), and reductions in energy lost to the environment through reduced surface-area-to-volume ratio, insulation, and winter aggregations. Relationships similar to those postulated to exist between cold-survival strategies may exist between resistance substrategies. An alternative to measuring resistance (or any axis) is to control for it. For instance, when comparing endotherms of similar size and diet, proportional tradeoffs between migration and torpor should be evident.

4. Conclusions

An integrated framework of cold-survival means comprehensive consideration of strategies that may be used in combination and to partial degrees, with the expectation that proportional tradeoffs should exist when controlling for geography and environment (Fig. 4-1). This framework encourages a quantified approach, can be altered for other environmental stressors (Körtner et al., 2016; Riddell et al., 2021), and makes testable predictions. For instance, species that collectively under-utilize all strategies (e.g., fall within the shaded area of Fig. 4-1) should be of conservation concern or extinct (or on a trajectory toward extirpation, if also occurring in more clement areas). Another prediction is that conspecifics across localities of variable winter severities should exhibit predictable differences in their overall extent of cold-strategy use. Although strategies are discussed here exclusively in the context of meeting minimum survival thresholds, it may also be that species use strategies to greater extents (e.g., occurring further into the unshaded area in Fig. 4-1) when there are corresponding payoffs (e.g., migrating farther to access abundant yet ephemeral resources).

Conducting research with intimate knowledge of focal taxa often mitigates pitfalls described above (e.g., considering only one strategy), highlighting the value of natural history perspectives. However, exclusive concentrations can blind researchers to broader contexts, especially for aspects of focal systems that are pervasive and consistent. Endothermy, for instance, is pervasive and consistent among birds and mammals. Therefore, it may be easy for those studying these animals to take for granted the tremendous degree of resistance this confers. This oversight may seem to only be problematic in the context of broader, comparative studies, but can also limit insights regarding focal taxa. For example, it might seem that hummingbirds break *proportional use* because they heavily use both migration and torpor (Eberts et al., 2021) (relative to other sympatric birds). However, *comprehensive consideration* reminds us of the third axis—cold resistance, and suggests hummingbirds may be compensating for low resistance capacities. Indeed, this seems likely—hummingbirds are relatively small and have limited winter food sources. Going forward, an integrated cold-survival framework encourages collaborations between scientists across taxonomic, scale-based, and methodological disciplines, thereby amplifying the power of natural history knowledge.

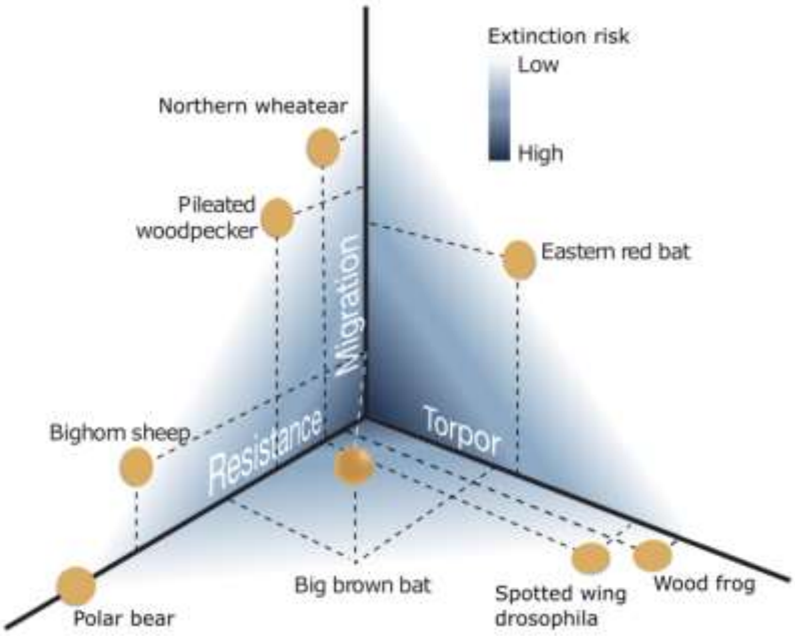

Fig. 4-1. **Cold-survival strategies in animals.** Species (tan circles) can utilize one of three axes (migration, torpor, or cold-resistance) to facilitate survival in freezing environments. Species may utilize just one axis, but would need to do so to a relatively high degree to avoid increased risk of extirpation or extinction (darker shades in center). Alternatively, species may also use multiple strategies to a lesser degree to reach the adaptive optimum (lighter shades). Species placements are informed approximations, and relationships are conditioned upon controlling for comparable environmental conditions in the winter extent of the range. Illustration credit John Megahan.

Glossary

Cold resistance: The capacity to remain active in cold temperatures (while maintaining normothermia). Substrategies (reviewed in Blix, 2016; Miller, 1982) include leveraging insulation, surface-area-to-volume ratio, caloric energy, and activity shifts.

Cold-survival strategy: Any strategy that facilitates use of an area which regularly experiences below-freezing temperatures—seasonal migration, torpor, and cold-resistance.

Seasonal migration: An annual movement that is often cyclically repeated (in terms of timing and geography) by individuals or populations (Dingle, 2014), often to avoid adverse climactic conditions.

Torpor: Any non-pathological reduction in body temperature below thermal ranges of standard activity levels. Used here to broadly include any type of adaptive heterothermy associated with tolerance of lowered body temperatures, including dormancy (Koštál, 2006) and hibernation. Nonadaptive, pathological reductions include cold injury or hypothermia.

Winter: A seasonal period that is colder, darker, snowier, and frozen (Studd et al., 2021, p. 2).

Acknowledgements

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Chapter V

Conclusion

The important of perspective shifts: Zooming in, zooming out, and stepping to the side

The microevolutionary and conservation processes considered in Chapters II and III are the building blocks of broader conservation and evolutionary outcomes, which are explored via a conceptual framework in Chapter IV (Fig. 5-1). Just as microevolutionary processes shape larger-scale, macroevolutionary patterns (Li et al., 2018), the conservation trajectories I consider (shifts in abundance, mobility, and fluctuations in range size) underlie a fundamental metric of evolution—species survival versus extinction.

As discussed in the concluding section of the previous chapter (IV), combining knowledge across disciplines and perspectives (including observed versus theoretical) can be a catalyst for scientific advances, and careful observations of focal species or populations (Chapters II and III) can provide key insights and gain increased importance when leveraged in broader frameworks. These perspectives are echoed in evolutionary biology's roots, when Darwin combined his vast, yet intimate, perspectives on natural history with emerging information from other disciplines, such as geology (Darwin, 1859; Lyell and Secord, 1998). Molecular mechanisms of inheritance, which Darwin was unable to discern at the time, later became clearer when Mendel's careful observation of inheritance in pea plants (*Pisum sativum*) were rediscovered (Mendel and Mangelsdorf, 1965). The importance of alternative perspectives continues, for instance in the contributions of Vermeij's insights of broad shifts in evolution of shell morphology towards anti-predator mechanisms in the Cretaceous (Vermeij, 1977). Many of Vermeij's observations, which were based primarily on tactile observations of shells because he was blind, may not have been as readily apparent to biologists who were more reliant on sight. "Disruptive scholarship" is perhaps increasingly important in our modern era of increased research output, which can otherwise be biased towards reinforcing, rather than challenging, scientific cannon (Chu and Evans, 2021).

Future multi-taxa directions

While organismal knowledge of species responses is important—say, that of a bat overwintering in a cave or a salamander living in mountaintops faced with glacial advance—integrating it in order to understand broader biological patterns is perhaps just as important. For example, to test explicit macroevolutionary hypotheses for factors underlying species extinctions in the Last Glacial Maximum. Glaciation cycles have been a key influence on speciation and extinction of modern bears (Hassanin, 2015). If we consider the expectations of proportional use (Chapter IV) in conjunction with the ecophysiological requirements of different species of bears, can we predict survival outcomes? Cave bears (*Ursus spelaeus*) are one species that went extinct during this time. In contrast, increase in cold-tolerance capacity in the polar bears (*U. maritimus*) occurred via a shift from an omnivorous to a carnivorous diet, allowing them to consume food throughout winter (key in their speciation from brown bears; *U. arctos*) (Rinker et al., 2019). At the same time, the bears reduced their use of torpor. The cave bear, however, only used shallow torpor and modest fat deposits to survive winter. They were likely unable to substantially increase levels of pre-winter fat reserves due to dietary constraints (due to their reliance exclusively on vegetative matter instead omnivory) (Bocherens, 2019; Pérez-Ramos et al., 2020). Neither could the species utilize deeper torpor (due to size constraints). Presumably, they also were unable to undertake long enough seasonal migrations (bears are not exceptionally mobile). Thus, cave bears lacked the ability to compensate for climactic changes by shifting along any axis (tolerance, torpor, or migration). Incorporating species' traits into predictions of evolutionary responses can result in more refined, informative, and statistically powerful analyses (Papadopoulou and Knowles, 2016, 2015), and the proposed cold-survival framework (Chapter IV) is an additional tool for doing this.

Another instance of where this framework may be useful is in resolving underlying tradeoffs. For instance, a rich research literature exists on partial migration in birds (e.g., Silverin, 2003; Townsend et al., 2018; Zúñiga et al., 2017), which could be leveraged to identify physiological or environmental constraints on the bounds of strategies used or drivers of tradeoffs between strategies. Whether these bounds and drivers are consistent or different in different taxa and at different scales (individual, population, or species level) is a line of research particularly well-suited to studies of partial migrants (and potentially their sister sedentary or fully migratory species, for-species comparative contexts).

