Caddisfly co-occurs in lakes & streams

Morphological plasticity in a caddisfly that co-occurs in lakes and streams

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ABSTRACT

Lake and stream fauna are frequently studied, yet surprisingly little is known about ecological and evolutionary dynamics of species that inhabit both lentic and lotic habitats. There are few examples of species co-occurring in the different habitat flow types, which raises questions on how this may impact their ability to adapt to changing climatic conditions. The aquatic insect *Limnephilus externus* Hagen (Trichoptera: Limnephilidae) is widely distributed in lakes of the Nearctic and Palearctic regions; in our study area of the northern Sierra Nevada mountains (California, USA), larval stages of this species co-occur in connected lakes and streams. We examined larval body and case morphology, interspecies phoretic associations, and the mitochondrial DNA cytochrome *c* oxidase I (COI) gene among lake and stream populations of *L. externus*. Further, we begin to explore potential morphological differences in distinct *L. externus* haplogroups. We observed differences between lake and stream populations in abundance, phenology, some aspects of body and case morphology, and abdominal mite presence, indicating that lakes and streams may yield distinct ecological phenotypes for the species. We also observed distinct regional differences in caddisfly body condition and sturdiness of case construction, as well as distinct communities of micro-invertebrates associated with the caddisfly and cases. Lake-stream *L. externus* did not show genetic divergence; however, three potentially distinct haplogroups were present across the research sites, as well as in sequences from North America and Canada which were imported from BOLDSYSTEMS. *Limnephilus externus* appears to exhibit wide geographic range and low geographic sequence structure which could account for the species' large variation in phenology and morphology at the lake-stream level. As the Sierra Nevada faces warming temperatures, reduced snowpack, and flow cessation, sensitive high elevation species will face potentially detrimental consequences. Aquatic insect

life history and phylogenetic structure provides valuable insight into the ecological and evolutionary dynamics that influence the adaptability of aquatic fauna to climatic change.

Keywords

Lentic-Lotic, Aquatic insect, DNA barcoding, Phenology, Morphology, Phoresy

INTRODUCTION

Lentic and lotic habitats are believed to differentially influence ecological and evolutionary dynamics. Indeed the distinction between these two hydraulic habitat types has been fundamental to the classification of aquatic ecosystems and has strongly influenced the way freshwater scientists conduct research and organize their disciplines (Wetzel 2001, Lottig et al. 2011, Allan et al. 2021). In riverine systems, mechanisms of upstream dispersal are a necessity for plant and animal species (Wubs et al. 2016), while dendritic network patterns create variation in metacommunities among headwater and mainstem habitats (Brown and Swan 2010). Lakes are commonly understood to favor greater dispersal traits, possibly because they are less stable over evolutionary timescales relevant to speciation. For example, lentic odonate species have larger latitudinal ranges than lotic species in the Nearctic and Palearctic (Hof et al. 2006). While some studies have found lotic species have greater genetic population differentiation and potential for cryptic diversity (Marten et al. 2006), this may not always be the case (Ribera et al. 2001).

There are few theoretical and empirical examples of studies on the ecological and evolutionary dynamics of individuals that can co-occur in both lentic and lotic habitats. The best examples of lake-stream eco-evolutionary comparisons thus far have come from fishes, especially work on three-spined stickleback (*Gasterosteus aculeatus* Linnaeus). In sticklebacks, co-occurrence seems possible due to morphologic variability and/or parapatric speciation (Thompson et al. 1997, Rennison et al. 2019, Paccard et al. 2020). Interestingly, in a case study transplanting lake-genotyped sticklebacks into streams, survival of lake-genotype fishes was poor and individuals with a hybrid lake-stream genotype had only moderately improved survival (Moser et al. 2016). In another case, freshwater drum (*Aplodinotus grunniens* Rafinesque)

exhibited more robust bodies in rivers and reservoirs with lower retention time (more flow), yet interestingly this species can show amenability to both lentic and lotic habitats beyond the age of ~12 years (Rypel et al. 2006). Minnows (*Phoxinus* spp. Rafinesque) from lakes and streams often also exhibit a similar morphologic pattern, though some evidence to the contrary suggests that in minnows this may be region-dependent (Ramler et al. 2017, Scharnweber 2020).

