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GENETIC PERSPECTIVES ON BIODIVERSITY IN ROCKY MOUNTAIN ALPINE STREAMS

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GENETIC PERSPECTIVES ON BIODIVERSITY IN ROCKY MOUNTAIN ALPINE STREAMS

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the University of Kentucky

By
Scott Hotaling

Lexington, Kentucky

Director: Dr. David Weisrock, Associate Professor of Biology

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2017

ABSTRACT OF DISSERTATION

GENETIC PERSPECTIVES ON BIODIVERSITY IN ROCKY MOUNTAIN ALPINE STREAMS

In alpine regions worldwide, climate change is dramatically altering ecosystems, affecting biodiversity across habitats and taxonomic scales. For streams, the associated recession of mountain glaciers and snowfields, paired with altered precipitation regimes, are driving shifts in hydrology, species distributions, and basal resources – often threatening the very existence of some habitats and biota. Globally, alpine streams harbor particularly substantial species and genetic diversity due to significant habitat insularity and environmental heterogeneity: however, anthropogenic warming threatens to homogenize habitats through the reduction of the cryosphere, thereby reducing biodiversity from micro- to macroscopic organisms and genes to communities. Still, alpine stream biodiversity, particularly in North America, is poorly understood, making it difficult to predict future changes without baselines for comparison.

For my dissertation, I used genetic tools to assess biodiversity in alpine streams of the central Rocky Mountains in North America. Here, I begin by reviewing the current state of alpine stream biology from an organismal perspective. Next, I provide two perspectives on macroinvertebrate diversity. The first, a population genetic comparison of three highly similar species, is followed by a fine-scale genomic study of one species, *Lednia tumana*. I follow these largely macroinvertebrate-centric chapters with a modern synthesis of the microbial ecology of mountain glacier ecosystems. Finally, I conclude with a study of microbial diversity that addresses how microbial diversity is shaped by geography, habitat, and hydrological source in North America.

Collectively, this research refines existing themes in alpine stream biology by revealing unexpected differences in population genetic patterns among closely related species, the influence of recent deglaciation on population genetic structure and demographic history of a threatened stonefly, and clarification of the environmental drivers shaping microbial diversity.

KEYWORDS: population genetics, global change, *Lednia tumana*, microbial ecology, demography

Scott Hotaling

July 7, 2017

GENETIC PERSPECTIVES ON BIODIVERSITY IN ROCKY MOUNTAIN ALPINE STREAMS

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Outside of my home department, I've been lucky to cross paths with many wonderful collaborators, and I would like to specifically acknowledge Joe Giersch, Deb

Finn, Lusha Tronstad, Lydia Zeglin, and Clint Muhlfeld. These five have acted, in many ways, like a second set of mentors guiding me through both the wilderness of professional science including the many nuances of collecting and analyzing data, as well as articulating my findings. I'm lucky to call them friends as well as collaborators.

It's impossible to acknowledge my family and friends to the degree that's equal to their importance in the completion of this dissertation. Above all, and perhaps more deserving of appreciation than anyone is my mother, Lynn Hotaling, who has read more of my academic writing – in its best and worst forms – than anyone ever should. She is also the reason why I'm writing this dissertation, as she cultivated a love of the natural world in me that runs as deeply as any other thread in my life. She always encouraged me to explore, be curious, and to pursue things I felt passionately about, whatever they wound up be being. Hiking to the Grinnell Glacier with her and my father, Richard Hotaling, in an early September snowstorm will always be a highlight of my life. Alongside my mother, my father, sisters – Elizabeth Hotaling and Ellen Girardi, and nephew, Jacob "Bobcat" Cline – have been immeasurable sources of support when I've needed it most. Many friends, both scientists and otherwise, have been important for a much needed laugh, opinion, or a supportive ear during this process (listed alphabetically) – Robin Bagley, Shishir Biswas, Jesse Brasher, Vincent Cassone, Jeremy Van Cleve, Varun Dwaraka, Christopher Eckert, Mary Foley, Tom Gawriluk, Delia Rose Gibbs, Danielle Herrig, Anna Hess, Paul Hime, Rachel Holsinger, Andrew Hope, Nikki Lawrence, Melissa Keinath, Taylor Kessinger, Justin Kratovil, Karla Lightfield, Dave Lytle, Rose Marks, Luke Moe, Taylor Moody, Mason Murphy, Schyler Nunziata, Susan Odom, Erin Richard, Jessica Santollo, Ashley Seifert, Tim Salzman, Jim Shafer, Jennifer

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While this collective work is largely my own, it would not have reached its present quality (or possibly even existence) without the input of many co-authors and collaborators. To this end, I would like to specifically acknowledge my co-authors on each chapter. For the first review chapter, what began as a discussion between sessions at the 2014 Joint Aquatic Sciences Meeting in Portland, Oregon, quickly transformed into a high-quality synthesis of alpine stream biology to which Deb Finn, Dean Jacobsen, Joe Giersch, and David Weisrock all contributed substantially. For chapter two, Deb Finn, Lusha Tronstad, Joe Giersch, and David Weisrock provided an array of input regarding our experimental design, analyses, and writing. For the third chapter, I was aided in data collection, analyses, and writing by Clint Muhlfeld, Joe Giersch, Omar Ali, Steve Jordan, Gordon Luikart, Michael Miller, and David Weisrock. Chapter four stemmed from a discussion between Trinity Hamilton and me that occurred when she gave a departmental seminar at the University of Kentucky in 2015. As the review began to develop, we included Eran Hood, who brought a timely source of creativity and biogeochemical expertise that greatly strengthened the final product. Chapter five was enhanced by efforts in the field and laboratory by Mary Foley, Lydia Zeglin, Lusha Tronstad, Joe Giersch, David Weisrock and the entire BIO198 independent research class from Spring 2016, who helped to generate and analyze the data.

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CHAPTER ONE

CLIMATE CHANGE AND ALPINE STREAM BIOLOGY: PROGRESS, CHALLENGES, AND OPPORTUNITIES FOR THE FUTURE

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Introduction

Alpine streams are often remote and represent some of the world's most pristine freshwater ecosystems, primarily due to limited anthropogenic impacts. Nevertheless, the highest rates of climate change are occurring above the permanent treeline in alpine and arctic ecosystems (Bradley *et al.*, 2006; Prowse *et al.*, 2006). Alpine biota are particularly vulnerable to rapid environmental warming due to the combined effects of high mountaintop insularity and upslope shifts of distributional ranges into increasingly smaller areas of suitable habitat (e.g., Walther *et al.*, 2005; Galbreath *et al.*, 2009; Rubidge *et al.*, 2012). Climate change is also substantially shrinking alpine glacier and snowfield mass (Hall & Fagre, 2003; Hansen *et al.*, 2005; Rauscher *et al.*, 2008; Pederson *et al.*, 2010), resulting in hydrologic shifts in existing streams (Milner *et al.*, 2009; Jacobsen *et al.*, 2014b) and new freshwater habitat taking the place of once-perennial ice (e.g., Finn *et al.*, 2010). Furthermore, the upslope advance of treeline into previously alpine habitat is likely to significantly affect the basal resources of aquatic food webs (Hauer *et al.*, 1997; Hood et al., 2015). Alpine streams in particular are important strongholds for biodiversity and production of food web subsidies in the comparatively harsh terrestrial environment. However, global alpine stream biodiversity is being negatively impacted across multiple levels of taxonomic resolution (Jacobsen *et al.*, 2012; Wilhelm *et al.*, 2013; Finn *et al.*, 2014).

Alpine streams are highly environmentally heterogeneous, even across small spatial extents (<1 km), primarily due to variation in hydrologic source contributions, including glacier melt, snowmelt and rain run-off, groundwater springs, and others. Each source type results in a unique signature of stream flow, temperature, sediment load, and chemistry (Ward, 1994), although individual sources rarely act in isolation, especially when seasonal melting is occurring (Füreder *et al.*, 2001; Smith *et al.*, 2001; Brown *et al.*, 2003). Therefore, alpine stream networks are habitat mosaics harboring significant beta diversity (differentiation among sites) both in terms of species diversity (Finn & Poff, 2005; Brown *et al.*, 2007a; Jacobsen *et al.*, 2012; Kubo *et al.*, 2013) and genetic diversity (Finn *et al.*, 2013; Finn *et al.*, 2014; Leys *et al.*, 2016; Leys *et al.*, 2017). Many alpine stream species are uniquely adapted to cold, harsh conditions (Lencioni *et al.*, 2009; Lencioni & Bernabò, 2015) and often endemic (Finn & Poff, 2008; Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016).

Diminishing hydrologic influence of glaciers and permanent snowfields is expected to ultimately result in environmental homogenization of alpine streams. Predicted biological effects at the regional scale include increased local (α) diversity as more-diverse assemblages shift upstream, but decreased among-stream (β) diversity as meltwater-specific assemblages are lost (Jacobsen *et al.*, 2012). The predicted erosion of regional-scale (γ) biodiversity associated with the homogenization of alpine stream habitat conditions includes both eukaryotes and prokaryotes (Wilhelm *et al.*, 2013), with associated negative implications for microbially mediated ecosystem function. And, although accelerated melting of the alpine cryosphere may initially benefit cold-adapted stream organisms as increased meltwater volume pushes harsh conditions farther

downstream (Jacobsen *et al.*, 2014b), these conditions will be short-lived as meltwater sources eventually dwindle (Jacobsen *et al.*, 2012).

Generally, species at risk of extirpation under changing environmental conditions have three options to persist: migrate to more suitable habitat, leverage a plastic response to the changing environment, or adapt (Hoffmann & Sgrö, 2011; Pauls *et al.*, 2013). For dispersal-limited alpine taxa, migration is an unlikely solution as species become caught in 'summit traps' at the upper, isolated end of available habitat (Pertoldi & Bach, 2007; Sheldon, 2012; Phillipsen & Lytle, 2013). As life history responses tend to be plastic in stream insects, an *in situ* plastic response to environmental change may be the most likely mechanism for population and species persistence, relative to long-distance migration or adaptation, at least in the short term (Treanor *et al.*, 2013; Lencioni & Bernabò, 2015; Madsen *et al.*, 2015). Furthermore, the potential for adaptation of alpine populations to changing conditions will depend upon standing genetic variation in genes that are relevant to the specific environmental changes (Barrett *et al.*, 2008; Hohenlohe *et al.*, 2010). Ultimately, local extinctions at the scale of individual streams can be expected for dispersal-limited taxa that are restricted to cold, meltwater-influenced alpine streams (Giersch *et al.*, 2015). However, with better understanding of both species' adaptive potential and the distribution of stream types (and associated environmental characteristics) that are expected to be more resistant to the effects of climate change, management strategies aimed at preventing extinctions may be possible.

A rich early history in alpine stream biology spanned multiple continents and emphasized the relationship between species assemblages and environmental conditions (Steinmann, 1907; Thienemann, 1912; Dodds & Hisaw, 1925; Dorier, 1937; Leger, 1937;

Mathews, 1951; Martinelli, 1959). The field began to develop substantially following reviews by Ward (1994) on the environmental and biological heterogeneity of alpine streams, and Milner and Petts (1994), who developed a conceptual model of the influence of glacier meltwater on stream biology in both high altitudes and latitudes. Because the field has continued to grow rapidly – potentially exceeded in pace only by the rate of climate change itself – specific topical reviews have appeared relatively regularly in the intervening two decades. These include a review of the sensitivity of alpine streams to various human impacts (McGregor *et al.*, 1995), revisits of the Milner and Petts (1994) model of glacier-melt influence (Brittain & Milner, 2001; Milner *et al.*, 2016), a review of our understanding of biological responses to hydrologic change in glacier-influenced streams (Milner *et al.*, 2009), and a summary of primarily biogeochemical effects of meltwater hydrology in alpine lakes and streams (Slemmons *et al.*, 2013).

Given the exceptionally high natural biodiversity contributed by flowing water habitat to alpine regions and the vulnerability of these systems to rapid environmental change, episodic reviews of the state of the science are essential. Here, for the first time since Ward (1994), we review the current state and future promise of organism-focused research in alpine streams, including all high-altitude lotic systems both with and without the presence of glaciers. We define 'organism-focused' as any research where the taxonomic identity of individual specimens is essential, including intraspecific genetic diversity, microbial community diversity, ecological responses of single species, and various ways of measuring eukaryotic community diversity. We build significantly on previous reviews that have emphasized glacier-influenced hydrology and macroinvertebrate species assemblages (Milner & Petts, 1994; Brittain & Milner, 2001;

Milner *et al.*, 2009). Our organism-focused review also complements the recent ecosystem-focused review of Slemmons *et al.* (2013). We begin with a summary of seven major approaches historically and currently applied to organism-focused ecology of alpine streams, including both long-standing, standard approaches and emerging approaches based on either newly developed methodological tools or novel concepts. We then take a forward-looking perspective on how alpine stream research could be bolstered through the thoughtful integration of long-standing approaches with emerging concepts and technologies, improved and more-standardized monitoring of alpine stream ecosystems on a global scale, and increased collaboration across disciplines (e.g., remote sensing and organismal biology). Ultimately, our aim is to provide a unified front to aid alpine stream biologists in overcoming conceptual and methodological hurdles in the field, while also confronting the pressing challenge of understanding the implications of rapid global change on sensitive alpine stream ecosystems in real time.

Current approaches

Linking organisms to environment

Alpine streams are biologically diverse due to their significant environmental heterogeneity and relative isolation, which limits the distribution of specialized taxa and promotes endemism (Brown *et al.*, 2007b; Füreder, 2007). Early research provided valuable descriptions of the spatial heterogeneity of alpine streams and their species assemblages (Ward, 1986), and this observational foundation linking organisms to environment has continued to expand in recent years (Muhlfeld *et al.*, 2011; Kubo *et al.*, 2013; Thompson *et al.*, 2013; Laursen *et al.*, 2015; Giersch *et al.*, 2016; Tronstad *et al.*,

2016). Generally, these studies integrate taxonomy and environmental parameters to describe patterns of species diversity with habitat. This approach has resulted in a global perspective on the environmental drivers of alpine stream biodiversity – as influenced both by local environmental filtering across the variety of alpine stream types and by limited dispersal among isolated alpine areas – and provided a plethora of snapshots to which future observational data can be compared. Much of the historical effort applying this standard, observational approach was focused in Europe, but alpine stream research on other continents has also been represented to varying degrees with North and South America receiving considerable recent attention (Figure 1.1). Still, many alpine regions – including mountain ranges within comparatively well-studied continents – have been understudied or overlooked.

Inferences about the effects of rapid environmental change on alpine stream biodiversity within this observational approach rely on a space-for-time framework, in which a gradient of spatial conditions is expected to represent the temporal trajectory of environmental change. Because a significant impact of climate change in alpine streams is the ongoing decline of glacier and snowfield mass (Oerlemans, 2005; Jacob *et al.*, 2012), many studies have emphasized biological responses to spatial gradients of hydrologic conditions, typically from glacier-fed to groundwater-dominated stream reaches (Milner *et al.*, 2008; Milner *et al.*, 2009; Finn *et al.*, 2010; Jacobsen *et al.*, 2012; Finn *et al.*, 2013; Cauvy-Fraunié *et al.*, 2014; Finn *et al.*, 2014). Various indices have been developed to quantify the proportional influence of glacier meltwater on local stream environments and biota (e.g., Ilg & Castella, 2006; Brown *et al.*, 2007b; Jacobsen & Dangles, 2012); reviewed by (Frenierre & Mark, 2014), and implementations of the

space-for-time approach in different alpine regions often support a general conclusion: that decline in meltwater conditions will likely decrease regional biodiversity (Figure 1.2).

Space-for-time studies also provide important starting points for identifying regions, stream types, or taxa most at risk from climate change, but correlations between extant biological diversity and current environmental conditions have limitations particularly when projecting future changes to single localities. They do not consider: changes in assemblage structure that could alter biotic interactions through time (Brown & Milner, 2012; Clitherow *et al.*, 2013; Khamis *et al.*, 2015); the adaptive potential of species to changing conditions (e.g., Barrett & Schluter, 2008; Hohenlohe *et al.*, 2010); or how changes in specific environmental factors might differentially influence altitudinal distributions of individual species (Jacobsen, 2008; Loayza-Muro *et al.*, 2013; Giersch *et al.*, 2015). Furthermore, many space-for-time predictions are based on comparisons between extant meltwater-fed and non-meltwater-fed (e.g., groundwater) streams. As such, they tend to not consider the potential for meltwater streams to become seasonally intermittent, a possibly fatal flaw considering that little is known about how dwindling meltwater sources influence groundwater aquifers in alpine regions (Haldorsen & Heim, 1999). Certainly, a complete disappearance of permanent stream habitat can be expected to have substantially greater impacts on biodiversity than, for example, a meltwater habitat transitioning to groundwater-fed habitat.

Tracking and modeling temporal change

Studies that resample the same locations through time or use historical occurrence data can demonstrate explicit biotic responses to environmental change and provide empirical data for testing model-based predictions of species occurrences and range shifts. For example, Sheldon (2012) collected two species of stoneflies along an elevation and stream-size gradient in the Great Smoky Mountains in both 1977–78 and 2006 to assess the magnitude of upstream range shifts in response to climate change in the region. With known rates of warming for the area (~0.72°C over the study period), these data provided an empirical assessment of a general model-based prediction (+11 m/decade) of upslope shifts for biotic assemblages (Chen *et al.*, 2011). Results revealed differential responses of the two study species, with evidence for an uphill shift in one but not the other, suggesting that factors other than water temperature influence elevational distributions of stream-dwelling species in the Great Smoky Mountains (Sheldon, 2012). In a similar example from alpine streams, Giersch *et al.* (2015) combined contemporary sampling of an alpine stonefly, *Zapada glacier*, with historical records and a known temperature increase in Glacier National Park (0.67–1°C) over a 52-year study period (1960–2012). The results pointed to a climate-change-induced range contraction of *Z. glacier* into the uppermost limits of the streams where it historically occurred. With alpine stream monitoring efforts becoming more common, exciting opportunities exist for resampling prior snapshots to analyze the rate and nature of single-site temporal change.

Glacier recession can also unveil virgin stream channels, providing an opportunity for temporal studies of colonization and succession in glacier-fed stream environments. Milner *et al.* (2008) monitored colonization and succession of an Alaskan stream

community over nearly three decades, and (Finn *et al.*, 2010) assessed the impact of rapid glacial recession over a 10-year period on macroinvertebrates and environmental features along an alpine stream gradient in the Swiss Alps. Both studies generally corroborated inferences from space-for-time research, showing that newly exposed and early successional glacier-fed stream habitats supported assemblages of cold-hardy species that likely colonized these habitats from downstream reaches with recently reduced meltwater influence. However, Finn *et al.* (2010) also demonstrated that the rate of temperature increase with stream distance below a glacial source had significantly steepened over a single decade, an observation that would not have been predicted in a space-for-time framework.

Beyond retrospective empirical studies, species distribution models (SDMs) predict future distributions by integrating occurrence data and associated environmental factors, and projecting these into the future under specific scenarios (e.g., climate change models). SDMs have become essential tools in conservation biology (Elith & Leathwick, 2009) but are currently underrepresented in alpine streams [but see Bálint *et al.* (2011), Muhlfeld *et al.* (2011), and Giersch *et al.* (2015)]. Although limited, these studies predict significant threats to alpine biodiversity due to loss of glaciers (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2015) or more general mountaintop habitat loss (Bálint *et al.*, 2011). Alpine stream biology is poised for increased application of SDMs to assess future threats to biodiversity, perhaps with a focus on developing implementations that account for more alpine-specific changes (e.g., shifts in meltwater influence and/or new stream habitat being uncovered as glaciers recede).

Predictive modeling has also been used to identify ecological tipping points in glacial streams that represent a point at which cold, stenothermic species assemblages may be extirpated. For instance, a Threshold Indicator of Taxa Analysis (TITAN; Baker & King, 2010) identified thresholds of < 5.1% glacier cover and < 66.6% meltwater contribution as tipping points where more generalist macroinvertebrates would begin to replace cold-adapted specialists in Pyrenean alpine stream communities (Khamis *et al.*, 2014). When viewed in isolation, it is impossible to make predictions about how strongly these results apply to other glaciated alpine regions, but this type of region-specific predictive modeling provides a valuable glimpse into potential points-of-no-return for alpine stream diversity. Future assessments carried out with the same methodology in additional regions hold the potential for clarifying alpine stream ecological tipping points from a global perspective.

Characterizing microbial diversity and function

Alpine glaciers and snowfields, and the extreme cryophilic habitat they represent, provide habitat for diverse microbial communities, including on the surface of glaciers (Anesio & Laybourn-Parry, 2012), below glaciers (Telling *et al.*, 2015), in meltwater streams (Wilhelm *et al.*, 2013), and the associated stream sediments (Fegel *et al.*, 2016). Until recently, microbial biodiversity and function in alpine headwaters had been largely unexplored. This disconnect is particularly noteworthy considering that stream microbial communities have been widely recognized for their general importance to biodiversity, ecosystem processes, and biogeochemistry (Zeglin, 2015; Battin *et al.*, 2016). Microbial biofilms in particular alter physical and chemical microhabitats, acting as living zones of

transient organic molecule storage. Moreover, because local-scale diversity of multicellular organisms is often relatively low in alpine streams, diverse microbial communities could play a disproportionate role in the ecology of alpine streams.

Structure and function of alpine stream microbial communities vary depending upon hydrology (Freimann *et al.*, 2013; Wilhelm *et al.*, 2013; Freimann *et al.*, 2014; Wilhelm *et al.*, 2014) and local habitat, whether streamwater, biofilm, sediments, or glacial snow and ice (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016). In Swiss alpine floodplains, microbial community structure and enzymatic function are influenced by sediment pH, conductivity, and other physicochemical conditions affected by the presence of glacial meltwater (Freimann *et al.*, 2013, 2014). To understand the connection between glacial ice and downstream microbial diversity better, (Wilhelm *et al.*, 2013) characterized 16S rDNA diversity of microorganisms in streamwater, biofilm, and source glacial ice for 26 glacier-fed streams in the Austrian Alps (Figure 1.3). This approach revealed that glacier retreat is likely to increase within-stream microbial α diversity while reducing among-stream β diversity, a pattern similar to that predicted for alpine macroinvertebrates as local environmental conditions become less harsh but more homogeneous (Jacobsen & Dangles, 2012; Jacobsen *et al.*, 2012). In a subsequent study, Wilhelm *et al.* (2014) used an RNA sequencing approach to compare the abundances of RNA (the product of cellular processes) to DNA for microbial life in the same Austrian streams. Their findings revealed that rare taxa (from a total DNA perspective; e.g., Wilhelm *et al.*, 2013) play a disproportionate role in microbial community dynamics of alpine glacier-fed streams (Wilhelm *et al.*, 2014). By comparing abundance of RNA *versus* DNA, it is possible to move beyond descriptions of biodiversity patterns to

understand the activity of microbial life in alpine streams better and identify which taxa are most important under a given set of sampling conditions (e.g., time of day, season, flow, etc.). Given the role of microbial life in dictating biogeochemical processes [e.g., carbon fixation (Singer *et al.*, 2012) or nitrogen cycling (Dodds & Smith, 2016), any clarification of microbial diversity and activity, as well as the environmental conditions both are linked to, will greatly improve understanding of how alpine stream ecosystems function.

A warming climate is also expected to affect basal resources (and microbial dynamics) in alpine streams, particularly in terms of organic carbon (OC), as glaciers recede, treelines rise, and stream energy inputs shift (Hood et al., 2015; Wilhelm *et al.*, 2015). Mountain glaciers store considerable OC, primarily within englacial (the glacial core where light does not penetrate) and basal ice (Hood et al., 2015). However, the implications of the accelerated release of this glacially derived OC on downstream ecosystems remains unclear (Hood et al., 2015), although links between glacially derived OC, microorganisms, macroinvertebrates, and fish have been made in Alaskan streams (Fellman *et al.*, 2015). This is particularly important for heterotrophic microbial communities as glacial OC is significantly more biologically available than dissolved OC from other inputs (e.g., vascular plants, Singer *et al.*, 2012). A slowly climbing treeline is also relevant to the availability of OC in alpine streams primarily because trees add an additional input of allocthonous dissolved OC. Below treeline, specialization by biofilm bacteria in response to more diverse allochthonous and authochthonous dissolved OC has been hypothesized to drive shifts from more-generalist biofilm communities (in terms of functional traits related to the utilization of resources) above treeline to specialist-rich

communities below. In a study of three alpine streams in the Swiss Alps, this hypothesis was partially supported with generalist microbiota dominating biofilm communities along an altitudinal gradient (including above and below treeline), and specialists gaining importance with increasing distance downstream of the treeline (Wilhelm *et al.*, 2015). Furthermore, questions of treeline shifts, energy inputs, and available OC may be further complicated in alpine streams by seasonality as energy inputs vary with magnitude of glacial discharge (Fenoglio *et al.*, 2014).

Life-history response to changing environments

Like many aspects of alpine stream biology, life histories of alpine macroinvertebrates are poorly understood. In the context of environmental change, population persistence could depend on the potential for life-history traits (e.g., development rate, emergence timing, size at maturity, and other reproductive traits) to respond rapidly to abiotic change. For aquatic insects, most life-history traits are highly plastic, at least within certain bounds (Vannote & Sweeney, 1980; Newbold *et al.*, 1994). Or, if adaptive, such traits may respond quickly to natural selection (Poff *et al.*, 2006). Hence, if selection pressures in alpine streams naturally vary spatially and/or temporally, resident insects could have a strong capacity to respond through phenotypic plasticity (Vannote & Sweeney, 1980; Stearns, 1989), adaptation (Hynes, 1976; Gray, 1981; Lytle & Schluter, 2001; Lytle & Poff, 2004; Lytle *et al.*, 2008), or a combination of the two.

Diversity in life-history traits may provide the raw material for selection, adaptation, and persistence of species in the face of rapid environmental change (*cf.* Schindler *et al.,* 2010), particularly if the characteristic environmental heterogeneity of

alpine streams across small spatial extents translates to intraspecific diversity in life histories. Indeed, many aquatic insects inhabiting steep temperature gradients (as is common in alpine streams) exhibit life-history variation along the gradient. For example, *Rhyacophila evoluta*, an alpine caddisfly, can enter diapause at any instar, which translates to a one-, two-, or three-year life cycle depending upon thermal conditions (Décamps, 1967). At high elevations, the alpine mayfly *Baetis alpinus* is semivoltine (two-year life cycle), larger at maturity, and females produce more eggs, while at low elevations, it is univoltine (one-year life cycle), smaller at maturity, and females produce on average approximately 25% fewer eggs (Lavandier & Décamps, 1983). Interestingly, *Allogamus uncatus*, another alpine caddisfly, showed life-history patterns more-or-less opposite of expectations in stream reaches 0.9–1.7 km downstream of a glacier (Shama & Robinson, 2009). Individuals closest to the glacier tended to reach pupation more rapidly and be smaller at maturity than those furthest away (Figure 1.4A). However, the study reaches were part of a complex alpine floodplain, and upstream–downstream patterns of environmental variation did not vary as expected with distance from the glacier, likely due to patchy groundwater inputs (Ward & Uehlinger, 2003) and minimal elevation difference (Shama & Robinson, 2009). These results highlight that spatial heterogeneity among alpine streams even over a very small spatial extent can significantly amplify intraspecific life-history variation.

Life-history traits of alpine stream insects might also respond quickly to temporal environmental variation. In a two-year study of insect emergence in a Rocky Mountain alpine stream, Finn and Poff (2008) found that emergence timing of four common species (a caddisfly, a stonefly, and two mayflies) was significantly later in a year following an

above-average winter snowpack, compared to one preceded by a below-average snowpack (Figure 1.4B). Documentation of these temporal differences in the same location suggests phenotypic plasticity, likely in response to degree-day accumulation, which depends heavily on duration of snowpack covering streams. Although largely unexplored, changes in snow accumulation and melt timing likely impact insect emergence, and therefore could affect connectivity among populations. Questions concerning life-history diversity and physiological limits on plasticity and adaptive potential in obligate alpine stream species remain underexplored but should be important foci in future studies addressing the potential for species persistence under rapidly changing conditions.

Population genetics

Population genetics has a rich history informing many aspects of evolutionary and conservation biology, but has been under-represented in alpine streams. To date, genetic studies on alpine stream organisms have focused on estimating population structure, demography, gene flow, and the impacts of glacier recession on intraspecific genetic variation (Monaghan *et al.*, 2001; Monaghan *et al.*, 2002; Finn & Adler, 2006; Bálint *et al.*, 2011; Elbrecht *et al.*, 2014; Finn *et al.*, 2014; Giersch *et al.*, 2015; Finn *et al.*, 2016; Giersch *et al.*, 2016; Jordan *et al.*, 2016). These studies have identified a general trend among alpine stream taxa of relatively high levels of genetic differentiation among populations, indicative of spatial isolation and limited gene flow (Finn *et al.*, 2006), as well as taxon-specific patterns including putative sex-biased dispersal (Elbrecht *et al.*, 2014) and variation in the influence of landscape features on population connectivity

(Geismar *et al.*, 2014). These molecular studies have tended to focus on single species, but some comparisons have been made across related or co-occurring species. For example, a multi-species comparison revealed that differences among species in dispersal behavior appear to be both order-specific (e.g., caddisflies were found to be better dispersers than mayflies) and dependent upon spatial scale (Monaghan *et al.*, 2002). In a study combining population genetics and SDM for nine montane macroinvertebrates, results indicated that loss of genetic diversity under future warming scenarios is predicted greatly to exceed that of more traditional metrics of biodiversity (e.g., morphologically defined species, Figure 1.5; Balint *et al.*, 2011).

An emerging genetic focus is understanding how decreasing habitat heterogeneity associated with glacial recession will impact intraspecific genetic variation. Using a space-for-time approach, Finn *et al.* (2013) classified 18 alpine stream reaches in the French Pyrénées as high-, mid-, or low-'glaciality', according to physicochemical variables linked to meltwater influence (Ilg & Castella, 2006). Population structure of *B. alpinus* was significantly greater among high-glaciality streams (Figure 1.6), indicating that decreasing habitat heterogeneity associated with shrinking glacial influence could lead to reduced regional-scale genetic variation. Furthermore, *B. alpinus* sampled from two recently deglaciated mountain ranges south of the Pyrénées had significantly lower regional-scale genetic diversity than at a similar spatial scale in the still-glaciated Pyrénées (Finn *et al.*, 2014). In another *B. alpinus* study, evidence from both mitochondrial DNA (mtDNA) and microsatellites revealed two distinct cryptic lineages that occurred in sympatry with differentiation between the two seemingly driven by elevation and habitat (one lineage was more abundant in groundwater-fed tributaries

versus glacier-fed streams, Leys *et al.*, 2016). It will be important to monitor and understand how climate-induced environmental homogenization of alpine streams might also erode existing patterns of genetic diversity, particularly given the role of genetic diversity as the template for natural selection.

Moving forward, significant opportunity exists to take advantage of next-generation sequencing (NGS) to address fundamental questions in alpine stream biology through the analysis of genome-scale data. While NGS inquiry is becoming commonplace within biological research, it is still under-represented in freshwater science at large (Pauls *et al.*, 2014), and particularly in the context of alpine streams. NGS data sets allow researchers to investigate the same questions described above but at finer scales and higher resolution while also providing the statistical power to address more complex questions (e.g., selecting models of demographic history, testing for signatures of natural selection). The potential of NGS in alpine stream biology is evidenced by two recent studies. A phylogeographic study of the montane caddisfly *Thremma gallicum* employed thousands of restriction-site-associated DNA sequencing (RADseq; Miller *et al.*, 2012; Andrews *et al.*, 2016) markers to assess models of demographic history and compare results to those inferred using mtDNA data (Macher *et al.*, 2015). The RADseq data had much greater statistical power than the mtDNA data to estimate genetic diversity, and to discern among alternative phylogeographic hypotheses. The second example used genotyping-by-sequencing (another restriction-site-associated method for generating large numbers of anonymous markers, see Elshire *et al.*, 2011) to take a genome-wide perspective on genetic differentiation among co-occurring winged and wingless stonefly species of the genus *Zelandoperla* (Dussex et al., 2016). The

results provided fine-scale evidence of the implications of flight loss on genetic differentiation as wingless populations of *Z. fenestrata* exhibited distinct genetic structure whereas populations of winged *Z. decorata* did not (Dussex *et al.*, 2015). Furthermore, signatures of low levels of hybridization between *Z. fenestrata* and *Z. decorata* were recovered raising questions regarding the fluidity of sympatric species and the possibility of dispersal-related phenotypes introgressing between taxonomically distinct taxa (Dussex *et al.*, 2016). Beyond extensions to both the power and diversity of analyses, NGS data sets also alleviate many limitations of mtDNA markers (e.g., matrilineal inheritance, no recombination), and application of these methods does not depend on previous genomic knowledge of the focal species. For these combined reasons, NGS data sets hold great promise for alpine stream biologists to describe existing genetic patterns better, address evolutionary questions in the field, and refine predictions of how climate change will affect alpine stream taxa.

Characterizing functional traits

Describing biological communities according to functional traits can provide a mechanistic understanding of the relationships between communities and their environment. This approach is used widely in stream ecology (Usseglio-Polatera *et al.*, 2000; Poff *et al.*, 2006) and has clear application to the heterogeneous environments of alpine streams. Rather than taxonomic descriptions, species are assigned traits related to habitat characteristics and environmental response (Lamouroux *et al.*, 2004; Statzner *et al.*, 2004). One requirement for a traits-based approach is that the ecology of the taxa under consideration is relatively well understood. This is a challenge for rare,

understudied alpine stream species that also tend to face atypical environmental filters compared to other stream types (Lencioni, 2004).

In glacier-influenced streams, resilience and resistance traits (e.g., streamlined bodies, high adult mobility, habitat and feeding generalism, clinging behaviour, short life cycles) are common among macroinvertebrates (Snook & Milner, 2002) and provide advantages for coping with harsh conditions (Füreder, 2007). Along gradients of decreasing glacial influence in streams, such coping traits tend to decrease in relative abundance, while others increase (Figure 1.7), with overall trait diversity rising in parallel with taxonomic diversity (Ilg & Castella, 2006; Milner *et al.*, 2009; Brown & Milner, 2012). To this end, the harsh conditions of glacier-fed streams may act as an environmental filter for both taxonomic and functional diversity, but strength of response to changes in glacial run-off appears to be highly taxon-specific (Jacobsen *et al.*, 2014a; Jacobsen *et al.*, 2014b). These differential responses are likely driven by varying types of coping traits among taxa (Füreder, 2007), with some species possessing traits better suited to one change (e.g., decreased suspended sediments), and others possessing traits better suited to a different change (e.g., increased water temperature). Certain ecophysiological traits [e.g., cold hardiness, metabolic performance, tolerance to ultraviolet (UV) radiation] are also likely drivers of species distributions in harsh alpine stream environments; however, the adaptive roles (and related physiology) of these traits have rarely been addressed. For macroinvertebrates in high-Andean streams for example, range shifts to higher elevation in response to warming temperature will likely be accompanied by a decrease in metabolic rate as a result of oxygen limitation (Jacobsen & Brodersen, 2008), or upstream range expansion might be limited by the negative effects

of increasing UV (Loayza-Muro *et al.*, 2013). These types of perspectives linking possible physiological limitations with scenarios of climate-change-induced range shifts can provide the foundation for hypothesis-driven experiments to assess the future viability of populations.

Two prominent selective agents in alpine streams are constant cold temperature and seasonal formation of ice (Lencioni, 2004; Danks, 2007). While water acts as a thermal buffer minimizing extreme temperature fluctuation and stream flow limits the formation of ice crystals (Danks, 1971; Füreder, 1999), aquatic insects still experience long periods of sustained cold during development which almost always takes place under snow or ice (Lencioni, 2004). For freeze-tolerant aquatic insects, one adaptive theme is the repeated evolution of elevated concentrations of glycerol and/or ice-binding factors (or anti-freeze proteins) in larval haemolymph (Danks *et al.*, 1994; Füreder, 1999; Lencioni, 2004; Danks, 2007; Lencioni *et al.*, 2008; Lencioni *et al.*, 2009; Lencioni *et al.*, 2013; Lencioni & Bernabò, 2015). For instance, Lencioni *et al.* (2008) and Lencioni and Bernabò (2015) comprehensively studied respiratory performance and thermal limits of the midge *Pseudodiamesa branickii* which inhabits glacier-fed streams in the Italian Alps. Larvae of this species are tolerant to freezing to temperatures as low as –16°C and although the upper lethal temperature of *P. branickii* may be as high as ~32°C, specimens appeared stressed above 12°C. These results suggest an adaptive strategy by *P. branickii* to thrive in extremely cold glacier-fed stream environments at the expense of effective competitive abilities for persisting in warmer downstream habitats. With increases in water temperature and associated decreases in dissolved oxygen availability, specialists such as *P. branickii* could lose their advantage in inhabiting glacier-fed streams and be

replaced by more generalist taxa with greater competitive ability. Species turnover dictated by competition in glacier-fed streams has been described previously for other midges, including those of the genus *Diamesa* (but see Sæther, 1968 and Nolte, 1991), and may represent a general mechanism applicable to cold-tolerant macroinvertebrate communities on a global scale (Flory & Milner, 2000). Furthermore, the extreme conditions associated with glacier-fed streams and the unique suite of traits required to withstand them likely exacerbate trade-offs between competitive (e.g., temperature generalism) and specialist traits (e.g., freeze-tolerant larvae).

Evaluation and monitoring of trophic (feeding) traits is also relevant to alpine stream biology as climates warm and basal food resources shift from predominantly autochthonous (*in situ* carbon fixation by algae and other primary producers) to allochthonous (carbon fixed by plants in the surrounding terrestrial environment, Hauer et al., 1997). Trophic traits in macroinvertebrates, including those driving specialization by consumers on either algae or leaves, tend to be phylogenetically constrained and therefore less likely to respond to natural selection than other, more evolutionarily labile traits (Poff *et al.*, 2006). As such, strong shifts in food resources might translate to relatively rapid changes in the functional and taxonomic structure of alpine stream assemblages and associated ecosystem-level processes (Robinson & Gessner, 2000; Cauvy-Fraunie *et al.*, 2016).

Field experimentation

Experimentation under natural conditions is a powerful tool for understanding the mechanisms of observed pattern and process and for predicting ecological responses to

climate change. However, *in situ* experimentation in alpine streams is rare, likely due to the logistical constraints of carrying out field experiments in remote, harsh environments. Furthermore, experiments in which natural conditions are manipulated for long time periods (>1 year) are particularly rare, and even short-term (single season or less) studies are uncommon. Nonetheless, alpine streams offer useful opportunities for field-based ecological experiments given their small size, limited taxonomic diversity and ecological complexity, minimal human impact, and natural habitat variation over small spatial extents. These advantages also ought to attract a broader swathe of ecologists looking for suitable ecological systems for experimentation.

In perhaps the only long-term ecological field experiment in alpine streams, Cauvy-Fraunie *et al.* (2016) conducted a four-year experimental flow manipulation in the Ecuadorian Andes, diverting one-third of natural discharge from a glacier-fed stream to assess how decreased run-off affects ecological pattern and process. Meltwater reduction increased benthic algal and macroinvertebrate herbivore biomass and changed macroinvertebrate community composition within a few weeks. After the diversion was terminated and the stream was returned to natural flow levels, the system did not return to its pre-perturbation state for over a year. From a climate change perspective, the rapid response to flow diversion suggests that as meltwater influence is reduced, impacts to alpine stream ecosystems will occur rapidly across a variety of biological metrics (Figure 1.8).

Short-term field experiments have evaluated biological drivers of organic matter decomposition rates in alpine streams. For instance, experimentally increased nutrient concentrations in an alpine groundwater-fed stream spurred an increase in shredder

(detritus-feeding macroinvertebrates) abundance and faster breakdown of detritus in leaf packs (Robinson & Gessner, 2000). The authors speculated that microbial assemblages colonizing leaves in these streams are nutrient-limited, such that when nutrients were supplemented, the microbially mediated quality of the leaf litter increased, leading to the observed increase in shredder abundance and feeding activity. In another alpine leaf-pack experiment focused on the relationship between shredder diversity and ecosystem function (i.e., decomposition rates), an interaction between shredder species richness and abundance had the strongest impact on decomposition rates in high-Andean streams (Dangles *et al.*, 2011). In this study, the three most abundant shredder species produced the greatest decomposition rates when co-occurring, a result that implies some degree of complementary resource use and/or facilitation among species (Dangles *et al.*, 2011). These findings suggest that differential climate-mediated range shifts (of both macroinvertebrates and terrestrially derived organic matter) could decouple important biological interactions in alpine stream ecosystems.

Experimental transplantation of species into novel communities and/or environments can help researchers understand how range shifts of alpine stream species might impact biotic interactions and ecosystem processes. In an Alaskan glacier-fed stream, transplanting stones colonized by midges of the genus *Diamesa* between reaches at different elevations demonstrated that the natural absence of this species from lower elevation sites was due to competitive exclusion rather than inability to tolerate the local environmental conditions (Flory & Milner, 2000). Similarly, Madsen *et al.* (2015) transplanted larvae of selected macroinvertebrates upstream of their natural altitudinal limit in a high-Andean glacier-fed stream to test the short-term (two weeks) effect on

survival. This treatment reduced survival by varying degrees among taxa, but the stonefly *Claudioperla* sp. survived well at a site where it did not naturally occur, suggesting that altitudinal limits are not always directly related to the abiotic environment, at least not in the short term. Khamis *et al.* (2015) tested the potential impacts of an introduced predator on natural species densities using side channels constructed next to an alpine stream in the French Pyrénées. By manipulating densities of the predacious stonefly *Perla grandis*, which occurs in the same streams but at slightly lower elevations, they simulated an upstream range expansion of *P. grandis* and found that some (but not all) prey species were reduced. The authors concluded that the extinction risk of range-restricted prey taxa could increase with upstream predator range shifts. From a broader perspective, these transplant studies demonstrate the utility of short-term field experiments in alpine streams, particularly in addressing the influence of rapid environmental change on species interactions and ecosystem processes.

Integrating multiple approaches

The seven approaches for organism-focused research in alpine stream biology described above are not mutually exclusive, and here, we argue that thoughtful integration of multiple approaches will lead to more robust understanding of these rapidly changing systems (Pauls *et al.*, 2014). Even in our discussion of each approach independently, it is clear that many examples bridged multiple approaches. For example, Shama and Robinson (2009) used common garden experiments to understand life-history variation of alpine caddisflies across a complex environment; Snook and Milner (2002) and others evaluated species traits to understand associations of species with

environmental conditions; and population genetic analysis of common species can be combined with the more traditional approach of evaluating spatial patterns of assemblage diversity to generate a more thorough understanding of the biological effects of environmental heterogeneity in alpine streams (Finn *et al.*, 2013). Future biological research in alpine streams should emphasize continued integration, with a particular emphasis on multiple levels of biological organization (genes to assemblages, and prokaryotes to eukaryotes) and a merging of the more traditional 'snapshot' observational approaches with experimentation and/or emerging technologies (e.g., NGS).

Recent studies showcase the power and promise of highly integrative research in alpine streams. For instance, Bálint *et al.* (2011) evaluated the contemporary spatial distributions of nine alpine/arctic macroinvertebrate morphospecies and three levels of cryptic biodiversity determined with population genetic methods for the same morphospecies. The authors then applied SDM according to two climate models based on future CO_2 emissions scenarios to each of the four levels of biodiversity independently. The results of their integrative analysis suggested that biodiversity loss under either climate model would be proportionally much greater in terms of genetic variation than morphospecies diversity (Figure 1.5). This outcome supports a broader hypothesis that rates of climate-related biodiversity loss (in fresh waters and otherwise) will be significantly underestimated if measured as impacts to morphological species diversity alone (e.g., Dudgeon *et al.*, 2006; Strayer & Dudgeon, 2010) without consideration of intraspecific genetic diversity (Bálint *et al.*, 2011; Pauls *et al.*, 2013).

Another promising example of integrating approaches is the resampling of the same systems and species through time and applying increasingly powerful population

genetic tools to understand specific biological effects of rapid environmental change, including biodiversity, population structure, and demography. To our knowledge, there have been just two published temporal comparisons of genetic variation in alpine streams. In the first, Shama *et al.* (2011) assessed genetic diversity both spatially and temporally for the caddisfly, *A. uncatus*, before and after an extreme drought in the Swiss Alps, revealing a significant decrease in overall genetic diversity but an increase in differentiation among populations over just two generations. In the second, Jordan *et al.* (2016) compared genetic diversity and patterns of population structure between historic (>10 years old) and modern (2010) samples of an endemic stonefly, *Lednia tumana*, threatened by climate change in Glacier National Park, USA. The results indicated decreased genetic diversity and increased subdivision among populations in just 10 years, an alarming finding given the near-term decline of the extremely cold glacier meltwater that comprises much of *L. tumana*'s habitat in the region. In the terrestrial alpine environment, a 90-year study of two alpine chipmunk species in Yosemite National Park revealed different population genetic responses to warming. One species maintained connectivity and gene flow through a largely unaffected range size over the study period, while the second species underwent significant fragmentation and genetic differentiation among populations likely as a result of a 500 m upslope range contraction (Rubidge *et al.*, 2012). In alpine streams, a related question remains to be addressed: will a changing climate affect population genetic structure across communities in a synchronous way or will changes be taxon-specific?