Conceptual frameworks, like the cold-survival one, can also reveal when a particular factor is *not* important. In other words, for many systems and questions, it may be that there is no importance of overall cold-survival strategy *per se*. For example, one study found that only 66% of migratory butterflies exhibited observable range shifts associated with temperature shifts (Sparks et al., 2005), while another found that nearly the same percent, 65%, of nonmigratory species are expanding their range northwards (Parmesan et al., 1999). Thus, it could be that overall seasonal strategy is not an important predictor at all of which species will undertake range expansions, but never comparing between strategies within the same study precludes this discovery.

Final perspectives in bats

Bats are a focal taxa in multiple areas of research and may be particularly apt for exploring the framework in Chapter IV. For instance, why are some species apparently shifting their ranges northward (e.g., Auteri and Kurta, 2015; Auteri et al., 2016)? Bats are also a taxa of interest for research on wildlife diseases, due to their abundance (which naturally means they carry many diseases; Mollentze and Streicker, 2020) and association with some highly-virulent or lethal diseases in humans (e.g., rabies and ebola). Although they do not necessarily carry disproportionately more diseases (Mollentze and Streicker, 2020), they may have particularly adept at withstanding the diseases they do carry (Hayman, 2019), and are disproportionately associated with diseases that are highly problematic when introduced to humans. Relationships between disease (or host responses to disease) and cold-survival strategies, including migration and hibernation, have been independently explored (Buckee et al., 2021; Eikenaar and Hegemann, 2016; Levesque et al., 2020; Owen and Moore, 2008; Rayl et al., 2021), including subcomponents of these strategies (O'Shea et al., 2014). However, a framework that considers these factors together in the context of bats could be informative for understanding host-pathogen dynamics in bats, and factors predicting spread of their pathogens to humans.

The main findings of adaptation in bats to white-nose syndrome in Chapter II (Auteri and Knowles, 2020) were shortly supported by other papers finding suggestions of similar outcomes (Gignoux-Wolfsohn et al., 2021; Lilley et al., 2020). Both studies, similar to Auteri & Knowles (2020), compared bats which were found dead to survivors, but considered little brown bats in different portions of their range and used low coverage, rather than individual-levels of certainty in SNP-calling; Gignoux-Wolfsohn et al., 2021; Lilley et al., 2020). These studies found suggestive SNPs associated with genes involved in disease resistance and tolerance mechanisms (Gignoux-Wolfsohn et al., 2021; Lilley et al., 2020). However, subsequent work also suggests adaptations may only be effective within a relatively narrow microclimate within hibernation sites (Grimaudo et al., 2022), effectively constraining the specie's niche.

Two of the characteristics that make system in bats tractable (e.g., that facilitated occurrence and observability of natural selection) is the likely high genetic diversity of the species before introduction of the disease, and very high death rate imposed by it (i.e., strong selective environment). These components, respectively, likely facilitated neutral genetic diversity (DeWoody et al., 2021) which potentially poses similarly strong selective forces. This is, quite likely, in contrast to the salamanders (Chapter III), which other studies suggested did not experience strong or tightly linked declines. However, understanding when evolutionary responses are, versus are not, readily apparent is one of the lessons we can learn from the Anthropocene, and its increased rate of change.

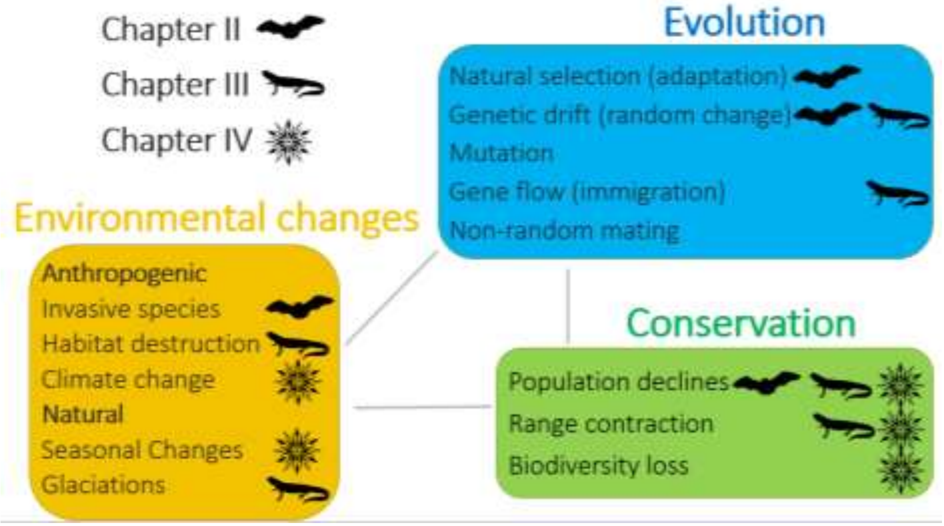


Fig. 5-1. Major Environmental, evolutionary, and conservation factors, including indicators of chapters in this dissertation touch on different subcomponents. Although Chapter IV does not focus directly on currently occurring evolutionary mechanisms in response to environmental change, it could be leveraged to do so.

Appendix A

Supplementary Tables and Figures for Chapter II

Decimated little brown bats show potential for adaptive change

Table A-S1. Overview of sampled bats. Sample ID names, in conjunction with survivorship group (survivor: S; non-survivor: NS), location (generalized to county in Michigan, USA), sex (M: Male, F: Female, U:Unknown), date of collection, whether the individual was excluded from the PCA due to missing data, and the method of collection (Surveillance: bats euthanized by the federal government for WNS disease monitoring; Salvage: bats found dead in the wild apparently due to WNS; Screening: bats euthanized by the state authorities for rabies inspection; Live: bats found in the wild that were sampled and released, with band numbers “EMU YPSI” followed by 7872, 7873, and 7875, sequentially).

ID	Group	County	Sex	Date collected (dd-mm-yyyy)	Included in PCA	Collection
14NWHC02	NS	Mackinac	U	23-03-2014	Yes	Surveillance
14NWHC03	NS	Alpena	U	22-03-2014	Yes	Surveillance
14NWHC05	NS	Alpena	U	22-03-2014	Yes	Surveillance
14NWHC07	NS	Dickinson	U	26-02-2014	Yes	Surveillance
16AM01	NS	Ontonagon	F	22-02-2016	Yes	Salvage
16AM02	NS	Ontonagon	M	22-02-2016	Yes	Salvage
16AM03	NS	Ontonagon	M	22-02-2016	No	Salvage
16AM04	NS	Ontonagon	M	22-02-2016	Yes	Salvage
16AM05	NS	Ontonagon	F	22-02-2016	Yes	Salvage
16CM01	NS	Ontonagon	M	20-02-2016	No	Salvage
16CM03	NS	Ontonagon	F	20-02-2016	No	Salvage
16CM04	NS	Ontonagon	F	20-02-2016	Yes	Salvage
16CM05	NS	Ontonagon	M	20-02-2016	Yes	Salvage
16CM06	NS	Ontonagon	M	20-02-2016	Yes	Salvage
16CM07	NS	Ontonagon	F	20-02-2016	No	Salvage
16CM08	NS	Ontonagon	M	20-02-2016	Yes	Salvage
16CM09	NS	Ontonagon	M	20-02-2016	Yes	Salvage
16CM10	NS	Ontonagon	M	20-02-2016	Yes	Salvage
16CM11	NS	Ontonagon	F	20-02-2016	Yes	Salvage
16CM12	NS	Ontonagon	F	20-02-2016	Yes	Salvage
16DM01	NS	Keweenaw	M	21-02-2016	Yes	Salvage
16DM02	NS	Keweenaw	F	21-02-2016	Yes	Salvage

16FS01	NS	Ontonagon	F	22-02-2016	Yes	Salvage
16FS02	NS	Ontonagon	M	22-02-2016	Yes	Salvage
16FS03	NS	Ontonagon	M	22-02-2016	Yes	Salvage
16FS04	NS	Ontonagon	M	22-02-2016	Yes	Salvage
16FS05	NS	Ontonagon	U	22-02-2016	Yes	Salvage
16RL01	NS	Gogebic	U	01-01-2016	Yes	Screening
16RL03	NS	Houghton	U	03-03-2016	Yes	Screening
16RL05	S	Houghton	M	12-04-2016	Yes	Screening
16RL06	S	Dickinson	M	04-04-2016	No	Screening
16RL07	S	Missaukee	F	18-04-2016	Yes	Screening
16RL08	S	Crawford	M	26-07-2016	Yes	Screening
16RL09	S	Mackinac	F	17-08-2016	Yes	Screening
16RL10	S	Menominee	F	20-09-2016	Yes	Screening
17PR01	S	Alger	F	17-07-2017	Yes	Live
17PR03	S	Alger	F	17-07-2017	Yes	Live
17PR04	S	Alger	F	17-07-2017	Yes	Live

Table A-S2. Information on the nine significantly differentiated SNPs with respect to the reference genome. The scaffold and position of the SNP can be used to identify the location in the reference genome (ftp://ftp.ncbi.nih.gov/genomes/Myotis_lucifugus/Gnomon/). F_{ST} is the AMOVA-corrected F_{ST} [84] as calculated in STACKS[67]. For SNPs not located within mRNA sequences, we located the nearest known annotated areas (upstream or downstream) in the scaffold.

Outlier	**Scaffold**	**Position**	F_{ST}	**Gene**	**Distance (bp) & region**	**Function**
1	NW_005871056.1	13633675	0.2800	—	52,169 upstream of exon	Unknown (similar to multiple proteins)
2	NW_005871075.1	3019927	0.2840	—	18,912 upstream of exon	Uncharacterized
3	NW_005871095.1	6433232	0.3033	—	116,727 upstream of exon	Unknown (similar to multiple proteins)
4	NW_005871096.1	98257	0.2929	GABRB1	Within mRNA intron	Regulates arousal from hibernation
5	NW_005871218.1	2491166	0.2800	—	25,913 downstream of exon	Uncharacterized
6	NW_005871219.1	1301763	0.6061	FOXP2	Within mRNA intron	Vocalizations, echolocation
7	NW_005871329.1	378057	0.2579	*PLA2G7**	2,747 upstream of PLA2G7 exon	Regulates release of histamine from mast cells
8	NW_005871536.1	273112	0.2778	cGMP-PK1	Within mRNA intron	Regulates breakdown of fats
9	NW_005871536.1	339572	0.28875	*cGMP-PK1**	3,387 upstream of cGMP-PK1 coding sequence	See above

*These SNPs do not fall within the listed genes, but adjacent to them.