Species that co-occur in lotic and lentic systems may be especially common in high altitude, glaciated mountain landscapes, where lakes are often hydrologically linked in chains by stream segments. High mountain lakes and streams are often oligotrophic, and wave action along rocky littoral zones of lakes produces microhabitats that can resemble headwater streams (Merritt and Cummins 1996, Baker et al. 2016). Stream-dwelling invertebrates have been observed to live in the inlet and outlet regions of high elevation lakes (Wissinger et al. 2016), yet the ecological and evolutionary dynamics of populations of aquatic organisms that co-occur in these mountainous lake and stream habitats remains poorly understood. Clarifying lentic-lotic population dynamics, especially in sensitive mountain ecoregions, would provide a basis to assess ecological and evolutionary behaviors of aquatic organisms and how these may alter in future climate change scenarios.

Here, we test whether populations of the caddisfly *Limnephilus externus* Hagen (Trichoptera: Limnephilidae) that co-occur in lakes and streams are evolutionarily and/or ecologically distinct. Specifically, we compare population genetic structure, abundance, larval phenology, larval body and case morphology, and interspecies phoretic interactions between lentic and lotic populations of *L. externus*. We follow this with a brief examination of morphologic differences between the three distinct *L. externus* haplogroups that emerged from this analysis.

METHODS

Study Organism

Limnephilus externus Hagen (Trichoptera: Limnephilidae) is a caddisfly whose larvae typically inhabit lentic habitats, such as lakes, permanent to semi-permanent shallow ponds, and wetlands (**Figure 1**) (Berté and Pritchard 1986, Wissinger et al. 2003, Jannot et al. 2008). The five larval instars and the pupa are aquatic; after pupation *L. externus* emerge as terrestrial winged adults (**Figure 2**). *Limnephilus externus* larvae create bulky cylindrical non-rigid cases ("hedgehog cases"; Johansson and Johansson 1992) assembled from fragments of vegetation, detritus, and other organic matter (Berté and Pritchard 1986, Wiggins 2004). While *L. externus* flight duration is not well documented, adults of this species likely live less than two months (Berté and Pritchard 1986, Wissinger et al. 2003). *Limnephilus externus* is well documented in lake habitats throughout the western North America, Canada, and the Palearctic (Morse 1993, Ruiter et al. 2013, Mendez et al. 2019). There are very few records of larvae of *Limnephilus* spp. in streams; in California *Limnephilus* spp. is widely known from lakes and outside of this study we are only aware of several documented stream site records (Pratha 2014).

Study Area and Sampling

Sampling occurred in two regions in the northern Sierra Nevada mountain range, California, USA. The Lakes Basin, in the northern Sierra Nevada, is a high elevation (2000 m) mountain region featuring a dendritic network of headwater streams and oligotrophic lakes. Six lakes and six streams of close proximity were selected from > 20 glacial lakes and their connecting streams (**Figure 3**). These lakes occur in the headwaters of two adjoining watersheds:

the Feather River (Silver, Little Bear, Big Bear, and Goose lakes) and the Yuba River (Upper and Lower Salmon lakes). To add context to the study, we also sampled *L. externus* populations from one additional lake (without inlet or outlet stream) in the Lakes Basin, Haven Lake (Feather River watershed), as well as a lake-stream pair in a second region ~100km south (Tamarack Lake and outlet stream, Upper Truckee River watershed); these contextual samples were only used in phenology and population genetics analyses.

In the winter preceding this study (2016 – 2017), California experienced above average rainfall and snowpack and thus above average streamflow (Guirguis et al. 2019). The first sampling event in late June 2017 occurred during peak snowmelt and streamflow. A second sampling event in July 2017 occurred after peak water levels had subsided.