There are a number of other opportunities for novel integrative research to address pressing questions in alpine systems facing rapid change, particularly within the context

of whether plastic or adaptive responses in life-history traits could have biological repercussions beyond local population persistence. For example, differences in emergence timing influenced by snowpack duration appear to affect flight activity of insect species (Finn & Poff, 2008), likely due to late-season colder air temperatures discouraging adult flight activity. Differential dispersal through time can affect regional-scale population persistence and genetic diversity, and potential responses of both can be measured or modeled with molecular methods (e.g., Bálint *et al.*, 2011; Shama *et al.*, 2011; Jordan *et al.*, 2016). Furthermore, spatial variation in development rates and emergence timing between streams with different temperature and flow regimes (e.g., a glacier-fed stream *versus* a groundwater-fed spring) has been hypothesized as a potential driver of reproductive isolation among stream insect populations (Finn *et al.*, 2013; Finn *et al.*, 2014). As alpine streams become more environmentally homogenous at the regional scale, these life-history traits will likely follow suit. Future research integrating the monitoring of temporal change in environmental variation, life-history traits, population structure, and genetic variation will improve understanding of how these aspects of population biology are interconnected in alpine streams.

Global perspectives, method standardization, and emerging methods

Beyond integration across the 'standard seven' approaches, we also see fruitful opportunity associated with recent and emerging technological advances. Examples of these include database management for long-term and universally accessible storage of samples and data, high-resolution remote sensing, and population genomics and

associated analytical tools (NGS). We elaborate here on methods and applications for seizing these opportunities in alpine stream biology.

Making the most of repeat sampling

Given the rapid pace of environmental change in alpine streams, temporal monitoring of environmental parameters and biota will be important to developing greater understanding of the degree of vulnerability of these systems to climate warming. To make the most of observational data henceforth, alpine stream researchers should ideally apply universally standard collection methods and proper storage of samples to serve as temporal comparisons for future studies. With the continued rise of powerful genomic tools (e.g., RADseq) and development of advanced morphometric approaches (e.g., micro-computed tomography, Friedrich *et al.*, 2014), properly stored and annotated biological specimens will no doubt provide useful genotypic and phenotypic reference points for understanding rates and mechanisms of evolutionary change as well as better understanding of existing variation. Furthermore, standardized monitoring of environmental parameters and biological assemblages will be essential for linking habitat change to biological change at a global scale.

In alpine stream biology, internationally accessible database(s) and associated collaborative networks have been proposed (Füreder & Schöner, 2013), but the idea has yet to be realized. The spatially limited temporal data sets discussed above (Milner *et al.*, 2008; Finn *et al.*, 2010; Shama *et al.*, 2011) provide useful precedents to justify a unified effort towards the development of such a network. Indeed, there are now international networks and databases set up to monitor long-term change in other ecological systems

that alpine stream scientists can look to for guidance. These include the Global Observation Research Initiative in Alpine Environments (GLORIA), which emphasizes the monitoring of terrestrial alpine plants (Grabherr *et al.*, 2000), and the Global Lake Ecological Observatory Network (GLEON), an international program with standardized protocols for documenting environmental change in lakes (Weathers *et al.*, 2013; Read *et al.*, 2016). As with any integrated monitoring effort, a networked alpine stream ecology database should provide explicit guidelines on standardized protocols for measuring and storing (if applicable) the unique suite of physicochemical, hydrologic, and biological variables relevant to the system. Discussions and previous large-scale research projects in the past [e.g., Arctic and Alpine Stream Ecosystem Research (AASER); Brittain & Milner, 2001] have emphasized a set of appropriate environmental variables (with a focus on hydrology) and collection methods for macroinvertebrate assemblage data. We suggest adding standardized protocols for storing and vouchering specimens for subsequent genetic and/or morphometric analyses. Furthermore, it will be important to recommend balanced spatial sampling strategies within each alpine region added to an international network (e.g., Füreder & Schöner, 2013), including multiple drainage basins and hydrologically defined stream types to address questions about landscape-scale connectivity, population genetic structure, and the interacting influences of local environment and spatial distance on biological responses. By putting these goals in a standardized global network, the field is poised to move beyond the story of one range or species that may be an anomaly of local variation, to the story of many species and ranges with the power to portend more significant trends.

Incorporating new and improved remote sensing and GIS

Opportunity also exists for alpine stream biologists to incorporate remote sensing technology and geographic information system (GIS)-based approaches into future research (Carbonneau, 2012). Through these tools, baseline measurements of landscape features specific to alpine stream structure and function can be assessed and serve as reference points for future research. Recent developments in remote sensing technology for spatial mapping (e.g., Light Detection and Ranging, LiDAR) as well as aerial infrared sensing provide more accurate (and more efficient) collection of stream and watershed attributes than previously possible. Example applications include, but are not limited to, monitoring of succession at the watershed scale in recently deglaciated basins (Klaar *et al.*, 2014), remote measurements of stream temperature (Handcock *et al.*, 2012), and remote characterization of watershed attributes (Hopkinson *et al.*, 2009). Glacial and snowfield margins, as well as corresponding stream networks, can also be digitized in GIS, quantified, and compared with other time periods or localities (e.g., del Río *et al.*, 2014; Hall *et al.*, 2015) or linked with existing biodiversity to assess ties between glaciers and species occurrences more clearly (Giersch *et al.*, 2016). From the perspective of modelling threats to biodiversity, finer-scale resolution of geologic and environmental change can directly bolster predictions about future distributions or environmental pressures. For all remote sensing projects in alpine streams, it is important that imagery be captured during later parts of the season to minimize ice coverage and maximize stream resolution. Monitoring of glaciers and the alpine environment – whether *via* remote sensing or other methods – is not new (e.g., Hall & Fagre, 2003). Rather, our take-home message is the potential for alpine stream biologists to cultivate collaborations

with researchers carrying out existing spatial monitoring and remote sensing efforts to inform links between abiotic (e.g., glacier size, water temperature) and biotic (e.g., algal growth) characteristics of alpine headwater ecosystems.

Applying genome-wide perspectives to better understand evolutionary processes

NGS and associated approaches (e.g., RADseq) for generating large genomic data sets can significantly improve our understanding of biological responses to rapid environmental change in alpine streams, specifically in an evolutionary context. Until recently, questions that required information from a genome-wide perspective (e.g., identifying genes under selection) were difficult even for model organisms and out of reach for all other taxa. This is no longer an issue for most species, thanks to the falling cost and rising efficiency of NGS data collection. Genome-scale data sets overcome many limitations of single- or few-marker studies (e.g., an overemphasis on the unique evolutionary history of the mitochondrial genome) that have been widely applied in alpine stream biology. However, with orders of magnitude more data and computationally intensive analytical methodologies, implementing NGS approaches requires specific laboratory and bioinformatic training. While a full review of NGS applications is beyond the scope of this review [but see Manel and Holderegger (2013) and Andrews *et al.* (2016)], we discuss specific topics below where an NGS toolkit could be particularly valuable for studying alpine stream biota from a climate change perspective.

A standing question in alpine stream biology is whether population connectivity will be substantially altered with the decline of cold meltwater habitat. Genome-scale

data sets are well suited to this challenge as they provide the necessary power to resolve fine-scale variation in migration among genetic clusters (Beerli & Palczewski, 2010) or can be used to characterize migration as a subset of parameters in an overarching demographic modeling framework (e.g., Gutenkunst *et al.*, 2009; Excoffier *et al.*, 2013). Indeed, this latter approach is particularly useful because, in addition to estimating parameters like migration, it also provides a means for simultaneous estimation of other aspects of population biology and history (e.g., divergence times among lineages, temporal changes in effective population sizes). For instance, given the important role that fragmented habitat plays in shaping genetic diversity, constructing a timeline of population divergence provides an outlet for linking divergence events with past landscape-level processes (e.g., recession of glaciers after the Last Glacial Maximum).

NGS data sets also represent a promising avenue for identifying ecologically relevant genetic diversity, a virtually unexplored realm in alpine stream biology. Using predominantly neutral, genome-wide markers to reconstruct the demographic history of populations provides a null model for identifying outlier markers that are either portions of genes responding to natural selection or are at least in linkage with them (Nielsen, 2005). For example, across a heterogeneous alpine stream network, if there is strong enough selection to drive adaptive divergence between populations in different habitats and enough time has passed for signatures of this selection to accumulate, divergent adaptation in outlier genes (e.g., a heat shock gene involved in chaperoning cellular processes under cold stress; Matz *et al.*, 1995, Lencioni *et al.*, 2013) may be observed. The potential for using genomic tools to link genotype to phenotype holds particular relevance to questions of ecophysiology in a changing climate. Specifically,

understanding the genomic mechanisms through which species have adapted to harsh conditions (e.g., the evolution of anti-freeze proteins) can also inform the degree to which those mechanisms may be flexible as conditions change, especially if closely related species or ecological gradients are available for comparison. A combined approach integrating analysis of population connectivity, demographic history, and detection of outlier loci that may be under selection all in the context of future distribution modeling, represents a framework for investigating how focal species may respond at the genomic level to changing environmental conditions. For instance, if many populations in a single mountain range are locally adapted to different thermal regimes then the rate and direction of migration (i.e., the potential for the spread of adaptive genetic diversity) among populations is a critical component of any broader adaptive response for the species.

Conservation

Despite the documented importance of alpine headwaters to biodiversity at the scale of whole stream networks (Finn *et al.*, 2013), there has been little emphasis on developing management strategies for biodiversity conservation in these systems (Khamis *et al.*, 2014). Unlike in lowland rivers where climate change is proceeding more slowly and conservation management and restoration practices typically emphasize returning systems back to some historical steady state from other types of anthropogenic impacts, rapid climate change is the single greatest threat to the integrity of otherwise pristine alpine streams (Hannah *et al.*, 2007). As such, conservation management in alpine streams is a daunting prospect, often perceived to be insurmountable due to the

limited potential to reverse the environmental effects of climate change (Khamis *et al.*, 2014). Therefore, the common conservation strategy of protecting individual, range-restricted or rare species and intraspecific genetic diversity (Allendorf & Luikart, 2007) may have minimal benefit in alpine streams. Instead, Khamis *et al.* (2014) call for a shift in focus from single species of concern to conserving ecosystem processes when possible, including maintaining or enhancing biological connectivity among alpine basins and limiting additional anthropogenic stressors.

However, even under this promising, more holistic conservation framework of maintaining ecosystem processes and broad swathes of heterogeneous habitat (Linke *et al.*, 2011), we can still expect a systematic disappearance of many headwater streams fed by the melting of ice or snow under future warming scenarios, along with the unique species and genetic variation they harbor. In terms of biodiversity conservation in alpine streams, then, a worthwhile goal is to identify and prioritize the protection of robust local populations of cold stenothermic species associated with meltwater habitat (Muhlfeld *et al.*, 2011), as well as meltwater-associated habitats that are likely to be most resistant to climate change. As part of this process, robust assessments of species boundaries from multi-locus genetic data will be an important component of conservation strategies (e.g., Grummer *et al.*, 2014; Hime *et al.*, 2016; Hotaling *et al.*, 2016). Although many alpine glaciers and associated meltwater habitats are expected to disappear in the near future (Hall & Fagre, 2003; Edmunds *et al.*, 2012), it is becoming increasingly apparent that there are other types of extremely cold meltwater habitat (not previously recognized in the alpine stream ecology literature) that might be more resistant to change and could serve as climate refugia for cold-adapted biota. Specifically, rock glaciers – subsurface

masses of ice and rock debris – act as hydrologic sources for some alpine streams and are likely more resistant to atmospheric warming due to the overlying layers of insulating debris (Millar & Westfall, 2008; Fegel *et al.*, 2016). Streams primarily fed by rock glacier meltwater might contain robust populations of cold stenothermic species of concern (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2015; Giersch *et al.*, 2016) and would therefore be ideal sites to prioritize for biodiversity conservation (as per Finn *et al.*, 2010). We advocate an integration of the approaches described above, including remote sensing for locating rock-glacier-fed streams and other potentially resistant alpine stream types, genetic methods for determining recent population demographics and species boundaries, and temporal sampling to evaluate population stability through time, as an effective toolkit for making conservation decisions for alpine stream biodiversity.

Conclusions

Alpine stream ecosystems contain disproportionately high biodiversity at multiple levels, from genes to communities. This biodiversity, along with extensive environmental heterogeneity, natural geographic isolation, generally low anthropogenic impact, and rapid climate change at high altitudes combine to make alpine streams sentinels of environmental change on a global scale. Increasing environmental homogenization of alpine streams with climate change, and potential ramifications from both biodiversity conservation and ecosystem function perspectives, are of major concern. It is timely and important to increase our understanding of the processes affecting alpine stream biodiversity and to make scientifically defensible conservation decisions for protecting the evolutionary legacy of these imperiled ecosystems.

We identify and summarize seven major, organism-focused research approaches that have been applied in alpine stream research both historically and more recently. These approaches vary from basic observational research of environmental conditions and macroscopic organisms to approaches emphasizing newly developed methods. Through awareness of existing methods and tools, future researchers are better suited to address research questions in an integrated, collaborative framework.

To develop a more robust understanding of the processes affecting alpine stream biodiversity under climate change, we advocate the following: (*i*) thoughtful integration of the seven approaches, with a specific focus on combinations of traditional and emerging approaches, to address complex hypotheses spanning multiple levels of biological organization; (*ii*) increased multidisciplinary collaboration, specifically to integrate useful tools outside of biology (e.g., remote sensing); (*iii*) systematic development and expansion of international research networks and the establishment of agreed-upon standards for sample collection, database management, and communication; and (*iv*) application of high-resolution genomic methods to address evolutionary, systematic, and conservation questions for alpine stream species.

Because the single greatest threat to alpine stream organisms is climate change, biodiversity conservation is a daunting challenge, and to date, minimal emphasis has been placed on this aspect of alpine stream biology. We suggest the best way forward for conserving threatened species is to identify and prioritize demographically stable, genetically diverse populations occupying stream sites with the maximum possible resistance to changing environmental conditions.

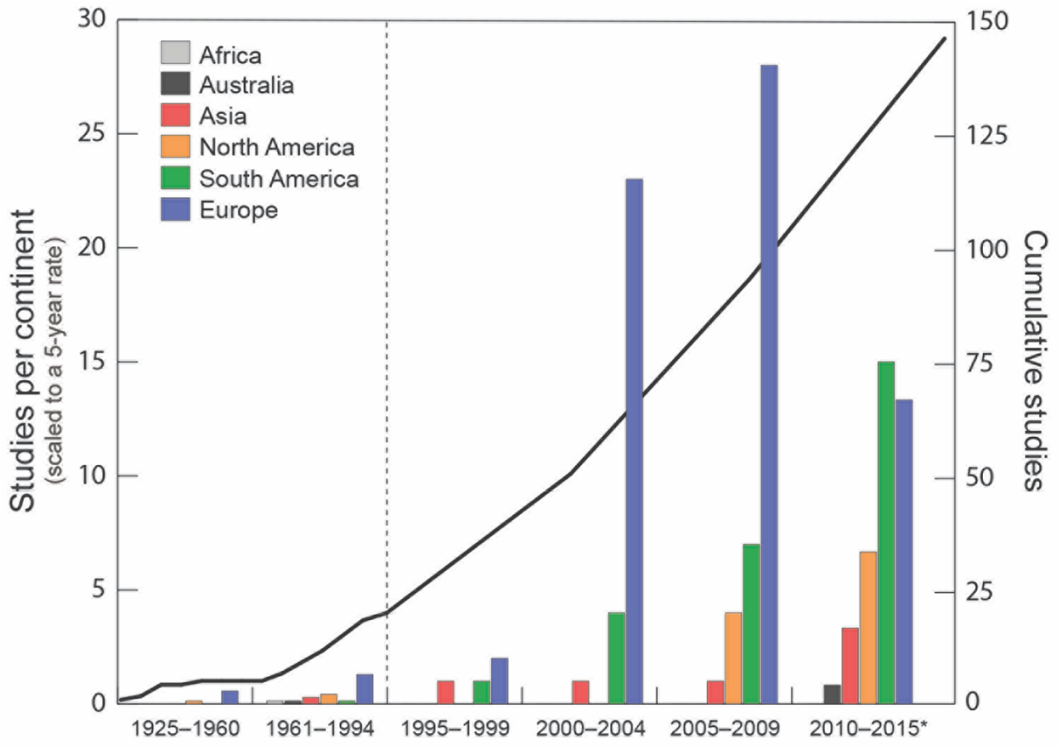

Figure 1.1. Continental distribution of alpine stream biology research published before and after seminal publications by Ward (1994) and Milner & Petts (1994), denoted by the vertical dashed line. The left *y*-axis and histogram bars are studies per continent across five-year intervals for the period 1995–2015 and ~35-year intervals for 1925–1994 (the asterisk indicates a six-year interval where data are scaled by a factor 0.833 to be equivalent to the other modern five-year periods). The right *y*-axis and black line reflect the cumulative number of studies on all continents through time. To generate a literature database, we first added known citations to a combined database. Next, this database was supplemented through two *Web of Science* searches: (1) for "alpine stream* ecology" OR "alpine stream* biology and (2) for "alpine" AND "stream" AND "gene*". All searches were conducted for the years 1925–2015. See Hotaling *et al.*, 2017b for complete details of the references included in this figure.

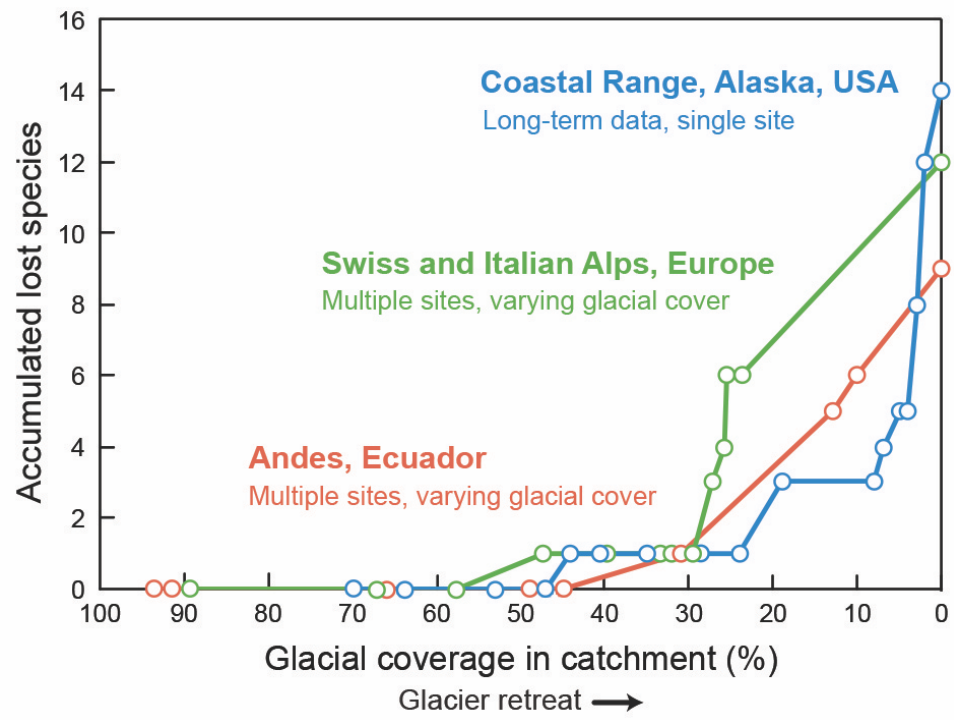

Figure 1.2. Cumulative decrease of regional species richness (γ diversity) as a function of glacial cover. Glacier-obligate macroinvertebrates begin disappearing when glacial cover drops below approximately 50%. Each data point represents a river site and lines are Lowess fits to the data. Figure modified with permission from Jacobsen *et al.*, (2012).

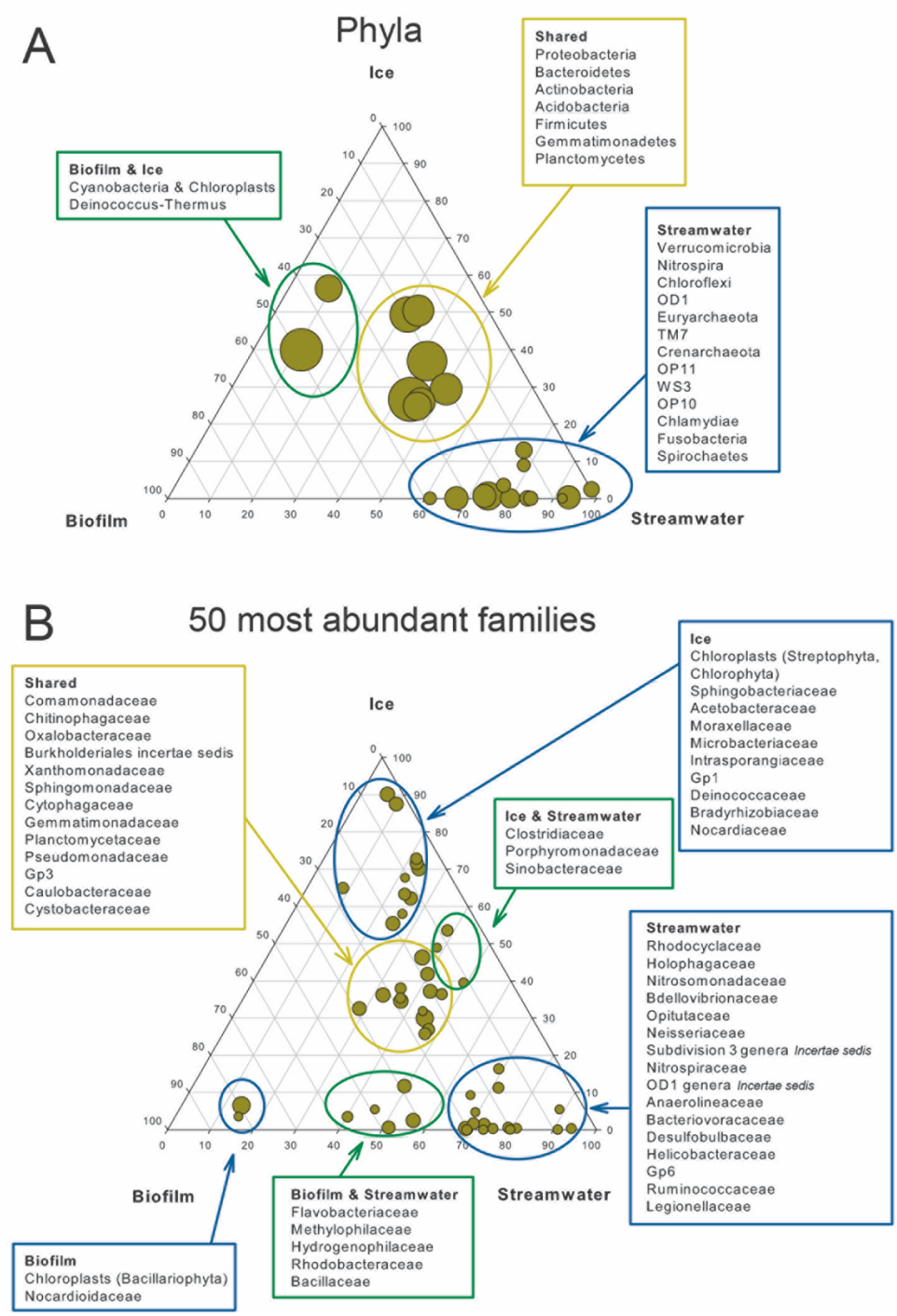

Figure 1.3. Distribution of microbial taxonomic groups in glacial ice, streamwater, and biofilm. The percentage of (A) phyla and (B) the 50 most abundant families associated with each habitat are visualized in ternary plots. Position within each triangle indicates the relative abundance of each taxon among the three habitats. Circle size represents the relative abundance of taxa overall. Figure modified with permission from Wilhelm *et al.* (2013).

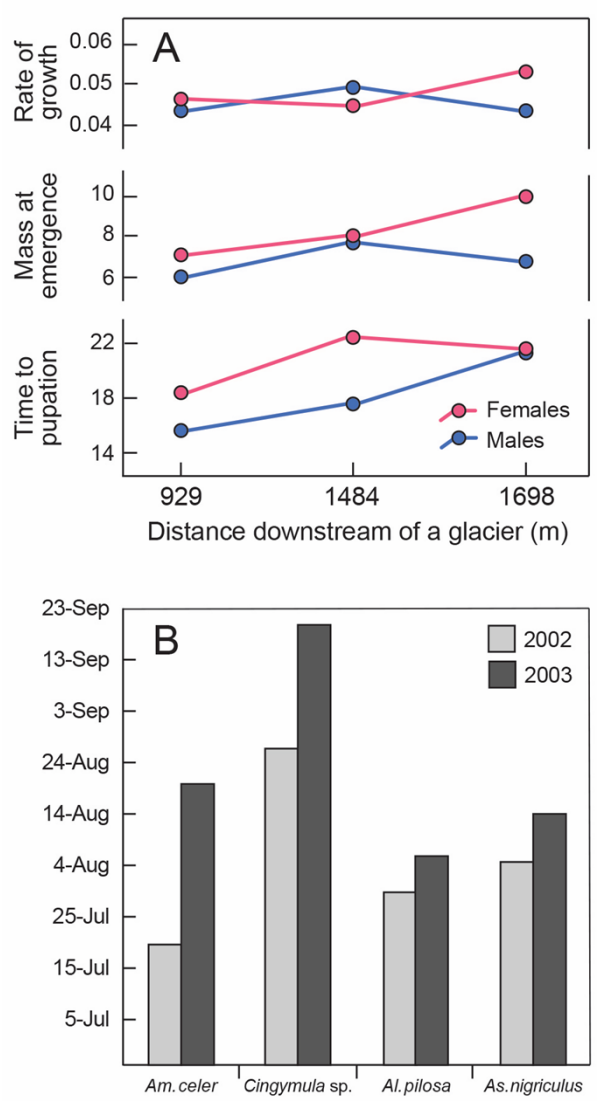

Figure 1.4. (A) Examples of fine-scale spatial variation in life-history traits of alpine stream insects. Plots indicate population-level differences in rate of growth [*ln*(mg)day^{-1}], mass at emergence (mg), and time to pupation (measured from third-instar larvae to the onset of pupation; days) for permanent stream populations of the alpine caddisfly, *Allogamus uncatus*, with increasing distance from glaciers in the Val Roseg floodplain of the Swiss Alps. Results were averaged across treatments in a common garden experiment. (B) An example of temporal variation in life-history traits. Histograms indicate the date at which 25% of the cumulative abundance of emerging adults was reached for four common alpine stream species (Ephemeroptera: *Ameletus celer* and *Cinygmula* sp., Plecoptera: *Alloperla pilosa*, and Trichoptera: *Asynarchus nigriculus*) along an alpine stream in the Rocky Mountains, USA. Samples were collected in two years: 2002 which followed an exceptionally dry winter, and 2003 which followed a winter with an above-average snowpack. Emergence timing was significantly earlier for all species in 2002. Data for A were redrawn (and approximated) from Figure 5 in Shama & Robinson (2009). Data for B are from Finn & Poff (2008).

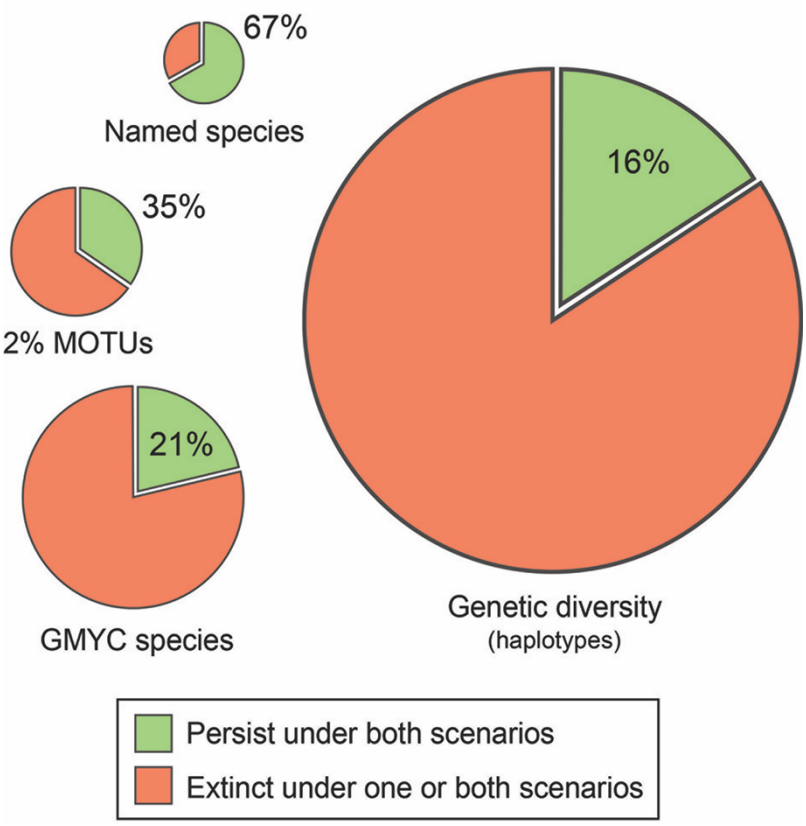

Figure 1.5. Projected loss or extinctions of morphologically diagnosed species, molecular operational taxonomic units (MOTUs) identified as having < 2% sequence divergence, general mixed Yule-coalescent (GMYC; Monaghan *et al*., 2009) species delimited using a model-based approach, and mitochondrial DNA (mtDNA) haplotypes for nine montane stream insect taxa in Europe under two IPCC 2080 CO_2 emission scenarios as inferred from future species distribution modelling. Green slices indicate units predicted to persist under both future emission scenarios and red slices indicate units predicted to go extinct under one or both scenarios. Circles are scaled proportionally by total units for each classification. Figure modified with permission from Bálint *et al.* (2011).

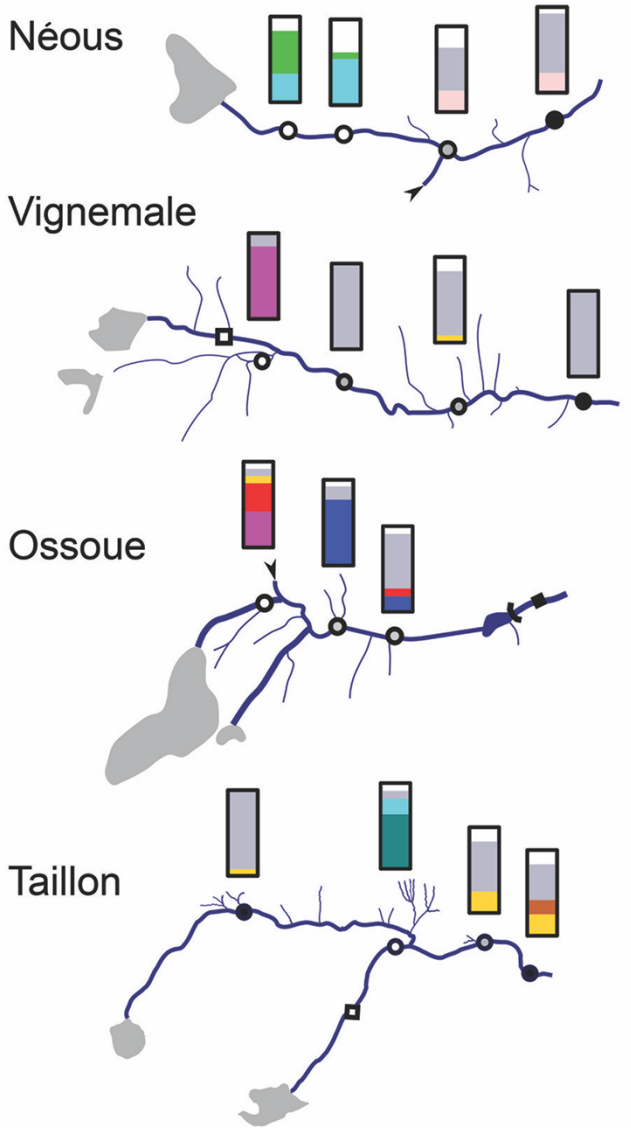

Figure 1.6. Distribution of common *Baetis alpinus* mitochondrial DNA (mtDNA) haplotypes along four streams of the French Pyrénées National Park. Stream drawings are approximations and are reoriented such that glaciers (grey) are to the left, and downstream-most sample reaches are to the right. Maximum downstream distance of a sample reach is 4.5 km (Vignemale basin). Coloured bars indicate haplotype abundance (total $N = 11–13$ per reach as indicated by height of bars). Sample reaches along streams are coded white for high-glaciality, grey for mid-glaciality, and black for low-glaciality. Squares indicate sites without *B. alpinus* populations. Figure modified with permission from Finn *et al*., (2013).

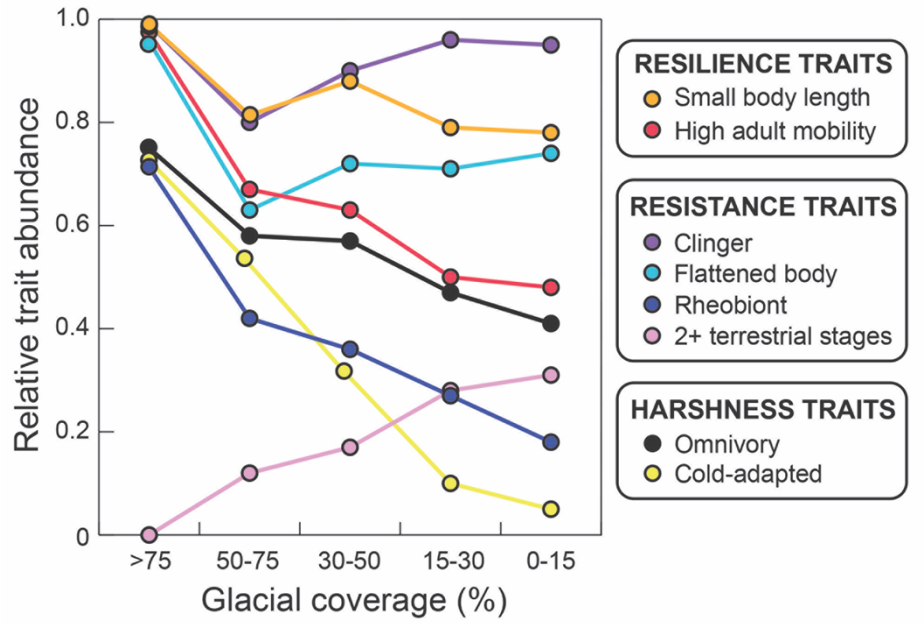

Figure 1.7. Mean relative abundance of eight species traits defined as either resilience, resistance or harshness traits for 297 aquatic invertebrate taxa collected from 60 sites along 37 different rivers representing a gradient of glacial cover in Hohe Tauern National Park, Austria. Resilience traits aid in rapid return to pre-disturbance population densities following a hydrologic disturbance. Resistance traits are linked to the capacity of organisms to withstand a hydrologic disturbance without significant loss of individuals. Harshness traits are linked to the capacity of individuals to survive cold temperatures or periods of low food availability. See Füreder (2007) for original data and additional discussion of specific traits.

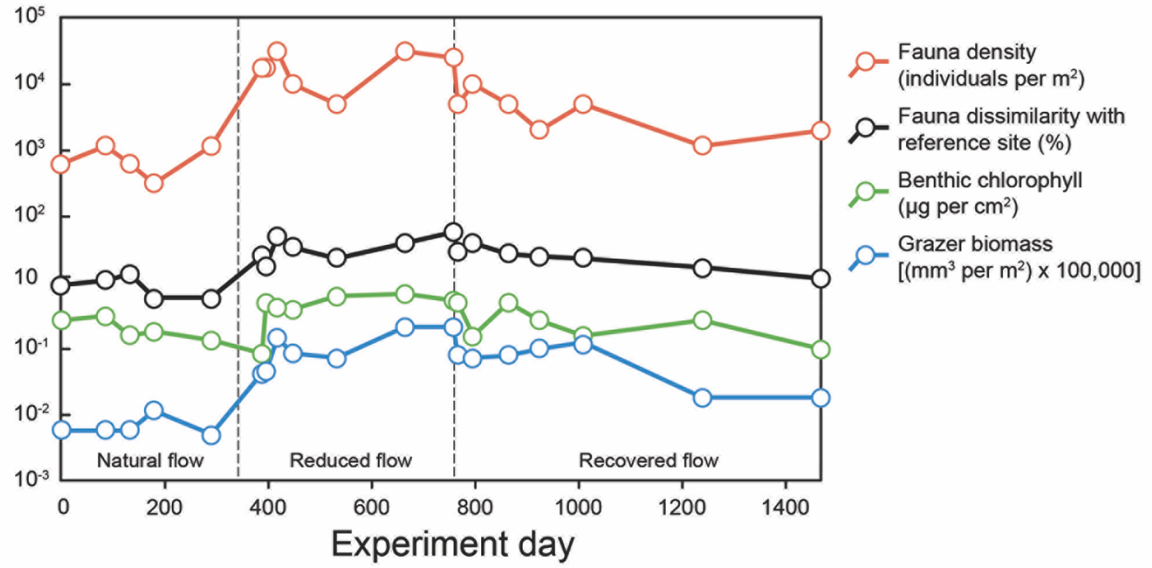

Figure 1.8. Results from a flow-reduction experiment in an Ecuadorian glacier-fed stream where benthic samples were collected at irregular intervals over a four-year study period. The experimental reach was sampled for approximately one year prior to flow manipulation ('natural flow'). After this initial period, approximately one-third of the discharge was diverted away from the reach for ~one year ('reduced flow'). Natural flow then was re-established and the reach was monitored for another two years ('recovered flow'). Data shown include density of benthic macroinvertebrates (red), fauna dissimilarity (Bray-Curtis; black) *versus* an unmanipulated upstream reference site, benthic chlorophyll (green), and total biomass of macroinvertebrate grazers (blue). Figure modified with permission from Cauvy-Fraunié *et al.*, (2016).

CHAPTER TWO

DISCORDANT AT THE TOP: LACK OF GENETIC CONGRUENCE DESPITE OVERLAPPING ECOLOGY AND GEOGRAPHIC DISTRIBUTIONS FOR THREE ALPINE STONEFLIES THREATENED BY CLIMATE CHANGE

Introduction

Many environmental, evolutionary, and historical factors dictate the contemporary distributions of species. Though perpetually in flux, contemporary distributions are akin to ecological snapshots, and provide the observational basis for hypotheses regarding how present-day diversity accumulated and is maintained. Because every individual, population, and species has its own evolutionary history, genetic data can provide a vital thread linking past processes to present distribution (Hewitt, 2000; Whiteman *et al.*, 2007). To this end, comparative population genetic studies are particularly powerful as they highlight the degree to which species have responded to various influences, including to past geological processes (e.g., glacial oscillation, Brunsfield et al. 2001) and/or variance in dispersal (Lourie *et al.*, 2005). Exemplar studies have shown that both shared geographic distributions and overlapping ecological requirements can be important predictors of similar shared evolutionary trajectories (Lapointe & Rissler, 2005; Whiteman *et al.*, 2007; Satler & Carstens, 2017). Thus, interspecies congruence in taxonomy, ecology, and geography should extend to highly similar population genetic differentiation and demographic history. However, rarely do communities of closely-related, biologically similar species exist in nature, thus a natural model for testing this hypothesis is rare. High-elevation streams provide a unique solution to this difficulty as they do contain many closely related species (e.g., invertebrates within the same family

or genus), and are environmentally harsh, thereby selecting for taxa with similar ecological requirements (Hotaling *et al.*, 2017b).

In the North American Rockies, two alpine stoneflies, *Zapada glacier* and *Lednia tumana* have been recommended for listing under the U.S. Endangered Species Act (ESA) due to climate change-induced habitat loss (U.S. Fish and Wildlife Service, 2016). A third stonefly endemic to the Teton Range – *Lednia tetonica* – is only known from a single stream fed by permanent subterranean ice. All three species are highly similar in many ways: they belong to the same order and family (Plecoptera: Nemouridae), are phytophagous with short (< 30 days) winged adult stages, and are tightly linked to the hydrologic conditions associated with melting glaciers, ice, and snow (Baumann, 1975; Muhlfeld *et al.*, 2011; Baumann & Call, 2012; Giersch *et al.*, 2015; Giersch *et al.*, 2016). Previous mitochondrial DNA (mtDNA) evidence has indicated genetic distinctiveness among populations of *Z. glacier* generally corresponding with mountain ranges, suggesting that *Z. glacier* may actually represent one or more mountain range-specific species, similar to the morphology-based descriptions of *L. tumana* and *L. tetonica* (Baumann & Call, 2012; Giersch *et al.*, 2015; Giersch *et al.*, 2016). Moreover, addressing these population genetic questions holds significant implications for conservation in the region, as the alpine streams that *Z. glacier*, *L. tumana*, and *L. tetonica* inhabit, as well as those worldwide, are under significant threat as rapid warming drives substantial glacier recession (Hall & Fagre, 2003; Hansen *et al.*, 2005; Pederson *et al.*, 2010; Roe *et al.*, 2016). Linked to this decline in the alpine cryosphere is the potential for loss of an entire community of meltwater-dependent alpine organisms (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016; Hotaling *et al.*, 2017b; Hotaling *et al.*, 2017a).

Taken together, these species present a rare opportunity to test the degree to which ecological, geographic, and taxonomic overlap extends to population genetic congruence. Specifically, we combined mtDNA sequence data with known geographic and ecological overlap to test two hypotheses: first, we tested whether overlap in ecology, geography, and taxonomy would extend to population genetic congruence between described species (*L. tumana* and *L. tetonica*) and populations (*Z. glacier*) inhabiting the same general geographic distribution.

Second, the discovery of cryptic speciation in aquatic insects has become commonplace (e.g., Gill et al. 2016; Leys et al. 2017), and given that our study species were described based solely upon the presence (*L. tumana* and *L. tetonica*; Baumann & Kondratieff 2010; Baumann & Call 2012) or absence (*Z. glacier*; Baumann 1975) of distinguishing morphological characters, we hypothesized that demographic modeling would reveal similar rates of contemporary gene flow among *Z. glacier* populations versus between species (*L. tumana* and *L. tumana*) across the same area, thereby lending support to the possibility that *Z. glacier* actually represents three mountain range-specific species. Beyond alpine stoneflies, our study sheds light on a larger challenge in conservation biology. To what degree can genetic insight for one species – perhaps fitting the "umbrella species concept" (Caro, 2010) – be applied to closely-related species sharing the same habitat, taxonomy, and ecological requirements?

Materials and Methods

Study species and field sampling

Zapada glacier (Plecoptera: Nemouridae; Figure 2.1) is known to occur in three mountainous regions: Glacier National Park (GNP) of northwestern Montana, the Absaroka-Beartooth Wilderness (ABW) of southern Montana, and the Teton Range of northwestern Wyoming (Figure 2.2; Giersch et al., 2016). Conversely, both *Lednia* (Plecoptera: Nemouridae) species are endemic to a single mountain range: *L. tumana* (GNP; Figure 2.1) and *L. tetonica* (Teton Range; Figures 2.1-2.2), and both species co-occur with *Z. glacier* in their respective ranges. Beyond *Z. glacier*, the *Zapada* genus is widely distributed, with seven recognized species in the western United States (Baumann, 1975; Baumann *et al.*, 1977) whereas *Lednia* contains only two other species, both of which are also mountain range endemics: *L. borealis* of the Cascades, and *L. sierra* of the Sierra Nevada (Baumann & Kondratieff, 2010).While no *Lednia* species overlap geographically, *Zapada* species overlap with one another extensively. As only *Zapada* nymphs are morphologically distinguishable from one another, these overlapping distributions raise a particular challenge for field identification (Baumann & Gaufin, 1971). To overcome this, we sequenced the cytochrome oxidase I (COI) "barcoding" locus for all newly collected *Zapada* specimens and compared these new sequences with those previously collected for known *Zapada* adults and nymphs (Giersch *et al.*, 2015) through mtDNA gene tree construction (see below).

In 2015 and 2016, we sampled alpine streams in GNP, the Absaroka-Beartooth Wilderness (ABW), and the Teton Range (Figures 2.1-2.2), with the goal of collecting *Zapada* and *Lednia* specimens. We also acquired specimens of *L. sierra*, *L. borealis*, and

Zapada from alpine streams in California, Washington, New Mexico, and Oregon (Figure 2.3). Despite efforts on multiple occasions, the genus *Lednia* has never been observed in ABW. Sampling information for all localities and species included are outlined in Table 2.1.