Table A-S3. Genotypes for each individual for the nine candidate SNPs. Major alleles were associated with non-survivors (the majority of our samples). Genotypes homozygous for minor alleles are shaded dark green, and heterozygotes are light green.

	Outliers								
Sample ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
mortality_14NWHC02	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_14NWHC03	—	—	TA	GG	GG	GG	CC	CC	TT
mortality_14NWHC05	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_14NWHC07	—	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16AM01	CC	AA	—	GG	GG	GG	CC	CC	—
mortality_16AM02	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16AM03	—	—	—	GG	—	—	CC	—	—
mortality_16AM04	CC	AA	TT	GG	GG	GG	CC	CC	—
mortality_16AM05	CC	AA	—	GG	GG	GG	CC	—	TT
mortality_16CM01	—	—	—	—	—	—	—	—	—
mortality_16CM03	CC	—	—	—	—	—	CC	—	—
mortality_16CM04	CC	AA	AA	GG	GG	GG	CC	CC	—
mortality_16CM05	CC	—	TT	GG	—	GG	CC	CC	TT
mortality_16CM06	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16CM07	—	AA	—	—	—	—	—	—	—
mortality_16CM08	CC	AA	TT	GG	GG	GG	CC	—	TT
mortality_16CM09	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16CM10	CC	AA	—	GG	GG	—	CC	CC	TT
mortality_16CM11	CC	AA	TT	GG	GG	—	—	CC	—
mortality_16CM12	—	AA	—	GG	GG	GG	CC	CC	TT
mortality_16DM01	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16DM02	CC	AA	TT	GG	—	GG	CC	—	TT
mortality_16FS01	—	AA	—	GG	GG	GG	CC	CC	—
mortality_16FS02	CC	AA	TT	GC	GG	—	CC	CC	TT
mortality_16FS03	CC	AA	TT	GG	GG	GG	CC	CC	TT
mortality_16FS04	CC	AA	TT	GG	GG	GG	CC	CC	—
mortality_16FS05	—	—	—	—	—	—	CC	—	TT
mortality_16RL01	CC	AA	TA	GG	—	—	CC	CC	TT
mortality_16RL03	CC	AA	TT	GG	GG	GG	CC	—	TT
survivor_16RL05	CC	AA	—	GC	GG	CC	GG	CC	TT
survivor_16RL06	—	—	—	—	—	—	—	—	—
survivor_16RL07	CC	AA	AA	GG	AA	—	GG	TT	AA
survivor_16RL08	CC	AG	AA	GC	GG	CC	CG	CT	TT
survivor_16RL09	—	—	TT	—	—	—	CC	—	—
survivor_16RL10	CC	AG	TA	GG	AA	CC	CC	CC	TA
survivor_17PR01	TT	AA	TA	GG	GG	GG	CC	—	TA
survivor_17PR03	TT	GG	TA	CC	—	GG	CC	CC	TA

survivor_17PR04	—	—	AA	CC	GG	CC	CC	CT	TT

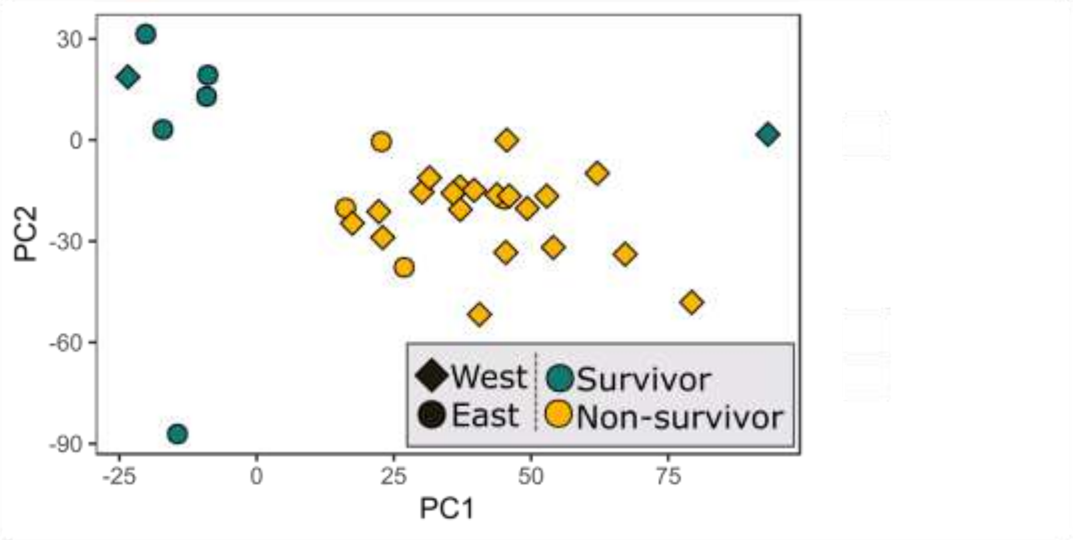


Fig. A-S1. A PCA with alternative filtering of data. A PCA performed in the same way as Fig. 2A, but with more stringent filters on missing data. Here, missing data is ≤ 8.7% per individual and ≤19% per locus (mean 1.9%), with a MAF limit 0.05. Applying these thresholds resulted in 13,666 loci and 31 individuals being included in the analysis. Survivors and non-survivors are shown in dark green and gold, respectively, with diamonds indicating individuals sampled from the western portion of the peninsula and circles indicating those from the east. PC1 explains 19% of the variance for survivors and 39% for the non-survivors, whereas PC2 explains 18% and 25%, respectively.

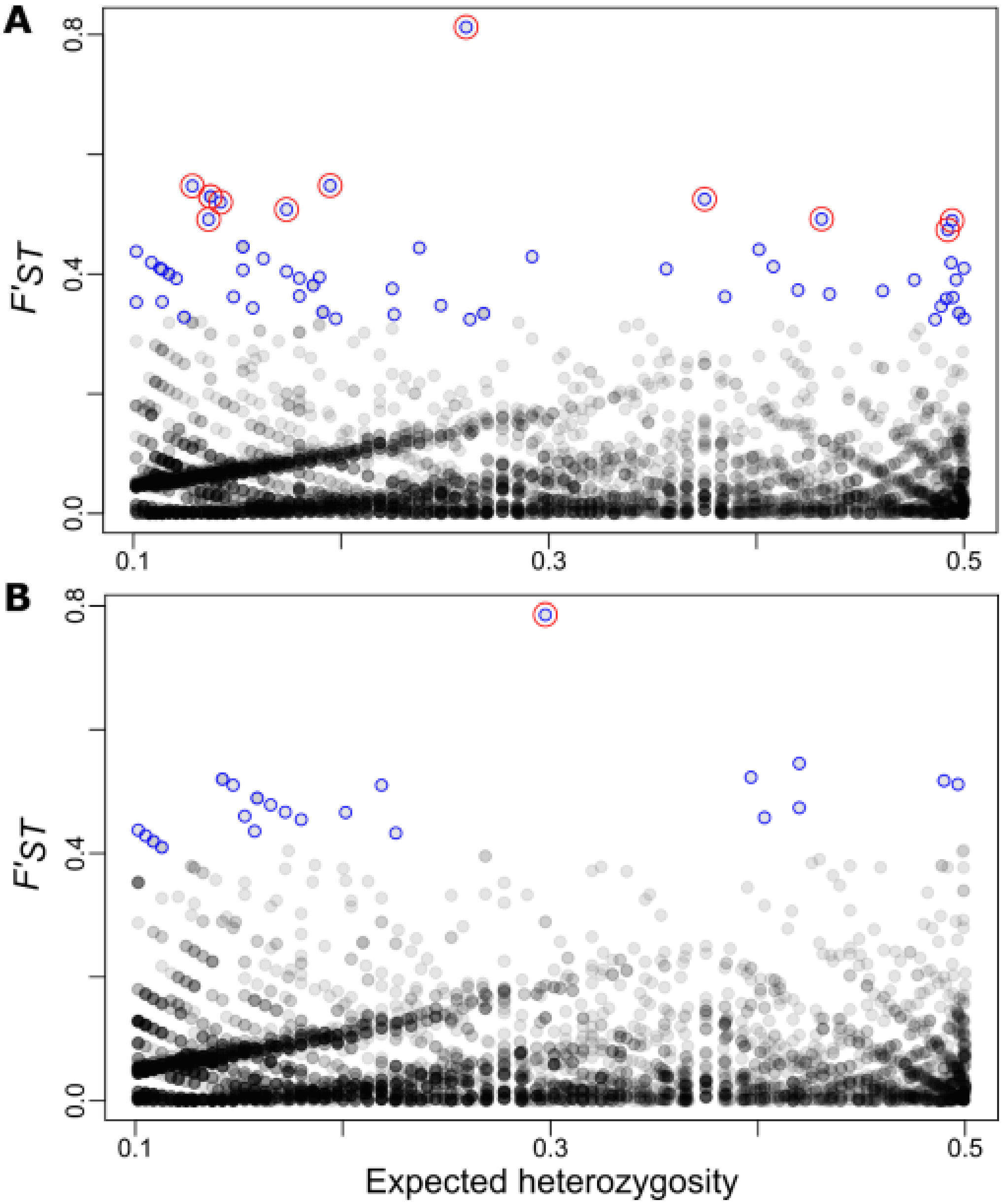


Fig. A-S2. Results showing OutFLANK SNPs of interest. Findings of OutFLANK, showing each SNP-site plotted in context of the expected heterozygosity (He) of the minor allele (x-axis) and the degree of differentiation between survivors and non-survivors (F'_{ST} on the y-axis, a version of F_{ST} not corrected for sample size as calculated in OutFLANK; Whitlock & Lotterhos, 2015) for analyses (A) of all individuals and (B) excluding individuals sampled in 2014. Loci in blue are significant at a threshold of alpha ≤ 0.05 (64 and 26 for A and B, respectively); loci with an additional red ring are significant at alpha ≤ 0.01 (12 and 1). Loci with low minor allele frequencies (He < 0.1) were excluded as per the developer's guidelines.