Water quality parameters, measured as spot samples during population and habitat surveys, were similar across all lake and stream study sites and typical of water quality in the higher elevations of the Sierra Nevada mountains. Conductivity was consistently below 25 µS/cm, while pH in both lakes and streams was neutral (pH 6.1-7.6). Dissolved oxygen levels were typically near saturation (70-90%), with lower values occurring during early morning hours, reflecting some moderate diurnal fluctuations. Water temperatures were similar among lakes and streams, and were higher, on average, in July (20.5 ^{o}C) than June (18.0 ^{o}C).

Sampling for *L. externus* took place in both lotic (stream habitats within 100 m of lake outlets or inlets) and lentic (lake habitats at least 100 m from the nearest inlet or outlet) habitats. Five 1 m^2 sampling areas were selected along the littoral zone of lakes and the benthic zones of streams in water depths of 5-50 cm. Sampling areas were spaced at least 1 m apart. Population surveys were performed for a timed interval (12 minutes per 1 m^2 area) by sampling a combination of cobble, boulder, and bedrock. At each site we examined and picked up 100-125

cobble-sized rocks to document the abundance of *L. externus*. All collected *L. externus* were preserved in 70% ethanol and taken to the lab for further analysis.

In the lab, *L. externus* larvae were roughly sorted into 5 instars based on case size. All subsequent analyses were performed using only individuals of the largest size class (presumed 5^{th} instar). *A posteriori* measurements of head capsule width of the largest size class (mean 1.57 mm) were similar to ranges for 5^{th} instar *L. externus* larvae reported in other studies (mean 1.62 mm, Berté and Pritchard 1986; mean 1.60 mm, Wissinger et al. 2003).

Population Genetics

We examined genetic variation among sampled *L. externus* populations through sequencing and analysis of the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene. We removed a single leg from twenty-nine individuals and placed each leg in a unique microplate well with 1-2 drops of 70% ethanol. Samples were sent to the Canadian Center for DNA Barcoding at the University of Guelph for standard DNA extraction, mtDNA COI gene isolation, and gene amplification, with established QA/QC standards. Forward primer C_LepFolF and reverse primer C_LepFolR were used to conduct PCR amplification on the marker COI-5P, a standard insect barcoding marker.

To visualize haplotype relationships and their spatial distribution for the Lakes Basin and Tamarack specimens, a haplotype network was created using the Minimum Spanning Network algorithm on twenty-eight sequences that had been trimmed to 633 base pairs with no missing data (Bandelt et al. 1999, Posada and Crandall 2001). To further understand these relationships and to examine genetic variation in our populations in the context of populations collected elsewhere, we aligned and compared the returned COI sequences to those found in

BOLDSYSTEMS (Ratnasingham and Hebert 2007). We searched the BOLDSYSTEMS Public Data Portal for nucleotide sequences belonging to "*Limnephilus externus*" and exported all 252 matching records and their metadata; data came from ten institutions, spanned three countries, and broke into three Barcode Index Number (BIN) clusters (i.e., algorithm-generated operational taxonomic units that are performed once per month based on diverging sequences) (Ratnasingham and Hebert 2013). We removed twenty-four sequences without BIN information and forty sequences with invalid residues. All sequences were aligned using a global alignment with free end gaps and a 65% similarity cost matrix. Additional sequences were removed if they showed many gaps in the nucleotide alignment, were too short relative to the other aligned sequences, or were of duplicate locations with identical (or nearly identical (< 0.002)) sequences congregated within the same haplogroup branch. The final nucleotide alignment comprised twenty-nine original sequences and twenty-five unique BOLDSYSTEMS sequences which may be found in **Dataset S1**.