DNA barcoding

In total, we sequenced the 'DNA barcoding' portion of the mtDNA genome, a 658-bp region of COI, for 79 newly collected specimens representing *Zapada* sp. (n = 34), *L. tetonica* (n = 43), *L. sierra* (n = 1), and *L. borealis* (n = 1). COI is commonly used in DNA barcoding as it is variable across species, yet retains conserved primer binding sites (Hebert *et al.*, 2003). Barcoding was performed by the Canadian Center for DNA Barcoding (CCDB) following established protocols for extraction (Ivanova *et al.*, 2006), polymerase chain reaction (PCR), and sequencing (Hajibabaei *et al.*, 2005; DeWaard *et al.*, 2008). For PCR, the primer sets LCO1490/HCO2198 (Folmer *et al.*, 1994) were used to amplify the target fragment of COI. Successful PCR amplicons were checked on a 2% agarose gel and products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, California, USA). Purified amplicons were cycle-sequenced using a Big Dye v3.1 dye termination kit, purified using Sephadex, and sequenced bidirectionally on an ABI 3730 sequencer (Applied Biosystems, Foster City, California, USA). Additional information on the methods and pipelines used for barcoding by CCDB are available at http://ccdb.ca/resources.php. Sample information, photographs, and sequences of newly barcoded specimens are available through the Barcode of Life Data System (BOLD; Ratnasingham & Hebert, 2007; project name = "LDZP"). After barcoding, COI

sequences were visually inspected, corrected, and aligned using MUSCLE (Edgar, 2004) as implemented in Geneious version 6.1.8 (Kearse *et al.*, 2012).

To identify *Z. glacier* specimens and generate a complete data set for population genetic comparisons, we combined the 79 new COI sequences with data from three published studies: two focused on *Zapada* sp. (Giersch *et al.*, 2015; Giersch *et al.*, 2016) and one on *L. tumana* (Jordan *et al.*, 2016). GenBank and BOLD accession information for all new and previously published sequence data can be found in Table 2.4. To limit any influence of temporal genetic change (e.g., loss of haplotypes, Jordan et al., 2016), only specimens for the focal species collected after 2010 were included, except for six specimens of *Z. glacier* from ABW that were collected in 2000. For *Zapada*, the final data set contained 460 specimens, with 256 sequences for *Z. glacier* and 204 sequences representing all other species in the western *Zapada* taxonomy. For *Lednia*, the final data set contained 115 specimens, with 70 *L. tumana* sequences, 43 *L. tetonica* sequences, and one sequence each for *L. borealis* and *L. sierra*.

Gene tree estimation, haplotype network construction, and population genetic analyses

For phylogenetic analyses, we analyzed the *Zapada* and *Lednia* data sets separately with *V. cataractae* serving as the outgroup for all *Zapada* specimens and *Z. glacier* as the outgroup for *Lednia*. To construct trees, we first used an Akaike information criterion (AIC) test implemented in MrModeltest (Nylander, 2004) to select the best-fit model of DNA substitution (GTR+I+G). Next, we used MrBayes version 3.2.4 (Ronquist *et al.*, 2012) to generate mtDNA gene trees for each data set using 5 chains analyzed for 10 million generations with a 1-million generation burn-in. Samples

were taken every 10000 generations for two replicates. Convergence was determined by inspecting effective samples size (ESS >200) values in Tracer v1.6.0 (Rambaut & Drummond, 2007). Retained poster distributions for each replicate were combined to generate a majority-rule consensus tree. For *Zapada*, the placement of our newly barcoded samples provided the evidence for which species or lineage our 34 new specimens represented.

We constructed haplotype networks by compressing sequences into common haplotypes using the ALTER web server (Glez-Peña *et al.*, 2010) and generating networks in POPART (Leigh & Bryant, 2015) with the TCS implementation (Clement *et al.*, 2000). We performed an analysis of molecular variance (AMOVA) in Arlequin 3.5 (Excoffier & Lischer, 2010) to assess how genetic variation is partitioned across multiple sampling levels. The AMOVA was performed separately on the *Z. glacier* and *L. tumana*+*L. tetonica* data sets using mountain ranges as the highest level of structure. We assessed significance and 95% confidence intervals using 5000 bootstrap replicates. We also calculated nucleotide diversity (π) for four spatially defined groups: *Z. glacier* across its full range, *Z. glacier* by mountain range, *L. tumana,* and *L. tetonica*.

Demographic model selection and gene flow estimation

For both the *L. tumana*+*L. tetonica* and *Z. glacier* data sets, we tested a range of demographic models and characterized gene flow (when applicable) under a coalescent framework in the program Migrate-n v3.6 (Beerli & Felsenstein, 2001). For *Z. glacier*, we tested eight three-population models that were similar to those tested for *Lednia* (Figure 2.4). For *L. tumana*+*L. tetonica*, we tested five two-population models ranging

from full, bidirectional gene flow to panmixia (Figure 2.4). For all Migrate-n analyses, initial parameter values were calculated using F_{ST} and model averaging was used to estimate migration rate (m) and θ. For the two models without migration, we followed Beerli and Palczewski (2010) in specifying a very small (m = 0.01), uniform custom migration rate among groups. We estimated the transition/transversion ratio (ti/tv) for both alignments via maximum likelihood model selection in jmodeltest2.1.10 (Darriba *et al.*, 2012). These ratios were 15.63 and 4.70 for *L. tumana*/*L. tetonica* and *Z. glacier*, respectively. For all runs, a static heating strategy with four short chains (temperatures of 1.0, 1.5, 3.0, and 1.0×10^6) and one long chain was used. We recorded 25,000 steps every 100 generations with 10,000 steps discarded as burn-in. To ensure Markov chain stationarity, we examined ESS values for each parameter with a minimum threshold of 200. To select among models, we used the Bezier Approximation Score (BAS) to calculate log Bayes Factors (LBFs) and probabilities for each model following Beerli and Palczewski (2010). We calculated number of migrants per generation using the equation, $Nm = M \times \theta$.

Results

Zapada *barcoding, systematics, and population genetics*

Our final COI alignment for *Zapada* was 658-bp long with 2.49% missing data across all specimens and 1.95% missing data for *Z. glacier* only. Phylogenetic analyses supported the seven recognized *Zapada* species as being monophyletic with posterior probabilities (PPs) of 1.0 (Figure 2.5). For our 34 newly barcoded *Zapada* specimens, 18 were identified as *Z. glacier.* These new specimens were from four streams where *Z.*

glacier had not previously been recorded: three in ABW and one in the Teton Range (Figure 2.2; Table 2.1), bringing the total number of streams known to contain *Z. glacier* to 13 (Giersch *et al.*, 2015; Giersch *et al.*, 2016).

Our phylogenetic analyses also identified four monophyletic *Zapada* lineages that may represent undiagnosed species lineages: (1) a "WY-NM" clade that is sister to *Z. haysi* and includes specimens from the Teton Range and the Wheeler Peak Wilderness ~1,075 km to the south in New Mexico. (2) A previously described "Sexton" clade named for its original discovery from specimens inhabiting the meltwater of the Sexton Glacier in GNP (Giersch *et al.*, 2015) and distributed across the Teton Range, ABW, and GNP. (3) A "*Z. oregonensis* WA" clade that is sister to *Z. oregonensis*, identified from the Goat Rocks Wilderness in western Washington, and distinct from Z. *oregonensis*, a wide-ranging *Zapada* species represented in this study by specimens collected in Wyoming and Montana. (4) A "Z. *columbiana* PNW" clade that may represent a pacific northwestern sister lineage of *Z. columbiana*, a species identified from Wyoming and Montana samples here.

A haplotype network connecting all *Z. glacier* specimens (n = 256) included 20 haplotypes from the three mountain ranges: GNP (n = 198 specimens; 14 haplotypes), ABW (n = 23 specimens; 2 haplotypes), and the Teton Range (n = 35 specimens; 5 haplotypes; Figure 2.6). Each mountain range was generally characterized by a distinct set of haplotypes, however, haplotypes were shallowly diverged within mountain ranges and only slightly more diverged among them. Interestingly, a single haplotype overlapped between the Grinnell Glacier site in GNP ($N = 1$) and ABW ($N = 22$). The maximum divergence among any two *Z. glacier* haplotypes was 1.22%. When the full

western *Zapada* taxonomy is connected in a haplotype network (Figure 2.7), relationships reflect those in the mtDNA gene tree (Figure 2.5). Described and potentially cryptic species-level *Zapada* lineages differed from those most closely-related to them by 4.26% (e.g., *Z. glacier* to *Z. haysi*) to 8.35% (*Z. cinctipes* to *Z. columbiana*; Figure S3). Among range differentiation explained 89.5% of the variation ($\Phi_{CT} = 0.89$) in the data and within population variation explained another 10.9% ($\Phi_{ST} = 0.89$; Table 2.3). Nucleotide diversity (π) for *Z. glacier* was 0.0696 and range-by-range π was: GNP ($\pi = 0.0203$), ABW ($\pi = 0.0003$), and the Teton Range ($\pi = 0.0066$; Table 2.3). In addition to *Z. glacier*, we also identified eight new 'Sexton' specimens from one location, seven new *Z. haysi* specimens from two locations, and a single new *Z. columbiana* specimen.

Lednia *barcoding, systematics, and population genetics*

Our final COI alignment for *Lednia* (n = 115) was 658-bp long with 1.27% missing data. We confirmed the presence of *L. tetonica* at its only previously known location, Wind Cave at the head of Darby Creek, in the Teton Range (Baumann & Call, 2012). Our field surveys expanded this known distribution to seven new sites, all within the Teton Range (Figure 2.1, Table 2.1). For other *Lednia* species, we did not identify any new localities beyond those previously described (Baumann & Kondratieff, 2010; Muhlfeld *et al.*, 2011; Baumann & Call, 2012; Giersch *et al.*, 2016; Jordan *et al.*, 2016). Phylogenetic analyses strongly supported the existing *Lednia* taxonomy with PPs of 1.0 for all nodes and described species resolved as monophyletic (Figure 2.5). The mtDNA gene tree placed *L. tetonica* and *L. tumana* as sister species, with *L. borealis* as the sister

lineage to the *L. tetonica*+*L. tumana* clade, and *L. sierra* as the sister species to the other three (Figure 2.5).

For all *Lednia* specimens, we identified five *L. tumana* haplotypes, seven *L. tetonica* haplotypes, and one haplotype each for the single specimens of *L. borealis* and *L. sierra*. A haplotype network connecting *Lednia* samples revealed strong divergence across described species (and by proxy, mountain ranges; Figure 2.6). When *L. tumana* and *L. tetonica* were grouped by species (i.e., mountain range), among species variation explained 95.3% of the variation ($\Phi_{CT} = 0.89$), differentiation within species explained a negligible amount (0.4%, $\Phi_{CT} = 0.08$), and within population differentiation explained the remaining 4.3% ($\Phi_{ST} = 0.95$; Table 2.3). Nucleotide diversity (π) for *L. tumana* and *L. tetonica* was 0.0035 and 0.0013, respectively (Table 2.3).

Demographic model selection and gene flow estimation

For *Z. glacier*, the best-supported demographic model was the "north-to-south" model, which included gene flow from GNP into ABW and from ABW into the Teton Range (model 2, model probability ~1; Figure 2.4, Table 2.4). All other models were strongly rejected (LBFs ≥ 12, model probabilities ≤ 2.4×10^{-3}). Interestingly, a no-migration model was one of the least supported (model 7; LBF = 47.3, model probability = 5.5×10^{-11}). For the best-fit model, the number of migrants per generation from GNP into ABW (mean Nm = 1.02, 95% confidence interval = 0 – 5.27) was estimated at twice the rate observed for ABW into the Teton Range (mean Nm = 0.5, 95% confidence interval = 0 – 2.75; Table 2.5).

For *L. tumana* and *L. tetonica*, the best-supported model included no migration between species (model 4, model probability ~1; Figure 2.4, Table 2.4). All models including a gene flow parameter were similarly rejected (LBFs ≥ 142.9, model probabilities ≤ 9.3×10^{-32}) as was the panmixia model (model 5, LBF = 529.5, model probability = 1.1×10^{-115}). Because the best-fit model did not include a gene flow parameter, we did not estimate migration rates between *L. tumana* and *L. tetonica*.

Discussion

Patterns of genetic differentiation among co-occurring species

Understanding the degree to which shared ecology and geography extends to shared evolutionary histories is a fundamental question in evolutionary biology. Comparisons of populations' genetic patterns for species at different points along a similarity continuum can collectively clarify the degree which predictions about species evolutionary histories, based upon their ecology and distributions, can be a made. From a conservation perspective, these comparisons directly inform the extent to which insight from one species can be applied to similar taxa that have received less research effort. Collectively, previous studies have indicated that shared distributions (Lapointe & Rissler, 2005; Barber *et al.*, 2006) and ecology (Whiteman *et al.*, 2007; Satler & Carstens, 2017) both play highly influential roles on population genetic differentiation, and can drive congruence in patterns independent of one another. In this study, we asked whether shared distributions and ecology for three stoneflies would translate to highly similar patterns of population genetic differentiation and demographic history, as previous research would suggest. This prediction was clearly rejected, as evolutionary

patterns did not align among our study species. This result indicates that instead of being a taxonomic artifact introduced during the taxonomic process, the morphological differentiation among nymphs of *Lednia* species, and lack thereof for *Z. glacier*, actually correlates with levels of mtDNA differentiation between the two groups.

Indeed, the most differentiation was observed between *L. tumana* and *L. tetonica* and corresponds with no evidence for contemporary gene flow between the two. Given the ~600 km distance between the two species, and lack of high-quality habitat (i.e., few cold, alpine streams) between their respective mountain ranges, this is an expected result. However, the patterns observed for *Z. glacier*, while aligning with previous studies (e.g., Giersch et al., 2015; Giersch et al., 2016), starkly contrast this pattern with demographic modeling rejecting a "no migration" model in favor of one supporting a "north-to-south" model of contemporary gene flow. This support for a north-to-south pattern of gene flow, falls in line with broader biogeographic hypotheses in North America, specifically the existence of a historical north-to-south immigration corridor along the spine of the USA Rocky Mountains from a Beringian refugia deep into the western United States (DeChaine & Martin, 2005; Finn & Adler, 2006). Beyond contrasts with *Lednia*, our comparative results also run counter to prevailing theory in alpine stream population genetics: that alpine specialists exhibit significant population subdivision, even over small spatial scales (Finn & Adler, 2006; Finn *et al.*, 2006; Jordan *et al.*, 2016)

Rejecting the hypothesis that shared natural history and ecology would extend to population genetic congruence has two primary implications: first, using shared natural history or ecology in conservation planning as a surrogate for shared genetic history should be done with caution. Two of the focal species in this study, *L. tumana* and *Z.*

glacier, have been recommended for listing under the ESA (Giersch *et al.*, 2016; U.S. Fish and Wildlife Service, 2016), and while it would be reasonable to assume conservation strategies would apply equally well to both, in the light of mtDNA, this wouldn't be totally appropriate assertion. However, it is true that given the patterns of general isolation occurring with mountain range, a conservation strategy developed from either *Z. glacier* or *Lednia* data alone would apply reasonably well to the other. Tied into this, a second implication of this study's results is the support for an as yet unknown difference between the two groups – perhaps in life history characteristics, geographic distributions, or both (see below).

Alpine stream population genetics

Studies comparing population genetic patterns for more than a single alpine stream species are rare (Hotaling *et al.*, 2017b), and have generally identified significant variation among co-distributed species with hypothesized links to dispersal ability or other biological traits that influence gene flow (Monaghan *et al.*, 2002; Dussex *et al.*, 2016). To this end, it appears likely that as yet unknown differences in life-history (e.g., dispersal ability) or a wider ecological profile for *Z. glacier* (e.g., in terms of thermal tolerance) are driving elevated levels of gene flow or a higher density of between range satellite populations, and thereby the observed genetic patterns. Such differences in life-history traits among co-occurring, closely related species have been observed for congeneric caddisflies (Jackson & Resh, 1991) and other aquatic insect taxa that are ecologically similar but vary in their dispersal abilities (Monaghan *et al.*, 2002; Finn & Poff, 2008). The patterns observed in this study could also reflect genus-specific

evolutionary trajectories as well. For instance, *L. tumana* and *L. tetonica* may have a longer history as cold-water specialists isolated in glacier associated refugia (e.g., before the last glacial maximum in the region ~20,000 years ago, Carrara, 1987). Conversely, *Z. glacier* may have only recently invaded headwaters due to more recent range shifts to higher elevations, perhaps following the retreat of glaciers or to avoid competition with lower elevation species. These hypotheses are not mutually exclusive and future studies will no doubt clarify the relative influences of any life-history variation as well as historical biogeography and divergence on contemporary patterns of genetic diversity among *Lednia* and *Z. glacier*.

Conservation of alpine stoneflies in North America

In light of the recent U.S. Fish and Wildlife Service decision to recommend listing of *Z. glacier* and *L. tumana* under the ESA due to climate change-induced threats (U.S. Fish and Wildlife Service, 2016), the results of our recent field surveys in the Teton Range and ABW provided important clarification of the distribution of *Z. glacier* and *L. tetonica*. We confirmed the presence of *Z. glacier* from four streams where it was not previously known to occur – three in ABW and one in the Teton Range – bringing the total number *Z. glacier* containing streams to 13 (Figure 2.2). For *L. tetonica*, the results were even more valuable: originally described from a single location, we expanded *L. tetonica*'s known distribution to seven streams, all still within the Teton Range and the surrounding mountains, and all fed by a permanent ice mass (i.e., subterranean ice, a rock glacier, or glacier). For *Z. glacier*, despite being more rare (in terms of known population density in a given area), we still found evidence for contemporary gene flow among

populations among isolated mountain ranges including the sharing of a single haplotype between specimens representing all populations sampled in ABW and a single specimen from GNP. Specifically, the support for a north-to-south pattern of gene flow from GNP through ABW and into the Teton Range highlights the likely importance of ABW as a stepping stone between GNP and the Teton Range. It appears likely that "stepping stone" populations of *Z. glacier* between GNP and ABW (e.g., the Crazy and Mission Mountains, Swan Range, or the Gallatin Range) remain to be discovered. The finding of a shared haplotype between geographically isolated ranges is not novel, rather it aligns well with patterns observed for Ecuadorian alpine mayflies (Finn *et al.*, 2016).

Whether or not imperiled species actually contain cryptic species-level lineages is a topic of significant interest in conservation biology with important implications for management planning (Hime *et al.*, 2016). Here, we find clear mtDNA support for both *L. tumana* and *L. tetonica* as separate, range-specific species with no contemporary gene flow between them. For *Z. glacier*, the reality is less clear: using similar mtDNA, Giersch *et al.* (2016) suggested that, like *Lednia*, the currently described *Z. glacier* may actually contain three separate range-specific species in GNP, ABW, and the Teton Range, as mtDNA haplotypes generally correspond with mountain ranges. Our extended mtDNA data, which included significantly more sampling from ABW and the Teton Range provide slightly less support for that assertion. Indeed, patterns of differentiation do correspond with mountain ranges, and there is a clear break between GNP+ABW and the Teton Range, but evidence for contemporary gene flow and a shared haplotype between GNP and ABW are clear evidence against additional species diversity within the *Z. glacier* complex. However, the possibility for discordance between mitochondrial and

nuclear genomes (where evolutionary patterns do not align between the two) in population genetic and systematic studies is well-known (Gompert *et al.*, 2008; Toews & Brelsford, 2012). Therefore, multi-locus nuclear data paired with coalescent-based species delimitation tools are needed before any robust conclusions can be made (Yang & Rannala, 2010; Grummer *et al.*, 2014; Hotaling *et al.*, 2016).

Conclusions

Understanding the degree to which shared ecology and geographic distributions extends to population genetic similarity is an important aspect of conservation biology. Beyond shedding light on taxon-specific patterns of differentiation and demography for alpine stoneflies, the results of this study also hold broader implications. The most overarching of these is the empirical evidence this study provides indicating that even when highly similar species are compared, population genetic patterns among them can starkly contrast. Generally speaking, these differences are difficult to link with causal variation – for instance, *Z. glacier* may simply be a more apt disperser than *Lednia* species – but highlighting the difference is important in its own right. Indeed, this study and others provide evidence that simplified "conservation by proxy" approaches (Roberge & Angelstam, 2004; Caro, 2010) where one species serves as a conservation surrogate for others are bound to overlook relevant interspecific patterns of genetic variation. In fairness, the goal of these approaches is not to accurately reflect all evolutionary histories and genetic boundaries in a given habitat, but rather to serve as a useful management tool, ultimately simplifying the process when resources are limited. In this study, we have shown that three confamilial alpine stoneflies that overlap in

ecology, habitat, geography, and climate change threats, can still differ dramatically in population genetic and demographic signatures inferred from mtDNA sequence data. Future studies incorporating more nuanced ecological perspectives – perhaps including a specific focus on dispersal potential and/or thermal constraints – and multi-locus, nuclear genetic data will no doubt clarify the timing and underlying influences driving these differences.

Table 2.1. Sampling information for *Zapada glacier*, *Lednia tumana*, and *Lednia tetonica* specimens included in this study. Region refers to the primary geographic area where specimens were collected. *N* is the sample size for a given locality. Elevation is reported in meters. Abbreviations include: GNP = Glacier National Park, ABW = Absaroka-Beartooth Wilderness, GRTE = Grand Teton National Park/Teton Range, π = nucleotide diversity averaged over the entire COI locus. All lake locations are referring to outlet streams unless otherwise indicated. Asterisks indicate populations newly identified in this study.

Species	Stream location	Range	*N*	Latitude, longitude	Elev.
Z. glacier	Piegan Pass	GNP	16	48.7294, -113.6972	1911
Z. glacier	Grinnell Lake	GNP	37	48.7574, -113.7248	1951
Z. glacier	Appistoki Creek	GNP	87	48.4589, -113.3489	2097
Z. glacier	Dry Fork Spring	GNP	55	48.5345, -113.3805	2207
Z. glacier	Buttercup Park	GNP	3	48.4237, -113.3844	1915
Z. glacier	*Jasper Lake	ABW	2	45.0233, -109.5785	3216
Z. glacier	*Timberline Lake	ABW	5	45.1325, -109.5077	2966
Z. glacier	Frosty Lake	ABW	6	45.0261, -109.5515	3194
Z. glacier	*W. Fork Rock Ck.	ABW	10	45.0962, -109.6040	3001
Z. glacier	*Delta Lake	GRTE	1	43.7325, -110.7750	2754
Z. glacier	Teton Meadows	GRTE	21	43.7259, -110.7904	2824
Z. glacier	S. Cascade Creek	GRTE	6	43.7285, -110.8373	2948
Z. glacier	Mica Lake	GRTE	7	43.7854, -110.8414	2886
L. tumana	Lunch Creek	GNP	23	48.7052, -113.7046	2156
L. tumana	Sexton Glacier	GNP	31	48.7003, -113.6281	1992
L. tumana	Siyeh Bend	GNP	4	48.7115, -113.6751	1943
L. tumana	Bearhat Mountain	GNP	10	48.6650, -113.7491	1957
L. tumana	Heavens Peak	GNP	1	48.7102, -113.8427	2042
L. tumana	Grant Glacier	GNP	1	48.3314, -113.7368	1606
L. tetonica	*Alaska Basin	GRTE	6	43.6895, -110.8327	3119
L. tetonica	*Sunset Lake Inlet	GRTE	6	43.7102, -110.8556	2949
L. tetonica	*Schoolroom Glacier	GRTE	6	43.7286, -110.8440	3039
L. tetonica	Wind Cave	GRTE	6	43.6657, -110.9561	2676
L. tetonica	*Teton Meadows	GRTE	6	43.7258, -110.7931	2845
L. tetonica	*N. Fork Teton Ck.	GRTE	6	43.7681, -110.8615	2780
L. tetonica	*Upper Paintbrush	GRTE	7	43.7852, -110.7941	2805

Table 2.2. GenBank and BOLD accession information for sequence data included in this study.

Species	Database	Project name or accession ID(s)	Study	Notes
Zapada sp.	BOLD/ Genbank	GNPZa / KM874110–KM874263	Giersch et al. 2015	
Zapada sp.	BOLD	GNPZP	Giersch et al. 2016	
Lednia tumana	GenBank	KX212679-KX212864	Jordan et al. 2016	Samples from 2010 or later only
Zapada sp. and *L. tetonica*	BOLD	LDZP	This study	

Table 2.3. Population genetic diversity metrics. Symbols include: Φ_{CT} = among region differentiation, Φ_{SC} = differentiation within mountain range or species, Φ_{ST} = total differentiation within populations (i.e., mountain range or species), and π = nucleotide diversity averaged over the entire COI locus. Numbers in parenthesis are the percent variation explained. For "*Z. glacier*, by range" the given π is for all *Z. glacier* specimens. ns = not significant at $P \leq 0.05$.

Group	π	Φ_{CT}	Φ_{SC}	Φ_{ST}
Z. glacier, by range	0.0696	0.89 (89.5%)	ns	0.89 (10.9%)
Z. glacier, GNP	0.0203			
Z. glacier, ABW	0.0003			
Z. glacier, Teton Range	0.0066			
Lednia sp., by species		0.96 (95.3%)	0.08 (0.4%)	0.95 (4.3%)
L. tumana	0.0035			
L. tetonica	0.0013			

Table 2.4. Phylogeographic model descriptions and selection results for (a) *Lednia tumana* and *Lednia tetonica* and (b) *Zapada glacier* tested in Migrate-n. LBF: log Bayes factor. GNP: Glacier National Park. ABW: Absaroka-Beartooth Wilderness. LBFs and model probabilities calculated following Beerli and Palczewski (2010). Arrows (>) indicate the direction of migration for a given model. The best-fit model is highlighted in bold.

Model	Description	LBF	Probability	Choice
(a) *Lednia tumana* and *Lednia tetonica*				
1	Full migration	255.6	3.0×10^{-56}	4
2	Unidirectional: *L. tetonica* > *L. tumana*	161.4	8.9×10^{-36}	2
3	Unidirectional: *L. tumana* > *L. tetonica*	142.9	9.3×10^{-32}	3
4	**No migration**	**--**	**~1**	**1**
5	Panmixia	525.5	1.1×10^{-111}	5
(b) *Zapada glacier*				
1	Full migration	64.5	1.0×10^{-14}	5
2	**North to south: GNP > ABW > Teton Range**	**--**	**~1**	**1**
3	South to north: Teton Range > ABW > GNP	12.1	2.4×10^{-3}	2
4	Out of GNP: GNP > ABW, GNP > Teton Range	14.0	9.0×10^{-4}	3
5	Out of ABW: ABW > GNP, ABW > Teton Range	31.5	1.4×10^{-7}	4
6	Out of the Teton Range: Teton Range > GNP, Teton Range > ABW	60.0	9.2×10^{-14}	6
7	No migration	47.3	5.5×10^{-11}	7
8	Panmixia	215.4	1.7×10^{-47}	8

Table 2.5. Rate of migration (M), direction, θ (mutation-scaled effective population size), and Nm (number of immigrants per generation) for the best-fit model (model 2) for *Zapada glacier* estimated using Migrate-n. All values are the mean estimate with 95% confidence intervals in parentheses. Provided θ values are for the region receiving migrants. GNP: Glacier National Park, ABW: Absaroka-Beartooth Wilderness, GRTE: Grand Teton National Park.

M	Direction	θ	Nm
636.5 (90–1296)	GNP > ABW	1.6×10^{-3} ($0–4.1 \times 10^{-3}$)	1.02 (0–5.27)
201.1 (0–529)	ABW > GRTE	2.5×10^{-3} ($0–5.2 \times 10^{-3}$)	0.5 (0–2.75)

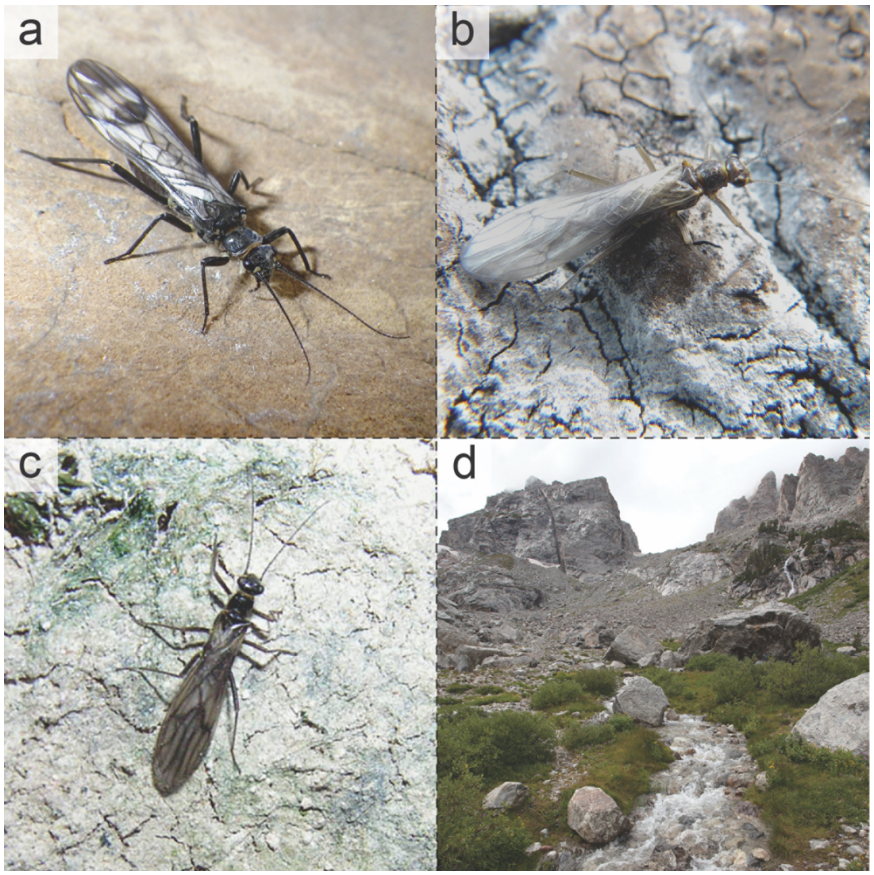

Figure 2.1. Photographs of adult (a) *Zapada glacier*, (b) *Lednia tumana*, and (c) *Lednia tetonica.* (d) Garnett Canyon in Grand Teton National Park, exemplar alpine stream habitat where *Z. glacier* and *L. tetonica* co-occur. The stream is primarily fed by the Middle Teton glacier above the cliff bands.

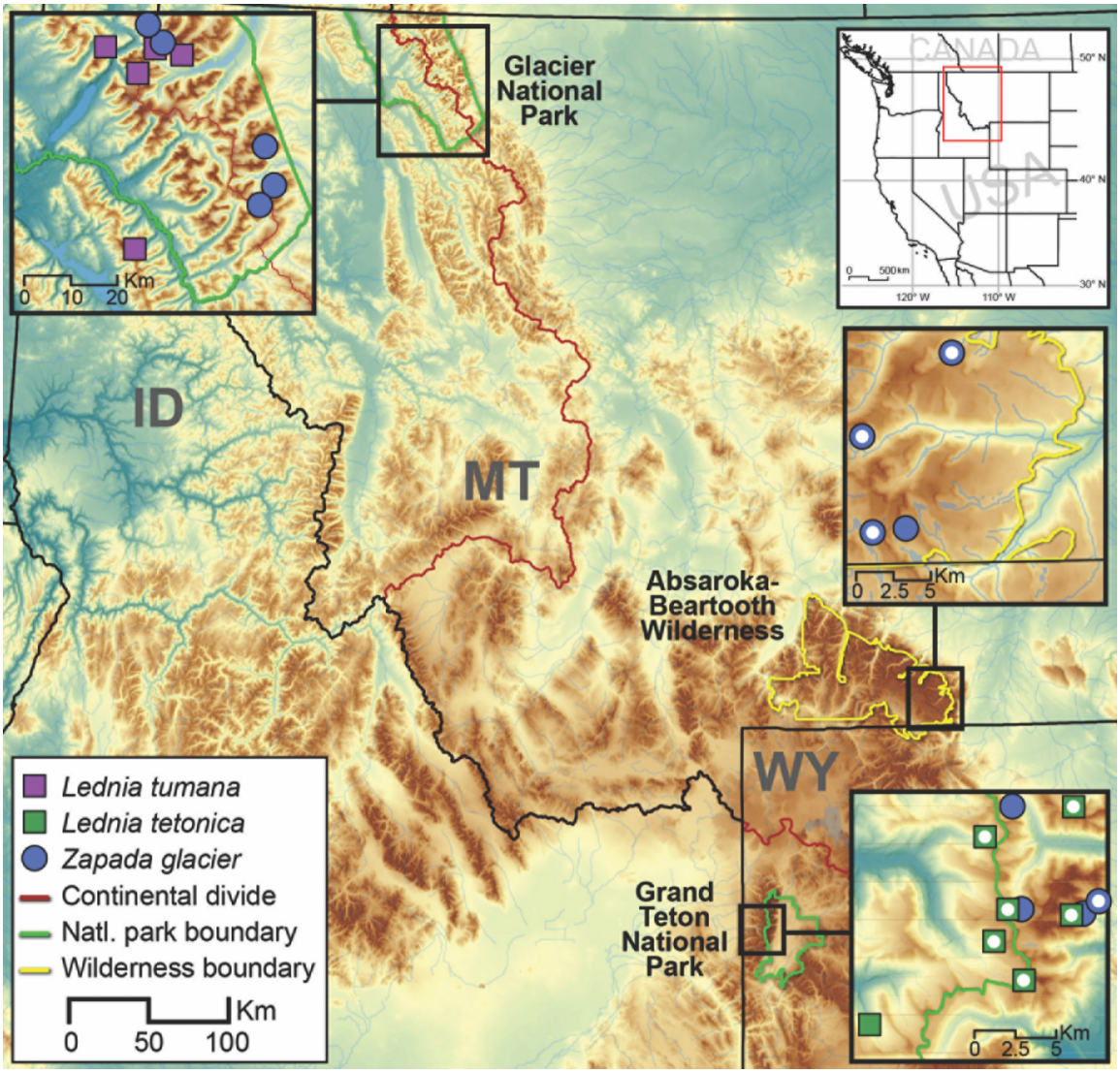

Figure 2.2. The distribution of *Zapada glacier*, *Lednia tumana*, and *Lednia tetonica* specimens included in this study. The study area shown includes Glacier National Park, the Absaroka-Beartooth Wilderness, and Grand Teton National Park superimposed on an elevation gradient. Detailed locality information is included in Table 1. Circles with white fill indicate the 10 new populations (four of *Z. glacier*, six of *L. tetonica*) identified in this study.

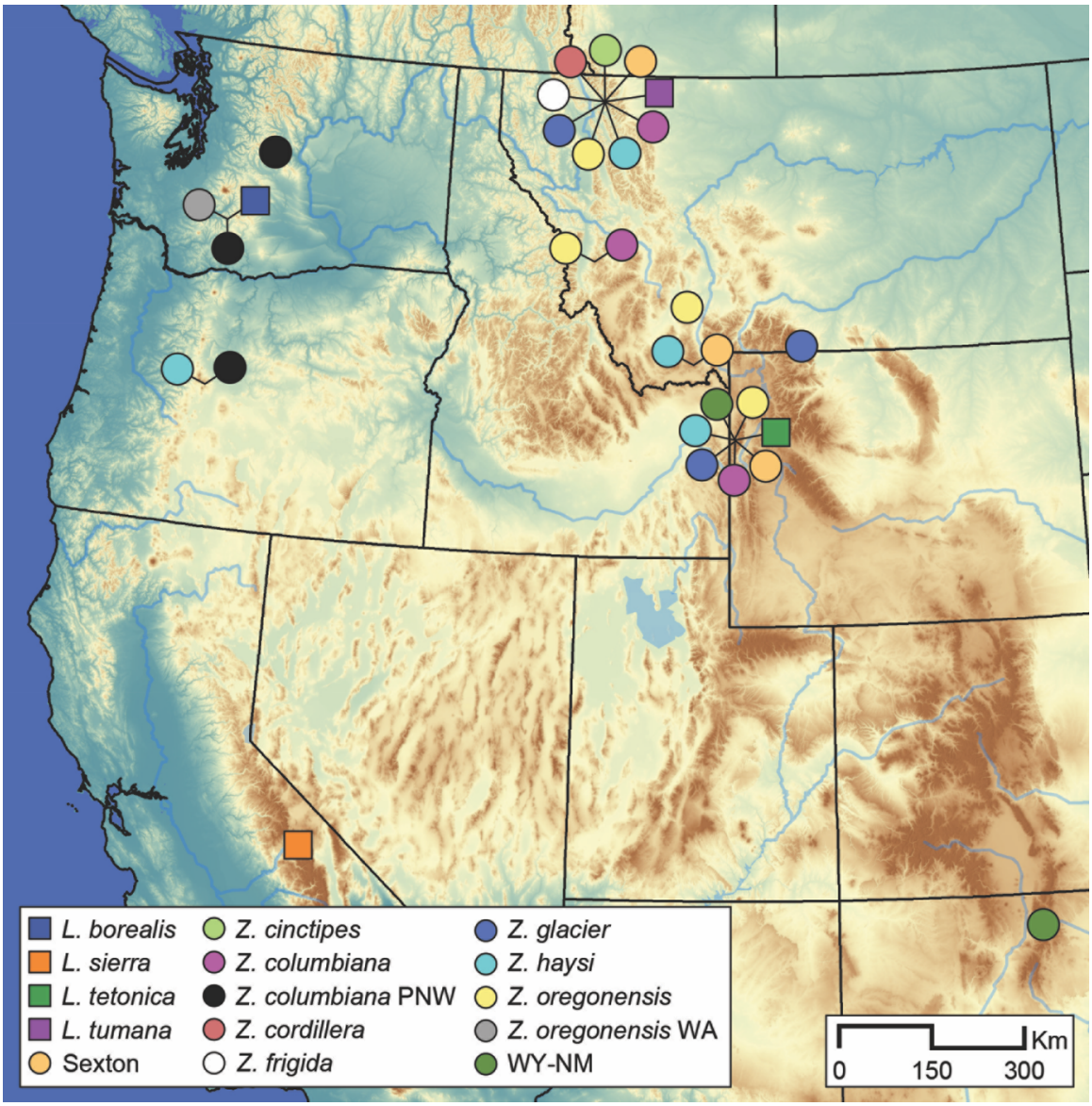

Figure 2.3. Distribution of all *Zapada* and *Lednia* specimens included in this study.

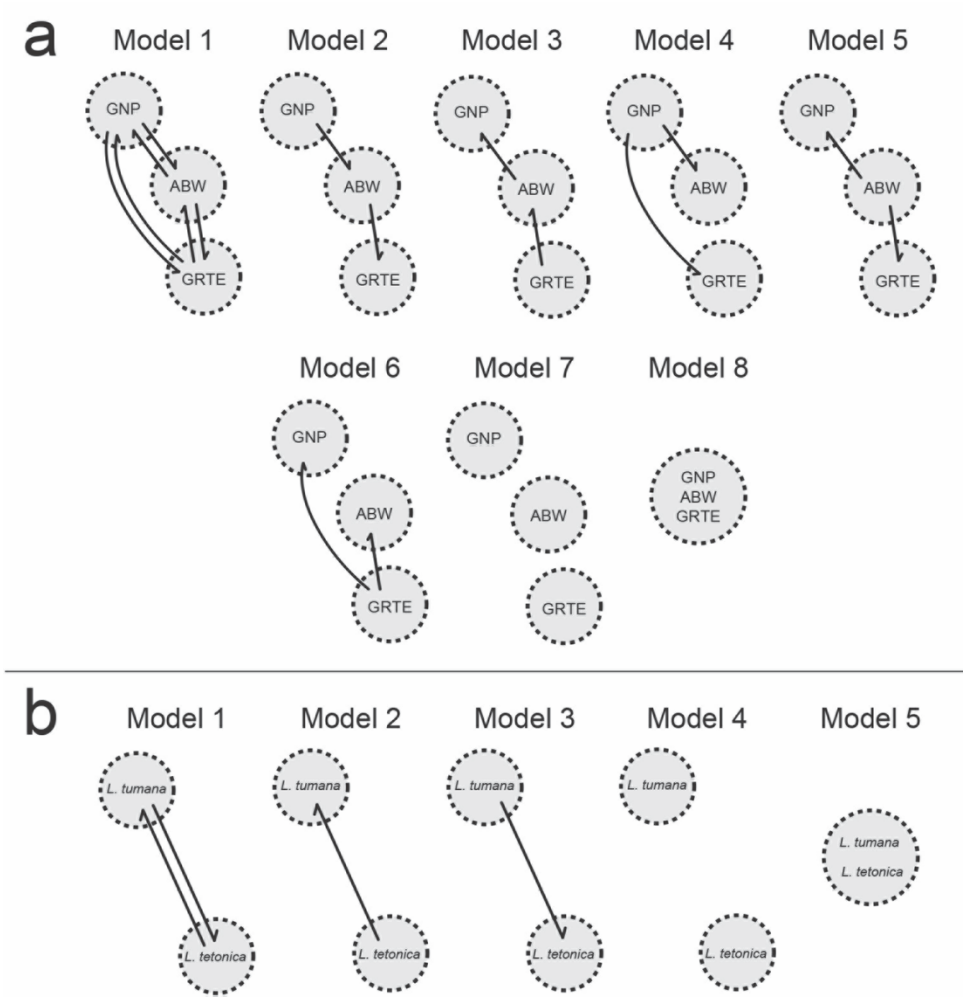

Figure 2.4. Phylogeographic models tested in Migrate-n for (a) *Zapada glacier* and (b) *Lednia tumana* and *Lednia tetonica*. GNP = Glacier National Park, ABW = Absaroka-Beartooth Wilderness, GRTE = the Teton Range. Black arrows indicate the direction of gene flow.

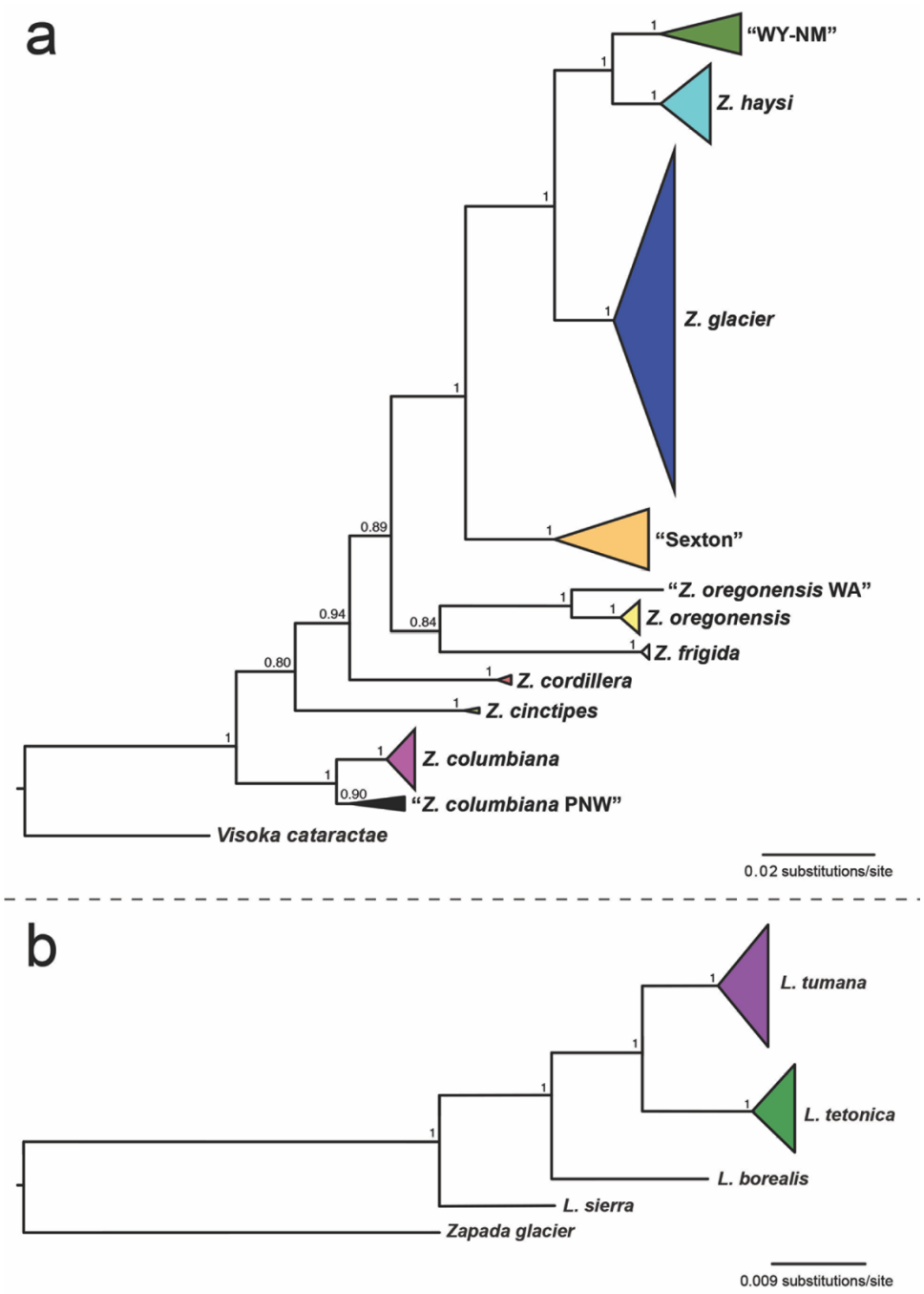

Figure 2.5. Cytochrome oxidase *c* subunit I (COI) gene trees of (a) the genus *Lednia* including 70 specimens from Jordan *et al.* (2016) and 45 newly barcoded specimens, and (b) western North American *Zapada*. Terminal nodes were compressed into triangles and scaled according to number of specimens. Node numbers indicate posterior probabilities.