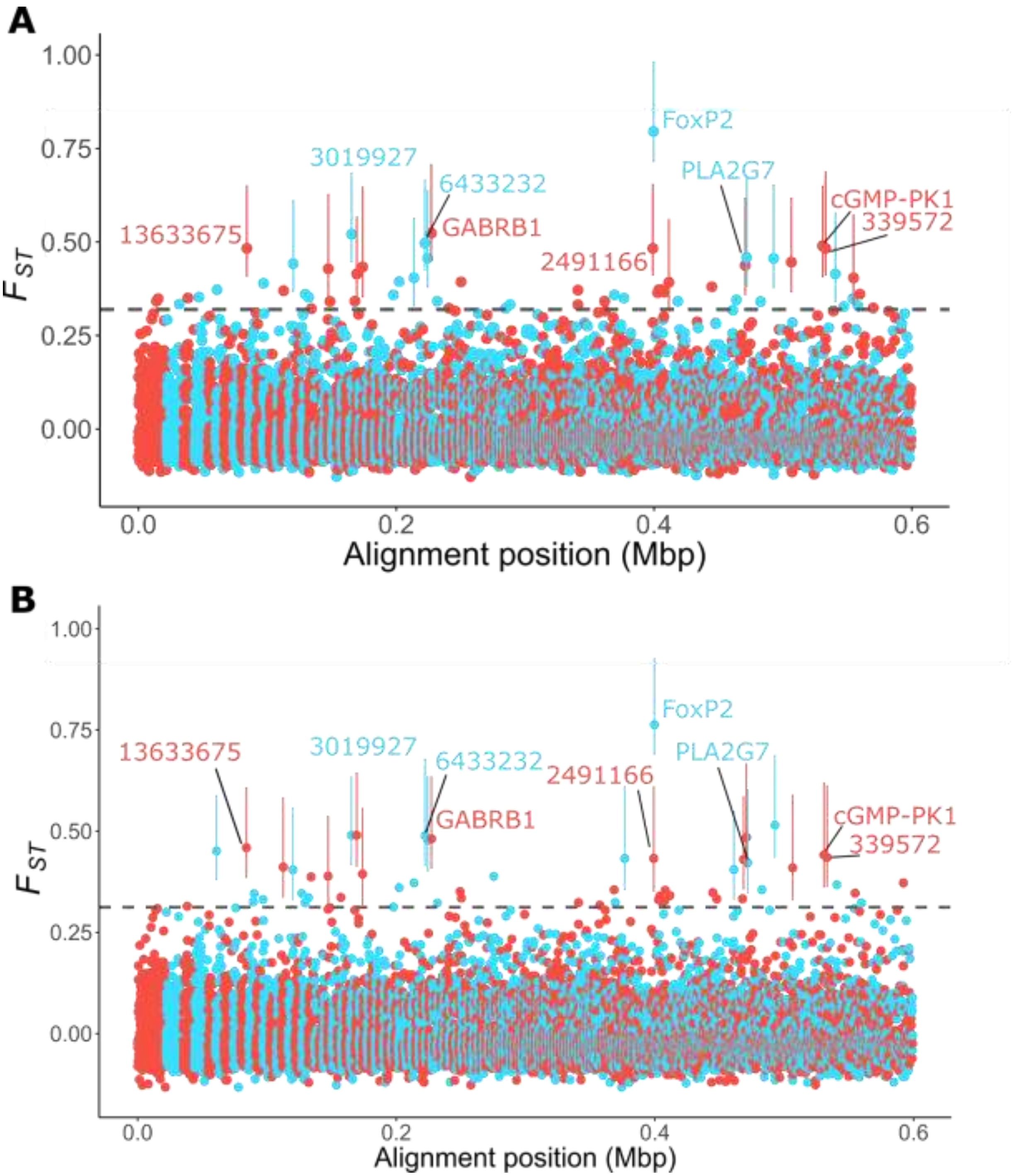


Fig. A-S3. Outlier plots with confidence intervals. F_{ST}-outlier plot with 95% confidence intervals based on analyses of (A) all the data, and (B) excluding the four bats from 2014 (see methods for details). The 9 top-candidate sites that were also identified in the other two tests (which are what we center the discussion upon) are labeled according to the gene name or position (see Table A-S2). In addition to these loci, 12 sites with CIs at least 5 standard-deviations from the mean were identified as significant in A (i.e., a total of 21 sites), and 13 additional sites were significant in B. However, these additional outliers are not discussed further due to their lack of identification via the two other outlier detection methods. Some outliers were moved horizontally for clarity.

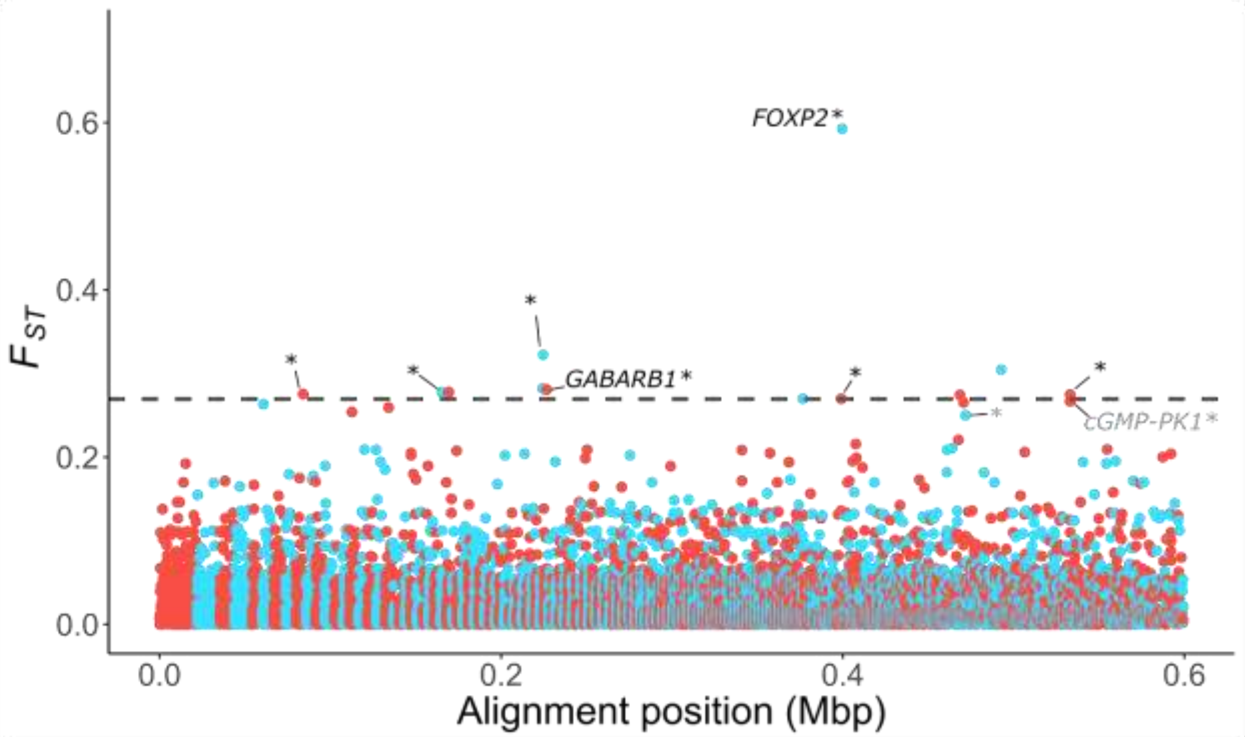


Fig. A-S4. Ourlier plots with alternative filtering of data. Results of the F_{ST}-outlier analysis (see also Fig. 3) excluding the four bats from 2014 (see methods for details). Here twelve (as opposed to nine with all the data) significant outlier SNPs were detected, and two SNPs previously identified as significant based on nine-standard deviation are significant by five-standard deviations given the slight drops in F_{ST} (i.e., PLA2G7 and cGMP-PK1).