Phylogenetic trees of the fifty-four COI sequences were constructed using both a distance-matrix method (UPGMA) and a Bayesian inference method (MrBayes version 3.2.6). We built trees using UPGMA for three different pairwise genetic distance models (i.e., *Jukes-Cantor, HKY, Tamura-Nei*) using a bootstrap resampling method (100 replicates). Bayesian analyses used both the *JC69* (nst = 1) and *HKY85* (nst = 2) substitution models (Huelsenbeck and Ronquist 2001). We selected the only two imported BOLDSYSTEMS sequences available from the Palearctic (Finland) as outgroups. All trees produced with the UPGMA and Bayesian analyses contained similar distinct clades and haplogroups, thus we only present results from the UPGMA Jukes-Cantor model that assumes equal rates of nucleotide substitutions as an inferred phylogenetic relationship. Algorithm-generated BIN assignments from BOLDSYSTEMS are

included in the branch label of exported BOLD sequences. We identified haplogroups using a criteria of ≥ 0.01 (1%) dissimilarity between parallel branches that resulted in substantially larger variation between groups than within groups.

Initial sequence metadata review was performed in R (version 4.2.0, R Core Team 2022). Haplotype networks were created using using PopART (Population Analysis with Reticulate Trees; Leigh and Bryant 2015). Nucleotide sequence alignments and phylogenetic tree construction used Geneious software (version 10.2.3). Original COI sequence data from this study are publicly accessible in the Barcode of Life Data Systems (http://dx.doi.org/10.5883/DS-LIMNEPH).

Morphology and Phoretic Associations

Limnephilus externus fifth-instar larvae (n = 44; 27 lake, 17 stream) and their associated cases were individually photographed, given unique identification codes, and examined under a dissection microscope at 10-20x magnification. Each individual was measured for head-capsule-width (HCW), body length, pronotum length, body width at both the pronotum and second abdominal segment, case length, and case width at its widest point. Body morphology was measured using a micrometer (± 0.01 mm) and case morphology was measured using calipers (± 0.1 mm).

We also qualitatively documented the following body and case morphologic features for each collected individual: abdominal condition, gill length, gill thickness, head capsule pigmentation, abdominal mites, case width type, presence of silt in the case, case material type, case sturdiness or fragility, case material length, lateral case extensions (Limm and Power 2011), case assembly uniformity, and case microinvertebrate hitchhikers (**Table 1**). Two distinct conditions of the ventral abdomen were also observed: even color tone with robust appearance,

and black spotting with a transparent cuticle. Finally, a variety of microinvertebrates (< 500 μm; e.g., Chironomidae, Acari, Oligochaetea, *Hydra*) were found attached to or embedded in caddisfly cases, or clinging to abdominal gills from within the case. These associated microinvertebrate taxa were coarsely identified, enumerated, and preserved in 70% ethanol.

To examine possible differences between lake and stream populations, we performed two-tailed t-tests assuming equal variance for the seven quantitative variables, and Fisher's exact tests of independence on the qualitative nominal data. We also performed two-way ANOVAs and Tukey's post-hoc tests to determine differences in the same seven quantitative variables among the three haplogroups identified in phylogenetic analyses, and Fisher's exact tests were used for qualitative differences among haplogroups (n = 26; 10 in group one; 6 in group two; 10 in group three). Six sequenced specimens did not have gill health criteria available. We report all p-values ≤ 0.05. Analyses and mapping were performed in R (R version 4.2.0, R Core Team 2022). Quantitative and qualitative data are provided with open access as part of this manuscript (**Dataset S2**). See supplemental material (**Figures S3**) for colored versions of all figures.