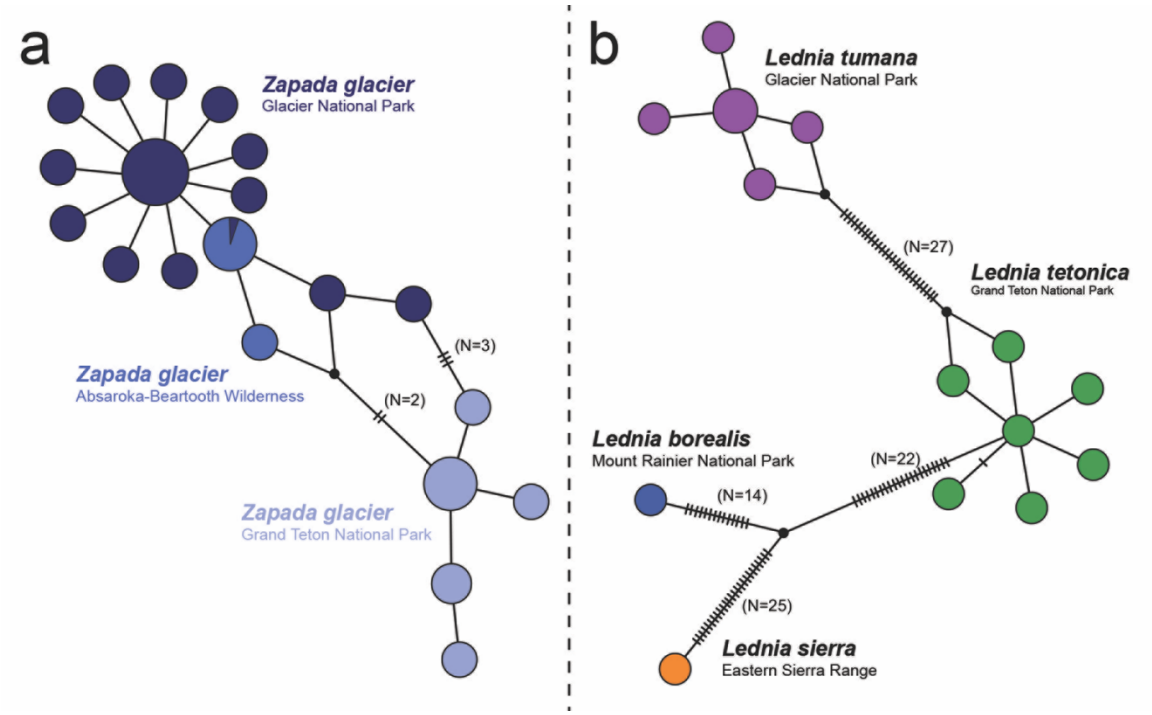

Figure 2.6. A COI haplotype network of (a) all *Zapada glacier* specimens and (b) the four species recognized by the current *Lednia* taxonomy. Colored circles represent compressed haplotypes (with higher frequency haplotypes as larger circles) with one mutational difference between them. Hashmarks between compressed haplotypes represent one additional mutational step. Connections with no hashmarks are one mutation apart.

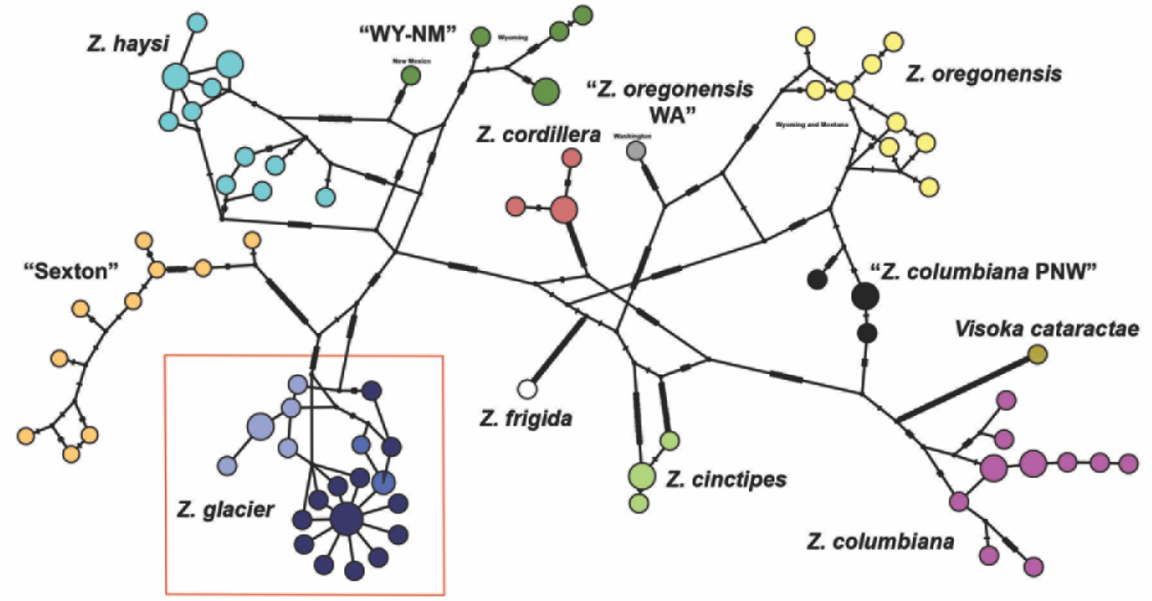

Figure 2.7. A COI haplotype network of all *Zapada* specimens. Colored circles represent compressed haplotypes (with higher frequency haplotypes as larger circles) with one mutational difference between them. Hashmarks between compressed haplotypes represent one additional mutational step. Connections with no hashmarks are one mutation apart. Depicted "clades" are putatively cryptic species-level lineages. The portion of the *Zapada* network depicted in Figure 2.6A is shown outlined by a red box.

CHAPTER THREE

DEMOGRAPHIC MODEL TESTING REVEALS A HISTORY OF DIVERGENCE WITH GENE FLOW FOR A GLACIALLY-TIED STONEFLY IN A CHANGING POST-PLEISTOCENE LANDSCAPE

Introduction

For species facing rapid environmental change, making predictions about future population persistence requires insight into the magnitude and distribution of intraspecific genetic diversity and the evolutionary forces shaping it. Generally, populations threatened by environmental change have three options: adapt *in situ* through phenotypic plasticity or genetic means, track suitable habitats through migration, or be extirpated (Hoffmann & Sgrö, 2011). Persistence through adaptation or phenotypic plasticity depends primarily on contemporary levels of genetic variation (i.e., the template for evolutionary change), past environmental variation and subsequent selection, and connectivity among critical habitats (e.g., the likelihood of an adaptive allele spreading as habitats change). Thus, better understanding of genetic structure, patterns of diversity, and evolutionary histories can inform our understanding of the future viability of populations and species. Moreover, discriminating recent changes in population structure and demography from more long-standing historical patterns can illuminate the impact of recent environmental change on genetic variation. Combined, these perspectives translate to a greater understanding of how genetic variation is shaped through time, a foundation for predicting response to future scenarios of global change, and to more informed conservation decisions for species at risk of climate-change-induced extinction.

Perhaps nowhere on Earth are species facing more rapid change than above the permanent treeline in alpine, meltwater-influenced streams (Brown *et al.*, 2007b;

Hotaling *et al.*, 2017b). In these habitats, climate change is progressing at two to three times the global average (Hansen *et al.*, 2005) causing major reductions of meltwater sources and significant changes to stream hydrology, biogeochemistry, and channel stability (Milner *et al.*, 2009). With significant environmental variation over small geographic scales (e.g., < 1 km), alpine streams provide critical habitat for an array of range-restricted communities (Ward, 1994; Hotaling *et al.*, 2017b). This unique habitat distribution is largely due to variation in primary hydrologic sources – whether glacier melt, snowfield melt, or groundwater-fed spring – and their heavy influence on downstream conditions. This environmental heterogeneity is a major contributor to alpine headwaters harboring significant beta diversity (i.e., biological differentiation among sites), both in terms of species and genetic diversity (Finn *et al.*, 2013; Wilhelm *et al.*, 2013; Finn *et al.*, 2014; Jacobsen *et al.*, 2014b; Giersch *et al.*, 2016; Jordan *et al.*, 2016; Hotaling *et al.*, 2017a). Consequently, a major predicted effect of climate change is the loss of this variation as glaciers recede and headwaters become more homogenous (Jacobsen *et al.*, 2012; Hotaling *et al.*, 2017b).

The impending threat of glacial recession on alpine stream ecosystems and biodiversity is exemplified in Glacier National Park (GNP), where glaciers and permanent snow masses could disappear by 2030 (Hall & Fagre, 2003). The meltwater stonefly, *Lednia tumana* (Plecoptera: Nemouridae; (Ricker, 1952), is endemic to GNP and has been recommended for listing under the U.S. Endangered Species Act due to climate-change-induced habitat loss (U.S. Fish and Wildlife Service, 2016). *Lednia tumana* only inhabits short sections (~500 m) of cold, meltwater streams directly below glaciers, permanent snowfields, and groundwater-fed springs (Muhlfeld *et al.*, 2011;

Giersch *et al.*, 2016). As glaciers and permanent snowpack decline, *L. tumana*'s range is expected to contract by more than 80% (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016). However, historical and contemporary patterns of genetic diversity and demographic history for this imperiled species remain underexplored. Mitochondrial DNA (mtDNA) evidence indicates low genetic diversity and significant subdivision across its range (Jordan *et al.*, 2016), a pattern similar to other mtDNA-based population genetic studies of alpine stream macroinvertebrates (Finn *et al.*, 2013; Geismar *et al.*, 2014). However, given the limited resolution of mtDNA as a population-level marker (Macher *et al.*, 2015; Rašić *et al.*, 2015), and the need to address metrics beyond differentiation, genome-wide data is well-suited to be applied in this study system.

Recent advances in DNA sequencing and marker generation [e.g., restriction-site associated DNA sequencing (RADseq)] have revolutionized the collection of genome-scale population genetic data for non-model species (Miller & Hammond, 2007; Andrews *et al.*, 2016), enhancing our ability to measure genetic diversity (Emerson *et al.*, 2010) and characterize evolutionary processes (Excoffier *et al.*, 2013). Moreover, methods leveraging the site frequency spectrum (SFS) provide new opportunities to statistically test models of demographic history from genome-wide single nucleotide polymorphism (SNP) data (Gutenkunst *et al.*, 2009; Excoffier *et al.*, 2013). Using the SFS simplifies genome-wide patterns of genetic variation, while retaining signatures of demographic history, thereby providing a powerful tool for historical inference (Sousa & Hey, 2013). Taken together, SNP data and coalescent-based demographic modeling provide the necessary framework to test and parameterize complex population histories.

Large SNP data sets also introduce novel challenges, particularly from an analytical perspective. RADseq (and similar) data sets can be filtered (and interpreted) in many different ways. Typically, filtering schemes focus on the level of missing data and the inclusion or exclusion of rare alleles, as both can influence important study objectives, such as the identification of the level of population structure (Baye *et al.*, 2011; Chattopadhyay *et al.*, 2014). Congruence of population structure results across filtered data sets provides evidence for robust conclusions regarding the number of genetic clusters, and these results are easily identified when clusters are highly differentiated with limited gene flow. However, for weakly differentiated groups – those that have recently diverged or are experiencing ongoing gene flow – the effects of different filtering may be more profound, with greater need to explore how levels of missing data and rare alleles impact inferences about population structure.

Here, we investigated genetic structure and demographic history of *L. tumana* using a genome-wide SNP data set. Specifically, we quantified genetic diversity and assessed population structure across *L. tumana*'s range, and then incorporated these results into a series of demographic model tests to identify the best-fitting population history for *L. tumana.* Given the complex glacial history of GNP and the surrounding area (Carrara *et al.*, 1986; Carrara, 1987; Hall & Fagre, 2003), we tested a wide range of demographic models to characterize historic and contemporary forces shaping genetic diversity. Furthermore, with strong links to permanent snow and ice, we expected that changes in glacial patterns have led to substantial population isolation and limited gene flow throughout *L. tumana*'s recent evolutionary history (i.e., since the end of the Pleistocene; Carrara et al., 1986). Also, because initial perspectives on population

structure from our data yielded unexpected results (i.e., more clusters identified than localities sampled), we took this result as an opportunity to explore the influence of missing data, singletons, and overall SNP number in the identification of levels of structure. Collectively, this study provides genomic insight into the demographic history of an organism directly tied to declining glacial mass as well as a broadly applicable empirical example of how data filtering can influence population genetic structure.

Materials and Methods

Genetic sampling

We collected ninety-six larval specimens of *L. tumana* from ten streams (eleven sites) throughout GNP and the surrounding areas between July 2010 and September 2011 (Figure 3.1; Table 3.1). Localities were from independent streams except for two sites along Clements Creek, denoted as 'high' and 'low'. All major alpine stream types (glacial, snowmelt, and groundwater-fed spring) were represented in our sampling (Figure 3.1). Samples were stored in > 80% EtOH and DNA was extracted using a Qiagen DNEasy Blood and Tissue Kit.

RAD sequencing and SNP calling

RADseq libraries were prepared following a modified version of published protocols (Appendix S1; (Baird *et al.*, 2008; Miller *et al.*, 2012). Briefly, samples were sonicated to 500 base pair (bp) fragments and size-selected using Agencourt AMPure XP beads (Beckman Coulter). Restriction digests used a single enzyme, SbfI. To uniquely mark each sample, we used six-base barcode adapters, each differing by at least three

nucleotides. The final 96-sample RADseq library was sequenced on one lane of an Illumina HiSeq 2500 to produce 100 bp, single-end reads.

Raw reads were demultiplexed and RADseq loci were assembled using several modules of the Stacks v1.13 pipeline (Catchen *et al.*, 2011; Catchen *et al.*, 2013). Quality filtering was performed using the process_radtags.pl script with default setting. Next, loci were assembled *de novo* in 'ustacks' with a maximum distance between stacks of 2 and minimum read depth of 5. A catalog of consensus loci was created in 'cstacks' and this catalog was compared to assembled loci using 'sstacks'. Finally, 'populations' provided a means for exporting datasets for downstream analyses and population genetic statistic calculations.

To maximize shared SNPs, we first filtered our data to remove individuals with > 60% missing data. We then removed specific loci with > 25% missing data, resulting in a final data set of sixty-five individuals from eleven localities (Table 3.1). All post-Stacks filtering steps and calculations of genotyping rate per individual for the final dataset were performed in PLINK (Purcell *et al.*, 2007). Tajima's D was calculated in δaδi, v1.6.3 (Gutenkunst *et al.*, 2009). At many stages of our analyses, we used PGDSpider v2.0.5.1 (Lischer & Excoffier, 2012) to convert data sets into program-specific formats. For fastsimcoal2 analyses (see below), we included one SNP per locus.

Testing for loci under selection

Using the 6819 SNP data set, we tested for outlier loci using BayeScan v2.1 (Foll & Gaggiotti, 2008). BayeScan uses a Bayesian model to estimate the likelihood that a given marker is under selection given background differentiation, while accounting for

differences in allele frequency due to small and/or varying sample sizes. We performed BayeScan analyses under default settings in two ways: (1) sampling localities and (2) for each genetic cluster (see Results). Outlier loci were identified as those that exceeded a false discovery rate of 0.05.

Assessing population structure and differentiation

Population genetic structure was inferred using a Bayesian clustering method implemented in the program Admixture v1.3.0 (Alexander *et al.*, 2009) and a discriminant analysis of principal components (DAPC) implemented in the R package *adegenet* (Jombart, 2008; Jombart *et al.*, 2010). In our analysis of population structure, we were also interested in how various aspects of data filtering (i.e., presence or absence of singletons) and missing data were affecting our results. To provide a systematic perspective on these factors, we generated five data sets for population structure analysis. These included: (1) the full 6819 SNP data set described above (~11% missing data), (2) a 2733 SNP data set with all singletons removed, which was equivalent to a minor allele frequency cut-off of 0.015 for 65 samples (~11% missing data), (3) a 1467 SNP data set with both singletons and any locus with > 10% missing data were removed (~5% missing data), (4) a 761 SNP data set with both singletons and any SNP with > 5% missing data removed (~3% missing data), and (5) an 86 SNP data set with all singletons removed and no missing data. All five of these data sets were analyzed using both Admixture and DAPC as described below.

Admixture analyses were performed using default settings under a range of cluster numbers (K) between 1-12. For each K, we calculated and plotted the cross-

validation error to select the best-fit (lowest cross-validation error) *K*. For DAPC analyses, we first used the *find.clusters* function to assess the optimal number of groups using the Bayesian information criterion (BIC) as our selection criteria with lowest BIC corresponding to the best-fit *K*. Because retaining too many discriminant functions with respect to the number of populations can lead to overfitting of the population structure model, we retained the optimal number of principal components according to the α-score for each data set (Jombart *et al.*, 2010). Next, we performed a final DAPC analysis for each data set using the best-fit *K* and optimal number of discriminant functions.

We calculated pairwise F_{ST} among sampling localities, tested for isolation-by-distance (Wright, 1943), and performed a hierarchical AMOVA for the 1467 SNP data set using the program GenoDive (Meirmans & Van Tienderen, 2004). For pairwise F_{ST} calculations, significance was assessed using 5000 permutations (or resamplings) of the observed data. To test for a signature of isolation-by-distance among sampling localities, we estimated Euclidean distances among localities with Google Earth and tested the correlation between geographic distance and F_{ST} using a Mantel test. The hierarchical AMOVA was performed on our best assessment of hierarchical population structure (K = 3, see Results and Discussion) to quantify how genetic variation was partitioned across different levels of sampling.

Demographic model testing

We sought to understand how *L. tumana*'s demographic history has changed in a temporal framework, including the roles of isolation and migration. This required the identification of population sets across its range that represent genetically distinct units.

Therefore, we developed demographic models around a $K = 3$ level of population structure, which we identified as the most likely scenario for population structure based on our thorough exploration of data filtering and missing data. These genetic clusters generally corresponded with geography and we refer to them as north, central, and south. When defining units for demographic modeling, we assigned samples based upon the majority genetic assignment of the locality, not on a per-sample basis.

We tested 20 demographic models (Figure 3.2). Models included scenarios specifying two divergence events for all possible topologies of the three genetic clusters (models 1-3, 8-13), trifurcation models where extant genetic clusters emerged simultaneously from a common ancestor (models 7 & 14), and models where admixture between two existing genetic clusters created the third (models 4-6, 15-20). For all models, we varied the potential for bidirectional gene flow both historically and recently.

We selected and parameterized the best-fit demographic model using fastsimcoal2 v2.5.2 (Excoffier *et al.*, 2013), a coalescent-based program which estimates demography from the SFS. Since demographic inference from the SFS is dependent on a complete SFS, and particularly the presence of rare alleles (i.e., singletons and doubletons; Gutenkunst *et al*., 2009), we used the full 6819 SNP data set for all demographic modeling analyses. Using fastsimcoal2, we performed 50 replicate runs for each model with each replicate using 50,000 coalescent simulations and a minimum (-n) of 20, and maximum (-N) of 80 cycles of a conditional maximization algorithm. A stop criteria of a 1.0E-4 difference between likelihoods was used to identify convergence. We specified the nuclear mutation rate at 3.5E-9 per site per generation following estimates for *Drosophila melanogaster* (Keightley *et al.*, 2009). To select the best-fit model, the

maximum expected likelihood (MEL) was compared to the maximum observed likelihood (MOL) for the data under each model for each replicate. The best-fit run was identified as the one that minimized the difference between MEL and MOL for each set of fifty replicates for each model. Using these best-fit runs, we calculated an AIC score for each model using the formula: $[(2k) - (2 \times ln(10) \times \text{MOL})]$, where k is the number of parameters included. Next, all model AICs were subtracted from the best-fit model to give a ΔAIC. Use of the AIC allowed for model comparison despite varying numbers of parameters.

To generate 95% confidence intervals (CIs) of parameter estimates for our best-fit model, we used a combination of the initial model selection runs and parametric bootstrapping. For 95% CIs, we simulated 100 replicates of the SFS from the *_maxL.par file (i.e., the parameter estimates that produced the maximum likelihood) for the best-fit run (minimized difference between MEL and MOL) of the best-fit model. Next, we performed the same fifty replicate analyses described above for each of the 100 newly simulated SFS files. Finally, we calculated mean parameter estimates and 95% CIs from the 100 best-fit bootstrapping replicates (i.e., using the best-fit run for each of the 100 simulated SFS files). We also assessed the influence of an order of magnitude higher and lower mutation rate on our parameter estimates.

To account for the diploidy of *L. tumana*, we divided the haploid fastsimcoal2 parameter estimates in half. Estimates of divergence time in fastsimcoal2 are calculated as number of generations before present. Field observations of co-occurring mature, late-instar nymphs and smaller, early-instar nymphs (S.H., personal observation) suggest a two-year life cycle. Therefore, we doubled divergence time estimates to convert from

generations to years.

Results

RADseq data

We generated 201,634,318 sequence reads with high variation in total reads per library (avg. = 1,012,499; min. = 2497; max. = 2,901,467). We identified 92,657 total RADseq loci across all 96 samples. After the overall filtering steps were completed, our final data set contained 65 samples, 3680 variable RADseq loci, and 6819 SNPs (mean = 1.85 per locus). Within this final dataset, 4043 SNPs were singletons (minor allele count = 1). The genotyping rate for the full data set was 91.0%, and this was similar for each genetic cluster (north = 91.8%, central = 92.3%, and south = 89.4%). Outlier tests identified six outliers in the full dataset (Figure 3.3) and none when genetic clusters were accounted for. Given this very small percentage (<0.01%) and lack of outliers when accounting for regional genetic clusters, these SNPs were not removed from downstream analyses.

Population structure

Across all five data sets, Admixture analyses favored grouping all samples into a single genetic cluster based on cross validation scores (Figure 3.4). In contrast, DAPC results favored a greater level of population structure, with the optimal level of genetic clustering influenced by singletons, missing data, and the total number of analyzed SNPs (Figure 3.5). Analysis of the full 6819 SNP data set produced BIC scores that identified K = 12 as the optimal level of population structure. When singletons were removed (2733

SNP data set), the BIC identified a $K = 5$ as the optimal level of structure. When the amount of missing data was trimmed to ~5% (1467 SNPs) or 3% (761 SNPs), the BIC identified $K = 3$ as optimal. Finally, for the 86 SNP data set featuring no singletons and no missing data, the lowest BIC corresponded to a $K = 4$.

Based on these results, we identified $K = 3$ as the best estimate of hierarchical population structure (see Figure 3.5 and the Discussion for justification of this decision). Genetic clusters largely aligned with geography and resulted in a "north" cluster including Upper Kintla samples only, a "central" cluster including samples from Swiftcurrent Glacier, Cracker Lake, and Sexton Glacier, and a "south" cluster, including samples from Lunch Creek, Clements Creek, Reynolds Spring, and Jackson Glacier (Figures 3.1, 3.5 & 3.6; Table 3.2).

Population differentiation

Average genetic differentiation (F_{ST}) among localities was 0.033 (SD = 0.016), and ranged from 0 (both sites along Clements Creek) to 0.067 [Sexton Glacier (main) to Upper Kintla; Table 3.3]. On average, the least differentiated locality was the high site along Clements Creek (mean $F_{ST} = 0.025$) and the most differentiated locality was Upper Kintla (mean $F_{ST} = 0.051$; Table 3.3). We detected a significant positive association between genetic and geographic distances ($r^2 = 0.42$, $P = 0.002$) when considering all pairwise comparisons. AMOVA results identified modest differentiation among the three genetic clusters ($F_{CT} = 0.020$, $P < 0.0001$; Table 3.4), but this only accounted for 2.0% of the total variation. Variation among localities within groups was also significantly differentiated, and this also explained a low amount of the total variation (3.6%). Most of

the observed variation (94.3%) was among individuals within sampling localities (Table 3.4).

For the full 6819 SNP data set, the number of private alleles (PAs; those observed in only one locality) varied widely (Table 3.5), with Cracker Lake averaging the most per sample (215.7) and Clements Creek (high) the least (25.3). Excluding intrastream localities, which may downwardly bias PA numbers due to increased migration, the lowest number of PAs per sample were observed for Swiftcurrent Glacier (37.2) and Reynolds Creek (38.4). When calculated for the full 6819 SNP data set, nucleotide diversity was highest in Cracker Lake samples ($\pi = 0.119$) versus the total average ($\pi = 0.087$; Table 3.5). Overall, Tajima's *D* was -2.26, suggesting a history of population expansion. For each genetic cluster, *D* was still negative, ranging from -0.25 to -1.58. The greatest magnitude of *D* for individual localities was observed for Cracker Lake (-1.40; Table 3.5).

For the full 6819 SNP data set, an interesting pattern was observed where H_{obs} was consistently lower than H_{exp} when calculated for localities, but reversed ($H_{obs} > H_{exp}$) when calculated for genetic clusters (aside from Upper Kintla which was the only locality in the north cluster; Table 3.5). However, when singletons were removed and missing data reduced to 10% (i.e., the 1467 SNP data set), H_{obs} was consistently lower than H_{exp} at both the level of sampling localities and regional cluster (Table 3.6).

Demographic model selection and parameter estimation

SFS-based analysis in fastsimcoal2 identified model M1 as the best-fitting model of demographic history (Figure 3.2; Table 3.7; model likelihood = 0.96). Model M1

included an initial divergence between the south and central+north genetic clusters, followed by a subsequent divergence between the central and north clusters with a bidirectional gene flow through *L. tumana*'s history. All other models, including those similar to M1, but with more restricted gene flow, were poorly supported ($\Delta AIC \geq 6.86$; model likelihoods $\leq$ 3.1E-2; Table 3.7).

Point estimates of demographic parameters for model M1 are provided with 95% CIs (Figure 3.7; Table 3.8). Our results indicate population expansions for all genetic clusters, with current effective population sizes (N_e) ranging from 246,743–410,562 (83,549–674,997), which is much higher than estimates for both the ancestor of all localities, $N_e^{N\_ANCALL}$, at 44,425 individuals (40,037–398,531) and the ancestral north+central cluster, $N_e^{N\_ANC01}$, estimated at 12,637 individuals (1,498–30,490). Changing the mutation rate influenced parameter estimates results by a similar amount (i.e., a 10-fold increase in the mutation rate corresponds with a 10-fold increase in parameter estimates; Table 3.9).

When accounting for ploidy and generation time, divergence time estimates are all less than 20,000 years ago (ya) with the ancestral north+central cluster splitting from South 17,551 ya (14,734–223,599), and north subsequently diverging from central 13,314 ya (10,751–15,450). Estimates of migration probabilities per generation between regional clusters varied greatly with the highest probabilities observed for north into south, 3.30E-5 (9.61E-7–2.07E-4), and central into south, 1.11E-5 (3.12E-10–6.90E-5). Ancestral migration probabilities were lower in both directions, with migration from north+central into south at 5.33E-11 (1.88E-10–6.22E-10) and the reverse at 1.19E-8 (8.16E-10–9.95E-

5). Migration probabilities in this context refers to the probability per generation that any gene from one population transfers to another (Table 3.8).

Discussion

Characterizing population structure for a weakly differentiated alpine stonefly

Our best estimate of population genetic structure is that contemporary *L. tumana* populations comprise three regional clusters that align with geography across its known range. We settled on this three-cluster model as the best fit for our data (Figs. 1, 3) after taking into account statistical support and clustering patterns across different data-filtering strategies. First, the lack of support identified by Admixture, and its stark contrast with the DAPC results, was in-line with other RADseq studies of poorly differentiated groups (e.g., lobsters, (Benestan *et al.*, 2015), suggesting that Admixture was likely to substantially underestimate population structure. Therefore, we focused on the DAPC results. Inspection of the $K = 12$ result from analysis of the 6819 SNP data set identified six clusters that are made up of single Cracker Lake individuals. This result clearly overestimated population structure and appeared to be linked to the influence of singletons, as Cracker Lake was an outlier in this regard, yielding the highest number of private alleles for any locality (Table 3.5). The influence of singletons was further seen with the analysis of the 2733 SNP data set, which removed all singletons and dropped the best-fit population structure model to a $K = 5$. Explorations of the effect of missing data by further filtering loci with greater than 10% missing data (1467 SNP data set) and 5% missing data (761 SNP data set) reduced the best-fit model to a $K = 3$. Interestingly, the $K = 5$ and $K = 3$ results are largely in agreement with one another, with the only difference

being whether to group individuals from Sexton Glacier, Cracker Lake, and Swiftcurrent into three clusters (2733 SNPs) or one (1467 and 761 SNPs). Ordination plots for the K = 5 model indicate some amount of overlap between the Cracker Lake and Sexton Glacier localities, indicating that these are two of the most weakly differentiated localities in this study (F_{ST} = 0.019-0.021 versus average F_{ST} = 0.033 for all pair-wise comparisons). On the other end of the spectrum, the removal of all missing data (86 SNP data set) produced a K = 4 result, although with an indication of a substantial loss of information, as nearly every locality comprised individuals assigned to multiple clusters, or with admixed or uncertain assignment.

Missing data has been previously shown to influence population structure analyses (Chattopadhyay *et al.*, 2014). Our results are consistent with this, although it appears that with missing data there may be a bit of a sweet spot when it comes to identifying patterns of population structure. A slight reduction of missing data from 11% to 5% had the biggest effect on inference of K, with similar results achieved with the 3% data set (although with greater influence of admixture or uncertainty in cluster assignments). It's possible that the general results here may be specific to this study system and data set. Nonetheless, we are confident in our choice to reject results based on the overall full data set and those from one in which all SNPs with any missing data are thrown out. Our choice of a preferred model was slightly more nuanced, and we elected to take a conservative approach in selecting between a model with three or five clusters, by choosing the K = 3 model, which best captures hierarchical structure across *L. tumana*.

Unlike studies focusing on wide-ranging and highly-differentiated species, we were faced with the challenge of identifying a best-fit model of population structure

without clear results. A lack of consensus in population structure across data sets and methods for large-scale SNP data sets is not unique to this study (e.g., Benestan *et al.*, 2015), but it is still an understudied finding in a field dominated by clear expectations and results [but see Janes *et al.* (2017) discussion of studies underestimating *K* when they achieve an initial and clear *K* = 2 result]. Specifically, our aim here was to attempt to better understand how the full 6819 SNP data set could give such opposing results when analyzed via DAPC (*K* = 1) or Admixture (*K* = 12). While our results still do not provide a perfectly objective method for describing population structure among contemporary populations of *L. tumana*, they clearly indicate that singletons can obfuscate the signal of population genetic structure, a pattern also observed for human populations (Baye *et al.*, 2011). Moreover, our results align with similar studies (e.g., Benestan *et al.*, 2015) in showing Admixture to be limited in its power to detect structure among weakly differentiated groups. While there may well be additional structure within the central cluster, our identification of *K* = 3 also serves a practical purpose. For three genetic clusters, our demographic models already included 15 parameters (Table 3.7). A five-cluster model would include 49 parameters, greatly escalating the computational complexity of the study and reducing the likelihood of generating biologically meaningful results.

Demographic history of L. tumana

Our results show that genetic structure, effective population size, and genomic variation in *L. tumana* likely accumulated over the last 20 ky, potentially in response to a rapidly changing, post-Pleistocene environment. Among the three identified geographic

genetic clusters, the deepest divergence occurred approximately 17 kya, and corresponds with the initial stages of ice retreat following the Wisconsin glaciation in northwestern Montana [ca. 20 kya; Carrara (1987)]. This was followed by a second divergence ~4 ky later between the present-day north and central clusters, a result that aligns well with a south-to-north pattern of ice sheet recession. Indeed, during this time glacial ice was receding across northwestern Montana, including GNP (Carrara, 1987), and divergence time estimates match well with the likely opening of new glacial stream habitat and its subsequent colonization by *L. tumana* from glacial refugia. Our resolution of three genetic clusters within *L. tumana* point to much less subdivision than identified from mtDNA sequence data (Jordan *et al.*, 2016); however, our results are based on thousands of nuclear loci, providing a more robust measure of variance across the genome. Moreover, while important assumptions must be considered when interpreting the temporal estimates presented here, particularly those surrounding the assumed mutation rate (see Table 3.9), our results provide important insight into how glacial dynamics at the end of the Pleistocene have influenced patterns of genetic variation for an alpine stonefly with direct ties to glacial ice.

Several additional lines of evidence support the hypothesis that extant genetic clusters of *L. tumana* originated and expanded from post-Pleistocene refugia. First, N_e estimates for ancestral nodes were substantially smaller than those for present-day regional clusters (Figure 3.7; Table 3.8). Second, Tajima's *D* calculated for all genetic clusters was negative, which indicates that each may have experienced a recent or ongoing population expansion. While the magnitude of these Tajima's *D* estimates are below the generally accepted threshold of a "significant" *D* ($\geq |\pm 2|$) for a population

expansion, when calculated for the full data set, *D* was -2.255 (Table 3.5). A rise in N_e may also be the product of undiagnosed additional population structure within identified genetic clusters (perhaps within the difficult-to-tease-apart central cluster described above). Greater population sampling that fully reflects our current understanding of *L. tumana* distribution (see Giersch *et al.,* 2016) could help to resolve this.

Interestingly, one sampling locality within the central cluster, Cracker Lake, contained a disproportionately high number of private alleles (Table 3.5) segregating at low frequency. While these PAs could be the product of sequencing error, this is unlikely as similar excess was not observed for other localities. An alternate explanation is that an expanding N_e in the Cracker Lake population (perhaps at a faster rate than other sampled localities) has led to an accumulation of younger haplotypes and an excess of low-frequency alleles. This alternate explanation is supported by the geology of Cracker Lake, with the Siyeh Glacier feeding the stream containing *L. tumana* surrounded by a horseshoe of 1400 m walls, likely limiting gene flow between Cracker Lake and nearby populations. Moreover, it has been posited that global warming may begin as a boon for alpine stream taxa, as accelerated glacial melt will push cold conditions downstream and expand existing habitat (Hotaling *et al.*, 2017b). To this end, rapid melting of the Siyeh Glacier – the smallest included in this study – may be driving a population expansion of *L. tumana* within the Cracker Lake inlet stream as increased meltwater expands its habitat area in the short-term.

Alpine aquatic biodiversity: an uncertain future

As climate change proceeds and alpine landscapes change, the need for accurate predictions of how species will respond becomes increasingly pressing. Understanding the potential for migration among populations is an important component of this discussion (Hoffmann & Sgrö, 2011). For alpine stream taxa, gene flow is challenged by fragmentation of suitable habitats, often resulting in population isolation and increased probability of extirpation (Hotaling *et al.*, 2017b). These expectations are supported by several mtDNA studies identifying strong isolation among headwater macroinvertebrate species (e.g., Monaghan *et al.*, 2001), including *L. tumana* (Jordan *et al.*, 2016). However, this study is the first to use genomic tools to understand historical and contemporary patterns of genetic diversity and connectivity in a range-restricted endemic that is acutely vulnerable to climate-change-induced loss of meltwater habitat.

With the addition of genomic data, demographic model testing unequivocally favored a history of gene flow over models that either excluded migration fully, or included fewer migration parameters. Both morphological (Garcia-Raventós *et al.*, 2017) and mtDNA (Finn & Adler, 2006; Finn *et al.*, 2006; Finn *et al.*, 2016; Giersch *et al.*, 2016; Jordan *et al.*, 2016) evidence suggests that stoneflies (and related alpine stream species) are poor dispersers. Consequently, the support here for gene flow occurring on large spatial scales is surprising and may provide some degree of optimism from a climate change perspective. Species exhibiting population structure with ongoing gene flow may be at an advantage in their response to climate change as potentially adaptive genetic variation from one geographic area or habitat type may spread to another (Hoffmann *et al.*, 2015). *Lednia tumana* appears to meet both criteria (population

structure and ongoing gene flow), thus indicating that it at least has the potential for an adaptive response to changing climate. Indeed, as a glacially-tied stream insect, *L. tumana* is representative of a global aquatic community that is directly at risk due to climate-change-induced habitat loss (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016; Hotaling *et al.*, 2017b; Hotaling *et al.*, 2017a).

As alpine glaciers recede, they tend to leave groundwater-fed springs in their wake (Baraer *et al.*, 2012; Chavez, 2013) and these warmer (but still very cold) groundwater springs may serve as refugia for cold-adapted, relict species (Ward, 1994; Williams & Williams, 1998; Hotaling *et al.*, 2017b). As is common for alpine stream species, *L. tumana* exists in a range of stream habitats, ranging from groundwater-fed springs (e.g., Reynolds Spring), to streams emanating from small glaciers that are likely transitioning from being meltwater driven to groundwater fed (e.g., Cracker Lake), and to streams still heavily influenced by large glaciers (e.g., Sexton Glacier). This variation in habitats, and possibly local selection, may be extremely important. For instance, if gene flow from a groundwater-fed spring occurs at a higher rate into a glacially-influenced population than the reverse, this differential migration could be a mechanism for genetic rescue if the glacially-tied population receives adaptive variation while in the midst of a habitat transition from current, harsh conditions to more stable groundwater spring-like conditions. Essentially, this variation in environmental conditions could act as a natural spectrum with some populations farther along the climate change timeline (i.e., those in groundwater-fed springs), with others much "earlier" in the process. However, the possibility of this rescue mechanism depends upon both the extent to which populations

of *L. tumana* are locally adapted (or leverage phenotypic plasticity) to specific habitat condition combinations and the scale of gene flow occurring between populations.

Future directions and conclusions

Ultimately, to form a comprehensive understanding of the evolutionary potential and persistence of *L. tumana* under future warming scenarios, additional studies will be required. Any future research would benefit from specific assessments of the role of isolation-by-distance (Wright, 1943) versus isolation-by-environment (Wang & Bradburd, 2014) in shaping extant genetic diversity, targeted efforts to identify ecologically-relevant genetic diversity that may be under selection (at a much finer genomic scale than in this study), and robust estimations of gene flow between streams, with a specific focus on those populations inhabiting opposite ends of the habitat spectrum. In all of this, it will remain important to not discount the role of phenotypic plasticity as a mechanism for population persistence as glaciers decline. A plastic response could act as an initial buffer, allowing populations to persist outside of their environmental optima while genetic adaptation accumulates (Reusch, 2014). To this end, laboratory tests of thermal tolerance for *L. tumana* nymphs suggested they can tolerate much warmer conditions than they experience in the wild (Treanor *et al.*, 2013). Most importantly, the survival of communities cannot be inferred from a single species. To better resolve the potential for glacially-tied communities to persist in a rapidly changing landscape, insight from a combined, multi-taxon perspective is required (Hotaling *et al.*, 2017b).

Despite the challenges associated with predicting the future of alpine stream taxa, this study demonstrates the promise of genomic tools for extending understanding beyond patterns of genetic differentiation to also include robust estimates of demographic history. Specifically, we showed the utility of genome-wide information to resolve population structure and recent demographic history for an alpine stonefly at high risk of local extirpation as climate change proceeds (Giersch *et al.*, 2016). As alpine stream species are facing similar threats globally, the results of this study have implications far beyond *L. tumana* or GNP. Indeed, studies like ours can easily be conducted for other species with no genomic resources to help quantify, monitor, and predict changes in population structure, connectivity, and ultimately persistence, in the face of environmental change.

Table 3.1. Sampling and locality information for population genomic and demographic history analyses of *Lednia tumana* in Glacier National Park, Montana, USA. The number of individuals (*n*) is given for the total number sequenced per locality and the number of individuals retained in the post-filtering 6819 SNP dataset.

Locality	GPS coordinates	Elev. (m)	*n*	*n*, post-filtering
Grant Glacier[a]	48°19'45.37", -113°44'28.10"	1836	3	0
Jackson Glacier	48°36'19.97", -113°41'44.65"	2006	7	6
Lunch Creek	48°42'35.40", -113°42'15.28"	2285	15	3
Sexton Glacier (main)	48°42'1.48", -113°37'24.12"	1847	9	4
Sexton Glacier (south)	48°41'58.33", -113°37'20.33"	1827	9	5
Clements Creek (high)	48°41'24.07", -113°44'0.95"	2173	9	8
Clements Creek (low)	48°41'16.33", -113°43'43.11"	2045	9	6
Reynolds Spring	48°41'4.77", -113°43'35.05"	2082	8	7
Upper Kintla	48°56'48.44", -114°8'24.59"	1826	9	8
Cracker Lake	48°44'21.42", -113°39'11.99"	1867	9	9
Swiftcurrent Glacier	48°46'41.52", -113°45'21.36"	2010	9	9
		Total	96	65

[a] Not included in downstream analyses due to limited coverage after filtering

Table 3.2. Results of population structure analyses for five data sets. Data set names correspond to the number of SNPs included after filtering. MAC: minor allele count. Filters: MAC >1 (singletons removed) and per locus missing (loci with >10%, >5%, or 0% missing data). PCs retained: number of principal components (PCs) retained according to α-score for discriminant analysis of principal components (DAPC) analyses. DAPC *K* and Admixture *K* indicate the number of genetic clusters (*K*) supported by each analysis based upon the lowest BIC score (DAPC) or cross-validation value (Admixture).

Data set	MAC	Per locus missing	Total missing	PCs retained	DAPC *K*	Admixture *K*
6819	n/a	n/a	~11%	8	12	1
2733	>1	n/a	~11%	9	5	1
1467	>1	>10%	~5%	7	3	1
761	>1	>5%	~3%	6	3	1
86	>1	0%	0%	11	4	1

Table 3.3. Population differentiation (F_{ST}) among localities calculated for the 1467 SNP data set. Values not significant at $P \leq 0.05$ are in bold. Mean values represent the average F_{ST} for each location (corresponding columns). Average F_{ST} overall was 0.033. Locality abbreviations: JKG = Jackson Glacier, LCK = Lunch Creek, SGM = Sexton Glacier (main), SGS = Sexton Glacier (south), CCL = Clements Creek (low), CCH = Clements Creek (high), REY = Reynolds Spring, KLA = Upper Kintla, CKL = Cracker Lake, SWG = Swiftcurrent Glacier.

	JKG	LCK	SGM	SGS	CCL	CCH	REY	KLA	CKL	SWG
JKG	--	0.046	0.045	0.033	0.018	0.011	**0.001**	0.055	0.034	0.037
LCK		--	0.056	0.024	0.026	0.028	0.034	0.058	**0.020**	0.048
SGM			--	**0.008**	0.047	0.054	0.051	0.067	0.019	0.033
SGS				--	0.035	0.032	0.033	0.055	0.021	0.033
CCL					--	**0**	**0.004**	0.046	0.027	0.026
CCH						--	**0.006**	0.053	0.028	0.030
REY							--	0.047	0.030	0.028
KLA								--	0.040	0.036
CKL									--	0.025
SWG										--
Mean	0.031	0.038	0.042	0.030	0.025	0.027	0.026	0.051	0.027	0.033

Table 3.4. Segregation of genetic variation according to an analysis of molecular variance (AMOVA) for 10 sampling localities (eight streams) grouped into three genetic clusters (north, central, and south). The AMOVA was calculated for the 1467 SNP data set with singletons removed and only loci with < 10% missing data retained.

Source of variation	Fixation index	Percentage of variation
Among groups	$F_{CT} = 0.020$	2.00
Among localities within groups	$F_{SC} = 0.037$	3.62
Within localities	$F_{ST} = 0.056$	94.38

Table 3.5. Population genetic statistics calculated from the full 6819 SNP data set. Statistics are provided for the sampling localities, genetic clusters, and overall. Nucleotide diversity (π), expected heterozygosity (H_{exp}), and observed heterozygosity (H_{obs}) were calculated for variable positions only. Additional abbreviations include: PA = private alleles, *D* = Tajima's *D*. Similar statistics calculated for the 1467 SNP data set which had all singletons removed and only retained loci with < 10% missing data are provided in Table 3.6.