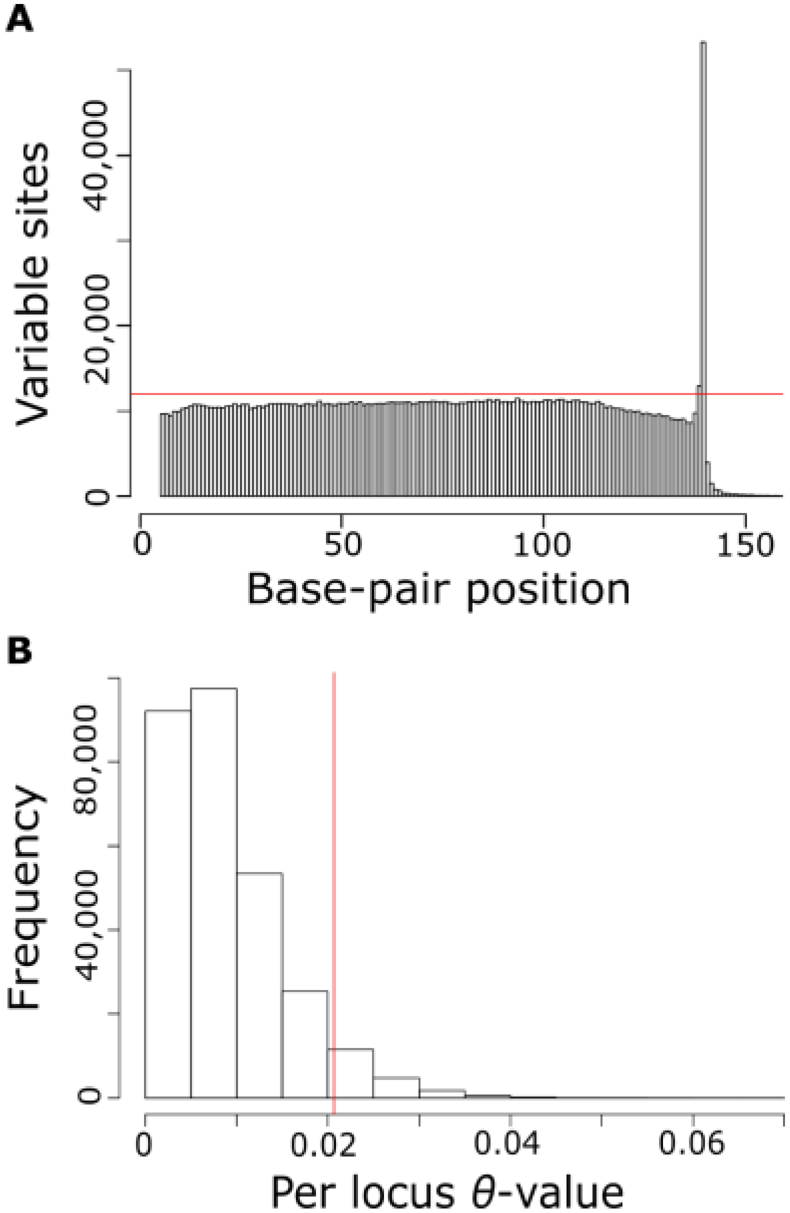


Fig. A-S5. Plots showing data filtering for sequence errors. A custom script in R was used to examine the number of variable sites per base-position across all reads (the ends of reads are more susceptible to sequencing errors). The pre-trimmed plot (A) indicates the threshold used for discarding SNPs (horizontal, at 4,500 variable sites) and (B) shows a subsequent trimming step in which the distribution of per locus θ-values was considered. Loci with θ-values above the 95% threshold (shown by the red line) were excluded in order to reduce the probability of including sites that were variable due to errors in sequencing or assembly.

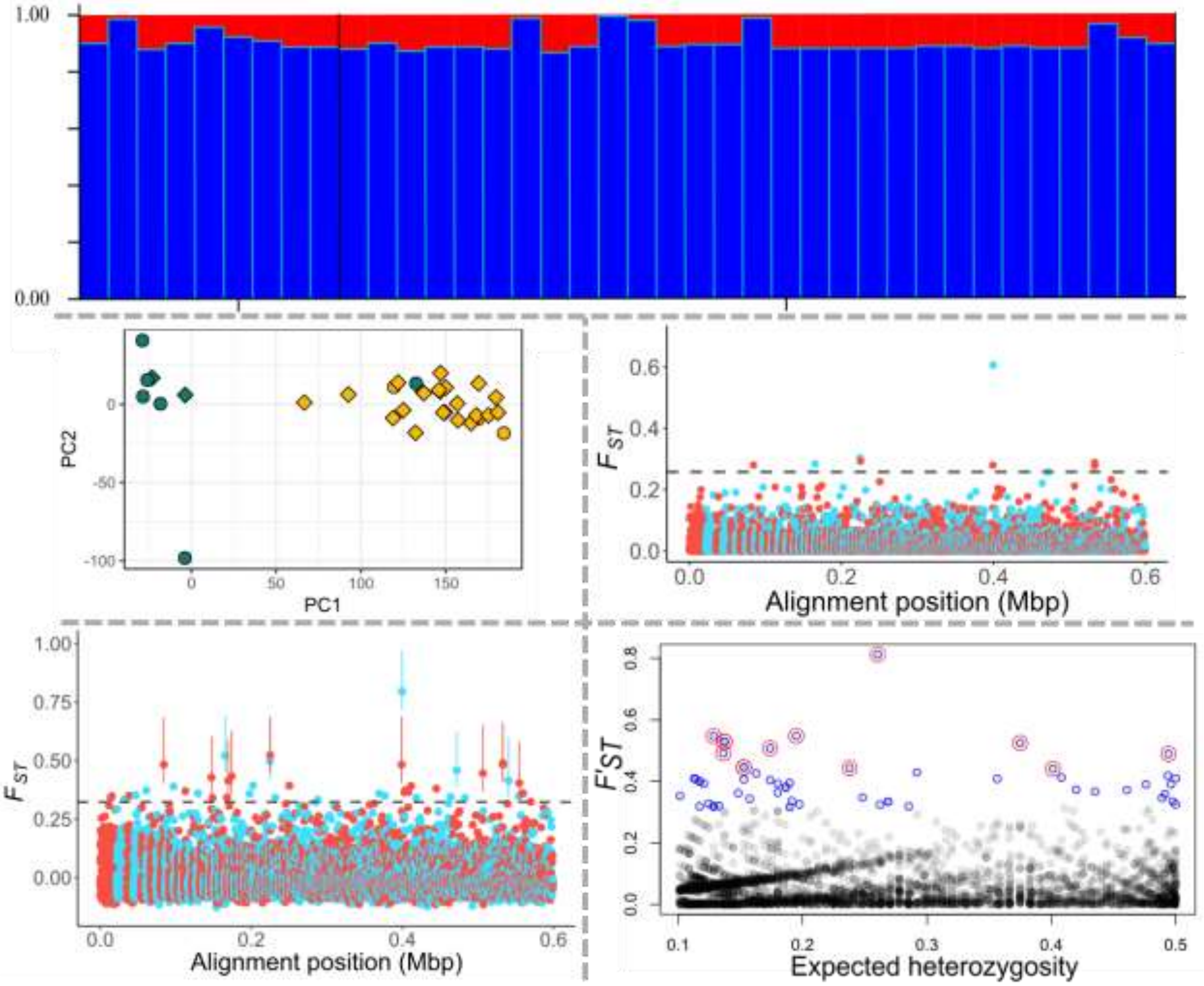


Fig. A-S6. **Results showing one SNP per locus.** The robustness of the results were confirmed by repeating analyses with a single randomly selected SNP per locus, except for the previously identified nine SNPs of interest. Evaluation of population subdivision in STRUCTURE[37] (top) shows a single, panmictic population (samples are in order corresponding to Table A-S1 with survivors on the left and non-survivors on the right). The PCA (middle left) corresponds to Fig. 2 with PC1 explaining 23% of the variance for survivors and 62% for non-survivors, and PC2 explained 13% for survivors and 9% for non-survivors. The estimated degree of drift using the *F*-statistic in STRUCTURE[36,37] (not pictured) remained similar at F=0.049 (SD±0.000624) for survivors and 0.0097 (±0.0007) for non-survivors. The outlier analysis (middle right; corresponding to Fig. 3) based on nine standard deviations from the mean (0.28) identified the same nine SNPs as significant, which were also significant in the bootstrap analysis (bottom left; see Fig. A-S3) and by OutFLANK[90] (bottom right; see Fig. A-S2).