RESULTS

Distribution and population genetic structure

Limnephilus externus was widely distributed in both lakes and streams but was more abundant in lakes. Although *L. externus* is known primarily as a lake-dwelling caddisfly, we documented its presence in 7 of 7 lakes and 5 of 7 streams (**Table 2**). We regularly collected twenty individuals per hour at 4 lakes in June (larvae were not observed from Upper Salmon Lake and Little Bear Lake) and at 5 lakes in July (larvae were not observed from Lower Salmon Lake). In contrast, no larvae could be found in the streams experiencing high snowmelt flows in

June. In July, one stream (Lower Salmon outlet) yielded at least 20 individuals per hour, while others had lower abundance (< 10 could be attained per hour). Many empty cases were observed in both lakes and streams in July. In lakes, larvae were commonly found on or near submerged vegetation (e.g., aquatic grasses), while in streams larvae were found primarily attached to stable substrates (e.g., fallen logs) in pools. While 5th instar larvae were present among all *L. externus* populations in July 2017, the proportion of instars varied greatly among sites (**Figure 4**). Fifth instars were the dominant size class at Big Bear Lake and Upper Salmon Lake. Lakes had roughly equal proportion of 4th and 5th instars (40.5% and 44.6%, respectively), yet streams had more 5th instars (59.6%) than 4th instars (14.9%). Few of the individuals we collected were 1st-3rd instars (lakes 14.9%, streams 25.5%).

Analysis of the mtDNA COI gene sequences indicates moderate intraspecies variation, low geographic structure, and wide geographic distribution of haplogroups. A haplotype network revealed eight unique haplotypes present between the Lakes Basin and Tamarack regions (**Figure 5**). Unique haplotypes are present at the following sites: H1 (Big Bear, Lower Salmon, Upper Salmon, Tamarack), H2 (Lower Salmon, Upper Salmon), H3 (Big Bear, Upper Salmon, Goose, Silver, Haven), H4 (Little Bear, Long), H5 (Big Bear), H6 (Lower Salmon), H7 (Upper Salmon), and H8 (Tamarack). There were no apparent lake-stream differences among haplotype groups. The three largest haplogroups (H1, H2, H3), comprising individuals from both Lakes Basin and Tamarack, also correspond with the three BOLDSYSTEMS algorithm-generated BINS (**Figure 5-6**). The haplotype network supported findings clarified by the phylogenetic analysis, confirming three primary haplogroups with the smaller sized groups nesting into one of these three primary groups on the tree. Within group dissimilarity (haplogroup one: range 0.1-0.5%, mean 0.2%; haplogroup two: 0.1-0.5, mean 0.2%; haplogroup three: range 0.1-0.4%, mean

0.2%) was much less than between-group dissimilarity (haplogroups one and two: range 0.8-1.1%, mean 0.9%; haplogroups one and three: range 0.8-1.4%, mean 1.1%; haplogroups two and three: range 0.8-1.2% mean 1.0%). In the phylogenic approach, all three haplogroups included individuals from both the United States and Canada, indicating that the three genetically distinct haplogroups are widely distributed. The first haplogroup includes multiple individuals from Lakes Basin, all the sampled individuals from the Tamarack study site, as well as individuals collected outside this study from other parts of the Sierra Nevada (Mono County, CA), Washington (USA), and Manitoba (Canada). The second haplogroup includes individuals predominantly from the Upper and Lower Salmon lake watershed and one individual from Big Bear Lake (Lakes Basin), as well as from the Rocky Mountains (Colorado) and individuals from across Canada (Alberta, Manitoba, New Brunswick). The third haplogroup includes individuals from the hydrologically connected system that includes Silver, Little Bear, and Big Bear lakes and streams, as well as nearby Goose and Haven Lakes (Lakes Basin), plus one individual from Manitoba (Canada).

Morphology

Limnephilus externus larvae exhibited significant differences in abdomen condition and gill thickness between lake and stream individuals (fisher test, $p = 0.0002$ and $p = 0.0008$, respectively). Black-spotted, transparent abdomens with attenuated gills were more common in lake individuals (100%) than stream individuals (28.6%) (**Figure 7**). Thick abdominal gills were also more common among lake individuals (72.7%) than stream individuals (8.33%) (**Figure 8**).

Caddisfly case construction and materials varied substantially among habitats and over time (**Figure 9**). Cases were significantly longer in lakes than streams in the Lakes Basin (t-test,

p = 0.0001). There were no other significant differences in cases among lake and stream individuals. Cases included more aquatic vegetation in June, while in July cases were constructed predominantly with twigs and bark. All cases from Tamarack Lake and outlet were fragile, bulky, and frequently had lateral extensions made with thin twigs; in contrast, all Lakes Basin *L. externus* cases exhibited stronger construction and no lateral case extensions.