	PA	PA per sample	*D*	π	H_{exp}	H_{obs}
Sampling locality						
Jackson Glacier	334	55.7	-0.200	0.092	0.082	0.074
Lunch Creek	177	59.0	0.229	0.082	0.066	0.056
Sexton Glacier (main)	206	51.5	-0.040	0.087	0.075	0.068
Sexton Glacier (south)	253	50.6	-0.019	0.087	0.077	0.061
Clements Creek (low)	363	60.5	-0.107	0.093	0.085	0.073
Clements Creek (high)	201	25.3	0	0.086	0.078	0.070
Reynolds Creek	269	38.4	-0.072	0.091	0.083	0.071
Upper Kintla	410	51.3	-0.249	0.090	0.083	0.067
Cracker Lake	1941	215.7	-1.404	0.119	0.111	0.084
Swiftcurrent Glacier	335	37.2	-0.104	0.087	0.081	0.070
Genetic cluster						
North	410	51.3	-0.249	0.090	0.083	0.067
Central	2180	77.9	-1.581	0.112	0.080	0.110
South	1276	47.3	-0.791	0.108	0.084	0.105
Overall	n/a	n/a	-2.255	0.098	0.097	0.071

Table 3.6. Population genetic statistics calculated for the 1467 SNP data set which had all singletons removed and only retained loci with < 10% missing data. Samples were grouped by locality, genetic cluster, or overall. ENA: effective number of alleles.

	ENA	H_{exp}	H_{obs}	F_{IS}
Sampling locality				
Jackson Glacier	1.291	0.197	0.159	0.192
Lunch Creek	1.257	0.199	0.116	0.415
Sexton Glacier (main)	1.261	0.188	0.142	0.244
Sexton Glacier (south)	1.279	0.196	0.128	0.346
Clements Creek (low)	1.311	0.207	0.172	0.171
Clements Creek (high)	1.282	0.192	0.158	0.177
Reynolds Creek	1.301	0.201	0.160	0.205
Upper Kintla	1.289	0.191	0.148	0.229
Cracker Lake	1.302	0.204	0.118	0.421
Swiftcurrent Glacier	1.295	0.193	0.158	0.180
Regional metapopulation				
North	1.289	0.191	0.148	0.229
Central	1.311	0.200	0.137	0.315
South	1.314	0.202	0.158	0.214
Overall	1.254	0.197	0.146	0.257

Table 3.7. Results of model selection analyses performed in fastsimcoal2 for three genetic clusters of *Lednia tumana*. *P* = number of parameters in the model. Model numbers correspond to those in Figure 3.2.

Model	Description	*P*	ΔAIC	Model prob.
Divergence with gene flow (recent and historical)				
1	(North, Central), South	15	--	0.96
2	(Central, South), North	15	12.36	1.99E-3
3	(North, South), Central	15	19.27	4.81E-9
Divergence with no gene flow				
8	(North, Central), South	7	6.86	3.10E-2
9	(Central, South), North	7	28.46	6.33E-7
10	(North, South), Central	7	26.60	5.91E-7
Divergence with gene flow (historical only)				
11	(North, Central), South	9	9.92	6.73E-5
12	(Central, South), North	9	32.38	8.96E-8
13	(North, South), Central	9	33.14	6.11E-8
Admixture with gene flow (recent and historical)				
4	North and South diverged, Central admixed	13	40.06	1.92E-9
5	Central and South diverged, North admixed	13	27.34	1.11E-6
6	North and Central diverged, South admixed	13	32.19	9.82E-8
Admixture with no gene flow				
15	North and South diverged, Central admixed	7	27.59	9.81E-7
16	Central and South diverged, North admixed	7	23.23	8.68E-6
17	North and Central diverged, South admixed	7	28.79	5.37E-7
Admixture with gene flow (historical only)				
18	North and South diverged, Central admixed	9	30.51	2.27E-7
19	Central and South diverged, North admixed	9	27.26	1.15E-6
20	North and Central diverged, South admixed	9	32.92	6.83E-8
Trifurcation with gene flow				
7	All lineages diverged at the same time.	11	35.91	1.53E-8
Trifurcation with no gene flow				
14	All lineages diverged at the same time.	5	24.69	4.18E-6

Table 3.8. Parameter estimates for the best-fit demographic model (Model 1). All estimates assume diploid cells, a one-year generation time, and a nuclear mutation rate of 3.5E-9 per site per generation (Keightley *et al.*, 2009). Point estimates are provided with 95% confidence intervals in parentheses. Point estimates are those identified in the best-fit run of the 50 model selection replicates. A schematic of this best-fit model is presented in Figure 3.7. Abbreviations include: N_e = effective population size, T_{DIV} = timing of divergence, m = migration probabilities (or, the probability that any gene from one population transfers to another on a per generation basis).

Parameter	Description	Estimate
Effective population sizes		
N_POP0	North N_e	410,562 (200,309 – 674,997)
N_POP1	Central N_e	246,743 (83,549 – 425,677)
N_POP2	South N_e	374,673 (52,864 – 475,344)
N_ANCALL	Ancestral N_e of all lineages	44,425 (40,037 – 398,531)
N_ANC01	Ancestral N_e of North+Central	12,637 (1,498 – 30,490)
Divergence times		
TDIV01	T_{DIV}, North+Central and South	13,314 (10,751 – 15,450)
TDIV2_ANC01	T_{DIV}, between North+Central	17,551 (14,734 – 223,599)
Migration probabilities		
MIG01	*m,* Central into North	4.8E-7 (6.6E-11 – 6.7E-6)
MIG10	*m,* North into Central	2.1E-7 (6.6E-11 – 3.7E-5)
MIG02	*m,* South into North	1.8E-8 (6.4E-11 – 9.9E-6)
MIG20	*m,* North into South	3.3E-5 (9.6E-7 – 2.1E-4)
MIG12	*m,* South into Central	3.7E-9 (7.5E-11 – 7.0E-6)
MIG21	*m,* Central into South	1.1E-5 (3.1E-10 – 6.9E-5)
MIGA2	*m,* South into North+Central	1.2E-8 (8.2E-1 – 9.9E-5)
MIG2A	*m,* North+Central into South	5.3E-11 (1.9E-10 – 6.2E-6)

Table 3.9. Parameter estimates generated from 50 runs of the best-fit demographic model (Model 1) for both a higher and lower mutation rate than the estimation from Keightley *et al.*, (2009) used for model selection To select the best-fit model, the maximum expected likelihood (MEL) was compared to the maximum observed likelihood (MOL) for the data under each model for each replicated. The best-fit run was identified as the one that minimized the difference between MEL and MOL for each set of fifty replicates for each mutation rate. Bold values indicate those from the best-fit run included in Figure 3.7.

Rate	N_POP0	N_POP1	N_POP2	TDIV1	TDIV2
3.50E-08	219488	34145	1031363	1118	1224
3.50E-09	**821124**	**493486**	**749345**	**13314**	**17551**
3.50E-10	4865058	2660727	5293620	141415	200175

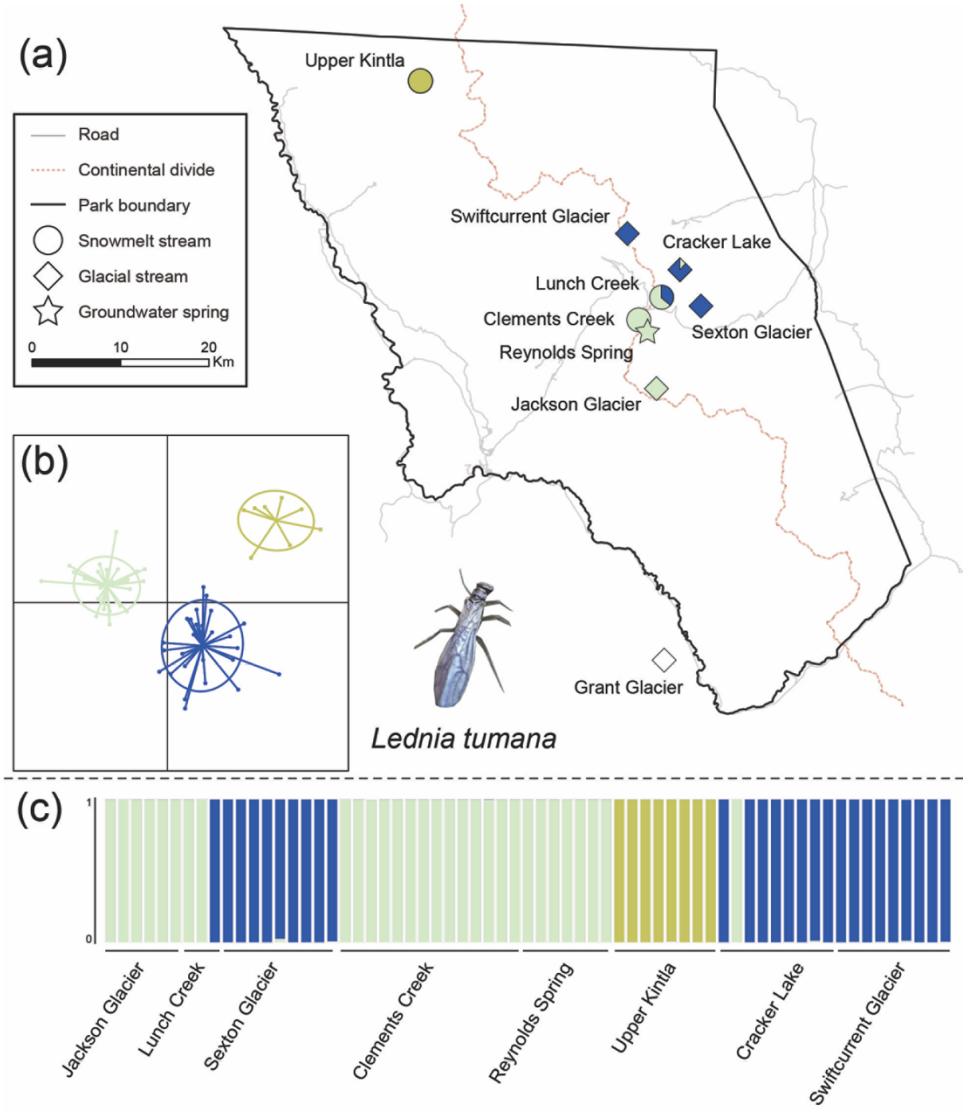

Figure 3.1. (a) Sampling localities across *Lednia tumana*'s known range within and near Glacier National Park, Montana, USA. Symbol shapes correspond with primary hydrologic source and colors indicate assignment to genetic clusters. The white triangle for Grant Glacier indicates a sampled population from which too few specimens passed filtering to be included in downstream analyses. (b) Results of a discriminant analysis of principal components analysis for $K = 3$ clusters. (c) DAPC-based assignment probabilities for all samples included in the full, post-filtered dataset.

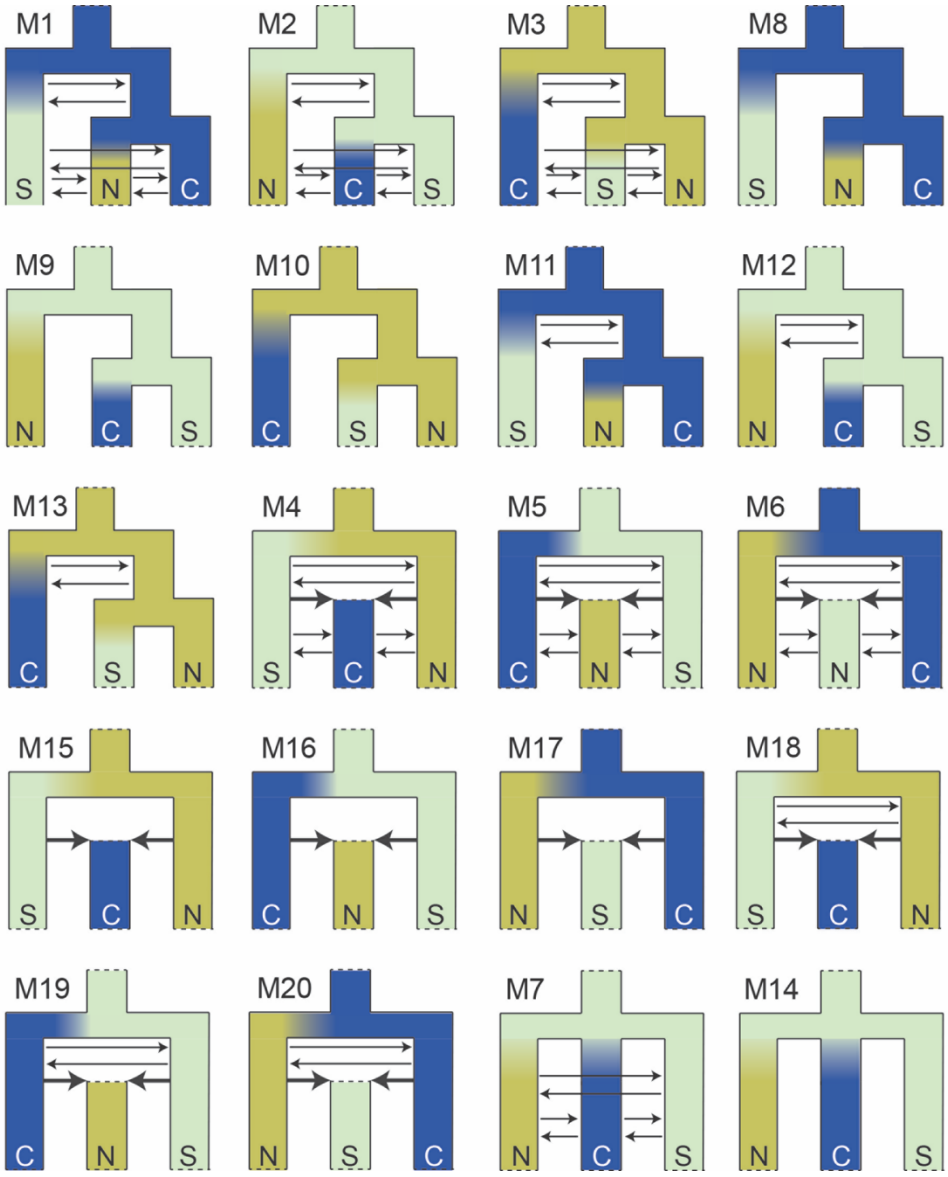

Figure 3.2. Schematics of the twenty demographic models tested in this study. Large arrows indicate admixture events, small arrows represent the presence of gene flow, and colors correspond to genetic clusters (N: northern, C: central, and S: southern) as defined in population structure analyses. Ancestral colors were arbitrarily defined for simplicity and model numbers reflect the order in which they were constructed (and the order of results in Table 3.7).

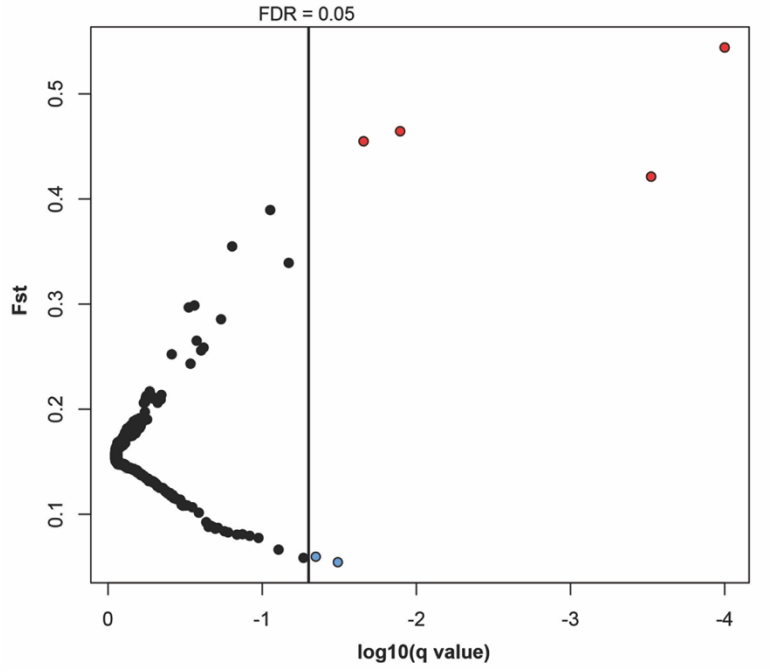

Figure 3.3. A plot of SNPs organized by F_{ST} to identify putative outlier loci. SNPs to the right of the dark vertical line are above a false discovery rate (FDR) of 0.05. SNPs were grouped by sampling locality for this comparison. Six (of 6819) SNPs were identified as outliers – four undergoing putative positive selection (red circles) and two undergoing putative purifying selection (blue circles). Analyses were performed in BayeScan v2.1.

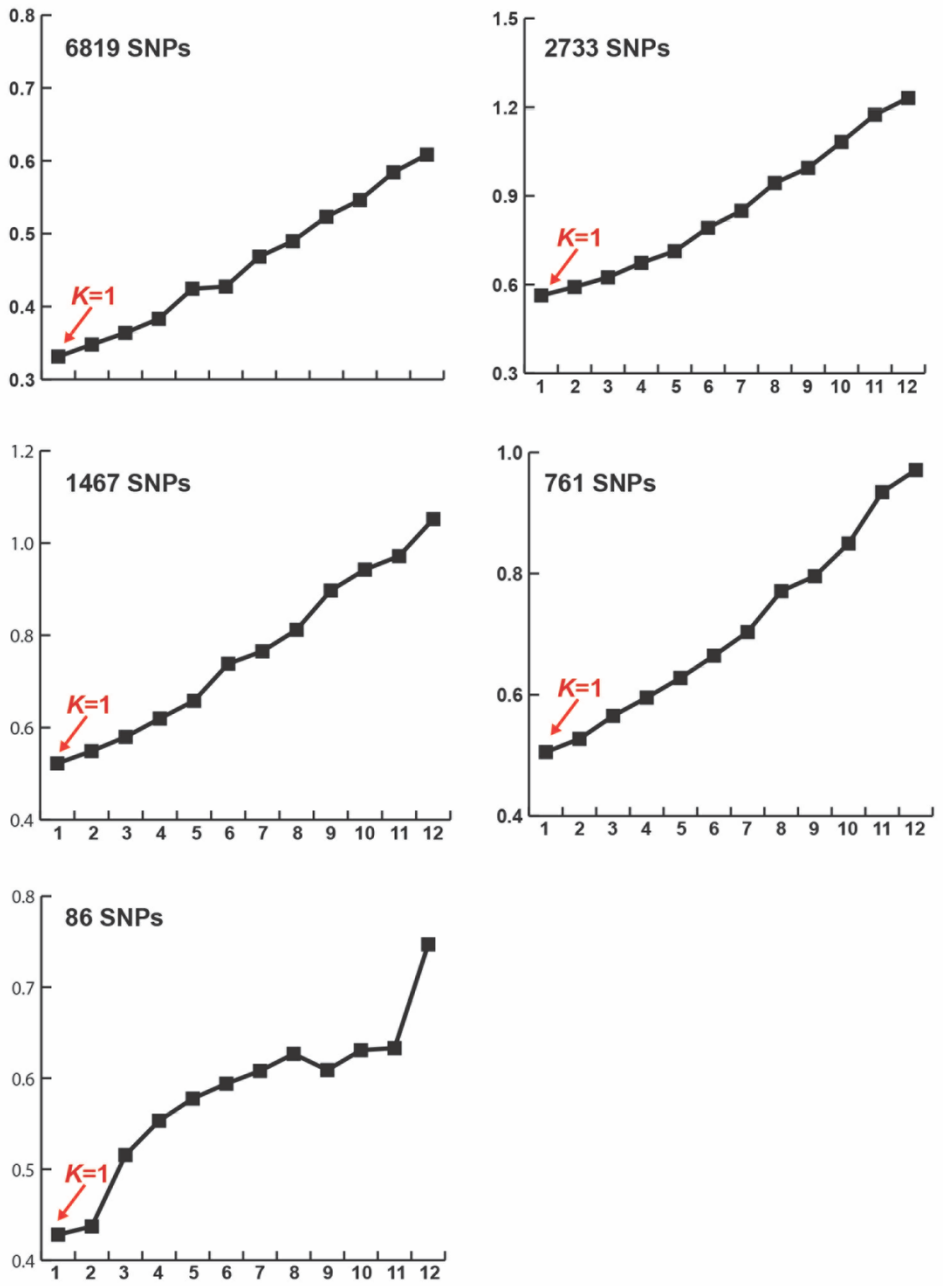

Figure 3.4. Comparisons of Admixture support for *K* clusters (x-axis) inferred from the five comparison data sets outlined in this study's methods. Support is ranked by cross-validation score (y-axis) with lower cross-validation scores indicate greater support for a model. All Admixture analyses support $K = 1$.

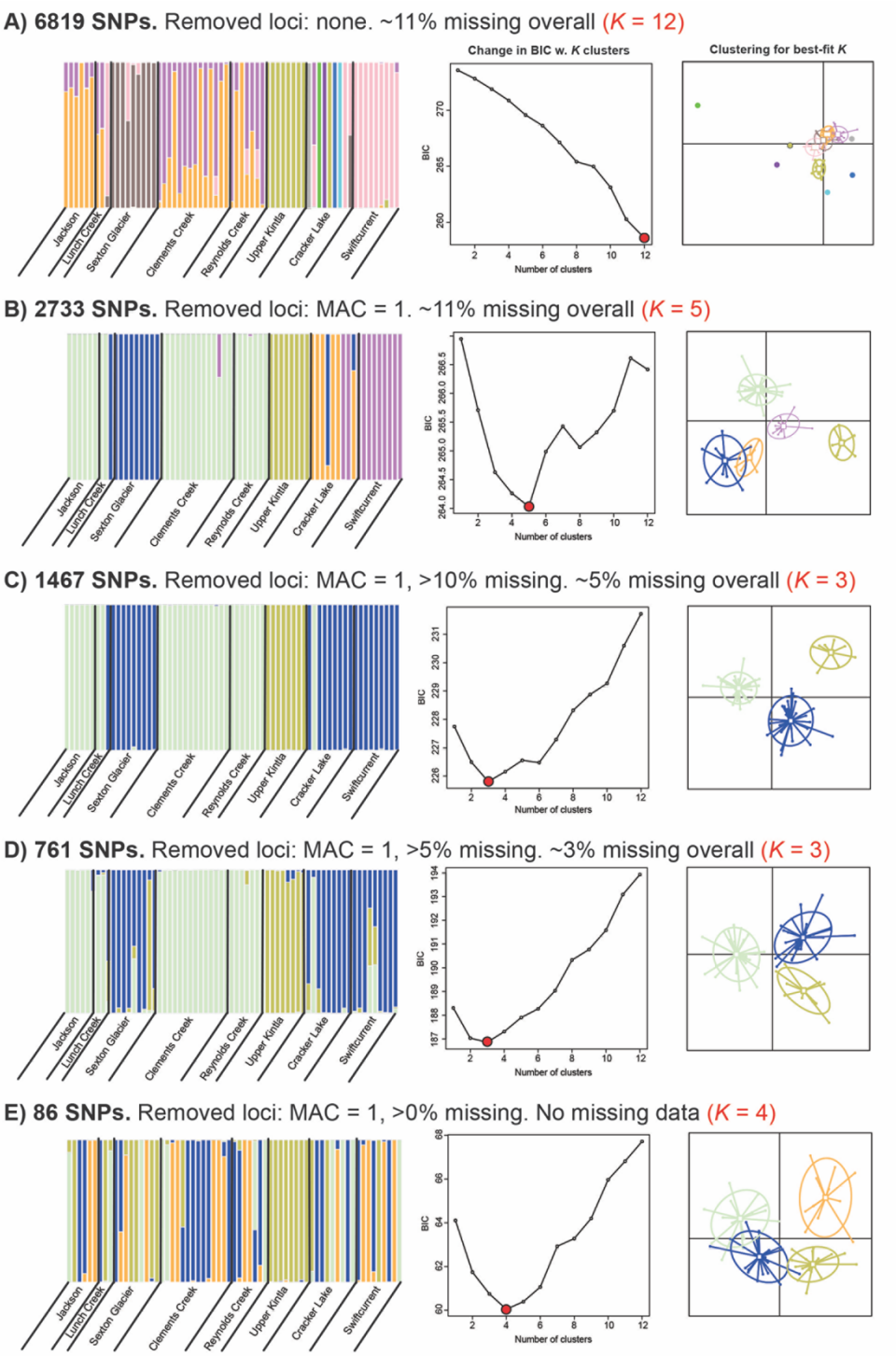

Figure 3.5. Comparisons of population structure results for DAPC analyses of five data sets with varying numbers of SNPs included. Filters are as described for each set of plots. MAC = 1: minor allele count of 1 (i.e., singletons) removed. The best-support *K* is in red. From right to left for each comparison: assignment plots where each vertical bar represents one individual, a plot of the Bayesian Information Criterion (BIC) for a range of *K* values (lower BIC indicates higher model support) and plots individual assignments to genetic cluster based upon the two most informative principal components.

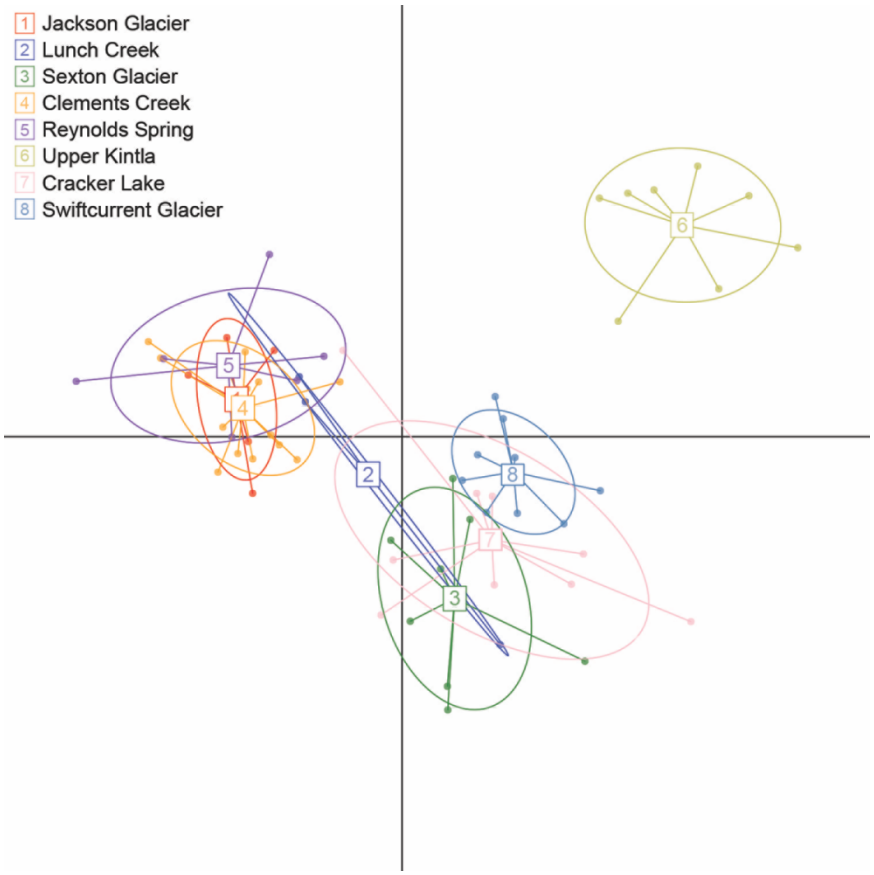

Figure 3.6. Results of a discriminant analysis of principal components for $K = 3$ clusters and 10 localities (eight streams) included in demographic modeling. Each point represents one sample and points are color-coded to streams. Note that this is the same clustering pattern presented in Figure 1b, except that here samples are coded by stream rather than genetic cluster. Samples belonging to clusters 2 (Lunch Creek) and 7 (Cracker Lake) highlight immigration between the south and central clusters

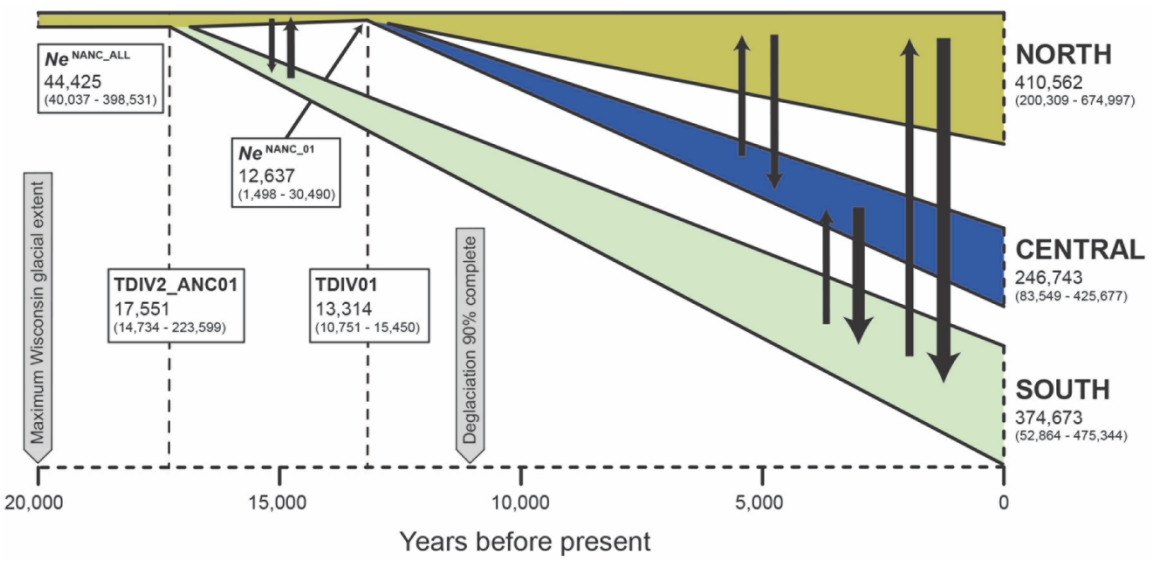

Figure 3.7. A schematic of the best-fit demographic model (Model 1) for *Lednia tumana*. Larger font parameter values are point estimates from the best-fit model selection replicate. Divergence time estimates are given in years and effective population size estimates (Ne) are given as individuals. 95% confidence intervals of parameter estimates are placed in parentheses. Black arrows represent relative estimates of migration probabilities with variation in arrow size based on a log scale. Ancestral coloration was arbitrarily chosen to simplify visualization. Geologic reference points were taken from Carrara (1987).

CHAPTER 4

MICROBIAL ECOLOGY OF MOUNTAIN GLACIER ECOSYSTEMS: BIODIVERSITY, ECOLOGICAL CONNECTIONS, AND IMPLICATIONS OF A WARMING CLIMATE

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Introduction

Mountain glaciers are globally distributed, developing where the accumulation of snowfall exceeds seasonal melting, which is often above treeline in the alpine biome. At low- to mid-latitudes, mountain glaciers tend to be warm-based with temperatures throughout generally equal to or exceeding the ice melting point. Like their polar counterparts, mountain glaciers are host to diverse microbial communities carrying out important ecosystem processes (e.g., carbon fixation; e.g., Anesio et al., 2009). However, in contrast to polar ice sheets, mountain glaciers are often characterized by steep elevational gradients (and high ice velocities), elevated levels of hydrological connectivity from surface to base (via fractures, crevasses, and moulins), and greater snow accumulation (and seasonal melting), particularly in maritime environments (Hodson *et al.*, 2008; Anesio & Laybourn-Parry, 2012).

Glacier ecosystems contain diverse habitats that we have divided into five ecological zones: supraglacial snow and ice (where sunlight penetrates); interior englacial ice; subglacial sediments at the bedrock-ice-meltwater interface; proglacial streams derived from supra- and subglacial runoff, as well as possible groundwater inputs; and glacier forefields where the recession of glacier ice exposes historically ice-covered soils to atmospheric processes (Figures 4.1-4.2). Supraglacial snow and ice, subglacial

sediments, proglacial streams, and glacier forefields support a diverse array of life, dominated by bacteria and eukarya (Anesio & Laybourn-Parry, 2012; Boetius *et al.*, 2015). In contrast, the englacial zone appears to contribute negligibly to broader ecosystem function and diversity (Boetius *et al.*, 2015), and primarily functions as a conduit delivering nutrients from supra- to subglacial habitat, and eventually, into proglacial streams (Hodson *et al.*, 2008; Anesio & Laybourn-Parry, 2012).

Ice sheets and glaciers are host to significant biodiversity despite cold temperatures, limited available water for cellular processes (particularly seasonally), and low nutrient availability (Fountain & Walder, 1998; Hodson *et al.*, 2008; Anesio & Laybourn-Parry, 2012; Boetius *et al.*, 2015). The ecology and productivity of microbial life on, in, and beneath mountain glaciers is largely dictated by a combination of proximity to sources of allocthonous organic matter (OM) and nutrients (e.g., an urban area, forest, or desert; Stibal et al., 2012), interactions between bedrock, ice, and subglacial sediments, solar radiation, and the magnitude of annual melting, which influences the scale of hydrological connectivity across ecological zones. While available sunlight is the most important component of supraglacial productivity (Hodson *et al.*, 2008; Anesio *et al.*, 2009; Chuvochina *et al.*, 2011; Boetius *et al.*, 2015; Lutz *et al.*, 2015). The supraglacial zone is also influenced, though perhaps marginally, by wind deposition of dust, ash, and particles from nearby bedrock (Chuvochina *et al.*, 2011). This limited influence of bedrock and elevated importance of solar radiation as an ecological control contrasts starkly with the subglacial zone where, in the absence of light, variation in ecological processes depends directly upon bedrock lithology, because different rock compositions vary in their ability to facilitate abiogenic chemical energy production (Telling *et al.*,

2015) and influence basal sliding (Sharp, 1988). The connection between hydrology and the supraglacial-englacial-subglacial axis is an important but underexplored component of mountain glacier ecology, as variation in water flowpaths – e.g., the extent of crevasses, moulins, or finer-scale fissures —within the supraglacial or englacial zones can greatly influence microbial activity in the subglacial zone. This high degree of influence stems from the vital role flowing water plays in linking the supraglacial to subglacial zones, through the englacial intermediary. Downstream, the microbial ecology of proglacial streams is influenced by basal and supraglacial meltwater in summer (when stream channels are most active) and by basal melting in concert with any groundwater inputs the rest of the year. Seasonal variation of basal meltwater discharge typically spans approximately two orders of magnitude (Fountain & Walder, 1998). Taken together, geomicrobiological and ecological activity anywhere on, within, or below a glacier can directly influence proglacial stream and lake ecosystem function (Figure 4.1). This includes carbon use across trophic levels (e.g., Fellman *et al.*, 2015), and these collective influences are likely highly seasonal.

Mountain glaciers respond strongly to climate change (Dyurgerov & Meier, 2000; Hall & Fagre, 2003; Barnett *et al.*, 2005; Wrona *et al.*, 2006; Brown *et al.*, 2009; Bolch *et al.*, 2012), and the implications of their ongoing recession are far-reaching. These impacts range from changes in the amount of available habitat (and taxonomic diversity) to both short- and long-term effects on the global carbon cycle (Wadham *et al.*, 2008; Anesio *et al.*, 2009; Jacobsen *et al.*, 2012; Hood et al., 2015). Despite slow doubling times and decreased rates of biogeochemical transformations (Anesio *et al.*, 2010), microbially mediated biogeochemical cycling is an important aspect of glacier ecology (Hodson *et*

al., 2008) and has been an emerging focus of glacier geomicrobiological research. Traditionally, the difficulty of culturing glacier microbes with slow doubling times and remote research locations has hindered geomicrobiological studies of mountain glaciers. Advances in sequencing technology (e.g., next-generation sequencing, or NGS), genomic data collection (e.g., microscale metagenomics, Rinke *et al.*, 2016), microcosm and isotope studies (e.g., single cell stable isotope probing, Wang *et al.*, 2016), and improved cultivation methodologies (e.g., microfluidic cultivation, Jiang *et al.*, 2016) hold significant potential for improving understanding of microbial diversity and function, as well as biogeochemical interactions in mountain glacier ecosystems. However, a comprehensive framework linking microbial ecology of mountain glaciers with the hydrological and ecological connections, within, nearby, and downstream of their influence, has not been developed.

Here, we provide a modern synthesis of microbial ecology of mountain glacier ecosystems with a focus on biodiversity and function. Specifically, we extend previous efforts that focused on the ecology and biodiversity of glaciers or ice generally (Hodson *et al.*, 2008; Anesio & Laybourn-Parry, 2012; Boetius *et al.*, 2015) and complement existing reviews centered upon the microbial ecology of specific components of the global cryosphere, including sea ice (Boetius *et al.*, 2015) and permafrost (Jansson & Taş, 2014). We also integrate broad perspectives on the interconnected nature of the mountain glacier ecosystem and highlight the inextricable ecological linkages between mountain glaciers and their proglacial aquatic habitats. We begin by synthesizing existing knowledge across five ecological zones – supraglacial, englacial, subglacial, proglacial streams, and glacier forefields. Next, we discuss the relevant ecological, hydrological,

and geological connections between our four ecological zones that are connected by hydrology – supraglacial, englacial, subglacial, and proglacial streams – and their influences on microbial ecology. We conclude by identifying areas for future research, possibilities for greater integration of emerging tools, and discuss the implications of a rapidly changing climate on mountain glacier ecosystems through the lens of microbial ecology.

Ecological zones

Supraglacial zone

Encompassing the uppermost, sun-lit layer of ice and snow, as well as surface streams, ponds, cryoconite holes, and moraines, the supraglacial zone is heavily influenced by solar radiation (Figures 4.1A-4.2) and inoculated with microbes and essential nutrients via atmospheric deposition (reviewed by (Xiang *et al.*, 2009; Rime *et al.*, 2016). When conditions are conducive to ice-melt, water becomes available and facilitates microbially mediated biogeochemical processes (Anesio & Laybourn-Parry, 2012). A significant portion of biological activity on glacier surfaces occurs in cryoconite holes – small, water-filled depressions (Figur 4.1B) – that form when dark material (e.g., soil or dust) is deposited on the glacier surface. The low albedo of the dark material (versus highly reflective snow and ice surrounding it) causes increased absorption of solar radiation and locally accelerated melting. Cryoconite holes are rich in OM, host diverse communities, and are an important habitat for glacial carbon cycling and fixation (Nylen *et al.*, 2004; Nkem *et al.*, 2006; Anesio *et al.*, 2009; Anesio *et al.*, 2010; Edwards *et al.*, 2014).

Surface life on mountain glaciers can be substantial with algal cell concentrations ranging from 4.4 x 10^4 to 9.9 x 10^5 cells per ml^{-1} of meltwater (Takeuchi, 2001; Anesio *et al.*, 2010). The pigmented cells of eukaryotic algae and cyanobacteria color snow and ice during the melt season, contributing up to 1.2 kg km^{-2} of biomass to the ecosystem (as observed in Alaskan mountains, Takeuchi *et al.*, 2006). Though dominated by autotrophic and heterotrophic bacteria, the supraglacial community also includes a number of eukaryotes: protozoa, diatoms, rotifers, fungi, and, in rare cases, even annelid iceworms (Tynen, 1970; Hodson *et al.*, 2008; Anesio & Laybourn-Parry, 2012). The most commonly observed heterotrophic bacteria are affiliated with Actinobacteria and Proteobacteria, but members of other phyla (e.g., Bacteroidetes) are also present (Simon *et al.*, 2009; Chuvochina *et al.*, 2011; Franzetti *et al.*, 2016). The bulk of supraglacial heterotrophic activity is supported by allochthonous carbon sources. In cryoconite holes for instance, microbial assemblages are equipped to degrade many different exogenous organic carbon sources (Simon *et al.*, 2009; Stibal *et al.*, 2012; Edwards *et al.*, 2013; Musilova *et al.*, 2015) as well as autochthonous extracellular polymeric substances (EPS) resulting from *in-situ* primary production. These EPS likely represent an important, high-density energy source for heterotrophs and light-limited photoautotrophs (Takeuchi *et al.*, 2010; Laybourn-Parry *et al.*, 2012). Though photoautotrophic communities of snow algae and cyanobacteria drive the majority of primary production on mountain glaciers, the recovery of 16S rRNA genes and transcripts affiliated with methanogens and ammonia-oxidizing archaea suggests that chemoautotrophic carbon fixation also occurs in the supraglacial zone (Hamilton *et al.*, 2013; Lutz *et al.*, 2015; Hamilton & Havig, 2016).

Moreover, recent metagenomic evidence suggests that anoxygenic phototrophy may also play a vital role in mountain glacier primary production (Franzetti *et al.*, 2016).

Primary productivity in supraglacial ecosystems occurs at rates that are relevant on the scale of the global carbon cycle (e.g., Anesio *et al.*, 2009). However, the balance between autotrophic and heterotrophic activity, and ultimately whether glaciers are net CO_2 sources or sinks, remains largely unknown. For polar glaciers, autotrophic production tends to exceed respiration (Anesio *et al.*, 2009; Telling *et al.*, 2011; Yallop *et al.*, 2012), and the same is likely true for mountain glaciers, but few data points exist. Indeed, significant algal and bacterial primary production have been observed on alpine snowfields and glaciers (Thomas & Duval, 1995) and on glaciers and snowfields in the Pacific Northwest (Hamilton & Havig, 2016), but respiration and heterotrophic activity have not been quantified for these habitats. In contrast to studies of surface blooms, observed rates of autotrophic production were lower than respiration rates in cryoconite holes in the Alps where microbial communities likely depend on the delivery of exogenous carbon (Edwards *et al.*, 2013). However, considerable variation in primary productivity measurements (differences of more than two orders of magnitude) have been reported for cryoconite holes in the Austrian Alps (Anesio *et al.*, 2009; Edwards *et al.*, 2013). Though intriguing, this wide variation was likely observed because one glacier, Stubacher Sonnblickes, was heavily influenced by the Chernobyl accident in 1984 through an accumulation of radionucleotides in the cryoconite (Anesio *et al.*, 2010). Regardless, the contributions of snow algal blooms and OM-rich cryoconite holes on mountain glaciers to the global carbon cycle remain poorly constrained. This is further complicated by the fact that the role of meiofauna in the carbon balance of mountain

glacier ecosystems is not well understood. In general, respiration rates decrease with decreasing temperature (Clarke & Fraser, 2004) and observed grazing rates of sea ice meiofauna are reported to be much lower than algal production (Gradinger, 1999; Nozais *et al.*, 2001). Still, meiofauna are regularly observed in supraglacial ecosystems including cryoconite holes, but studies to characterize the contribution of these heterotrophs to carbon mineralization are lacking.

Even less is known regarding other biogeochemical cycles (e.g., N, Fe, S and P) in supraglacial ecosystems. Debris, rock flour, and volcanic ash can be important sources of essential nutrients to the ice and snow surface (Lutz *et al.*, 2015) as well as the delivery of fixed nitrogen via atmospheric precipitation. Metagenome sequencing of cryoconite microbial communities recovered abundant evidence for efficient nutrient acquisition including genes for assimilating Fe, inorganic and organic S, and ammonia (Edwards *et al.*, 2013). Moreover, the recovery of nitrifying archaea (Hamilton *et al.*, 2013) and *nifH* sequences, a gene encoding a structural protein necessary for nitrogen fixation (Boyd *et al.*, 2011), suggests active nitrogen cycling occurs in surface microbial assemblages. The observation of nitrate production, presumably via nitrification, within a supraglacial stream reach in coastal Alaska also supports the notion that N is actively cycled in supraglacial ecosystems (Scott *et al.*, 2010).

Englacial zone

Located in the interior of the glacier where light doesn't penetrate, the englacial zone is characterized by high pressure (Price, 2000), limited interstitial space in the ice matrix, a near absence of available water for cellular processes, and few energy sources

(Priscu *et al.*, 2006). Since biological activity in the englacial is likely negligible compared to other glacial habitats, meltwater channels (i.e., moulins, crevasses, and any small interstitial spaces) are the englacial zones' most important contribution to the broader glacial ecosystems (Hodson *et al.*, 2008). Specifically, through these meltwater channels, englacial zones provide an important, albeit strongly seasonal (both in scale and structure), conduit for delivering nutrients, water, atmospheric gases and viable cells from the glacier surface to bed, and eventually into proglacial streams and lakes. This delivery of resources is greatly dependent on several factors: incidence of crevasses and moulins on the glacier surface (and their respective depth), seasonal snow coverage, ambient temperature, and solar radiation. Mountain glaciers experience significant variability in all of these factors, and especially the scale of seasonal melt; thus the englacial network of nutrient delivery pathways may be especially important in these ecosystems. Additionally, though discussions of viable microorganisms entombed in glacial ice is beyond the scope of this review, see Hodson *et al.* (2008), Anesio and Laybourn-Parry (2012), and Boetius *et al.* (2015) for general discussions stemming from polar studies (e.g., Lanoil *et al.*, 2009) that likely also apply to mountain glaciers.