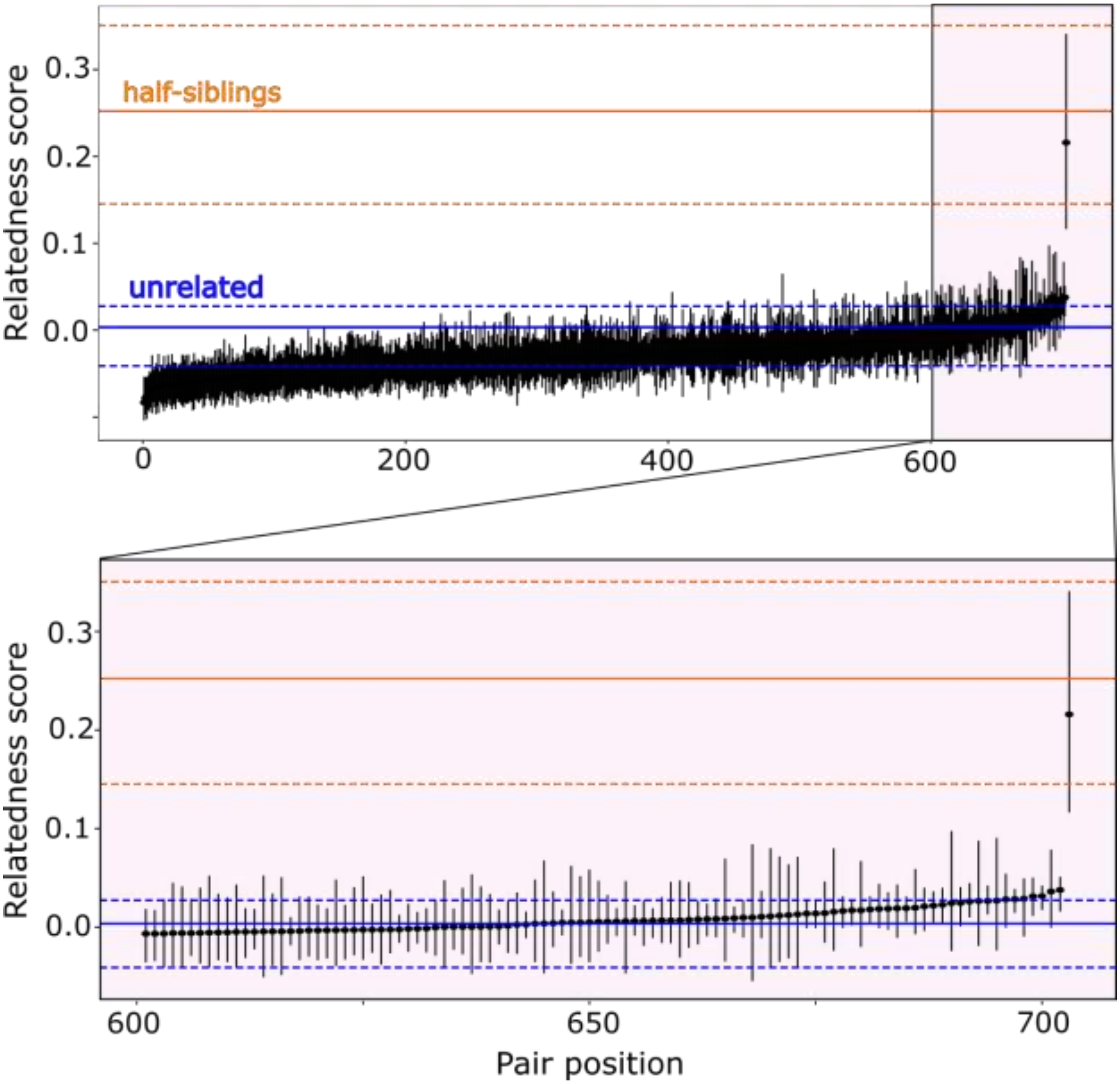


Fig. A-S7. Assessment of relatedness among sampled bats. Estimated relatedness of pairs of sampled bats (dots) with 95% confidence intervals (CIs, vertical lines), arranged along the x-axis from least to most related. The y-axis is Ritland's estimator of relatedness[77], which theoretically ranges from 0 to 1, with one representing a clone. Horizontal lines indicating expected scores for unrelated individuals (blue) and half-siblings (orange). Solid horizontal lines represent the mean expected value, whereas the dashed lines represent the 1st and 3rd quantiles. Estimates of expected and observed relatedness were generated using the package *related*[76]. Expected values were simulated from 250 randomly chosen loci from our dataset for 100 pairs of individuals per relationship category (parent-offspring, full sibling, half sibling, and unrelated), then estimated for each pair of samples based on all 1,242 loci used in the analyses (i.e., loci with a minor allele frequency greater than 0.01, and not missing from more than 2 individuals, with a minimum of 25% missing per individual). Only a single pair of individuals (sample IDs 16CM03 and 16CM11; see Table A-S1) was estimated to be related (as half-siblings; Ritland score = 0.216, 95% CI 0.12 – 0.34).

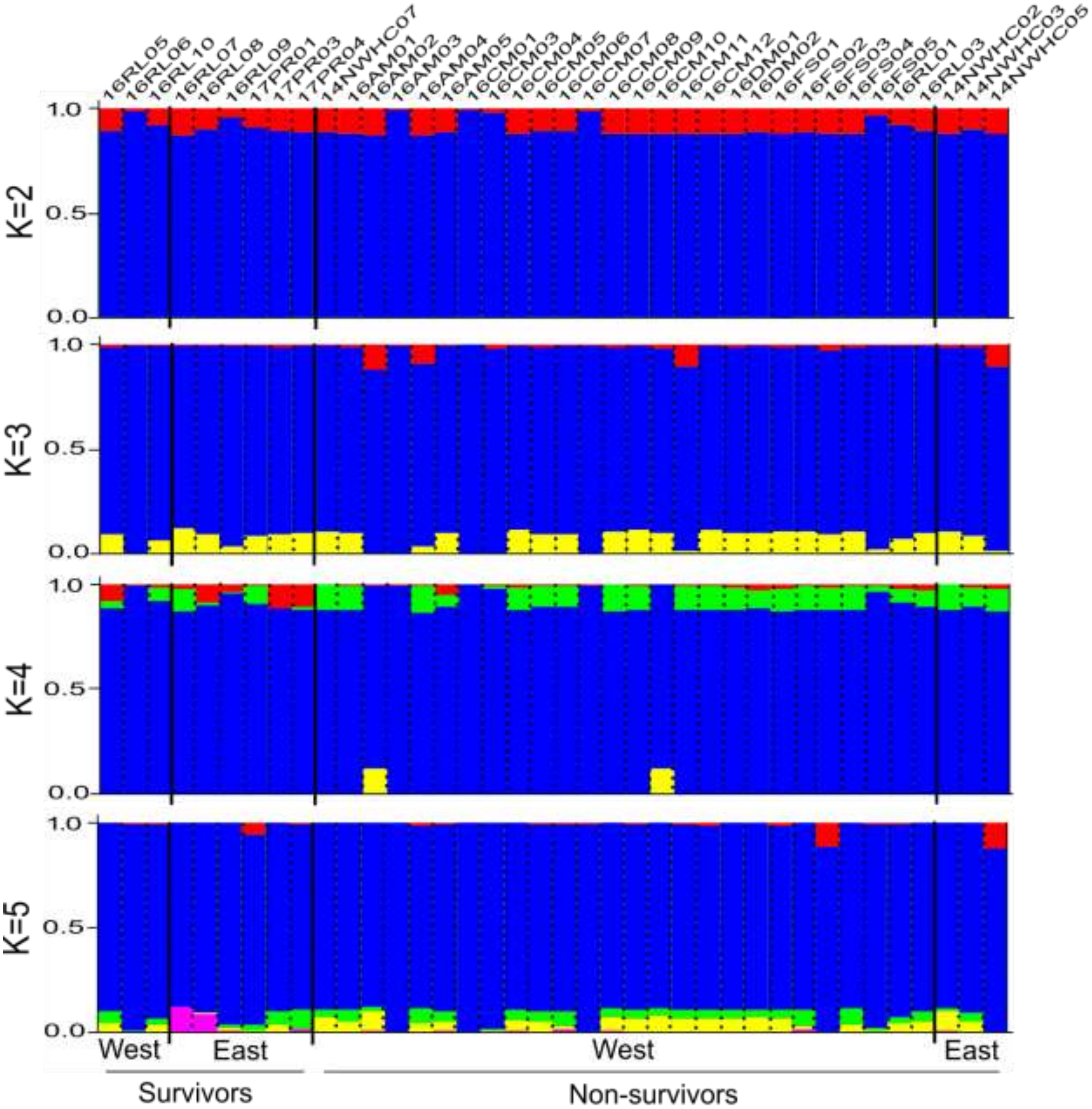


Fig. S8. Assessment of population structure in sampled bats. A single genetic group is suggested by STRUCTURE[37], with no evidence of multiple ancestral source populations for different geographic areas. Results for the different *k*-genetic clusters are shown, where each individual (separated by dashed black lines) is represented by a bar and inferred ancestry (posterior probabilities of different ancestral makeups) is represented by different colors. Individuals are grouped by survivors (left) or non-surviving bats (right) and the geographic sampling region (West or East) are labeled. Sample labels (top) correspond to Table A-S1.

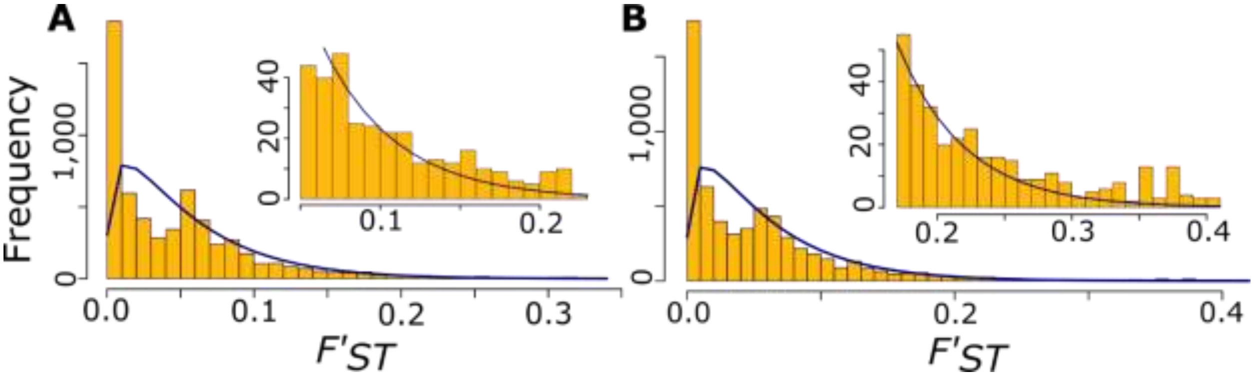


Fig. A-S9. Evaluation of fit for OutFLANK models. Evaluations of the fit of the chi-squared distribution to the distribution of F'_{ST} values for purportedly neutral alleles in our dataset in OutFLANK[90] for (A) all individuals and (B) excluding individuals sampled in 2014. Insets show a focus on the fit on the right tail of each distribution, which is the main concern (Whitlock & Lotterhos, 2015)—F'_{ST} values beyond this are considered outliers.