Among the three haplogroups, there were significant differences in pronotum length (F = 6.31, p = 0.0068), body length (F = 4.64, p = 0.0208), and case length (F = 4.98, p = 0.0183). A Tukey's post hoc test revealed pronotum length was shorter in haplogroup one compared to haplogroups two-three, and body length was shorter in haplogroup one than haplogroup two; haplogroup three exhibited similarities with haplogroup one and two in different characteristics (**Figure 10**). Head pigmentation and case structure sturdiness also were significantly, or nearly significantly, different across haplogroups (fisher test, p = 0.0626 and p = 0.0287, respectively).

Phoretic Associations

Hydra, nematodes, oligochaetes, chironomid midges (three morphospecies), and water mites were found securely fastened to many caddisfly cases, either stuck to the surface or buried into case silt (**Figure 9D & 11**). These case-associated microinvertebrates were phoretically associated with both lake (36.6%) and stream (50%) caddisfly cases across the three primary haplogroups. We did not observe differences in the microinvertebrate community composition between cases from lake and stream individuals.

Abdominal mite presence was significantly different between lake-stream habitats and among haplogroups (fisher test, p = 0.013 and p = 0.0397, respectively). Abdominal mites were

only found on individuals from lakes (40.9%), not streams (0%); however abdominal mite association was only observed at Upper Salmon and Big Bear Lakes which make up haplogroup one and two. The highest abdominal mite association was 31 mites on a single individual; inflicted individuals had a mean of 4 mites. All individuals with water mites on their abdomen were observed to be less robust, had dark and transparent abdomens, and attenuated black spotted gills. However, nearly half of the larvae that lacked water mites at the time of collection also had some of these characteristics.

Water mites observed on the exterior of caddisfly cases were identified as adult oribatids (Acariformes: Sarcoptiformes: Oribatida), possibly in the family Trhypochthoniidae or Malaconothridae, while those clinging to the abdomen were identified as larval hygrobatoid water mites (Acariformes: Parasitengona: Hydrachnidiae: Hygrobatoidea), possibly in the family Hygrobatidae or Unionicolidae (Heather Proctor, University of Alberta, personal communication).

DISCUSSION

We formally documented the presence of *Limnephilus externus*, a caddisfly widely known from lentic habitats throughout North America, in both lake and stream habitats in the Sierra Nevada. We examined the degree to which *L. externus* occurring in lakes and streams are evolutionarily and ecologically distinct by comparing: (1) population genetic structure, (2) abundance, (3) larval phenology, (4) larval body and case morphology, and (5) interspecies phoretic interactions. Further, we briefly explored the potential for morphological differences between distinct haplogroups of *L. externus*.

The use of the mitochondrial cytochrome *c* oxidase I (COI) gene (i.e., DNA barcoding) helps to reveal patterns in biodiversity (Hebert et al. 2003). Studies connecting the techniques of DNA barcoding with traditional taxonomy have increasingly reported higher cryptic diversity than previously suspected (Sheth and Thaker 2017, DeSalle and Goldstein 2019). Indeed in some cases, use of the COI gene has revealed relatively high genetic diversity and low geographic structure in other aquatic insect species (Heilveil and Berlocher 2006, Ståhls and Savolainen 2008, Pessino et al. 2014). This study found no genetic differences between lake and stream *L. externus.* Instead, we found eight unique haplotypes, at least three of which are geographically widespread and distinct, separated by 1-2% genetic difference. These putative haplogroups may have potential to represent distinct subspecies (White et al. 2014), but likely are not biologically meaningful without additional multilocus data (Dasmahapatra et al. 2010) as a minimum 2-3% genetic divergence is often used to distinguish haplogroups as distinct subspecies or species. We note that the three primary haplogroups in our analysis do match with the three algorithm-generated BINS identified by BOLDSYSTEMS, which are intended to nearly approximate species, suggesting further work would be valuable to explore these relationships. *Limnephilus externus*' three primary haplogroups are widely distributed throughout the United States and Canada. Our findings suggest *L. externus* has a wide geographic range and low geographic structure that could support phenotypic plasticity between habitat types, and possibly genotypic and phenotypic variation at the haplogroup level. These results also suggest *L. externus* exhibits potentially high morphological plasticity and may be well adapted to disperse long distances. Relative to other insect species the COI mtDNA gene evolves quickly within the *Limnephilus* genus, supporting its use when exploring recent divergences (McCullagh et al. 2015, Steinke et al. 2022). Our results support and expand on the extensive genetic analysis and findings of *L.*