Subglacial zone

Temperate glacial ice is permeated by a network of hydraulically linked fractures (Fountain *et al.*, 2005). Water percolates and flows through these fractures and cavities from the supraglacial surface, through the englacial zone, and accumulates below glaciers at the ice-bedrock interface when mean subglacial temperatures reach the pressure melting point (Nye & Frank, 1973). For polar glaciers, this mix of available water and

nutrients accumulates in saturated sediments, often forming subglacial lakes. The steep elevational gradients that typify mountain ecosystems often preclude the development of subglacial lakes, though under the right conditions, they can form, often at glacier margins (Capps *et al.*, 2010; Livingstone *et al.*, 2016). Therefore, meltwater streams and sediments at the glacier-bedrock interface are the most common subglacial habitat in montane habitats. Energy sources in subglacial ecosystems are both diverse and abundant, with basal melting, rock comminution, supraglacial input, and, in some cases, geothermal energy, all contributing to ecosystem function and associated services (Hodson *et al.*, 2008; Hamilton *et al.*, 2013; Boyd *et al.*, 2014; Boetius *et al.*, 2015). Moreover, redox potential is a key control on microbial community structure and function in the subglacial zone, and depends primarily on hydrological flowpaths and connectivity between chemical weathering and oxygen sources (Tranter *et al.*, 2005).

Diverse, active communities of archaea, bacteria, and even fungi live beneath mountain glaciers with observed cell counts ranging from ~10^6–10^7 cells mL^{-1} of meltwater (Sharp *et al.*, 1999; Skidmore *et al.*, 2000; Foght *et al.*, 2004; Price & Sowers, 2004; Montross *et al.*, 2013). While viable bacteria, archaea, and eukarya have been recovered and cultured from subglacial sediments (Sharp *et al.*, 1999; Foght *et al.*, 2004; Margesin *et al.*, 2005; Turchetti *et al.*, 2008; Buzzini *et al.*, 2012; Hamilton *et al.*, 2013), NGS technologies have greatly clarified the scale of diversity and functions of subglacial microbes (e.g., Hamilton *et al.*, 2013). Specifically, 16S rRNA sequences affiliated with chemolithoautotrophs and autotrophic methanogens have been recovered from alpine subglacial sediments suggesting primary production within the subglacial system (Skidmore *et al.*, 2005; Boyd *et al.*, 2010; Hamilton *et al.*, 2013). Further evidence for

lithotrophic primary production in subglacial sediments includes the recovery of RuBisCO mRNA transcripts (Boyd *et al.*, 2014) and the isolation of *Thiobacillus* sp. RG5 (a member of the Betaproteobacteria subphyla) from subglacial sediments in the Canadian Rockies (Harrold *et al.*, 2016). In polar and subpolar regions, chemolithoautotrophic activity can fix several micrograms of carbon per m^2 per day (Gaidos *et al.*, 2004; Mikucki & Priscu, 2007; Christner *et al.*, 2014). While similar measurements are not available for mountain glaciers, the recovery of abundant transcripts affiliated with chemolithoautotrophs and autotrophic methanogens from mountain glacier subglacial sediments (Boyd *et al.*, 2010; Hamilton *et al.*, 2013) suggests this activity is likely significant enough to be highly relevant to broader ecosystem function.

Evidence for phylogenetically diverse heterotrophic bacteria and eukarya has also been recovered from mountain subglacial sediments including sequences affiliated with Proteobacteria, Bacteroidetes, ciliates (Strichotrichida) and amoebae (Tectofilosida; Hamilton *et al.*, 2013). Subglacial heterotrophs are most likely supported by a diversity of sources: labile carbon deposited in pre-glacial times, organic outputs from the activity of chemolithoautotrophs, ancient OM in sediments, and organic carbon delivered from the supraglacial zone (Mikucki & Priscu, 2007). While conditions in subglacial sediments – specifically, anoxia (Bottrell & Tranter, 2002; Wadham *et al.*, 2004; Wynn *et al.*, 2006) and the availability of labile carbon substrates (Wadham *et al.*, 2008) – are often referenced as favorable for organic carbon degradation via methanogenesis, recent evidence suggests the majority of subglacial methanogenesis may be hydrogenotrophic (autotrophic; Boyd *et al.*, 2014; Telling *et al.*, 2015). Evidence for active cycling of Fe,

fixed N, and S has been observed in mountain subglacial sediment flowpaths where variable redox conditions are common. Specifically, sulfate reduction and sulfide oxidation (Bottrell & Tranter, 2002; Wadham *et al.*, 2004; Lanoil *et al.*, 2009), ferric iron reduction (Foght *et al.*, 2004), and nitrification and denitrification (Hodson *et al.*, 2005; Wynn *et al.*, 2006; Wynn *et al.*, 2007; Boyd *et al.*, 2011) all occur however, the spatial extent and magnitude of the cycling for these elements remains poorly constrained.

Glacial bedrock comminution, or the grinding of bedrock into particles by a glacier, generates fresh mineral surfaces capable of sustaining chemotrophic microbial communities including autotrophic mineral-based metabolism with reduced Fe and S as key electron donors (Mitchell *et al.*, 2013; Boyd *et al.*, 2014; Figure 4.1D). In the Canadian Rockies, chemical energy generated during the oxidation of pyrite (FeS_2) and nitrification likely fuels a significant portion of primary productivity in the subglacial zone (Boyd *et al.*, 2010; Boyd *et al.*, 2014). The oxidation of pyrite produces hydrogen ions – key drivers in chemical weathering – that promote dissolution of bedrock calcite and dolomite, thereby contributing to microbially mediated weathering (Raiswell, 1984; Schlesinger & Jiang, 1991; Fairchild *et al.*, 1993; Tranter *et al.*, 1994). Subglacial abiotic hydrogen production may also support microbial metabolism of both autotrophic bacteria (Boyd *et al.*, 2014) and methanogenic archaea (Boyd *et al.*, 2010; Stibal *et al.*, 2012; Dieser *et al.*, 2014; Telling *et al.*, 2015).

To date, the breadth of functional and phylogenetic diversity beneath mountain glaciers has likely been underestimated due to multiple factors: undersampling of the full range of bedrock lithologies that drive habitat conditions, limited spatial variability in study sites at individual glaciers, and relatively few studies have incorporated NGS

approaches to characterize diversity. Moreover, the potential for bedrock communition to provide a sustainable energy source to subglacial ecosystems has broad implications for not only the present-day landscape of biodiversity, but also Earth's ancient past and the possibility of life on other planets. This is supported by stable climatic conditions provided by glacial beds that may have enabled biodiversity to persist during periods of inhospitable climatic, atmospheric, or geologic conditions (Skidmore *et al.*, 2005; Hodson *et al.*, 2008).

Proglacial streams

Proglacial streams and lakes are a prominent component of glacierized ecosystems, exerting strong controls on the geomorphology and ecology of these systems, while also serving as a link between glacial processes and downstream habitats, both freshwater and marine (Battin *et al.*, 2003; Battin *et al.*, 2004; Besemer *et al.*, 2009; Wilhelm *et al.*, 2013; Wilhelm *et al.*, 2014; O'Neel *et al.*, 2015). Though not explicitly discussed in this review, proglacial lakes form between glacial ice and downstream habitats, significantly influencing abiotic stream conditions (Jacobsen *et al.*, 2012; Freimann *et al.*, 2014). Proglacial lakes are an important habitat in need of future study, particularly in the context of anthropogenic climate change (Peter & Sommaruga, 2016). Still, streams are the overarching constant in high-elevation freshwater ecosystems, yet despite their importance as biogeochemical conduits, microbial diversity and function in proglacial streams has not been well studied (Hotaling *et al.*, 2017b). To date, most research on proglacial stream ecosystems has focused on macroinvertebrate species and genetic diversity (Brown *et al.*, 2007b; Jacobsen *et al.*, 2012; Giersch *et al.*, 2016; Tronstad *et al.*,

2016). In contrast to general research in cryobiology which has largely focused on polar latitudes, much of our existing understanding of proglacial streams stems from mountain glacier ecosystems.

The microbial ecology of proglacial streams is geographically diverse with studies from the Austrian Alps (Wilhelm *et al.*, 2013; Wilhelm *et al.*, 2014), Swiss Alps (Freimann *et al.*, 2013, 2014), and North America (Sheik *et al.*, 2015; Fegel *et al.*, 2016). These studies have collectively revealed that proglacial streamwater and biofilms tend to be dominated by Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, and algae (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016). Microbial alpha (within site) diversity in streamwater and biofilms decreases with elevation, possibly due to harsher higher elevation habitat conditions, less diverse sources of microorganism input (i.e., aside from the glacier itself), or a combination of the two (Wilhelm *et al.*, 2013). In contrast, beta (between site) diversity decreases with increasing streamwater temperature (and correspondingly lower elevation), which indicates that as glaciers recede, a more homogenous microbial community will shift to higher elevations (Wilhelm *et al.*, 2013). This predicted change in microbial diversity of proglacial streams adds a third layer to a larger, more ominous, trend in alpine stream biology. That is, there is combined evidence that as glaciers recede, gamma (regional) and beta (between site) diversity will decline in proglacial headwaters for macroinvertebrate species diversity (e.g., Jacobsen *et al.*, 2012), macroinvertebrate genetic diversity (e.g., Finn *et al.*, 2013; Jordan *et al.*, 2016), and microbial diversity (Wilhelm *et al.*, 2013). Bacterial diversity and richness of glacier-fed streams appears to depend heavily upon microhabitat (sediment, streamwater, or biofilms) and hydrological composition, whether purely glacier-fed or also influenced by

other hydrological sources (e.g., groundwater, Wilhelm *et al.*, 2013). However, other stream characteristics, particularly Ca, Fe, SiO_2 concentrations, appear to be specific to local geology and bedrock regardless of source contributions and may be largely dictated by basal geochemical processes rather than surface inputs (Fegel *et al.*, 2016). From a temporal standpoint, microbial communities in proglacial streams have been observed as being relatively stable across summer months (May-September) with only fine-scale day-to-day variation, likely in response to shifting geochemistry and water residence times (Sheik *et al.*, 2015).

Despite supraglacial melt acting as a significant hydrological source for proglacial ecosystems, microbiota from supraglacial environments contribute only marginally to biofilm and streamwater community composition (Wilhelm *et al.*, 2013). Instead, microbial communities in proglacial streamwater and subglacial sediments are most similar, a finding which is consistent with the majority of suspended sediment in glacier-fed streams being derived from subglacial comminution (Mitchell *et al.*, 2013). This limited supraglacial microbial footprint in proglacial stream ecosystems is likely twofold in origin. First, as described above, potential differences in routing of meltwater stemming from supraglacial versus subglacial environments dictates the amount of input contribution for each source. For temperate glaciers, most meltwater is routed through glacial fractures (Fountain & Walder, 1998; Fountain *et al.*, 2005), and therefore passes through the subglacial environment before exiting into proglacial streams. Second, differences in the physical and chemical characteristics of ice, streamwater, and/or biofilms are significant and likely promote specialization by microbiota for each, thereby limiting colonization across habitats, particularly from ice to streamwater.

An additional link exists between glaciers and the physicochemistry of downstream ecosystems which correlates with changes in microbial community composition in both streamwater and biofilms (Wilhelm *et al.*, 2013). Glacial meltwater, which scales with catchment glacier coverage, exerts a strong control on the environmental properties of mountain streams, manifested in variations of streamwater electrical conductivity, pH, and temperature (Hood & Berner, 2009; Wilhelm *et al.*, 2013; Fellman *et al.*, 2014). All three significantly influence biofilm community composition (Wilhelm *et al.*, 2013), and stream microbial assemblages are also sensitive to warming, even at small magnitudes. This sensitivity will be exacerbated by future decreases in glacial influence and increasing fragmentation of the alpine stream landscape, which will expose more stretches of streams to flowing over bedrock (and associated solar radiation; Vincent, 2010). In glacial floodplains, structure and function of microbial communities is again linked to ecosystem physicochemistry, with a prevalence of specialist taxa in harsh glacier-fed streams versus a greater abundance of generalists in more stable, warmer groundwater-fed springs (Freimann *et al.*, 2013, 2014).

While studying microbial diversity in mountain glacier streams has been gaining interest, the functional role of these organisms remains understudied; however, this gap is also narrowing, albeit more slowly. For example, when relative abundances of rRNA versus rDNA are compared, some relatively rare taxa (i.e., comprising a small amount of rDNA abundance) are significantly more abundant in rRNA samples, suggesting that rare taxa play a disproportionate functional role in mountain proglacial streams (Wilhelm *et al.*, 2014). However, given the range of metabolic states that microbes exist in, results of this comparative approach should be interpreted with caution (Blazewicz *et al.*, 2013).

While seasonality affects physical conditions in the other glacier zones discussed above, none are subject to the scale of variability inherent to proglacial streams. This variability spans both daily and seasonal temporal scales, and includes changes in volume of stream discharge, temperature, sediment load, and redox conditions, all of which have important implications for resident biota (Hannah *et al.*, 2007; Milner *et al.*, 2010; Hotaling *et al.*, 2017b). The possibility of observed variation in metabolic activity (rRNA abundance) versus total diversity (rDNA abundance) in hyper variable proglacial streams supports the idea that a microbial 'seed bank' (Pedrós-Alió, 2006) may provide community-level functional plasticity with different taxa being transcriptionally active in response to different sets of environmental parameters (Wilhelm *et al.*, 2014).

As mountain glaciers recede and contribute less to streamflow, there will be corresponding shifts in the export of basal resources and nutrients (i.e., C, N, P, and Fe) from glaciers to proglacial streams (Hood & Scott, 2008; Saros *et al.*, 2010; Hood et al., 2015). This is in part because glaciers can act as an important source of rock-derived elements such as P and Fe (Hodson *et al.*, 2004; Schroth *et al.*, 2011). In addition, OM derived from glacier ecosystems is compositionally unique – it is N-rich and highly bioavailable compared to OM derived from terrestrial ecosystems that are dominated by higher plants (Hood *et al.*, 2009; Singer *et al.*, 2012). Glacier OM can function as a source of C and energy for heterotrophs and ultimately higher trophic level consumers including macroinvertebrates and fish (Figure 4.1C; Fellman *et al.*, 2015). Future changes in treeline elevation and vegetation succession in glacier forelands will impact the magnitude and character of OM inputs to proglacial streams with implications for the structure of in-stream microbial communities. To this end, recent evidence suggests

biofilm bacteria below treeline tend to be more specialized, possibly in response to the higher diversity of allocthonous and autochthonous organic carbon inputs to mountain streams below treeline versus above (Wilhelm *et al.*, 2015). Thus, shifts in microbial community assemblages in proglacial streams may occur as treelines creep higher and new vegetation communities develop in glacier foreland riparian zones where they have not existed, at least since prior glacial cycles.

Glacier forefields

As mountain glaciers continue to retreat on a global scale (Roe *et al.*, 2016), retreating ice fronts will expose newly deglaciated forefields to colonization. The sequence of colonization, scale and sources of energy inputs, and how these forefields transition from newly deglaciated terrain to supporting higher-level organisms, remains underexplored (Bradley *et al.*, 2014). Based on comparative sequencing of microbial communities from the Damma glacier in the Swiss Alps, atmospheric input (through snow, rain, or aeolian dust) of microorganisms to mountain glacier forefields is not a significant factor driving colonization patterns (Rime *et al.*, 2016). Instead, bacterial community composition of newly exposed soils is more similar to endogenous supra- and subglacial habitats, indicating that microbiota from the existing glacial ecosystem are the initial constituents in newly exposed soils. However, the same pattern is not present for fungal communities, suggesting differential dispersal abilities between bacteria and fungi (Rime *et al.*, 2016), likely due to differences in size and physiological capabilities between the groups (Schmidt *et al.*, 2014). Although atmospheric deposition is not a significant source of microorganisms in the Damma glacier forefield, it was an important

source of carbon, nitrate, and ammonium to these ecosystems. Thus supra- and subglacial habitats may play a large role in seeding glacier forefields while atmospheric deposition may be important source of nutrients for these ecosystems. Both fungi and bacteria have been identified as important in primary succession of glacier forefields, establishing organic carbon and nitrogen pools prior to vegetation (Brown & Jumpponen, 2014). As mountain glaciers continue to recede, understanding the biological and nutrient dynamics of glacier forefields, particularly in the context of global nutrient cycles, will directly inform our collective capability to anticipate ecosystem-level changes.

Ecological linkage among zones connected by hydrology

Mountain glacier ecosystems encompass a unique combination of hydrological sources, largely microbially mediated ecosystem services, and geologic activity dictating the structure and function of distinct yet fundamentally linked habitats. Essentially, the physical properties of glaciers influence the microbial communities that reside within them, and these communities drive ecosystem services, the products of which are exported into aquatic habitats (Figure 4.2). Beyond immediate connections between supraglacial, englacial, subglacial, and proglacial habitats, mountain glacier-fed streams flow from headwaters, through many biomes, and eventually into the world's oceans. While beyond the scope of this review, focused investigation and synthesis regarding the influence of mountain glaciers on downstream biomes, aquatic or otherwise, is urgently needed (but see (Moore *et al.*, 2009; Slemmons *et al.*, 2013; O'Neel *et al.*, 2015; Arimitsu *et al.*, 2016; Hotaling *et al.*, 2017b)0. In this section, we outline a framework describing

the relevant hydrological and ecological connections in mountain glaciers upon which future syntheses can build.

The supraglacial-subglacial ecological connection depends upon the hydraulic configuration of glacial drainage. This connection is mediated by the englacial zone, and primarily the layout of fractures and caverns within, which dictate the flow of OM, nutrients, and micronutrients throughout the ecosystem (Fountain *et al.*, 2005; Laybourn-Parry *et al.*, 2012). At the height of summer, meltwater either runs off the supraglacial surface directly into proglacial streams, or more likely, drains through a constantly evolving matrix of crevasses, moulins, fractures, and other spaces within the ice. Depending on drainage dynamics, ephemeral ponds can form on the surface and margins of mountain glaciers. Under the right conditions, these ponds can drain rapidly, delivering a pulse of meltwater and nutrients to the subglacial zone (Laybourn-Parry *et al.*, 2012). Ice velocities, their variation from surface to base, and local topography largely determine the degree of crevassing, and represent an important control on intra-glacier hydrology (Sharp, 1988). For warm-based mountain glaciers, high ice velocities yield the highest incidence of crevasses in the global cryosphere (Benn & Evans, 2014) and this high crevasse incidence can translate to ~100% delivery of surface water and nutrients to subglacial sediments (Fountain & Walder, 1998; Hodson *et al.*, 2008). This potential for high supra-to-subglacial delivery of nutrients contrasts starkly with the cold-based, valley glaciers of polar latitudes where almost no surface water or nutrients are delivered to the subglacial zone (Hodson *et al.*, 2008). The supraglacial-subglacial connection in mountain glaciers fluctuates on daily and seasonal timescales. During the melt season, intraday increases in delivery of nutrients and water from the surface occurs

in parallel with daily temperature fluctuations (and solar radiation) on ice and snow surfaces. Outside of the melt season, only basal melting and the possibility of groundwater input can provide water and nutrients to subglacial ecosystems and thereby to proglacial streams.

As the literal source of many riverine networks globally, mountain glaciers and the microbial communities they support hold significant potential for ecosystem influence both near (e.g., proglacial streams) and far (e.g., oceans). Accelerated input (and ultimate decline) of glacier-derived OM as glaciers recede will directly impact the availability of carbon in proglacially influenced stream ecosystems as warming proceeds (Hood *et al.*, 2009; Singer *et al.*, 2012; O'Neel *et al.*, 2015). And, while the Antarctica Ice Sheet is the major repository of organic carbon on a global scale, mountain glaciers are responsible for much of its release on an annual basis. By 2050, it is estimated that mass loss from mountain glaciers will release ~15 Tg of dissolved organic carbon (DOC; Hood *et al.*, 2015), equivalent to roughly half the annual DOC flux from the Amazon River (~27 Tg; Moreira-Turcq et al., 2003). Much of this labile glacier-derived DOC will likely be respired in glacier-fed streams (Singer *et al.*, 2012), although in regions with abundant tidewater and near-marine terminating glaciers, DOC released from glaciers will contribute to heterotrophic productivity in coastal ecosystems. This finding underscores that importance of proglacial streams in modulating global land-to-ocean biogeochemical fluxes.

Conclusions

Despite considerable progress, mountain glaciers remain one of the most understudied, yet arguably most imperiled and rapidly changing, ecosystems on Earth. Retreating ice directly translates to loss of habitat and species diversity, but beyond biodiversity implications lie myriad additional concerns. Chief among these is developing a better understanding of the role that microbial communities play in the cycling of carbon fixation and nutrients on, within, and beneath glaciers, as well as how shifts in the release of these resources from glaciers may affect downstream ecosystems. And, these research foci should extend to the changing net area of glacier ice versus glacier forefields and how shifts in this ratio will affect many of the same large-scale processes. For example, if photosynthesis exceeds respiration on the surface of mountain glaciers (similar to conclusions drawn from polar ice sheets), then these habitats represent a contemporary sink for CO_2. If the opposite is true, then mountain glaciers are instead acting as net CO_2 sources. Both possibilities hold important climate change implications, particularly for atmospheric feedback loops driving the extension or retreat of glaciers themselves, and for any research efforts aimed at quantifying global CO_2 source-sink dynamics (Anesio *et al.*, 2009). Regardless, many factors linked to climate change may favor snow algae growth both temporally and spatially, ultimately decreasing surface albedo and accelerating both glacial melt and the release of stored carbon (Box *et al.*, 2012; Hood et al., 2015; Lutz *et al.*, 2016). Retreating glaciers will also expose new stream channels, sediments, and bedrock, significantly altering mountain landscapes, as well as the nutrients and cells transported via atmospheric deposition and precipitation. For subglacial environments, anoxia and darkness are defining habitat characteristics

(Bradley *et al.*, 2014). Thus, exposure to light and oxygen will drastically shift energy inputs and selective pressures driving microbial assemblages, thereby altering diversity, ecosystem services, and function (Wadham *et al.*, 2004; Wadham *et al.*, 2008).

Here, we have presented mountain glacier ecosystems as the product of five ecological zones, four of which are interconnected by hydrology. We also discussed the physical conditions, biotic makeup, and linkages among them. This synthesis underscores several gaps in our basic knowledge of these difficult to study habitats. These include: the effects of large seasonal shifts in meltwater and nutrient delivery among zones; the role of bedrock composition in driving microbial community composition and function – to date, the majority of glacier geomicrobiological research has focused on glaciers that override primarily carbonate or granitic bedrock types, with little known of the processes that support microbial life for glaciers overriding volcanic terrains (e.g., basalt or andesite); and, the general microbial ecology of closely related mountain cryosphere habitats (i.e., snowfields and rock glaciers). With this in mind, we offer three aspects of mountain glacier microbial ecology that are ripe for future study:

1. For mountain glaciers, and particularly those below polar latitudes, there is a pressing need to improve understanding of how intra-annual shifts in hydrological flowpaths and sources impact microbial community structure and biogeochemical function. The transport of water from supraglacial to subglacial environments exhibits extreme seasonality. Similarly, the melt season supports light-dependent primary producers for only a fraction of the year. In contrast, basal melting, rock comminution, and geothermal energy sources should be less seasonally affected

and, in most regions, snowfall covers the supraglacial surface and proglacial streams for large portions of the year. These colder ambient temperatures paired with filling of crevasses, moulins, and other interstitial spaces should reduce supraglacial-subglacial-proglacial meltwater and nutrient connections to near zero. Therefore, seasonal shifts in intraglacial water transport, which alter both nutrient availability and redox potential (Tranter *et al.*, 2005), are likely key controls on the structure and function of microbial communities in mountain glacier ecosystems..

2. The extent to which atmospheric dynamics and material deposition influence the microbial ecology of supraglacial and glacier forefield ecosystems deserves further attention. In particular, the degree to which exogenous material, particularly nutrients, is derived from local versus distant sources remains poorly constrained. While communition and redox potential largely affect subglacial ecosystems, atmospheric particle deposition (e.g., black carbon, dust, or volcanic ash) delivers key nutrients, organic matter, and possibly microbiota to both zones, and ultimately this material can be released to downstream marine environments (Stubbins *et al.*, 2012; Doherty *et al.*, 2013; Dumont *et al.*, 2014; Gabbi *et al.*, 2015; Lutz *et al.*, 2015; O'Neel *et al.*, 2015).

3. Mountain glaciers contribute substantially to the global cryosphere, particularly above treeline in the alpine zone. While the microbial ecology of mountain glaciers remains understudied compared to high-latitude glaciers and icefields, research into other components of the alpine cryosphere is largely absent, especially when

investigations beyond measures of biodiversity are considered. Indeed, perennial snowfields and rock glaciers are often contiguous with mountain glaciers; thus, a better understanding of their microbial dynamics has direct relevance to both glacier and mountain ecosystems. Moreover, snowfields and rock glaciers may provide important points of comparison. Snowfields do not exhibit rock communition on the scale of glaciers and have much lower crevasse incidence, two key differences that could allow for better resolution of how those two contribute a wide array of variables that may be of interest. Similarly, rock glaciers and the debris-covered glaciers commonly found in the Himalaya have layers of organic and mineral debris, which provide insulation as well as a source of nutrients, thus serving as useful examples of naturally "nutrient-loaded" glaciers that may be less susceptible to anthropogenic warming.

Mountain glaciers are vital components of total biodiversity, carbon cycling, food web dynamics, and ecosystem services on a global scale (Hotaling *et al.*, 2017b), with microbial life acting as the dominant biogeochemical force in these extreme, ice-laden ecosystems. As they are among the most imperiled habitats on Earth, it is imperative that we continue to refine existing understanding of the role mountain glaciers play in geomicrobiological, biogeochemical, and ecological processes. This understanding stands to be bolstered by continued incorporation of emerging technologies with established approaches. Though specific details and applications are beyond the scope of this mini-review, these technologies may include NGS tools for assessing biodiversity through metagenetic (single marker, many taxa; e.g., Wilhelm *et al.*, 2013) and metagenomic

approaches (many markers, many taxa; e.g., Edwards *et al.*, 2013), transcriptional activity (e.g., Wilhelm *et al.*, 2014), particulate matter quantification through remote sensing (e.g., Di Mauro *et al.*, 2017), characterizing biodiversity via improved culture techniques (e.g., diffusion chambers, gel microdroplets, and hollow-fiber membrane chambers, reviewed by Vester *et al.*, 2015), and measuring *in-situ* microbial activity (e.g., improved chemical sensors, Bagshaw *et al.*, 2016).

Given the links between microbial ecology, local bedrock, and seasonal melting, individual data points from single glaciers or mountain ranges are useful but difficult to synthesize. Perhaps the field's most pressing need is that of collaborative globalization. Indeed, there is immense opportunity for researchers working in glacier ecosystems to establish standardized protocols, coordinate sampling efforts, and integrate comparable data to build cross-continental perspectives. Similar calls have been raised for alpine streams (Hotaling *et al.*, 2017b), and increased coordination between stream biologists, microbial ecologists, and glaciologists could greatly enhance geographic sampling scopes with little added cost. Ultimately, our collective goal should be a more general understanding of the extant contributions of mountain glaciers to microbial biodiversity and ecosystem function, as well as how these patterns may be altered as climate change proceeds.

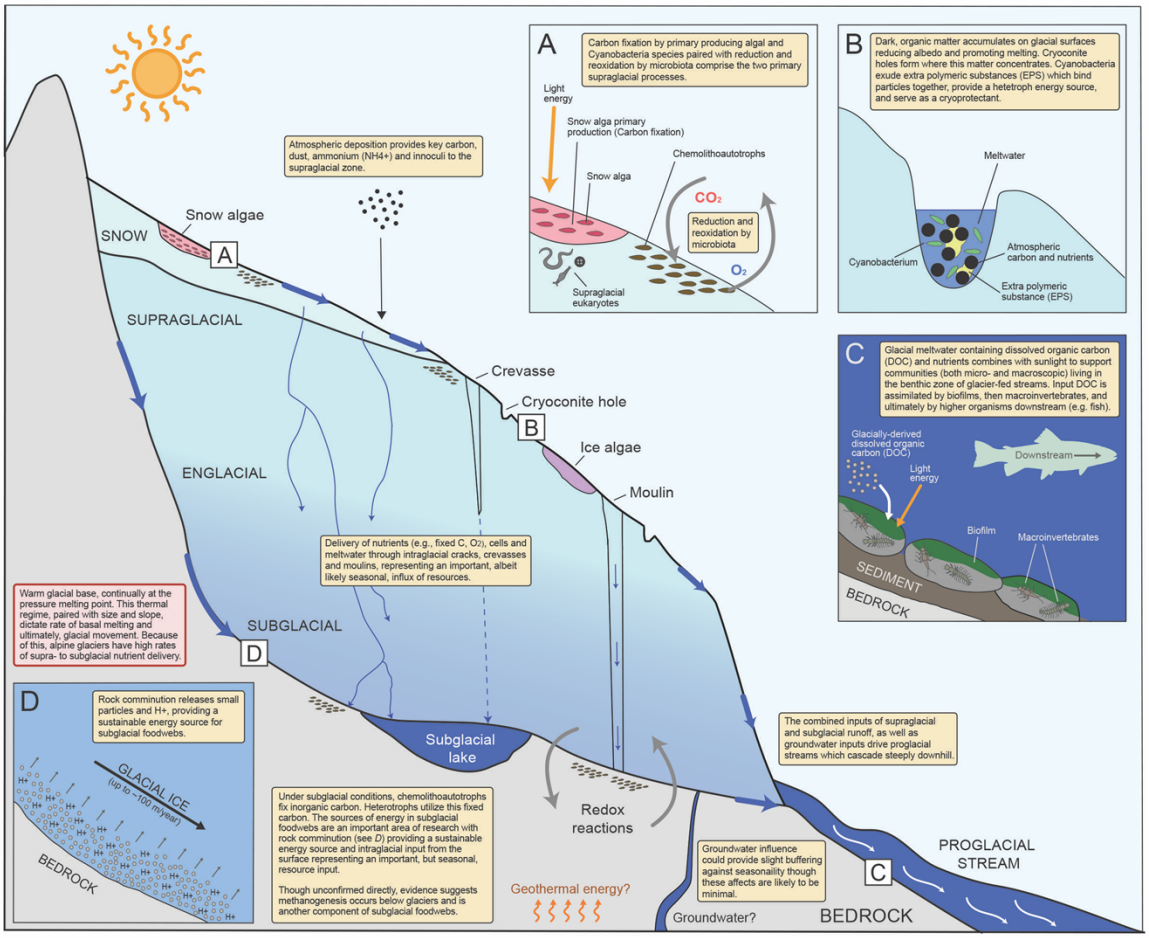

Figure 4.1. A schematic of mountain glacier ecology, hydrology, and geomicrobiology. Black arrows represent atmospheric deposition, orange arrows (A, C) indicate solar radiation, blue arrows indicate meltwater moving on, through, and under the glacier, eventually emanating from the glacier terminus into a proglacial stream, and the black arrow (D) shows the flow of glacier ice downhill. This figure is based on Figure 2A in Boetius *et al.*, (2015), and has been extended to reflect mountain glaciers specifically.

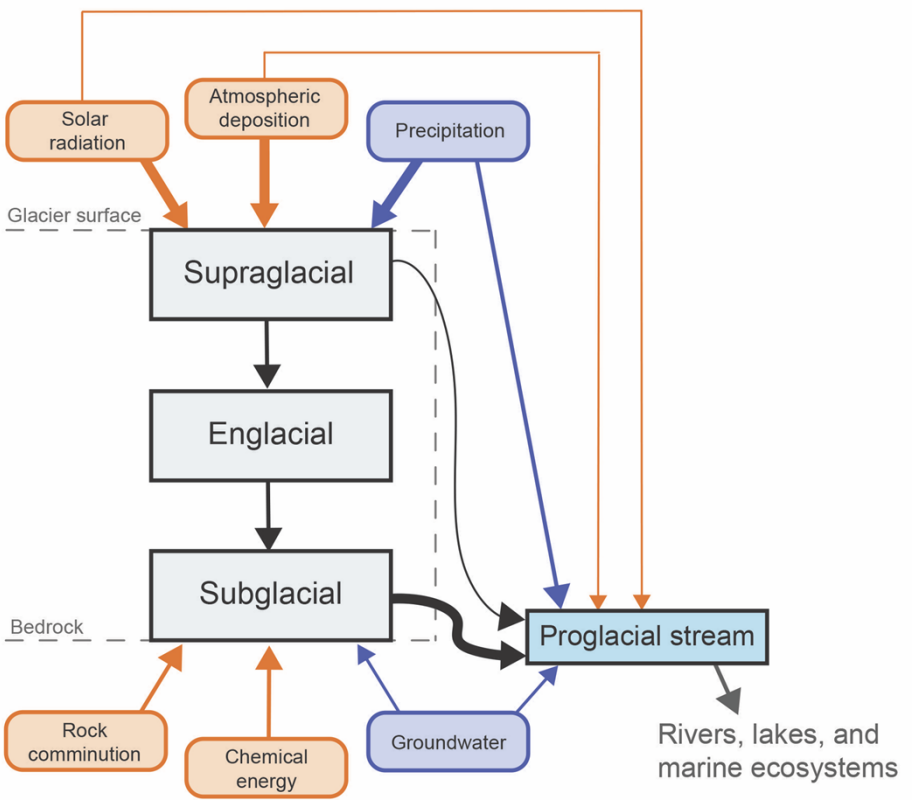

Figure 4.2. A schematic of energy and nutrients (orange) and hydrologic (blue) inputs and flow through a mountain glacier. Black arrows indicate combined flow of both. Differential flow, up to ~2 orders of magnitude, is shown by darker versus lighter arrows between the supraglacial-subglacial-proglacial connections.

CHAPTER 5

MICROBIAL DIVERSITY OF ALPINE STREAMS VARIES WITH HYDROLOGICAL SOURCE AND MICROHABITAT BUT NOT SUBRANGE IN THE CENTRAL ROCKY MOUNTAINS OF NORTH AMERICA

Introduction

Alpine streams harbor significant habitat diversity over small spatial scales, largely due to substantial variation in hydrological source (Hotaling *et al.*, 2017b). As traditionally described, these primary hydrological sources range from stable, groundwater-fed springs to harsh, glacier-fed streams, with snowfield-derived streams intermediate in terms of environmental harshness (Ward, 1994). A fourth class of stream emanates from permanent subterranean ice sources and these streams tend to be as stable as groundwater-fed springs yet as cold as traditional glacier-fed steams. These "icy seeps" are most commonly associated with rock glaciers – masses of subterranean ice covered in a thick layer of organic debris (Janke, 2007; Janke, 2013; Millar *et al.*, 2013), making them perhaps the most resistant to climate change of all meltwater-associated alpine stream types (Hotaling *et al.*, 2017b). As a result of this hydrological (and subsequent environmental) variation, alpine streams exhibit correspondingly high beta (between site) diversity across many taxonomic levels from macroinvertebrate species (Brown *et al.*, 2007b; Finn *et al.*, 2011; Jacobsen *et al.*, 2012) and genetic diversity (Finn *et al.*, 2013; Finn *et al.*, 2014; Leys *et al.*, 2016) to microbial diversity (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016; Hotaling *et al.*, 2017a). While alpine stream ecosystems, and particularly the biodiversity they contain, have been understudied in comparison with their lower elevation counterparts, investigations of microbial diversity and function of these habitats are particularly rare (Hotaling *et al.*, 2017a).

Glacier ice (Anesio & Laybourn-Parry, 2012), snow (Lutz *et al.*, 2016), and stream ecosystems (Zeglin, 2015) all harbor diverse microbial communities carrying out important functional roles including primary production, carbon fixation, and nutrient cycling (Anesio *et al.*, 2010; Hotaling *et al.*, 2017a). As the literal headwaters of many rivers, alpine streams serve as the vital hydrological link between cryospheric processes and downstream habitats, both freshwater and marine (Hood et al., 2015; O'Neel *et al.*, 2015; Hotaling *et al.*, 2017a). To date, most studies of microbial ecology in alpine headwaters have focused on proglacial streams, including those fed by traditional glaciers (Freimann *et al.*, 2013; Wilhelm *et al.*, 2013) or rock glaciers (Fegel *et al.*, 2016), with far fewer investigations of other stream types (e.g., groundwater-fed springs, Esposito *et al.*, 2016). Generally, these studies have identified alpine streamwater and biofilm communities – complex clusters of microbial cells attached to underwater substrate (Besemer, 2015) – to be dominated by Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, and algae (Hotaling *et al.*, 2017a). In glacier-fed streams, within stream (alpha) diversity in both streamwater and biofilms decreases with higher elevation, perhaps due to harsher, more variable conditions closer to the ice, less diverse sources of microbial input (i.e., only the glacier and atmosphere), or more likely, some combination of the two (Wilhelm *et al.*, 2013). In contrast, between stream (beta) diversity decreases at lower elevations (and correspondingly elevated stream temperatures), supporting an ominous broader trend in alpine stream biology. That is, as climate change proceeds and glaciers decline, within stream (alpha) diversity will increase as a warmer elevation community shifts into the higher reaches of existing streams while regional (gamma) and between stream (beta) diversity will simultaneously decline as habitats become

increasingly homogenized (Hotaling *et al.*, 2017b). Existing evidence supports this trend across taxonomic scales, from macroinvertebrate species and genetic diversity (Jacobsen *et al.*, 2012; Finn *et al.*, 2013; Finn *et al.*, 2014; Jordan *et al.*, 2016) to microbial diversity (Wilhelm *et al.*, 2013).

Local conditions are a driving force dictating the structure of microbial communities in glacier-fed streams (Hotaling *et al.*, 2017b). This is true for streams emanating from both traditional surface and rock glaciers (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016). Perhaps no greater factor dictates local conditions than where taxa exist, particularly for microorganisms that could be dispersal limited. Local conditions in stream microhabitats (e.g., biofilm, sediment, streamwater) drive unique suites of physiochemical variation which could select for specific groups of organisms (Zeglin, 2015). This is true for alpine, glacier-fed streams where both microhabitat and physiochemical conditions have been identified as major predictors of bacterial diversity and richness (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016). Moreover, more nuanced characteristics such as the concentrations of specific elements and compounds (e.g., Ca, Fe, SiO_2) also influence prokaryotic community, at least in rock glacier-fed streams (Fegel *et al.*, 2016). This is intriguing as these stream characteristics, while partially dependent on hydrological makeup, are thought to be largely dictated by basal geochemical processes below glaciers – a component of mountain glacier ecosystems that is heavily dependent on local geology (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016).

Climate change is predicted to greatly alter alpine stream habitats as glaciers recede, stream hydrology shifts, and in some instances, streams convert from being primarily glacier-fed to groundwater spring-fed (Hotaling *et al.*, 2017b). Indeed, as alpine

glaciers recede, they tend to leave groundwater-fed springs in their wake (Baraer *et al.*, 2012; Chavez, 2013). However, the degree to which microbial diversity varies across all of the existing alpine stream types has been unexplored. In this study, we clarified the driving forces behind how microbial diversity is structured in North American alpine streams through 16S rRNA sequencing and three levels of comparison: 1) between subranges of the Rocky mountains in northwestern Wyoming and Montana, 2) among microhabitats including biofilms and streamwater, as well as source ice when applicable, and 3) between the full range of primary hydrological sources (glaciers, snowfields, groundwater-fed springs, and a new category we call "icy seeps"). Alluding to broader themes in global change biology, the results of this study have clear implications for the future of alpine stream microbial diversity as climate change proceeds, the global cryosphere declines, and in-stream physiochemical conditions shift. Following similar studies in the Austrian Alps (Wilhelm *et al.*, 2013), we expected microhabitat to be the most important factor dictating microbial community composition with primary hydrological source acting as a secondary factor. Furthermore, we expected to observe some influence of mountain subrange (and therefore local geology) on microbial diversity in line with Fegel *et al.* (2016) who identified clear differences in microbial communities among mountain ranges of the western United States. From a conservation perspective, as icy seep streams fed by either debris covered rock glaciers or other forms of subterranean ice may be the most resistant meltwater-associated stream types to climate change, these streams may represent important future strongholds of microbial diversity suited to harsh, icy stream conditions.

Materials and Methods

Study sites and sample collection

During the summer of 2015, microbial samples were collected from alpine streams in Grand Teton National Park (GRTE) and the surrounding mountains of northwestern Wyoming as well as Glacier National Park (GNP) in northwestern Montana (Figures 5.1, 5.2; Table 5.1). All field sampling was performed within a six-week interval from early August to mid-September, when alpine habitat in North America is the most free of seasonal snowmelt. In addition to improving access, this reduced influence of snowpack also allows for stream conditions and communities that most closely reflect their primary hydrology. All study sites were contained within three subranges of the Rocky Mountains: the Teton Range (GRTE) and the Lewis and Livingston Ranges (GNP). For purposes of comparison, these subranges will hereafter be referred to as GRTE and GNP, as the Lewis and Livingston ranges are much more similar to one another than either us to the Teton Range. In total, we sampled 13 streams: six in GRTE and seven in GNP. The primary hydrological sources for streams was determined by comparing a suite of environmental variables, satellite imagery, and field observation (see additional details below).

For each stream, we sampled three microhabitats: streamwater, biofilm, and source ice (when possible). Streamwater was sampled by collecting one liter of streamwater into sterile Whirl-Paks (Nasco, Salida, CA, USA) from three or more representative places within the stream where water was clearly flowing. Biofilm samples were collected by scrubbing a ~4” x 4” section of three representative rocks from the stream bottom into an ethanol-sterilized plastic dish containing ~25 mL of sterile PCR

water. A wire brush used for scrubbing was flame-sterilized between sites. When source ice was present and safely accessible, we sampled subsurface ice (~1 ft. depth) from three locations that appeared typical of the glacier or snowfield surface. For each ice collection site, we first removed the upper ~1 ft. of ice using a flame-sterilized ice axe adze. Next, we re-sterilized the adze and collected ~3 L of ice into sterile Whirl-Paks. These samples were transported to the next basecamp and allowed to melt. We had one exception to this general sampling regime – Wind Cave – a unique site in the Teton Range where subterranean ice emanates from a cave. Here, we collected two separate streamwater samples, one from the main channel and another from a small seep in a cave wall.

After the varied collection methods, all samples were filtered using BD Luer-Lok sterile syringes and filter holders (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and 0.2-μm filters (Millipore, Billerica, MA, USA). For streamwater and ice, ~0.5-1 L was typically filtered. For biofilm supernatants, ~2-20 mL were filtered. After sample collection, filters were immediately placed into sterilized sucrose lysis buffer (SLB; 20mM EDTA, 400 mM NaCl, 0.7 M sucrose, 50 mM Tris, pH 9.0). For each site and microhabitat, three replicate samples were collected. In addition, a "field negative" was collected for every site by using forceps to briefly expose a sterile filter to atmospheric conditions before preserving it in SLB.

Categorizing streams by hydrological source

To assess the influence of stream hydrology – and specifically primary source – on microbial diversity both within and between subranges of the Rocky Mountains, it was important that we were able to accurately categorize streams into one of four categories:

glacier-fed, snowfield-fed, groundwater-fed, or an icy seep. For GNP, with a lower prevalence of rock glaciers, categorizing streams by hydrology was relatively straightforward. Groundwater-fed springs were only deemed as such when the source could be identified, both maximum and average temperature profiles were higher than other streams in the range, and a clearly stable stream channel was present. For glacier and snow-fed streams, our classification criteria followed those outlined in Giersch *et al.* (2016) with glacier-fed streams identified when National Agricultural Imagery Program satellite imagery from September 2005 (a low snow year in the park) clearly showed crevasses visible in their ice sources. Moreover, presumed glacier locations cross-checked with georeferenced glacier boundaries included in the publicly available GNP glacier layer (NPS Geospatial Dataset #1019881). Snowfield-fed streams included any that emanated from a permanent snow feature that was not identified as a glacier based upon the above criteria. We assessed channel stability (an important factor in determining the relative influence of glaciers on stream ecosystems) for all sites using a modified version of the Pfankuch Index for streambed stability, which has been demonstrated to work well in mountain streams (Peckarsky *et al.*, 2014).

In contrast, GRTE and the Teton Range are rife with subterranean rock glaciers (Fegel *et al.*, 2016) that can look outwardly like groundwater springs but with very different environmental composition. Therefore, to characterize sites by hydrology in GRTE, we measured a suite of environmental variables including four variables commonly used to characterize the degree of glacier meltwater influence (Ilg & Castella, 2006; Finn *et al.*, 2013). These key variables included: water temperature [year-round range (T_{range}), average temperature between the summer and autumn solstices (T_{summer}),

and average for the full year (T_{year})], electrical conductivity (measured with a YSI Professional Plus Multiprobe, YSI Incorporated, Yellow Springs, OH, USA), channel stability (via the Pfankuch Index described above), and suspended solids. For suspended solids, we filtered water through pre-ashed and weighed 25-mm, type-A/E glass fiber filters (Pall Life Sciences, Port Washington, NY, USA) until the filters clogged or 600 mL had been filtered, whichever came first. After being dried at 50 °C for 24 hours and subsequently burned at 500 °C for four hours, filters were placed in a desiccator with charged desiccant for one hour. Filters were then weighed and pre-filtered mass was subtracted. To assess variation in water temperature throughout the year, we deployed *in-situ* temperature dataloggers (HOBO, Onset Computer Corporation, Bourne, MA, USA) in all but one stream (Sperry Glacier) and allowed them to run for approximately one year. For all GNP streams, temperature data was collected in the 2012-2013 year except for Grinnell Glacier (2013-2014). For all GRTE streams, temperature data was collected in 2015-2016. Data were recorded at one-hour intervals for the full year. Our identification of rock glaciers versus groundwater-fed springs was strengthened through the use of a rock glacier inventory database generated for the western United States (Johnson & Fountain, 2016).