Appendix B

Supplementary Notes, Tables, and Figures for Chapter III

Vulnerability of coastal giant salamanders (*Dicamptodon tenebrosus*)? The threats that may obscure effects of land-use, fire frequency, or river drainage effects on genetic variation

Results excluding locus D6

Because locus D6 did not pass the test for Hardy Weinberg Equilibrium, results without the locus are presented here. These results do not differ substantially from those presented in the main manuscript. Pairwise F_{ST} between sites ranged from 0.0 to 0.28. The PCA and STRUCTURE (Falush et al., 2003; Pritchard et al., 2000) results did not differ substantially, with the former still showing reduced genetic in northern populations and the latter still supporting $K = 4$, generally divided by region. Similarly, IBD was still significant across regions ($P \approx 0.002$), but not within regions ($P = 0.888$), and water catchments were not significantly related to IBD. When residuals were considered in the context of the five different watershed delimitation methods, watersheds separated by category 1 (large) streams was still significant ($P = 0.022$) before, but not after Bonferroni correction for multiple comparisons. Another watershed classification (drainage basin) was significant, but the trend was in the opposite direction we would expect if different drainages led to higher-than-expected F_{ST} between sites (both residuals and F_{ST} tended to be higher if sites were sampled in the same Drainage River match).

B-S1 Table. Summary statistics of the five loci used in this study. Loci (far left column) are summarized per sampled site (1–23). The summary statistics described are number of individuals with information on that locus (*n*), observed heterozygosity (H_o), expected heterozygosity (H_e), expected heterozygosity under Hardy-Weinberg equilibrium (HWE), the number of alleles (NA), the allelic richness (AR), and inbreeding coefficient (F_{IS}).

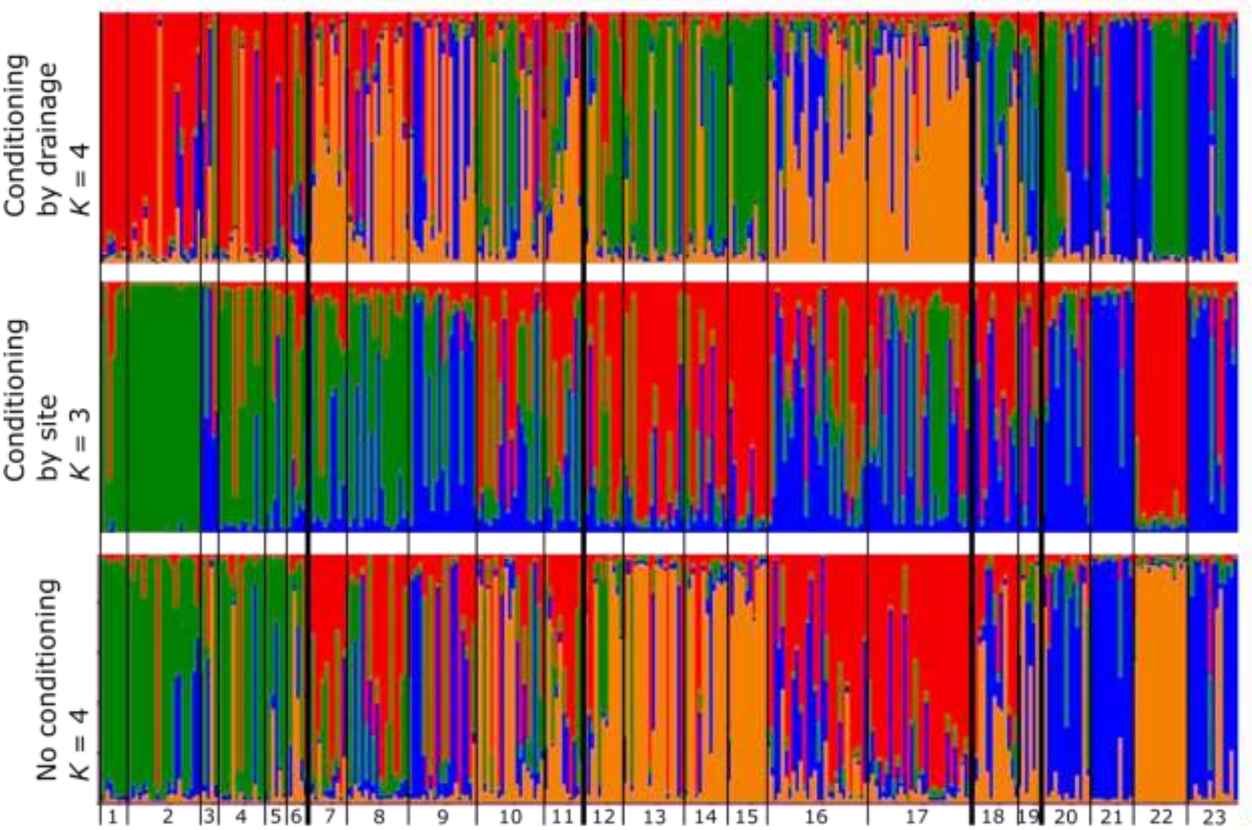
		Region A						**Region B**					**Region C**						**Region D**		**Region E**			
Loc		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**
D6	n	8	18	5	13	6	4	10	17	19	18	11	11	17	11	11	25	29	13	6	14	12	15	15
	H_o	0.13	0.00	0.00	0.15	0.17	0.00	0.40	0.41	0.32	0.39	0.36	0.36	0.41	0.36	0.55	0.44	0.21	0.62	0.50	0.50	0.33	0.93	0.53
	H_e	0.86	0.88	0.90	0.88	0.87	0.83	0.81	0.80	0.78	0.91	0.90	0.90	0.92	0.84	0.77	0.72	0.64	0.79	0.92	0.89	0.85	0.87	0.85
	HWE	0.75	0.82	0.73	0.81	0.79	0.64	0.73	0.70	0.75	0.88	0.85	0.82	0.89	0.88	0.82	0.74	0.77	0.77	0.89	0.81	0.78	0.83	0.83
	NA	5	8	4	7	5	3	5	5	6	11	8	7	11	9	7	6	7	6	7	7	6	8	8
	AR	3.71	4.27	3.60	4.17	3.81	2.93	3.60	3.64	3.58	4.68	4.42	4.32	4.77	4.09	3.67	3.33	2.87	3.68	4.52	4.33	3.97	4.40	4.13
	F_{IS}	0.85	1.00	1.00	0.82	0.81	1.00	0.50	0.49	0.60	0.57	0.60	0.59	0.55	0.57	0.29	0.39	0.68	0.22	0.46	0.44	0.61	-0.07	0.37
D14	n	8	12	3	13	5	5	9	17	19	19	11	11	17	12	11	27	29	13	6	14	12	15	15
	H_o	0.75	0.92	1.00	0.69	0.60	0.60	0.78	0.77	0.63	0.84	0.82	0.82	0.88	0.92	0.73	0.78	0.90	1.00	0.50	0.64	0.58	0.60	0.73
	H_e	0.96	0.89	0.92	0.93	1.00	0.78	0.93	0.92	0.87	0.91	0.91	0.86	0.88	0.75	0.59	0.83	0.92	0.89	0.85	0.90	0.81	0.73	0.91
	HWE	0.93	0.87	0.93	0.93	0.95	0.82	0.92	0.91	0.87	0.90	0.92	0.85	0.89	0.82	0.78	0.87	0.91	0.92	0.85	0.88	0.72	0.76	0.89
	NA	10	9	5	13	8	5	10	13	10	12	11	8	11	7	6	11	15	12	6	10	5	6	11
	AR	5.16	4.63	5.00	4.95	5.33	3.67	4.94	4.86	4.38	4.77	4.82	4.25	4.56	3.69	2.99	4.05	4.95	4.75	4.04	4.57	3.66	3.42	4.71
	F_{IS}	0.22	-0.03	-0.09	0.26	0.40	0.23	0.16	0.17	0.28	0.07	0.10	0.04	0.00	-0.22	-0.24	0.06	0.03	-0.12	0.41	0.28	0.28	0.18	0.19
D17	n	8	20	5	13	6	5	11	17	19	19	11	10	17	10	10	23	28	12	4	11	10	13	13
	H_o	0.88	0.55	0.20	0.54	0.50	0.60	0.27	0.35	0.32	0.74	0.82	0.40	0.18	0.60	0.50	0.44	0.64	0.25	0.75	0.55	0.40	0.23	0.15
	H_e	0.80	0.80	0.80	0.79	0.58	0.68	0.69	0.82	0.82	0.86	0.87	0.88	0.81	0.86	0.92	0.89	0.85	0.83	0.92	0.93	0.89	0.91	0.87
	HWE	0.75	0.74	0.73	0.77	0.79	0.82	0.73	0.75	0.75	0.84	0.85	0.83	0.79	0.83	0.89	0.84	0.87	0.78	0.86	0.88	0.86	0.84	0.77
	NA	5	6	4	6	5	5	5	6	6	9	8	7	7	7	9	9	11	6	5	9	8	8	6
	AR	3.71	3.72	3.33	3.62	3.00	3.40	3.14	3.72	3.79	4.26	4.40	4.22	3.73	4.18	4.67	4.46	4.20	3.74	4.39	4.76	4.39	4.43	4.04
	F_{IS}	-0.09	0.31	0.75	0.31	0.14	0.11	0.61	0.57	0.61	0.14	0.06	0.55	0.78	0.30	0.46	0.51	0.24	0.70	0.18	0.41	0.55	0.75	0.82
Dte 11	n	8	20	5	13	6	5	11	17	19	19	10	11	17	12	10	28	29	13	6	13	12	15	14
	H_o	0.13	0.75	0.40	0.54	0.67	0.60	Monomorphic	0.41	0.11	0.53	0.10	0.36	0.65	0.17	0.10	0.04	0.35	Monomorphic	Monomorphic	0.62	0.67	0.40	0.50
	H_e	0.34	0.67	0.55	0.58	0.68	0.60		0.47	0.20	0.54	0.10	0.31	0.45	0.40	0.10	0.07	0.38			0.71	0.76	0.57	0.82
	HWE	0.33	0.48	0.38	0.51	0.58	0.60		0.49	0.48	0.61	0.31	0.31	0.28	0.30	0.31	0.47	0.46			0.63	0.72	0.62	0.71
	NA	2	3	2	3	3	3		3	3	4	2	2	2	2	2	3	3			4	5	4	5
	AR	1.79	2.75	2.00	2.48	2.76	2.57		2.29	1.57	2.61	1.30	1.75	1.93	1.86	1.30	1.21	2.05			3.05	3.47	2.35	3.76
	F_{IS}	0.63	-0.12	0.27	0.08	0.02	0.00		0.13	0.47	0.03	0.00	-0.18	-0.46	0.59	0.00	0.50	0.09			0.13	0.12	0.29	0.39
Dte 14	n	8	18	5	13	6	6	11	17	19	19	11	10	17	12	5	18	18	13	6	13	6	15	10
	H_o	0.00	0.11	0.40	0.00	0.00	0.17	0.00	0.18	0.05	0.42	0.82	0.40	0.18	0.17	0.00	0.33	0.17	0.39	1.00	0.46	0.17	0.47	0.30
	H_e	0.25	0.46	0.38	0.28	0.53	0.57	0.47	0.49	0.60	0.72	0.59	0.65	0.41	0.71	0.70	0.54	0.50	0.77	0.75	0.66	0.47	0.61	0.71
	HWE	0.33	0.49	0.60	0.29	0.36	0.36	0.52	0.49	0.49	0.61	0.52	0.53	0.61	0.52	0.60	0.49	0.49	0.71	0.71	0.51	0.58	0.50	0.65
	NA	2	3	3	2	2	2	3	3	3	4	3	3	4	3	3	3	3	5	4	3	3	3	4
	AR	1.63	2.05	2.20	1.68	1.97	1.99	2.23	2.19	2.38	3.06	2.45	2.63	2.15	2.78	2.73	2.14	2.10	3.39	3.35	2.71	2.27	2.60	2.93
	F_{IS}	1.00	0.76	-0.07	1.00	1.00	0.71	1.00	0.64	0.91	0.41	-0.39	0.39	0.57	0.76	1.00	0.39	0.67	0.50	-0.33	0.30	0.64	0.23	0.58