externus in the Manitoba province of Canada (Zhou et al. 2011, Ruiter et al. 2013). This rapid evolution of the COI gene, or the widespread gene flow hypothesis, could account for why three widely distributed haplogroups had large variation in morphology and may be found distributed across entire countries. This phylogenetic finding alludes to hidden biodiversity patterns and the need to further identify species boundaries in aquatic insect taxa.

Lake and stream populations exhibited distinct ecological phenotypes in abundance, phenology, some aspects of body and case morphology, and abdominal mite presence. Fifth instar *L. externus* were present at all lake-stream sites, while other instars varied in proportion. All lake individuals had abdomens that were transparent (tracheae were visible), black-spotted, and with more attenuated gills, whereas a small fraction of stream individuals had these characteristics. Lake individuals were also observed to have thicker abdominal gills. These morphological differences could represent adaptations resulting from several possible abiotic factors that differ between lakes and streams (e.g., lower levels of dissolved oxygen in lakes). Similarly, gill breadth and visible tracheae have been key factors in distinguishing the lentic *Baetis tracheatus* Keffer Müller & Machel from the lotic *B. bundyae* Lehmkuhl, which has narrow gills and invisible tracheae (i.e., abdomen not transparent) (Engblom 1996, Ståhls and Savolainen 2008). On the other hand, research has linked altered and atrophied tracheal gills (i.e., black speckling) in caddisflies to the introduction of pollutants or bacteria in a headwater stream (Simpson 1980).

Lake *L. externus* constructed cases using longer pieces of material than those in streams, and cases from the Tamarack region had weaker construction. Caddisfly case construction is highly dependent on the availability of materials in the surrounding habitat, yet the observed differences in case structure could also reflect adaptations to abiotic or biotic factors (i.e., flow,

predator defense). For example, *L. externus'* stout cases are reported to be a better deterrent to predation by beetle larvae than some more tubular cases of other species (Wissinger et al. 2006), while another study reported that differences in case structure between two *Limnephilus* species *(L. pantodapus* McLachlan and *L. rhombicus* Linnaeus) affected the behavior of predaceous dragonfly larvae (Johansson and Johansson 1992). Indeed the construction of more protective cases has been found to be a resource allocation trade-off inducible by predator chemical cues (Correa-Araneda et al. 2017).

Across haplogroups, pronotum length, total body length, case sturdiness, and presence of abdominal mites were significantly different between at least two haplogroups. Haplogroups also exhibited a nearly significant difference in head pigmentation, which has previously been used to distinguish between *Limnephilus* species (Ruiter et al. 2013). We consider these morphological haplogroup differences to suggest that real clade-level differences may exist and should be further studied. This study was designed to investigate differences between lakes and streams in one region, and therefore a representative sampling of each haplogroup may not have been achieved.