DNA extraction, PCR amplification, and sequencing

In the laboratory, replicate samples for the same site and microhabitat were pooled and DNA was extracted from the SLB-preserved filter slurry after a brief vortex following the direct-to-PCR method outlined in Flores *et al.* (2012). The 515f/806r PCR primer sets developed for the Earth Microbiome project (Gilbert *et al.*, 2014) and

described in Bates *et al.* (2011) were used to target the highly variable V4 region of the 16S rRNA gene. PCRs were performed in 20-µL reactions containing 10-µL of Extract-N-Amp Ready Mix (Sigma-Aldrich, St. Louis, MO, USA), 1-µL mixtures of both forward and reverse primers for each library at 5 µM concentrations, 4-µL of extracted DNA, and 5-µL of PCR grade H_2O. Samples were amplified using an initial denaturing step of 3 minutes at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C, 60 s of primer annealing at 50 °C, and a final elongation of 60 s at 72 °C. Each PCR included a negative control and PCR amplicons were gel-checked to ensure amplification. Amplicons were sequenced on an Illumina MiSeq at the University of Kentucky Advanced Genetic Technologies Center using 250-bp paired-end chemistry. In total, 48 libraries (36 samples, 11 field negatives, 1 PCR negative) were sequenced on two MiSeq lanes. The first lane included all 48 libraries and the second was dedicated to seven non-negative libraries for which the total reads recovered was below 40,000 in the first sequencing run.

Raw sequence analyses and OTU calling

After sequencing, reads were demultiplexed and reads from both lanes of sequencing were combined into one file per library. Next, forward and reverse reads were combined in FLASH (Magoč & Salzberg, 2011) with a minimum and maximum of 20- and 250-bp of overlap allowed, respectively. Reads that did not merge were not included in downstream analyses. Next, reads were filtered for quality using the Fastx-Toolkit (Gordon & Hannon, 2010) with reads retained that had an average quality score of 24 or greater across 80% of the read. After read merging and quality filtering, all other

analytical steps were performed within the QIIME v.1.8.0 pipeline unless otherwise noted (Caporaso *et al.*, 2010). Sequence files were converted from FASTQ to FASTA (convert_fastaqual_fastq.py) and combined for downstream processing (add_qiime_labels.py). Using the default 97% similarity threshold, operational taxonomic units (OTUs) were picked (pick_de_novo_otus.py). This process wrapper script includes sequence alignment (align_seqs.py) and taxonomy assignment (assign_taxonomy.py) by comparing aligned sequences to the Greengenes reference database (DeSantis *et al.*, 2006). As part of the OTU picking process, we also filtered our database in three ways: first, to remove singleton OTUs. Second, to remove possible contaminant OTUs based upon the results of sequencing both field and PCR negatives, many of which included some degree of positive amplification. And, third, because chloroplasts evolved from Cyanobacteria (Martin *et al.*, 1998), 16S rRNA sequencing still identifies significant chloroplast diversity, however, as these are not true prokaryotes, we removed chloroplast-associated OTUs.

Statistical analyses

To assess biodiversity differences within and among groups, whether source, microhabitat, or subrange, we calculated within site (alpha) diversity using the Shannon diversity index (H) metric. We also estimated differences in microbial assemblages between sites (beta diversity) using Bray-Curtis dissimilarity distances. Based on our alpha diversity results, we rarefied our beta diversity estimates to 20725 reads, the lowest number of sequences recovered for a non-negative library after quality filtering and OTU removal. We summarized samples by dominant taxonomic groups (phyla to genus) using

the summarize_diversity_through_plots.py script. To compare total alpha diversity of defined groups to each other, we used a two-sample, nonparametric t-test implemented with compare_alpha_diversity.py, using H as our biodiversity metric and 999 permutations. Since environmental variables, and particularly temperature, can play a significant factor in determining biodiversity, we assessed the degree to which alpha diversity correlated with stream temperature (T_{range}, T_{summer}, and T_{year}) using Pearson's correlation implemented in the R package 'Hmisc' (Harrell Jr, 2008).

To test whether microbial assemblages differed among defined groups (whether subrange, source, or microhabitat), we performed 'adonis' analyses (which are analogous to a PERMANOVA analyses) using the R package 'vegan' (Oksanen *et al.*, 2007). Because our study design included inherently nested structure, we incorporated defined strata (groups) to constrain permutations to within groupings and thereby control for the influence of one treatment on another. Specifically, we tested three factors of interest: subrange, source, and microhabitat. For each test, we constrained permutations into one of three groupings. For subrange, we tested the effect of this difference after defining our strata to include both source+microhabitat. For source, our defined strata were subrange+microhabitat. And, for microhabitat, our strata were subrange+source. For all adonis tests, we used a nonparametric approach to partition distances among sources of variation that allows for both the strength and significance of a specific factor to be determined. For our comparisons, we used Bray-Curtis dissimilarities and 9999 permutations to assess significance. We also quantified the dispersal (or variation) within defined clusters using the make_distances_boxplots.py script with samples grouped by source or source+microhabitat. Significance of within group beta diversity distances

departing from the average distances overall was assessed using two-sample Student's t-tests comparing the group of interest (e.g., beta diversity within biofilms to the overall mean distance with defined groups. For all comparisons, non-parametric *P*-values were generated for 999 permutations.

We visualized how microbial taxonomic abundance and frequency corresponded with treatment groupings using ternary plots generated via the R package 'ggtern' (Hamilton, 2015). Specially, we constructed plots comparing microhabitats as well as primary source. For primary source, we included glacier-fed streams, icy seeps, and groundwater-fed springs to best cover the variation of hydrology present in our sampling design as well as assess how icy seeps compare to both extremes of previously described alpine stream types.

Results

In total, we generated 13,093,344 sequences for 48 libraries. After quality filtering, we retained an average of 56,328 reads per library. For non-negative libraries only, the minimum reads retained was 20,725 and maximum was 312,514. Sequencing depth was determined to be sufficient for appropriately resolving the amount of diversity present based upon rarefaction curves of H with relatively little new diversity being discovered after 5,387 sequences per sample. Overall, we identified 50,179 OTUs which were classified into 52 phyla, 187 classes, and 598 families (Table 5.2). Hereafter, unless specifically stated, all results will pertain to the 36-library treatment data set only (i.e., with no negative samples included).

Stream hydrological classifications

Based upon the environmental, spatial, and field data collected for each subrange, we classified our 13 sample streams into four categories of primary hydrological influence. For GRTE, we identified two glacier-fed streams, two icy seeps, and two snowmelt streams. We did not observe any groundwater-fed springs in GRTE. For GNP, we identified two glacier-fed streams, one icy seep, two snowmelt streams, and two groundwater-fed springs (Figure 5.3, Table 5.1). Though the same environmental data was not collected for both mountain ranges, we did measure the Pfankuch index (PI) for all sites. In GRTE, where we had additional environmental data to support our hydrological classifications (Figure 5.3), streams clearly fell into groups based upon PI: glacier-fed streams were the highest (> 40), and therefore least stable. Snowmelt-fed streams were intermediate (PI = 21-24) and icy seeps the most stable (PI = 15-18). Our GNP classifications largely aligned with this. Our two glacier-fed streams also had the highest PI (36 and 39), snowmelt-fed and icy seeps were intermediate, 15-25 and 19, respectively. And, as expected, groundwater-fed springs were the most stable (PI = 11; Table 5.3). When our four key 'glaciality' variables were viewed in principal component space for GRTE, we observed the widest variability between our two glacier-fed streams with the opposite pattern indicated by snowmelt streams (Figure 5.3). Finally, GNP streams were slightly colder on average (2.9 versus 3.5°C) than GRTE streams. The coldest stream overall was an icy seep, Piegan Spring (T_{summer} = 1.1°C) and the warmest was the south fork of Teton Creek (T_{summer} = 7.1°C; Figure 5.12, Table 5.3).

While we grouped three streams into the icy seep category, it is useful to note the unique characteristics of each. In GRTE, one icy seep emanated from Wind Cave, the

outlet to a cave system that remains cold enough year-round to support subterranean ice, and therefore has very cold water that belies its relatively low, glacier-less elevation (2692 m, the lowest study site in GRTE; Table 5.1). The other icy seep in GRTE emanates from a debris-covered rock glacier. In GNP, the only icy seep included – Piegan Spring – is somewhat similar to the Wind Cave site in that despite it's appearance as a spring, it is actually fed by the subglacial meltwater from the Piegan Glacier on the opposite mountainside from where the spring emerges (Figure 5.2).

Microbial diversity in alpine streams

Among sources, both icy seeps and groundwater-fed springs contained the most phyla (45). However, when total families observed was considered, snowmelt-fed streams were the most diverse, containing 474 families (Table 5.3). When samples were grouped by both source and microhabitat, snowmelt-fed streamwater contained the most families (441). Icy seep biofilms contained at least 25% fewer phyla (20), classes (41), and families (149) compared to all other combinations (Table 5.2). For alpha diversity, we observed no difference in average microbial diversity of each mountain subrange (mean H, GNP = 7.07 vs. mean H, GRTE = 6.87, $P = 0.808$). For microhabitats, streamwater (mean H = 8.82) was more diverse than both biofilms ($P = 0.006$) and ice ($P = 0.006$) but no significant difference was observed for total diversity of ice versus biofilms ($P = 0.147$; Figures 5.4, 5.5A; Table 5.4).

When source, microhabitat, and subrange were all taken into account, the least diverse samples were biofilms in glacier-fed streams and icy seeps of GNP (mean H = 2.71 and 2.88; Figure 5.5B). Conversely, the most diverse samples were from

streamwater in groundwater-fed springs (mean H = 10.04, GNP only; Figure 5.5B, Table 5.5). Generally, diversity of biofilms and streamwater scaled with source. That is, in higher diversity habitats like snowmelt-fed streams, both biofilms and streamwater were correspondingly more diverse than in lower diversity habitats like glacier-fed streams. Interestingly, the largest disparity in alpha diversity between microhabitats within a single source type was observed for icy seeps. In these habitats, streamwater was an average of 5.97 units more diverse than biofilms – a much higher differential than the average of all other sources (mean H difference, BF to SW = 3.25). We also observed a clear signature of temperature influencing within stream biodiversity. For streamwater samples, higher values of both T_{summer} ($P = 0.017$) and T_{year} ($P = 0.004$) were associated with increased alpha diversity. For biofilms, higher values of T_{range} ($P = 0.012$) and T_{summer} ($P = 0.011$) were both associated with increased alpha diversity (Figure 5.13).

We observed no subrange-specific signatures of microbial community assemblage ($P = 0.072$; Table 5.6). In contrast, both source ($P = 0.013$, 22.59% of variance) and microhabitat ($P = 0.013$, 22.59% of variance) were significant drivers of microbial community structure (Table 5.6). When viewed in principal coordinate space, these patterns were supported with samples grouping closely with those from the same source and microhabitat (Figure 5.6). However, we did observe one instance of a possible subrange-specific assemblage pattern in glacier-fed biofilms. For this treatment group, both samples for each subrange clearly partitioned into tight clusters, separate from one another. The only other anomaly of note was a single streamwater sample from the terminus of the Petersen Glacier in GRTE (Figure 5.6).

When samples were grouped by source, average beta diversity distance among samples was highest for those in glacier-fed streams (0.89) and lowest for groundwater-fed springs (0.74). Microbial communities taken directly from ice, while not a source type per se, were even more tightly constrained with an average distance among samples of 0.63 (Table 5.7). When samples were grouped by both source and microhabitat, both glacier-fed groupings (streamwater and biofilms) remained the most variable (0.84 and 0.85, respectively). However, like alpha diversity, a clear disconnect in icy seep groupings was again clear. Where icy seep+streamwater samples are highly variable (0.82), icy seep+biofilm samples were the most similar of any grouping, including samples from source ice (0.42; Table 5.7). When group variability were compared to the average variation within all groups, four of five source groupings differed significantly from this mean expectation. Only groundwater-fed spring samples did not fit this pattern (Figure 5.7A). When source+microhabitat were considered, all glacier-fed and icy seep groupings differed from overall average (Figure 5.7B).

Dominant microbial taxa

Regardless of mountain range, source, or microhabitat, microbial diversity was dominated by OTUs belonging to two phyla, Proteobacteria and Bacteroidetes. Within Proteobacteria, the most dominant classes were α- and β-proteobacteria with α-proteobacteria particularly abundant in glacier-fed and icy seep biofilms (Figures 5.8-5.9). Cyanobacteria-associated sequences were recovered from all samples, most dominant in biofilms, and at conspicuously low levels in icy seep biofilms. When grouped by microhabitat and source, dominant phyla clearly differed between

streamwater samples from glacier-fed streams versus all other source types. But, this difference did not extend to biofilms where snowmelt- and groundwater-fed streams were clearly much more similar than they were to other sources (Figures 5.8-5.9).

Of the 25 most common phyla, only four (Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria) were generally equally distributed across all three microhabitats (Figure 5.10). Six phyla were primarily shared between ice and streamwater and the rest of the most common phyla (13) were most abundant in streamwater (Figure 5.10). From a more targeted perspective, much greater variability was observed for the 50 most common families among microhabitats with eight families well-distributed across all three, with only one family (Neisseriacae) shared between streamwater and biofilms, and three families primarily associated with ice (Microbacteriaceae, Chitinophagagaceae, and Sporichthyaceae). The majority of common families (23 of 50) were shared between streamwater and ice. At both the phylum and family levels of classification, no taxa were shared exclusively between ice and biofilms (Figure 5.10).

When samples from glacier-fed streams, icy seeps, and groundwater-fed springs were compared, no phyla stood out as being unique to icy seeps however, two phyla (Firmicutes, Tenericutes) appeared to be primarily associated with glacier-fed streams. Another six phyla were shared among all three source types but only Actinobacteria and Proteobacteria were shared in comparisons of both microhabitat (Figure 5.10) and source (Figures 5.11). While five phyla were primarily associated with groundwater, the bulk (11) were shared between icy seeps and groundwater-fed springs (Figure 5.11). When families were considered for the same comparison, we observed a much more balanced

distribution with a number of families primarily associated with specific sources: glacier-fed streams (5), icy seeps (4), and groundwater-fed springs (15; Figure 5.11). Again, aside from shared families overall (11), most shared families were associated with icy seeps and groundwater-fed springs. While only one family was shared between glacier-fed streams and icy seeps (Enterobacteriaceae), it was conspicuously abundant overall (~21% of all OTUs), but nearly absent in groundwater-fed springs (0.003% of all OTUs; Figure 5.11). Enterobacteriaceae were also only at 1.47% frequency in snowmelt-fed streams (not shown). Another family of note is Pseudomonadaceae which was much more abundant in icy seeps that glacier-fed streams (~6x more abundant), groundwater-fed springs (~100x), and snowmelt-fed streams (~4.5x). For groundwater-fed springs, two families (Cytophagaceae and Phormidiaceae) were particularly abundant in these environments and at low frequency elsewhere. For glacier-fed streams, five families were more associated with these environments than others, but only one (Exiguobacteraceae) was nearly absent from all other stream types (Figure 5.11).

Discussion

The global retreat of the alpine cryosphere – including glaciers, permanent snowfields, and rock glaciers – is causing seismic shifts in biotic communities, affecting organisms large and small, and ecosystems from headwaters to oceans (O'Neel *et al.*, 2015; Hotaling *et al.*, 2017b). Under this overarching umbrella of global change are direct threats to the persistence of many organisms as unique habitats are lost: however these threats are impossible to quantify without underlying knowledge of current community composition, and more importantly, drivers of extant biodiversity. Microbial

communities of alpine streams represent one of the largest disconnects between possible communities under near-term threat of large-scale biodiversity change and existing knowledge of their diversity, biogeography, and function. Development of molecular approaches to characterize microbial communities regardless of culturability (Schmidt *et al.*, 1991; Caporaso *et al.*, 2010; Bates *et al.*, 2011) has spurred a corresponding expansion in knowledge of alpine stream microbial ecology (e.g., Wilhelm *et al.*, 2013). And, recent alpine-stream specific efforts have identified a diverse, active microbial community residing in glacier-fed streams, with local diversity between streams heavily influenced by temperature and mountain range (Wilhelm *et al.*, 2013; Wilhelm *et al.*, 2014; Fegel *et al.*, 2016). Moreover, streams emanating from traditional, surface glaciers and rock glaciers differ biogeochemically, and these differences extend to microbial community structure (Fegel *et al.*, 2016).

Our study sites included both surface and rock glaciers, as well as the full gradient of hydrological conditions present in the Rocky Mountains. Within GRTE, environmental variation clearly partitioned streams into three categories, which include a previously undescribed alpine stream type that we have termed icy seeps. These streams, include rock glacier runoff as well as other streams fed by subterranean ice and are very cold (< 3°C mean temperature, like their surface glacier counterparts, but have high conductivity (>60 μS cm^{-}1) and intermediate channel stability (Figure 5.3; Table 5.3). Based on all available data, one site in GNP, a spring emerging from the southwest face of Piegan Mountain actually appears to be fed by subglacial meltwater from the Piegan Glacier on the northeast side of the mountain (Figure 5.2K), fitting the definition of being fed by subterranean ice. This spring is also very cold and has intermediate channel stability

according to the Pfankuch Index. Beyond environmental overlap, icy seeps contained correspondingly unique microbial communities with diverse streamwater assemblages, clustering with samples from many sources and microhabitats (Figures 5.6, 5.8-5.11) but seemingly much more tightly constrained biofilm assemblages which all cluster closely together along with the two glacier-fed biofilm samples from GNP (Figures 5.6, 5.8). Moreover, at least one prokaryotic family, Pseudomonadaceae, was particularly enriched in icy seeps, though the underlying mechanism for this increase is unclear.

Despite differences in underlying geology between GNP and GRTE, mountain subrange had little to no influence on both total diversity and microbial community assemblage. It should be noted that this finding contradicts, at least to some degree, Fegel *et al.* (2016), who identified clear signatures of mountain range-specific microbial diversity both in total OTUs observed and microbial assemblage composition between the Rocky Mountains, Cascades, and Sierra Nevada Range. This difference is likely because our comparison was made within the Rocky Mountain range, but still, given the ~600 km and geological differences between the two subranges, this finding is surprising. Another explanation may be the focus in Fegel *et al.* (2016) on surface and rock glaciers, which both engage in active glacial comminution, or grinding of bedrock into particles, which can substantially influence stream biogeochemistry (Telling *et al.*, 2015). This focus on streams so inherently linked to active geological processes may have elevated signals of mountain range differences that are otherwise reduced in our study containing a much wider array of stream sources. Regardless, our results indicate that the processes driving the assemblage of microbial diversity in alpine streams is controlled by much more significant local or geological processes than mere isolation by distance or variation

within the same mountain range, a pattern supported by general studies of microbial biogeography (Fierer *et al.*, 2007).

Rather, the most significant drivers of microbial diversity in alpine streams appear to be microhabitat, primary hydrological source, and temperature. Within stream microhabitats (or compartments) have been identified as important to microbial diversity in alpine streams previously (Wilhelm *et al.*, 2013), though our results are more clear in this regard, likely because we included a wider array of hydrology beyond glacier-fed streams alone. For temperature, we observed a strong relationship of higher microbial diversity in warmer streams for both streamwater and biofilms. This space-for-time substitution of temperature across streams for the direction of future change lends additional credence to the premise that warming stream temperatures will yield higher diversity communities at the local (within stream) scale.

Taken together, these collective perspectives indicate that biodiversity is highest among glacier-fed streams and the lowest for groundwater-fed springs, and that this diversity is correlated with stream temperature, adds a rare microbial data point, in support of a larger, ominous trend in alpine stream biology. That is, reduced meltwater influences will homogenize streams on regional scales (Ward, 1994; Hotaling *et al.*, 2017b) in terms of both environmental variation and between stream beta diversity. This process, while expected to increase within site (alpha) diversity as more diverse, lower-elevation assemblages shift upstream (Jacobsen *et al.*, 2012; Hotaling *et al.*, 2017b) will ultimately yield lower regional (gamma) diversity and may span multiple taxonomic scales from macroinvertebrate species (Brown *et al.*, 2007b; Jacobsen *et al.*, 2012) and genetic diversity (Finn *et al.*, 2013; Finn *et al.*, 2014) to microbial diversity (Wilhelm *et*

al., 2013). Our results extend the microbial component of this broader story to include the full suite of primary hydrological sources of alpine stream ecosystems.

The most common phyla observed in North American alpine streams were not surprising as they fell in line with other examples from alpine and/or glacier-fed streams (Wilhelm *et al.*, 2013; Sheik *et al.*, 2015; Fegel *et al.*, 2016) and are typical components of freshwater stream microbial communities broadly (Zeglin, 2015). Moreover, many of the same phyla (e.g., Actinobateria, Firmicutes), classes (e.g., α- and β-proteobacteria), and families (e.g., Exiguobacteraceae) have been identified in other cryosphere-focused studies showing them to be common members of ice-associated habitats (Chaturvedi & Shivaji, 2006; Anesio & Laybourn-Parry, 2012; Hotaling *et al.*, 2017a). Given the role of Cyanobacteria in primary production of streams, it was unsurprising that they were most abundant in biofilms (Battin *et al.*, 2016), however the absence of Cyanobacteria in all three icy seep biofilm samples is difficult to explain. All three icy seep streams were relatively stable (PI = 18-21, Table 5.3), unlike both GNP glacier-fed streams which also contained little to Cyanobacteria associated sequences (Grinnell and Sperry Glaciers, PI = 36 and 39). At both the phylum and family level, streamwater is likely acting as the link between source ice microbiota and downstream communities developing in stream biofilms. This pattern does not necessarily indicate significant long-term residence of ice-associated taxa in streamwater but rather likely reflects the high influx and low residence time of meltwater-associated taxa in alpine streamwater, particularly during the summer melt season (Wilhelm *et al.*, 2013). However, while it would be easy to dismiss point estimate patterns of microbial diversity from meltwater-fed streams as being largely ephemeral due to the rapidly changing nature of hydrological influence on both daily and

seasonal timescales, evidence from Alaskan streams indicates that glacier-fed streams – the most variable of all alpine stream types in terms of discharge – are actually quite stable, particularly in their phylogenetic composition through time (Sheik *et al.*, 2015). Aside from environmental seasonality, there is wide latitude in the range of metabolic states microbial cells exist in (Blazewicz *et al.*, 2013) which complicates any inference regarding the functional influence of OTUs observed from DNA-based approaches on alpine stream communities. For instance, when relative abundance of rRNA was compared to rDNA, some relatively rare taxa (i.e., comprising a small amount of total rDNA) were significantly more represented in rRNA, suggesting that these rare taxa were contributing much more to relative ecosystem function than actual cell count abundance would indicate (Wilhelm *et al.*, 2014).

When considered in the context of global climate change, and particularly the rapid retreat of alpine glaciers and snowfields worldwide, our findings indicate that regional losses of microbial diversity will follow described patterns of macroinvertebrate species and genetic diversity (Brown *et al.*, 2007b; Jacobsen *et al.*, 2012; Finn *et al.*, 2013; Finn *et al.*, 2014; Hotaling *et al.*, 2017b). Our results also align with other recent perspectives (Wilhelm *et al.*, 2013; Fegel *et al.*, 2016) indicating that observed trends may be global in scope. Moreover, our results provide an important expansion of previous efforts to incorporate the full suite of hydrological influences on known alpine stream microbial diversity. As part of this, we have identified icy seeps as an important alpine stream type, distinct from those previously described in the alpine stream literature (e.g., Ward, 1994; Hotaling *et al.*, 2017b) in terms of geology, environmental variation, and microbial diversity. As climate change proceeds, icy seep sources (e.g., rock glaciers)

will likely be much more resistant to warming due to their layers of insulating organic debris (Millar *et al.*, 2013; Millar *et al.*, 2015; Fegel *et al.*, 2016), thereby serving as important refugia for meltwater-associated macro- and microscopic taxa (Hotaling *et al.*, 2017b). Similarly, as groundwater-fed springs already exhibit warmer, more stable conditions than any other alpine stream type, groundwater-fed springs along with lower-elevation communities in meltwater streams may represent the future of microbial diversity in alpine headwaters. Monitoring microbial communities in possible refugia (icy seeps) for changes in their makeup and any increasing beta diversity versus those associated with other stream types will be an important component of alpine stream research going forward. In addition, more targeted perspectives of microbial diversity from South America and Asian mountain ranges with a focus on hydrological variation as a surrogate for temporal change will provide greater resolution of how diversity is organized globally and how it may change. These future perspectives should also take cues from other recent cryosphere-related studies (e.g., Edwards *et al.,* 2013 and Wilhelm *et al.*, 2014) and include functional perspectives, as well as true metagenomic perspectives when possible, to better resolve the functional role microbial diversity plays in alpine stream ecosystem services.

Table 5.1. Sampling information for all sites included in this study. Sample abbreviations include: biofilm (B), streamwater (S), source snow or ice (I), and negative (N). Elevations are in meters.

Stream	Subrange	Source	GPS	Elev.	Samples
Clements Creek	GNP	Snowmelt	48.6899, -113.7335	2170	B, S, I
Oberlin Spring	GNP	Groundwater	48.6983, -113.7305	2113	B, S, N
Reynolds Spring	GNP	Groundwater	48.6823, -113.7311	2162	B, S, N
Lunch Creek	GNP	Snowmelt	48.7068, -113.7043	2189	B, S, I, N
Piegan Spring	GNP	Icy seep	48.7031, -113.6941	2370	B, S, N
Grinnell Glacier	GNP	Glacier-fed	48.7603, -113.7249	1917	B, S, I, N
Sperry Glacier	GNP	Glacier-fed	48.6259, -113.7634	2318	B, S, I, N
Petersen Glacier	GRTE	Glacier-fed	43.7818, -110.8463	2922	B, S, I
South Cascade Ck.	GRTE	Icy seep	43.7217, -110.8377	3152	B, S, I[a], N
S. Fork Teton Ck.	GRTE	Snowmelt	43.6908, -110.8434	2987	B, S, I
N. Fork Teton Ck.	GRTE	Snowmelt	43.7774, -110.8595	2955	B, S, I, N
Wind Cave	GRTE	Icy seep	43.6661, -110.956	2692	B, S[b], N
Middle Teton	GRTE	Glacier-fed	43.7277, -110.7954	2955	B, S, I, N

[a] Ice samples were collected but the stream is primarily fed by a subterranean rock glacier
[b] Two streamwater samples collected, one in the main channel and one seeping from a cave wall into the main channel

Table 5.2. Total number of phyla, classes, and families observed overall, for each hydrological source, and for each source + microhabitat grouping.

Group	Phyla	Classes	Families
Overall	52	187	598
Glacier-fed	39	128	391
Icy seep	45	153	453
Snowmelt	42	153	474
Groundwater	45	145	402
Glacier-fed + streamwater	39	122	346
Glacier-fed + biofilm	26	73	246
Icy seep + streamwater	45	150	441
Snowmelt + biofilm	33	98	300
Snowmelt + streamwater	42	151	458
Groundwater + biofilm	26	67	207
Groundwater + streamwater	44	143	383
Icy seep + biofilm	20	41	149

Table 5.3. Environmental data collected for all streams included in this study. Subranges: Grand Teton National Park (GRTE) and Glacier National Park (GNP). T_{range} = difference between minimum and maximum temperatures for a calendar year. T_{summer} = mean temperature from summer to autumn solstices (21 June – 22 September). n/m = variable not measured. C = conductivity. Units: temperature (°C), ss = suspended solids (g/L), C (μS cm^{-1}), stability (Pfankuch index units). Overall, the average summer temperature for GRTE and GNP streams were 3.5 and 2.9 °C, respectively.

Stream	T_{range}	T_{summer}	T_{year}	ss	C	Stability
Petersen Glacier	13.7	1.4	0.6	0.2096	2	42
South Cascade Ck.	10.1	1.4	0.5	0.033	67.1	21
S. Fork Teton Ck.[a]	17.7	7.1	1.9	0.0132	55.6	24
N. Fork Teton Ck.	15.2	6.6	2.5	0.0241	7.9	15
Wind Cave	4.4	2.9	1.7	0.0229	101.1	18
Middle Teton	7.1	1.7	0.7	0.1053	2.6	42
Clements Creek	12.4	4.2	1.5	0.0199	n/m	15
Oberlin Spring	10.3	3.5	1.3	n/m	n/m	11
Reynolds Spring	11.5	4.7	1.4	0.0196	n/m	11
Lunch Creek	13.1	3.6	1.6	0.0203	n/m	25
Piegan Spring	1.1	0.3	0.2	0.0214	n/m	19
Grinnell Glacier[b]	6.3	1.1	0.8	n/m	n/m	39
Sperry Glacier	n/m	n/m	n/m	n/m	n/m	36

[a] Temperature logger failed on 24 June 2016

[b] Temperature data from 2013-2014

Table 5.4. Comparisons of alpha diversity by mountain subrange and microhabitat using a two-sample, nonparametric t-test. Values are in units of the Shannon diversity index and standard errors are in parentheses. All comparisons of different source types were not significant at $P < 0.05$. Samples were rarefied to 20725 reads before comparison.

Comparison	Group, 1	Mean, 1	Group, 2	Mean, 2	*P*-value
Mountain range	GRTE	7.07 (2.26)	GNP	6.87 (2.40)	0.808
Microhabitat	Ice	6.71 (0.59)	Biofilm	5.00 (1.95)	0.147
Microhabitat	Streamwater	8.82 (1.66)	Biofilm	5.00 (1.95)	0.006
Microhabitat	Ice	6.71 (0.59)	Streamwater	8.82 (1.66)	0.006

Table 5.5. Alpha diversity for microhabitat + source groupings. Diversity was calculated using the Shannon diversity index (H) metric. All samples were rarefied to 20725 reads.

Microhabitat	Source	H	H error
Biofilm	Glacier-fed	3.88	1.69
Biofilm	Icy seep	3.07	0.41
Biofilm	Snowmelt	6.96	0.75
Biofilm	Groundwater	6.21	0.67
Streamwater	Glacier-fed	6.83	1.66
Streamwater	Icy seep	9.04	1.10
Streamwater	Snowmelt	9.93	0.33
Streamwater	Groundwater	10.04	0.10
Ice	Ice	6.71	0.59

Table 5.6. Results of nonparametric tests of beta diversity assessing the amount of observed variation explained by a given factor when a specified strata (group) was taken into account. Bray-Curtis distances between samples were used and 9999 permutations were performed to assess significance.

Factor	Strata	*P*-value	Variance explained
Subrange	Source + microhabitat	0.072	Not significant
Source	Subrange + microhabitat	0.013	22.59%
Microhabitat	Source + subrange	0.038	19.14%

Table 5.7. Average beta diversity distances among samples within source+microhabitat groups ranked from highest to lowest. Higher distances correspond to more variable communities within groups. Distances are Bray-Curtis dissimilarities.

Grouping	Distance
Glacier-fed	0.89
Icy seep	0.86
Snowmelt	0.78
Groundwater	0.74
Ice	0.63
Glacier-fed + streamwater	0.85
Glacier-fed + biofilm	0.84
Icy seep + streamwater	0.82
Snowmelt + biofilm	0.74
Snowmelt + streamwater	0.70
Groundwater + biofilm	0.62
Groundwater + streamwater	0.61
Icy seep + biofilm	0.42

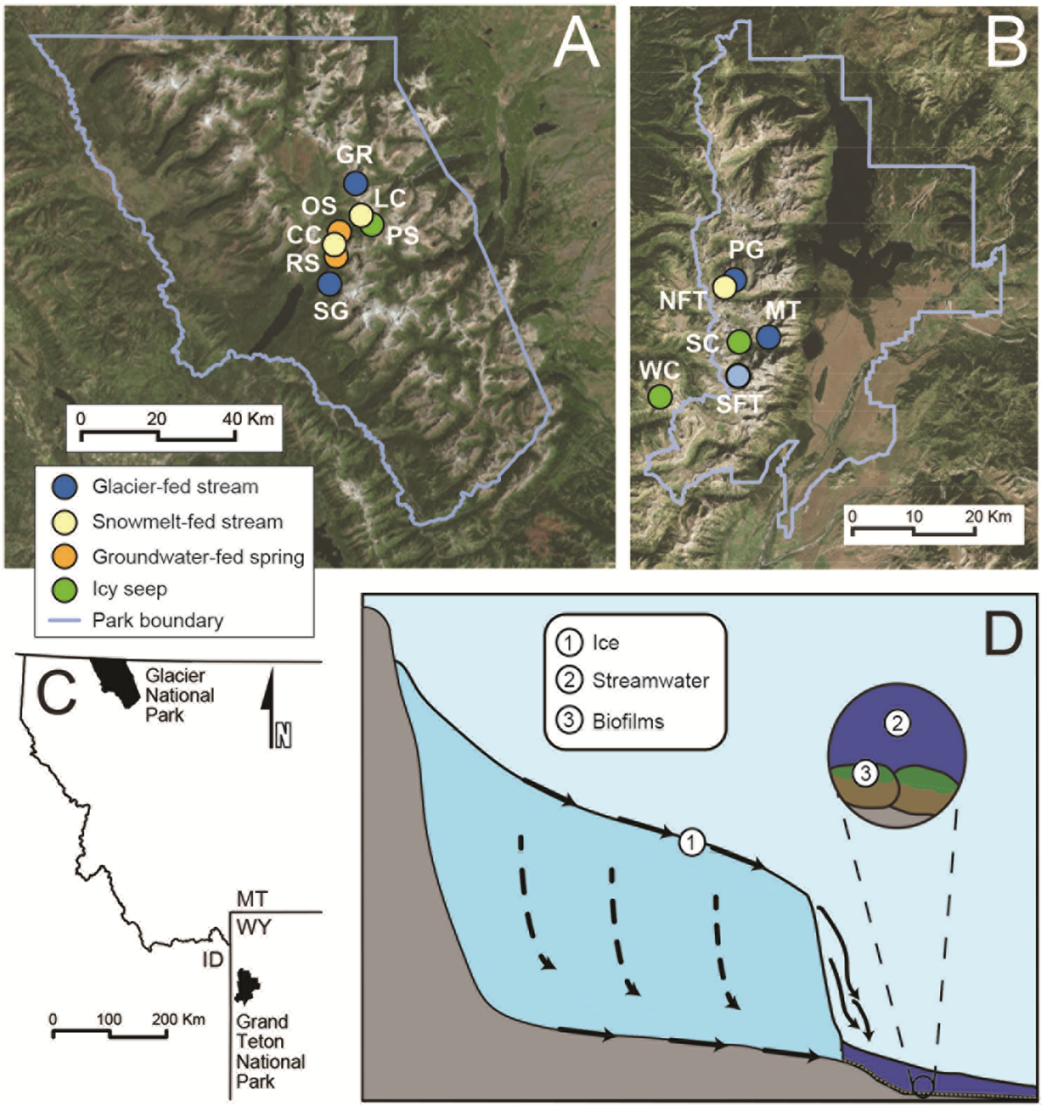

Figure 5.1. Sampling locations and site classifications included in this study: a) Glacier National Park, Montana, b) Grand Teton National Park, Wyoming, and c) geographic proximity of the two locations which represent distinct subranges of the Rocky Mountains. d) Sampled microhabitats. Acronyms include: Grinnell Glacier (GR), Lunch Creek (LC), Piegan Spring (PS), Oberlin Spring (OS), Clements Creek (CC), Reynolds Spring (RS), Sperry Glacier (SG), Petersen Glacier (PG), Middle Teton Glacier (MT), North Fork Teton Creek (NFT), South Fork Teton Creek (SFT), South Cascade Creek (SC), and Wind Cave (WC).

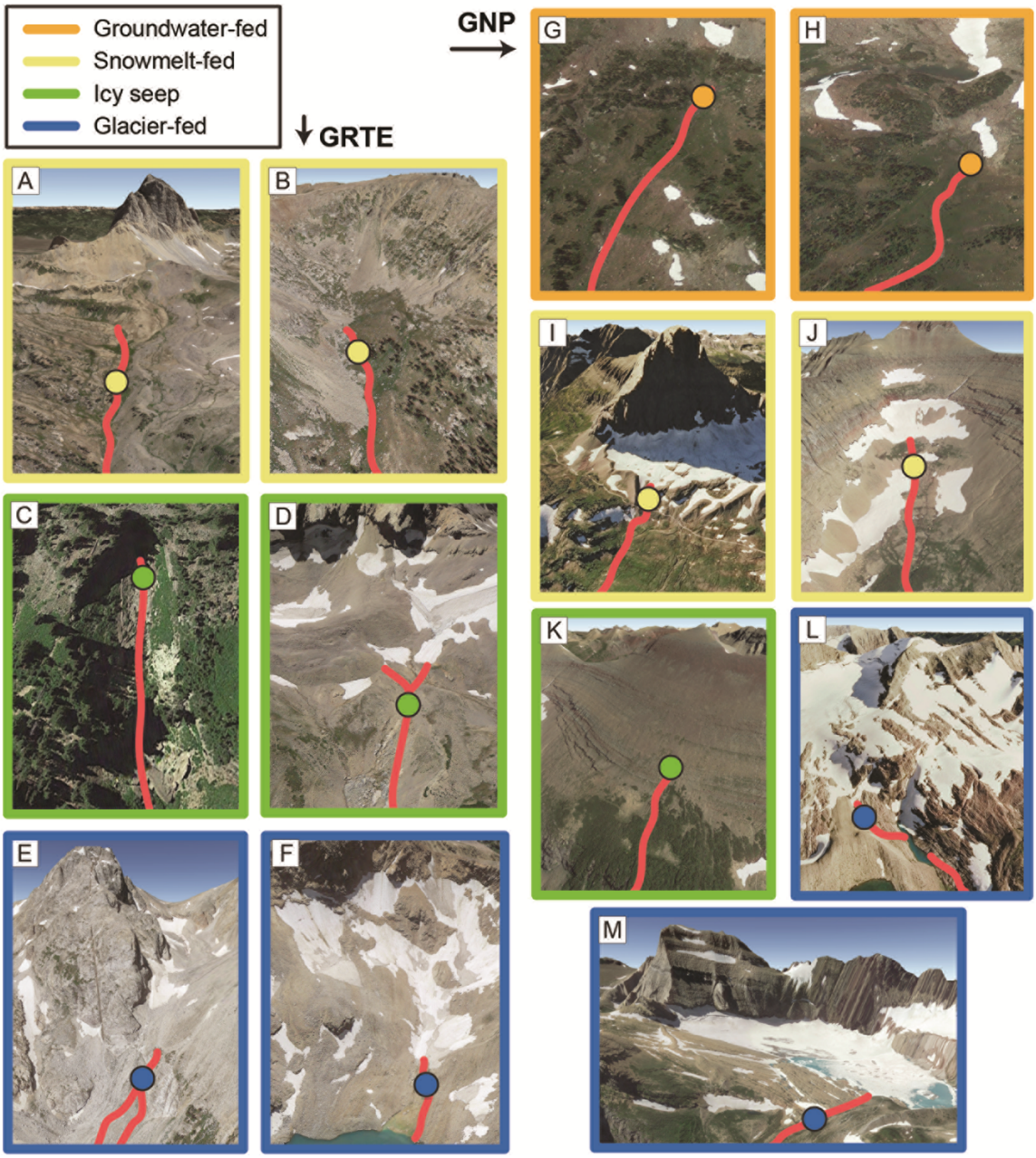

Figure 5.2. Aerial imagery of sampling streams (red lines) and sites (circles) included in this study. GRTE: a) South Fork Teton Creek, b) North Fork Teton Creek, c) Wind Cave, d) South Cascade Creek, e) Middle Teton Glacier, and f) Petersen Glacier. GNP: g) Reynolds Spring, h) Oberlin Spring, i) Clements Creek, j) Lunch Creek, k) Piegan Spring, l) Sperry Glacier, and m) Grinnell Glacier. Imagery was taken from Google Earth Pro and covered a range of years from 2011-2016 depending upon lighting and snow coverage.

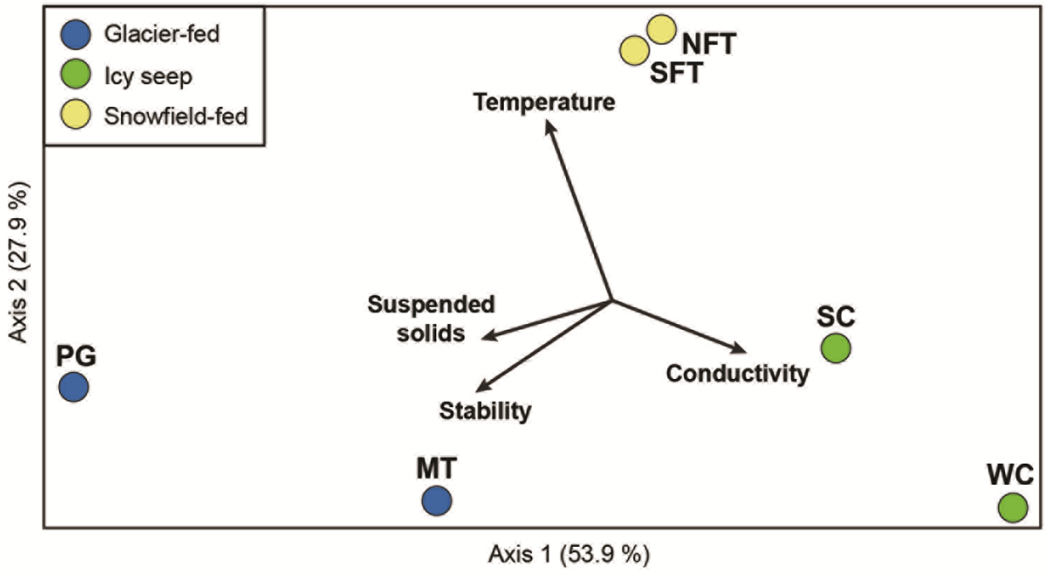

Figure 5.3. Grouping of streams in Grand Teton National Park according to four key environmental variables commonly used to assess the influence of glacier meltwater on stream ecosystems. Abbreviations include: North Fork Teton Creek (NFT), South Fork Teton Creek (SFT), South Cascade Creek (SC), Wind Cave (WC), Middle Teton (MT), Petersen Glacier (PG). No groundwater-fed spring sites were identified in GRTE.

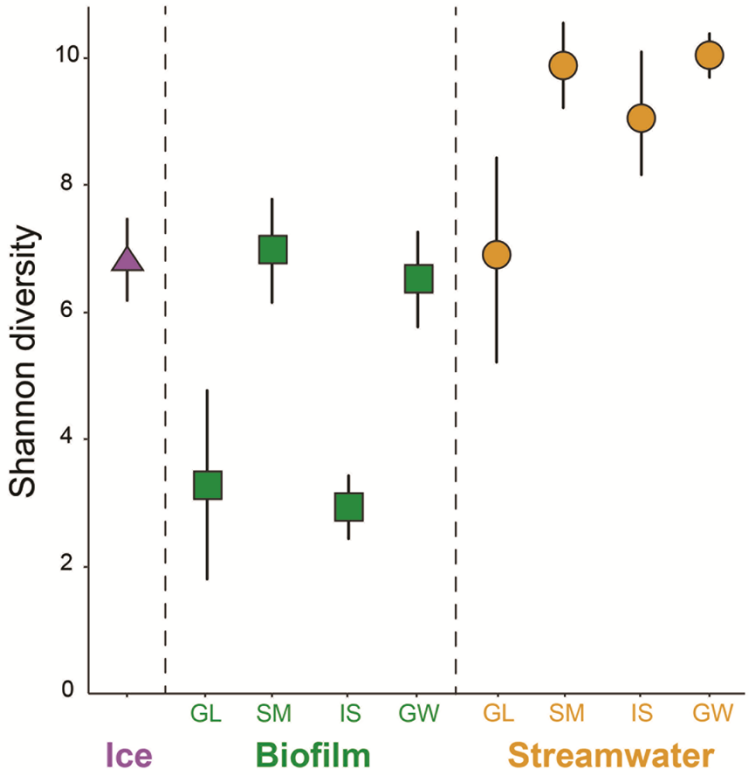

Figure 5.4. Comparisons of total alpha diversity for each microhabitat included in this study. Symbols indicate the mean and bars are standard errors.