B-S2 Table. Corresponding hydrology information for sampled sites (1–23). These include corresponding watershed units: (HUs 08, 10, and 12; U.S. Geological Survey, 2001), drainage basin, or watershed subdivided by Level 1 streams (Lev1; Esri, 2010). For HUs, numbers correspond to the unique HU identifier codes designated by the U.S. Geological Survey (2001). Numbers associated with "Drainage" and "Lev1" classifications are arbitrary, but similarly intended to show subdivision membership.

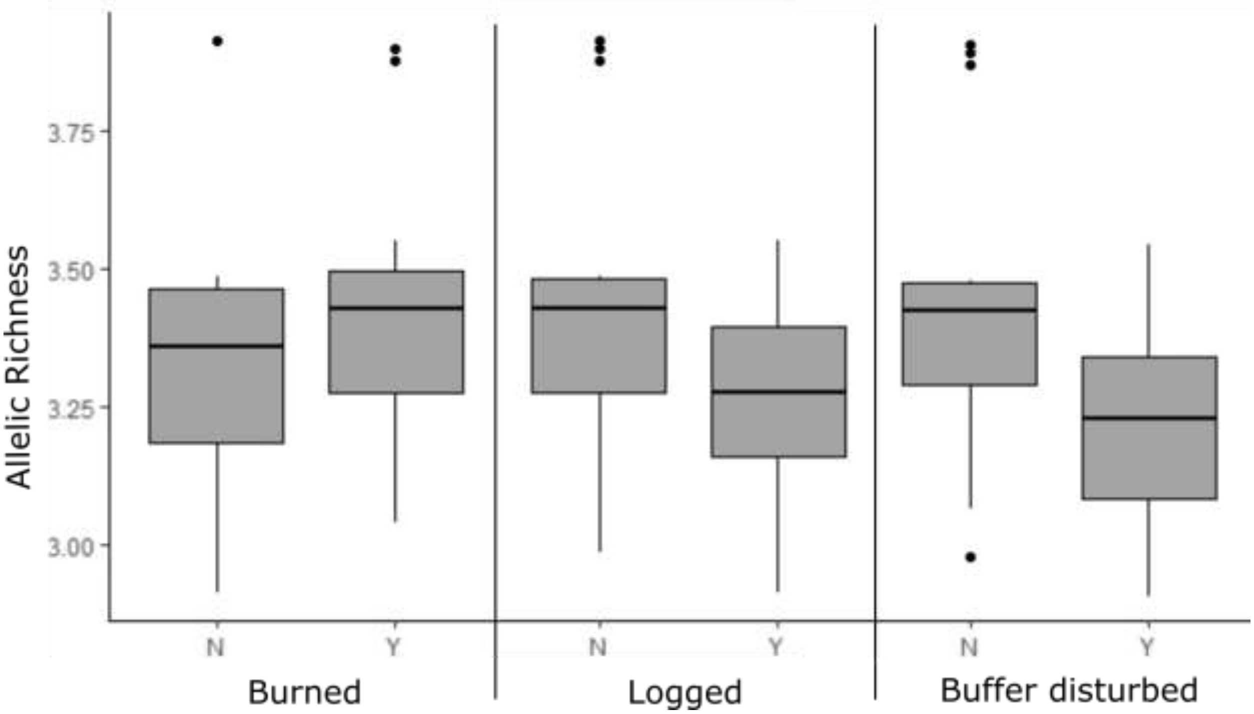
Site	HU08	HU10	HU12	Drainage	Lev1
1	238	2097	9113	2	1
2	238	2097	9113	2	1
3	238	2097	9113	2	1
4	238	2097	9115	2	2
5	238	2097	9115	2	2
6	238	2097	9114	2	1
7	195	1837	11067	3	3
8	195	1837	11067	3	3
9	240	2101	9176	4	4
10	195	1837	11065	3	3
11	238	1817	8416	2	5
12	240	2107	9185	1	6
13	240	2107	9185	4	7
14	240	2107	9185	4	8
15	240	2107	9185	4	9
16	240	2107	9185	4	10
17	240	2107	9185	1	11
18	241	2121	9074	5	12
19	179	2046	10196	6	13
20	180	2049	11268	7	14
21	181	2060	10635	8	15
22	181	2060	10635	8	15
23	180	2049	11266	7	16

B-S3 Table. Genetic diversity metrics for each population and landscape characteristics. For each site (1–23), mean H_o, mean H_e, and mean F_{IS}, and the total number of alleles (NA). Estimates for expected heterozygosity given mutation-drift equilibrium (H_{eq}) and whether the populations has likely experienced a genetic bottleneck (*P*-values) are based on the program BOTTLENECK (Piry et al., 1999). Dashes represent populations which were not evaluated due to low sample sizes, and asterisks denote populations with significant likelihood of having experienced a bottleneck. Also recorded is whether or not (Y/N; yes/no), in the past 70 years, there was evidence of disturbance (Fire or Logging) the forested buffer remained undisturbed (i.e. Y means yes, the buffer remained intact).

Region	Site	H_o	H_e	H_{eq}	*P*	F_{IS}	NA	Fire	Logging	Buffer
A	**1**	0.38	0.62	—	—	0.42	24	N	Y	Y
	2	0.47	0.73	0.68	0.03*	0.37	29	N	N	Y
	3	0.4	0.68	—	—	0.44	18	N	N	Y
	4	0.38	0.68	0.66	0.31	0.44	31	N	Y	N
	5	0.39	0.7	—	—	0.47	23	N	N	Y
	6	0.39	0.65	—	—	0.43	18	N	Y	N
B	**7**	0.29	0.57	—	—	0.5	23	N	N	Y
	8	0.42	0.69	0.67	0.11	0.4	30	N	N	Y
	9	0.28	0.64	0.67	0.31	0.57	28	N	N	N
	10	0.58	0.78	0.77	0.11	0.26	40	Y	N	N
	11	0.58	0.67	0.69	0.41	0.14	32	N	N	Y
C	**12**	0.47	0.71	0.67	0.05*	0.35	27	Y	Y	N
	13	0.46	0.69	0.69	0.59	0.34	35	Y	N	N
	14	0.44	0.7	0.67	0.59	0.38	28	Y	N	N
	15	0.37	0.6	—	—	0.39	27	Y	N	N
	16	0.4	0.61	0.68	0.89	0.34	32	Y	Y	N
	17	0.45	0.65	0.7	0.92	0.31	39	Y	Y	N
D	**18**	0.45	0.65	—	—	0.31	29	Y	Y	N
	19	0.55	0.67	—	—	0.2	22	N	N	Y
E	**20**	0.55	0.8	0.74	0.02*	0.32	33	Y	N	N
	21	0.43	0.74	—	—	0.43	27	Y	Y	N
	22	0.53	0.73	0.71	0.08	0.29	29	Y	N	N
	23	0.44	0.82	0.77	0.02*	0.47	34	N	N	Y



B-S1 Fig. Plot of genetic STRUCTURE results. Individuals (vertical bars) are grouped by population and organized according to sampled site (labels in three horizontal rows; see Fig 1 for geographic details). Results are presented for the most likely number of genetic clusters for each scenario (either $K = 4$ or $K = 3$) as shown by the different colors. The three scenarios include *a prior* categorization of individuals (i.e. conditioning) by (i) watershed (based on HU 08 categorization) and (ii) population, as well as with (iii) no *a priori* information for individuals.



B-S2 Fig. Boxplots of Allelic Richness versus disturbance history. Allelic richness (y-axis) refers to the average allelic richness for each site, and this was compared to whether or not the sites experienced a disturbance event (burning, logging, or having the riparian buffer disturbed by fire or logging) within the last 70 years. Kruskal-Wallis tests (three) did not identify statistically significant effects of these factors on allelic richness.

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