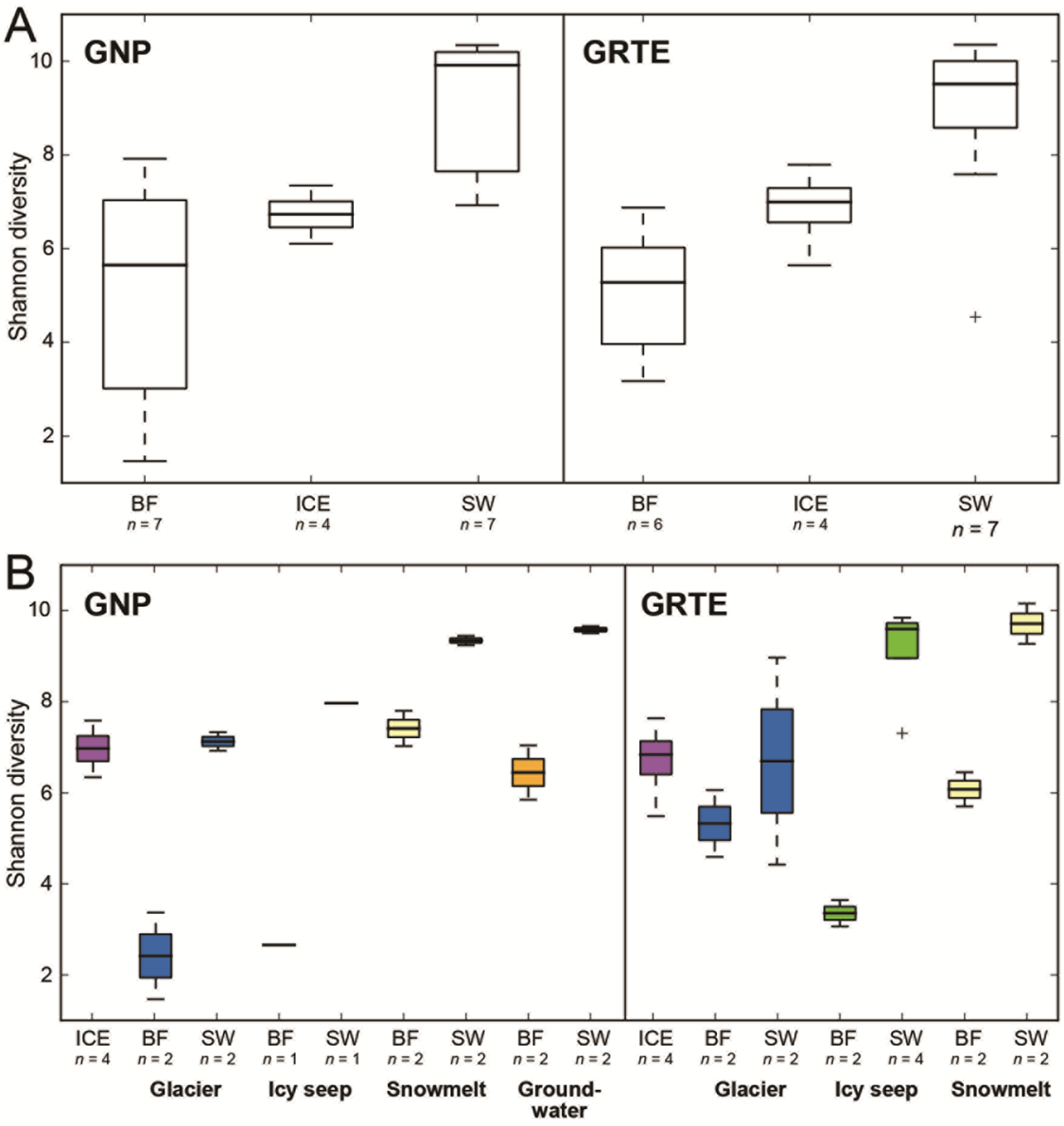

Figure 5.5. Box-and-whisker plots of alpha diversity by a) mountain range + microhabitat and b) mountain + source + microhabitat. Upper and lower for each group indicate the highest and lowest values observed. Dark lines represent the median value. Outliers are denoted with crosses.

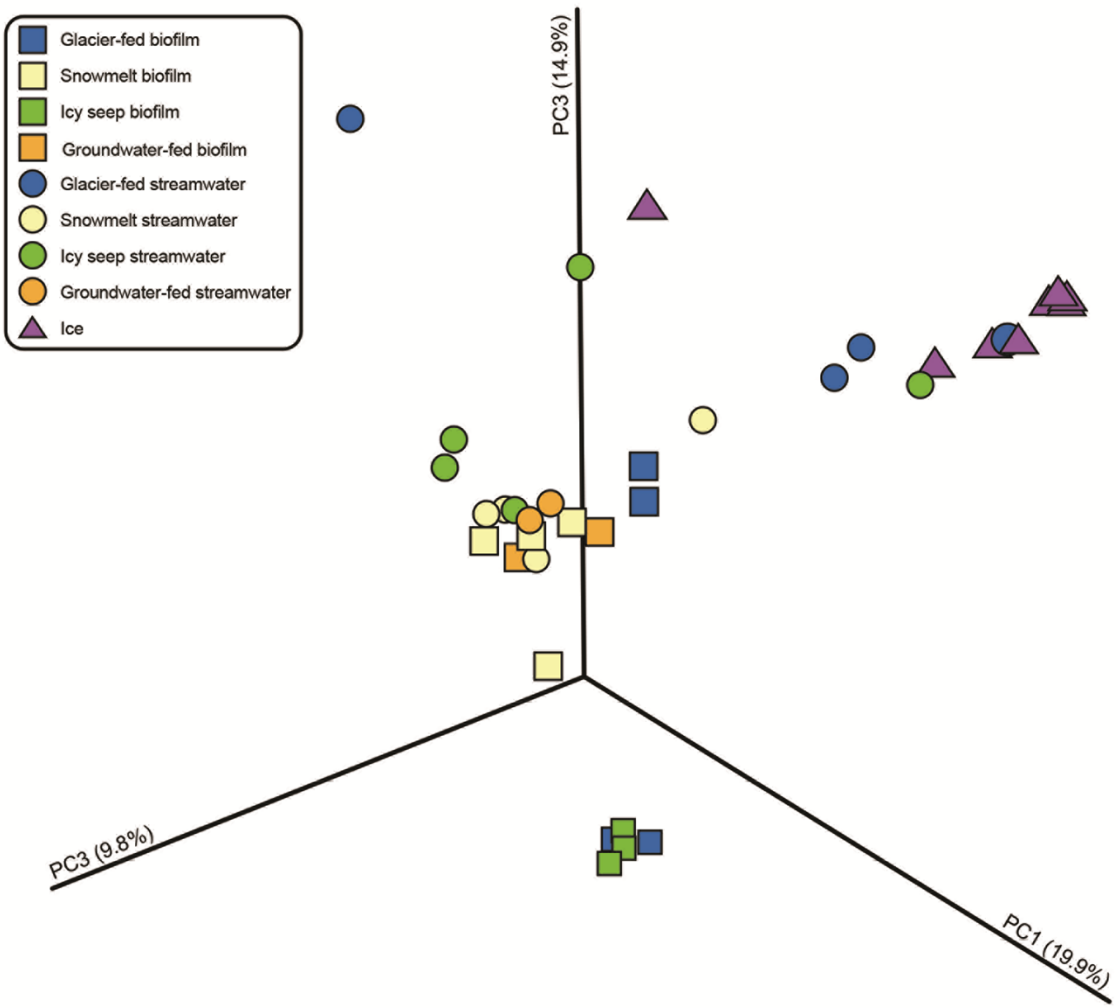

Figure 5.6. Beta diversity of all 36 treatment samples viewed as a 3D principal coordinates analysis plot. Samples are color-coded by source and shapes correspond with microhabitat. Samples were rarefied to 20725 reads and distances between samples are Bray-Curtis.

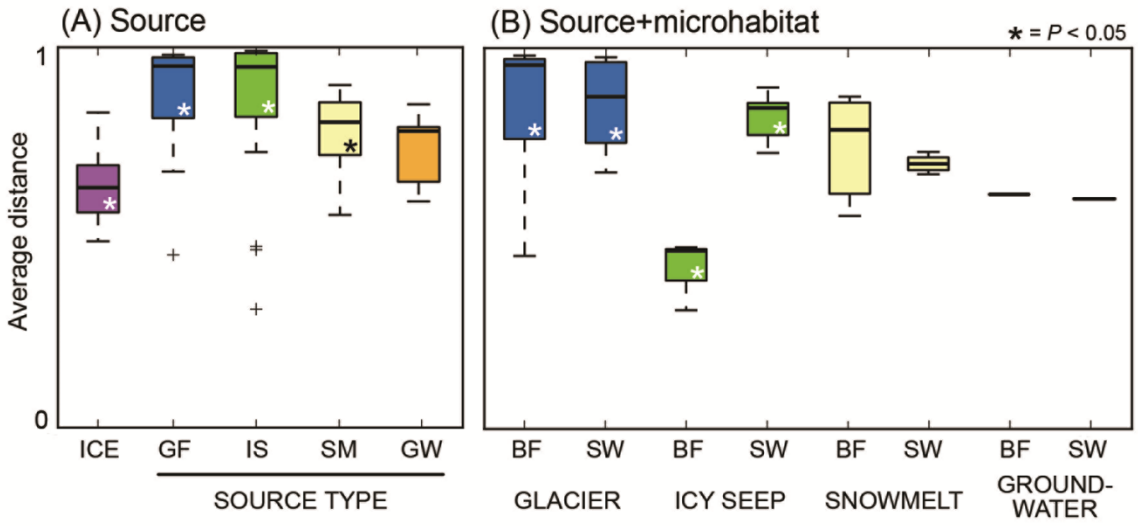

Figure 5.7. Box-and-whisker plots of beta diversity distances within a) source and b) source+microhabitat groupings. Asterisks indicate significant differences in average distance between sites versus all sites combined for a) source or b) source+microhabitat, respectively. Upper and lower for each group indicate the highest and lowest values observed. Dark lines represent the median value. Outliers are denoted with crosses.

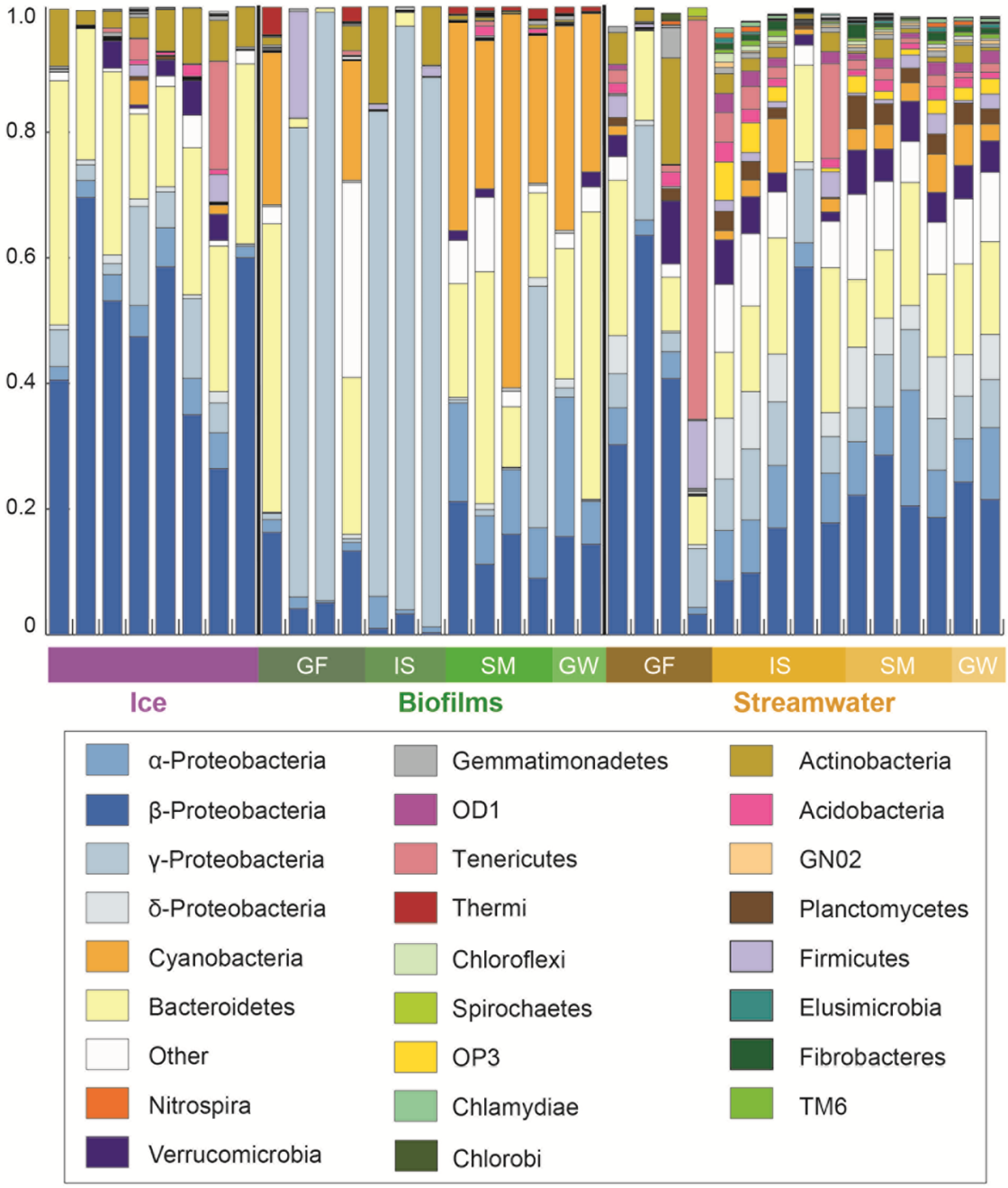

Figure 5.8. Histogram plot of prokaryotic phyla across microhabitats and sources. Bars represent one sample and phyla relative abundances are color-coded. Only phyla present at 1% or greater frequency are included, therefore bars vary in height, depending upon how many sub-1% phyla were identified.

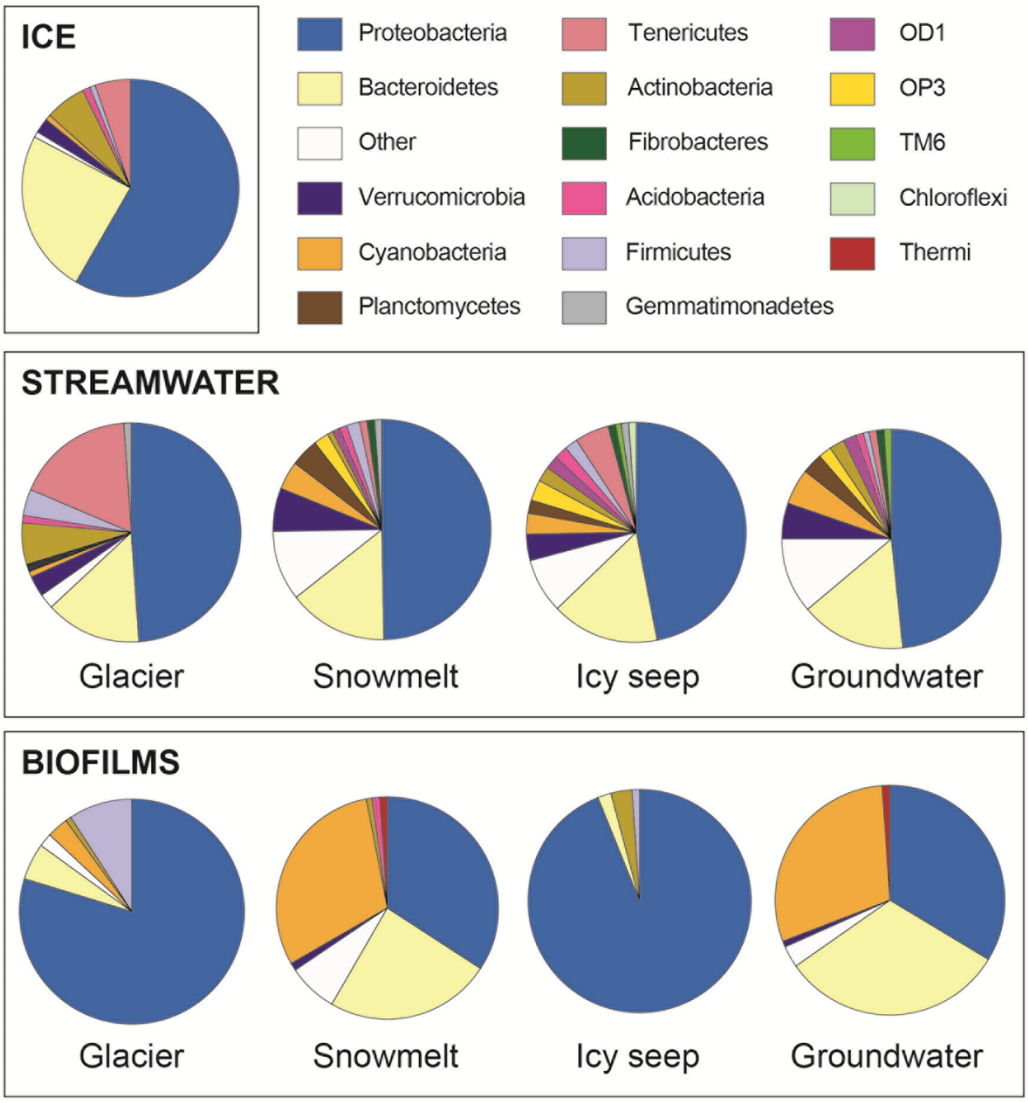

Figure 5.9. Relative abundance of the most common phyla (present at >1% frequency) in each source+microhabitat combination.

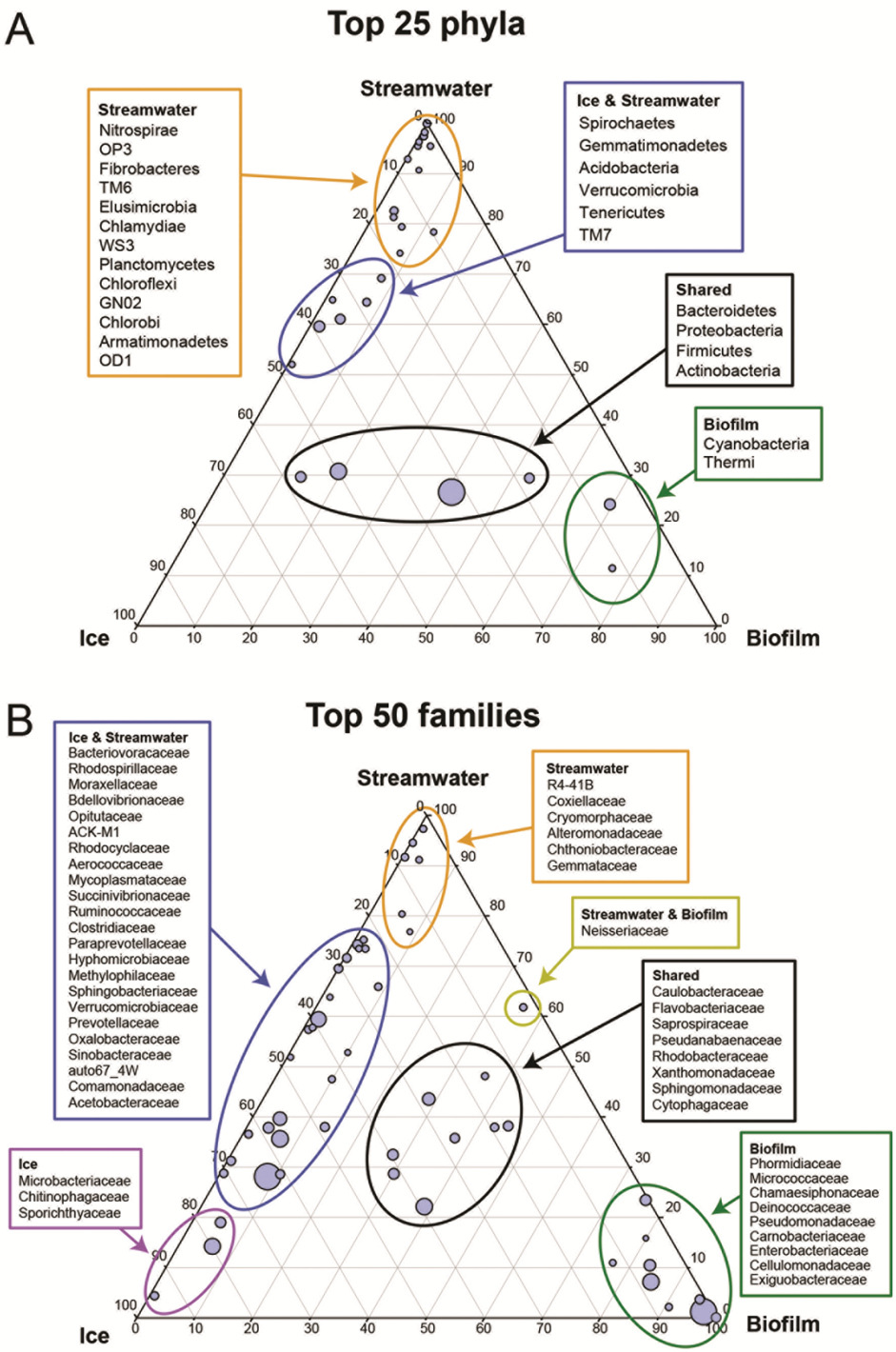

Figure 5.10. Distribution of taxonomic groups in streamwater, ice, and biofilm visualized in ternary plots by percentage of a) the 25 most abundant phyla and b) the 50 most abundant families for each microhabitat. Position of each point indicates the relative abundance of that taxon among the three microhabitats. Relative size of the circles represents their relative abundance overall.

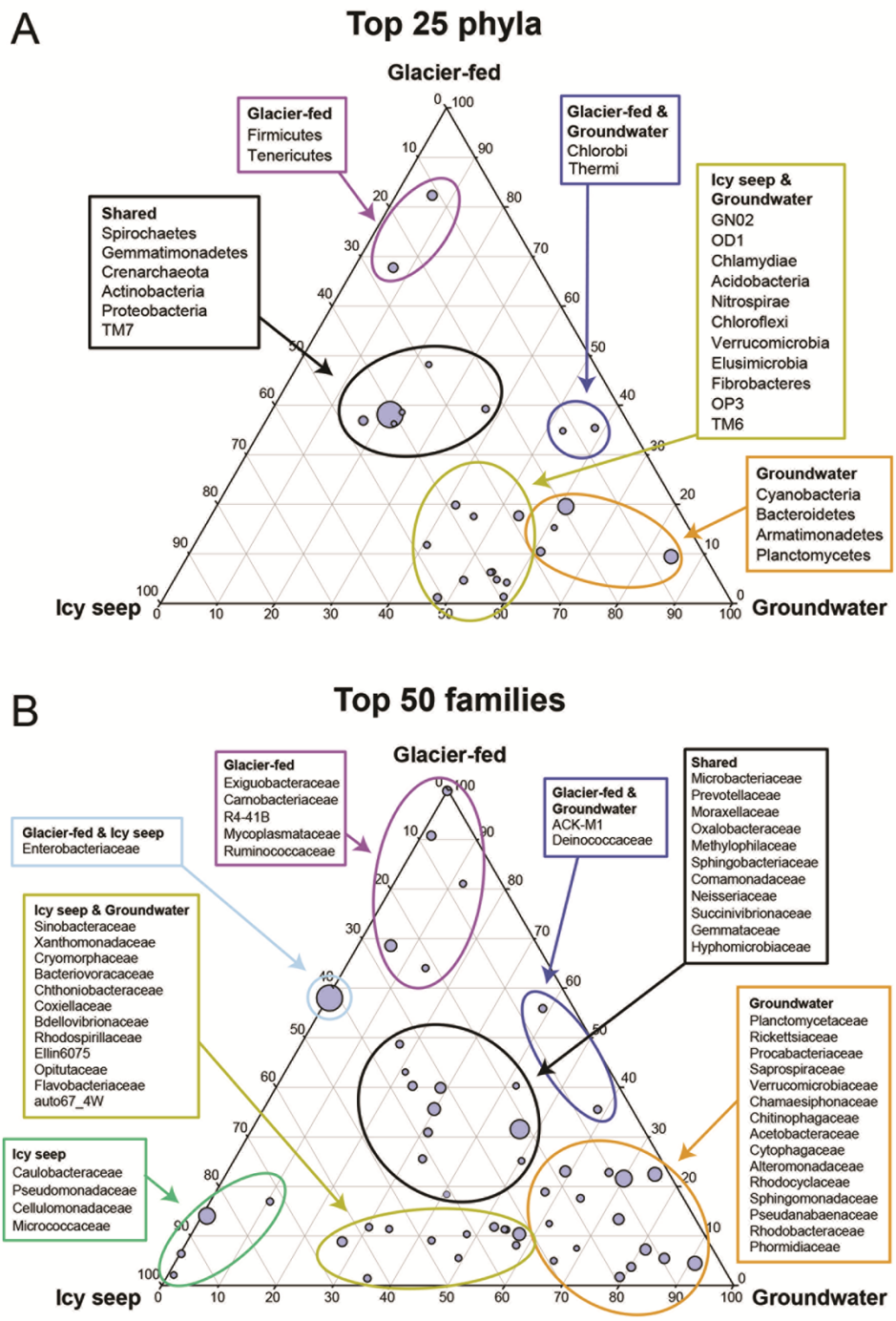

Figure 5.11. Distribution of taxonomic groups in glacier-fed streams, icy seeps, and groundwater-fed springs visualized in ternary plots by percentage of a) the 25 most abundant phyla and b) the 50 most abundant families for each microhabitat. Position of each point indicates the relative abundance of that taxon among the three sources. Relative size of the circles represents their relative abundance overall. Snowmelt-fed streams are not shown.

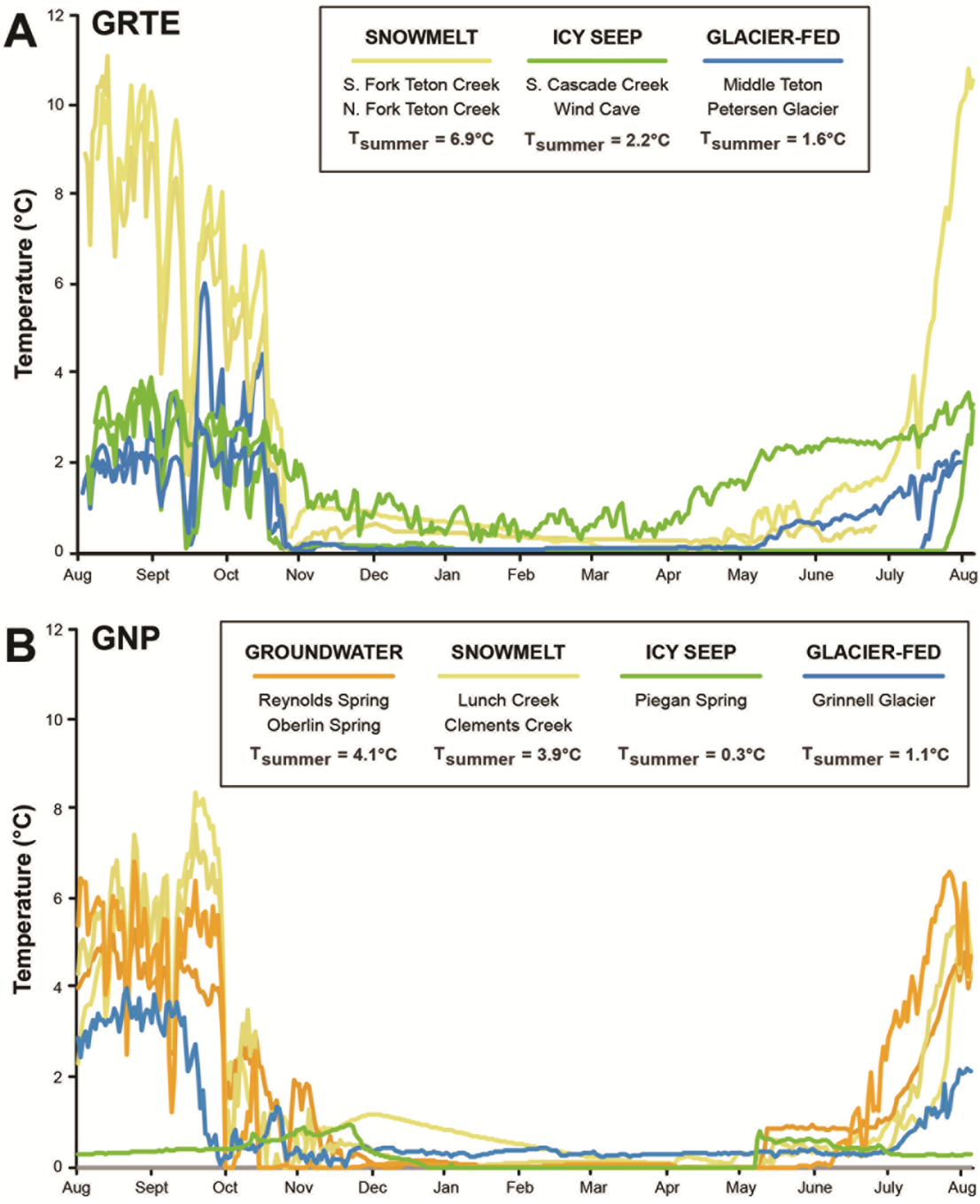

Figure 5.12. Thermographs of average daily temperatures for six study streams apiece in a) Grand Teton National Park (GRTE) and b) Glacier National Park (GNP) over a calendar year. T_{summer} = average daily temperature between the summer and autumn solstices (21 June – 22 September). Streams are color-coded by primary source. Not shown: Sperry Glacier (GNP). Data ends for S. Fork Teton Creek (GRTE) because the logger failed on 24 June 2016. All GRTE profiles are from 2015-2016. All GNP profiles are from 2012-2013 except for Grinnell Glacier which was recorded from 2013-2014.

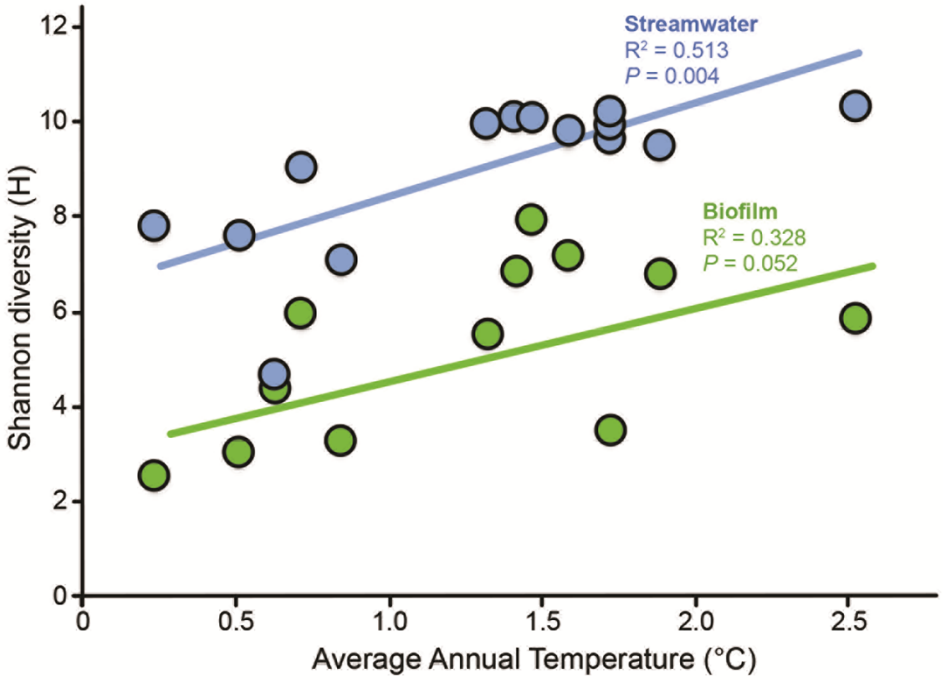

Figure 5.13. Alpha diversity (Shannon diversity index) is positively correlated with stream temperature averaged over a calendar year for both non-ice microhabitats (streamwater, biofilm) included in this study. Sperry Glacier samples (Glacier National Park) were excluded from the comparison because no temperature data was available. *P*-values were calculated from Pearson's correlation coefficients. Circles represent one sample and color-coded by microhabitat.

CONCLUSIONS AND FUTURE PERSPECTIVES

The results of my dissertation clearly reveal the utility of integrating next-generation sequencing approaches into the study of how biodiversity is structured, and perhaps in flux, in alpine stream ecosystems. While not a fundamental clarification of a specific research question, providing examples of how modern molecular tools can be applied to alpine streams is a necessary step in bridging one of the most obvious research lacunas for both alpine streams (Hotaling *et al.*, 2017b) and freshwater science (Pauls *et al.*, 2014). Indeed, through these tools, my collaborators and I were able to clarify the underlying geological drivers of extant population structure for an alpine stonefly (*Lednia tumana*) which is tightly linked to glacial meltwater (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016). We outlined evidence that ice recession following the Pleistocene glaciation has shaped modern patterns of genetic differentiation for *L. tumana*, with no support for both deeper (e.g., pre-Pleistocene) and more recent (e.g., the Little Ice Age, ~150 years ago) events influencing *L. tumana*'s genetic structure. This result indicates that short pulses of glacier extensions, like those that occurred during the Little Ice Age, may not leave genomic signatures. If they do, any signature is certainly much smaller in magnitude than those following major shifts in ice expansion or retraction. And, though not a multi-locus, genomic perspective, results of the comparative population genetic study detailed in Chapter Two provides a rare direct comparisons of population genetic patterns among alpine stream insects in North America. Moreover, it also provides an important cautionary note: when interpreting our results, even for the most similar species (e.g.,

Lednia tumana and *Zapada glacier*), extrapolating from one species to another should be done with caution.

Beyond population genomics, microbial diversity in alpine streams has been woefully understudied in comparison to macroinvertebrate species and genetic diversity despite the clear relevance microbiota have to overall ecosystem health. The emergence of 16S sequencing and associated tools, has forever altered the landscape for studying microbial diversity. No longer is culturability a requisite factor when probing bacterial diversity, with tens of thousands of taxa now readily identifiable in parallel. Since this methodological advancement, there have been a handful of examples applying these tools to glacier-fed stream generally (e.g., Sheik *et al.*, 2015) but only two in alpine streams (Wilhelm *et al.*, 2013 and Fegel *et al.*, 2016). These perspectives shed important early light on how microbial communities are structured in glacier-fed ecosystems, with Wilhelm *et al.*, (2013) revealing that microhabitat (or "compartment" in their words) as well as environmental factors (e.g., temperature) are the most overarching determinants of microbial community structure. Next, Fegel *et al.*, (2016) took an important second step, extending perspectives from Europe to North America and adding direct comparisons between the microbiota associated with surface glaciers and subterranean rock glaciers, as well as how the underlying biogeochemical elements influenced them. Our efforts extended this knowledge base to include all known alpine stream types (glacier-fed, snowmelt-fed, and groundwater-fed spring) and the same microhabitats as Wilhelm *et al.*, (2013). Through this study we defined a possible new class of alpine stream (see below), confirmed many of the patterns observed by the previous studies (e.g., microbial diversity increases with temperature), and showed that subrange is

possibly not a major factor dictating microbial community assemblages. The former adding an additional layer to Fegel *et al.*, (2016) who identified strong biogeographic patterns of microbial assemblages across mountain ranges in the Pacific Northwest.

While alpine streams are still understudied compared to their lower elevation counterparts, my hope is that the contributions of this dissertation – both basic research and synthesis – have contributed to greater understanding of their ecology, population genetics, and biodiversity. However, given the onslaught of climate change in these habitats, continued efforts are urgently needed as the field moves towards translating the wide baseline of knowledge developed over the last ~100 years into robust predictions of how a declining alpine cryosphere will affect associated biota and ecosystem function going forward.

Icy seeps, a new class of alpine stream

The identification of a possible new class of alpine stream – the "icy seep" – is a particularly interesting, and unexpected, result of this work. For more than 20 years, stream ecologists have classified flowing waters above treeline into one of three categories based upon primary hydrological source: glacier-fed, snowmelt, or groundwater-fed (Ward, 1994). These classifications have held up well over the intervening years with new data reaffirming their utility. However, as more streams have been explored, outliers like Wind Cave in the Teton Range – a stream fed by cave-associated subterranean ice – and Piegan Spring in Glacier National Pak where subglacial meltwater flows through a mountain to emerge as an icy spring on the opposite face, have challenged these traditional classifications. In both cases, these streams are much colder

than their groundwater origins would belie, but much more stable with fewer suspended particles than their glacier- and snowfield-fed counterparts. Moreover, specific conductivity, a measure of groundwater influence, is high in icy seeps – suggesting much greater influence of groundwater over precipitation like seasonal snow and rain. Taken together, we can identify a few preliminary factors that define an icy seep – cold water (< 3°C during summer months), elevated specific conductivity (> 60 μS cm^{-1}), a relatively stable channel (Pfankuch Index < 24), and perhaps most importantly from a climate change perspective, deriving their primary hydrological input from a subterranean source.

The fact that everything from true rock glacier-fed streams (South Cascade Creek in GRTE) to outliers like Wind Cave and Piegan Spring all fit under the same environmental umbrella is an exciting concept for alpine stream classification and monitoring. Additionally, the finding that this classification is supported by microbial communities adds further evidence that we are not merely dividing existing groups into ever smaller, but likely inconsequential, groupings. Perhaps nowhere is this more clear than when icy seep biofilm communities are considered – three samples from a rock glacier-fed stream, an icy seep emanating from a subterranean ice-filled cave system, and an intramontane, subglacially-derived spring exhibited the lowest beta diversity (i.e., distance to one another) of any sample grouping in the study. That means that icy seep biofilms were more similar than biofilm samples from any of the traditional stream classes (and it was not particularly close, see Table 5.7).

Beyond classifications for the sake of clarifying habitat diversity in alpine streams, icy seeps hold significant potential to affect how biologists assess the future of alpine stream biodiversity. Because these ecosystems are fed by subterranean ice, they

should be much less affected by anthropogenic warming (Fegel *et al.*, 2016), possibly acting as refugia for cold-adapted organisms as meltwater sources dwindle. While it's unclear the degree to which macroinvertebrate species and genetic diversity are shared between icy seeps and other stream types (though this is a research question we're actively pursuing in the Rocky Mountains), there is clear overlap between icy seeps and described stream classes for at least 11 of the most abundant microbial families (Figure 5.11). Aside from how biodiversity is segregated, the next layer of this question lies in actually measuring the rate of temporal change in icy seep versus other ecosystems. As the best time to plant a tree was 20 years ago, these temporal comparisons take time to bear fruit, but we are optimistic that monitoring sites established in 2015 in both Grand Teton and Glacier National Parks will begin to reveal answers to this pressing question in the near future.

A forward-looking perspective on forward-looking perspectives in alpine streams

Predicting the evolutionary trajectories of populations remains a central challenge in evolutionary biology, and perhaps nowhere is it more relevant than in the context of global change (Hoffmann & Sgrö, 2011). While my dissertation research does not address this topic in alpine streams directly, this predictive framework is an overarching thread throughout. I'm optimistic that the realization of such a goal is much closer than it may appear from the outside. And, moreover, I would argue that *L. tumana* and Glacier National Park may be the perfect arena for proof of concept research that could be translated broadly. Here, I outline what such a research effort may look like, existing

resources that could be integrated, and what questions remain to be addressed before a such an integrative perspective can be realized.

Previous studies have shown genetic diversity in alpine streams to vary with hydrological source (e.g., Finn *et al.*, 2013; Leys *et al.*, 2017). And, under future warming scenarios, extensive losses of montane insect diversity have been predicted (Bálint *et al*., 2011). In Glacier National Park, some degree of alpine stream monitoring has been occurring since at least the mid-1990s and over the intervening period my research and the collaborative efforts of the USGS ecology and glaciology groups have identified a robust baseline of glacier and permanent snow extent, as well as the species associated with this permanent ice and alpine groundwater-fed springs (Muhlfeld *et al.,* 2011; Giersch *et al.*, 2015, 2016). Past changes to exemplar glaciers have changed in the past, and may continue to change in the future, have been presented predictive model form (Hall & Fagre, 2003) and how loss of alpine glaciers will alter alpine flow regimes have also been described (Clark *et al.*, 2015). Adding to this, my dissertation research has clearly shown the utility of genome-scale data in addressing population change through time, particularly in terms of connectivity, highlighting general patterns of divergence in the face of gene flow among existing *L. tumana* population genetic clusters.

From an environmental standpoint, there are stark differences across alpine stream types, even for streams that are geographically very close. For example, consider average summer temperature and stability of a groundwater-fed spring (Oberlin Spring: 3.5°C, very stable), snowmelt-fed stream (Clements Creek: 4.2°C, intermediate stability), and an icy seep (Piegan Spring: 0.3°C, intermediate stability). All three support robust populations of *L. tumana* (Muhlfeld *et al.*, 2011; Giersch *et al.*, 2016) and are less than

3.5 km apart, which is likely close enough for dispersal to occur, even for stoneflies which tend towards being dispersal-challenged (Garcia-Raventós *et al.*, 2017). While selection against migrants has not been directly tested for *L. tumana* or any similar species, ecological variation (including temperature differences) can have significant ramifications for stream insects, both from life history (Lavandier & Décamps, 1983) and genetic diversity (Leys *et al.*, 2017) perspectives. Moreover, we know the direction of environmental change (towards less influence of meltwater), could be faster than space-for-time studies suggest (Finn *et al.*, 2010). More importantly, we know the most likely environmental result: that groundwater-fed springs tend to remain in the wake of glaciers (Baraer *et al.*, 2012; Chavez, 2013).

An obvious next step is to move beyond patterns of genetic differentiation estimated from largely neutral loci to clarify the degree to which populations of *L. tumana* residing in different stream types are locally adapted to those conditions. We know *L. tumana* populations exhibit a pattern of isolation-by-distance (Wright, 1943), but if differentiation is also the product of environmental isolation (Wang & Bradburd, 2014) then understanding the genomic basis of that pattern would be highly relevant to future predictions. To this end, the tools for detecting adaptation from genome-scale data are rapidly maturing, with many examples leveraging natural replication (e.g., stickleback invasions of freshwater lakes) to understand how populations have repeatedly adapted to the same environmental regimes from standing genetic variation (Hohenlohe *et al.*, 2010; Lescak *et al.*, 2015; Reid *et al.*, 2016) or used *de novo* mutations to adapt to novel environments as they emerge in real time (Linnen *et al.*, 2009; Linnen *et al.*, 2013). By employing a similar comparative genomic tactic to clarify how *L. tumana* and other

species may be adapted to different hydrological conditions, it is possible to identify ecologically relevant genetic diversity (e.g., perhaps genes involved in a cellular stress response) contributing to isolation of populations by environmental factors. Also, it should be noted that it is certainly possible that an alternative result would stem from such inquiries. Indeed, *L. tumana* populations may not be locally adapted (and thus gene flow among populations is not hindered by environmental differences) and are instead persisting through phenotypic plasticity. In many ways, this finding would be equally relevant, as it would imply threats to the persistence of *L. tumana* are exaggerated, at least in the near-term, as populations will still inhabit groundwater-fed springs in a post-glaciation landscape and perhaps a rapid intrastream transition from glacierized to groundwater-fed conditions would be less formidable that our current understanding would predict.

To summarize, there are dozens of named glaciers still present in Glacier National Park, along with many snowfields, groundwater-fed springs, and icy seeps. The majority of these headwater streams contain robust populations of *L. tumana* (and similar taxa: e.g., *Zapada glacier*, Giersch *et al.*, 2016). Models for how glaciers will change in the future (Hall & Fagre, 2003) and how this will affect runoff (Clark *et al.*, 2015) have been established. It is clearly possible to predict fine-scale patterns of neutral genetic differentiation, gene flow, and population size change from genomic data (Excoffier *et al.*, 2013; Nunziata *et al.*, 2017). Identifying ecologically relevant genetic variation contributing to local adaptation in wild populations through genome-scale data is also viable (e.g., Hohenlohe *et al.*, 2010), even for non-model species (Andrews *et al.*, 2016). And finally, we know alpine stream hydrology will generally trend towards groundwater-

fed springs for the foreseeable future. Putting this together, it is straightforward to imagine a scenario where gene flow among *L. tumana* populations in ecologically distinct streams is quantified, individuals are genotyped for possible adaptive variation (i.e., functional variation that appears to be associated with persisting in groundwater-fed spring habitats), and environmental changes to study streams of interest are predicted. From this template, it will be possible to assess the degree to which adaptive variation may already exist in populations inhabiting an alpine stream of interest, how this stream can be expected to change in the decades to come, the rate at which ecologically relevant genetic diversity may spread, and finally, possible evolutionary outcomes for threatened biota.

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