Finally, a collection of microinvertebrates (i.e., chironomid midges, water mites, hydrae, oligochaetes) were discovered buried within *L. externus* cases. In addition, water mites were found on the abdomen of only lake individuals. We observed at least three morphospecies of chironomid midge on the cases, suggesting that the microinvertebrate community on the cases may be diverse. Water mites observed on case exteriors were identified as adult oribatid (Acariformes: Sarcoptiformes: Oribatida) mites, possibly in the family Trhypochthoniidae (Heather Proctor, University of Alberta, personal communication). Oribatids commonly feed on detritus, algae, and occasionally macrophytes (Behan-Pelletier and Hill 1978, Proctor and

Pritchard 1989). The association of Oribatid mites on the organic cases suggests a commensal relationship in which the mites could be benefiting by living in or feeding on the cases. The nature of these ecological associations at these locations is not known, however, *L. externus* did not appear to be negatively affected or parasitized by any of the microinvertebrates on the exterior of their cases. Therefore, in these instances, we suspect a phoretic (non-harmful) association. In contrast, mites found on the abdomen of *L. externus* larvae may pose greater threat. Abdominal water mites were identified as larval hygrobatoid water mites (Acariformes: Parasitengona: Hydrachnidiae: Hygrobatoidea), possibly in the family Hygrobatidae or Unionicolidae (Heather Proctor, University of Alberta, personal communication). Hygrobatoid mites are known to engage in pre-parasitic attendance of caddisflies, remaining near the host until it is close to pupation and feeding on it when it emerges as an adult (Proctor and Pritchard 1989).

The occurrence of phoretic and parasitic relationships is common among aquatic organisms. Other aquatic insects have been documented to play host to midge and water mite travelers in relationships that vary along the gradient of ectoparasitism, predation, and phoresy (Tracy and Hazelwood 1983, Henriques-Oliveira and Nessimian 2009, Buczyńska et al. 2015). In Quebec, Canada, *Limnephilus* has been documented to have water mite larvae (Hygrobatoidea), with prevalence ranging from 4-42% (Fairchild and Lewis 1987). Other aquatic organisms, like the fish *Ancistrus multispinis* Regan in Atlantic forest streams in Southeastern Brazil, have chironomid larvae in phoretic association (Mattos et al. 2018). Understanding the role of associated macroinvertebrates on aquatic organisms is a challenging topic to study; (Grabner 2017) found testing for parasitic taxa using PCR might be an efficient and cost-effective method to identifying links between host feeding type and prevalence. Additional

studies would be needed to identify the nature of these associations and their consequences to *L. externus*.

CONCLUSION

In this study, we documented the presence of *Limnephilus externus* in both lake and stream habitats. Lake populations had conspicuous abdominal tracheae, thicker gills, and black spotting. Lake populations exhibited longer case construction, and only caddisfly cases from the Tamarack region were significantly more fragile in construction. Microinvertebrate hitchhikers found on the cases of the caddisflies are presumed to maintain a phoretic relationship, while mites on the abdomen may be demonstrating pre-parasitic attendance behavior. Finally, while lake populations were not genetically different from stream populations, we did find eight unique haplotypes present. Of these eight haplotypes, three are distinct and geographically widespread in the Sierra Nevada as well as throughout western North America and Canada. These putative haplogroups exhibited some significant morphological variation but further research is needed to validate these results. Overall, our observations and analyses suggest that environmental differences between lake and stream habitats may produce variation in plastic traits, but dispersal and gene flow are likely preventing genetic differentiation.

The frequency of aquatic invertebrate species that co-inhabit lentic and lotic ecosystems is unknown, and reflects the paucity of studies of aquatic fauna across habitat types. Our findings suggest that species with plastic traits amenable to both flow types may be overlooked in aquatic research. As a result, we may be missing valuable information on ecological and evolutionary behaviors of aquatic organisms, especially in light of anticipated climatic changes.

While lotic and high elevation lake shoreline habitats have been recognized for their ecological similarities, the way these two distinct ecosystems will respond to climatic changes will be vastly different. Indeed (Wissinger et al. 2016) observed cold-water stream insects inhabiting rocky and wave-swept alpine lake shorelines of Colorado, Switzerland, and New Zealand, and evidence that other freshwater fauna may be amenable to both hydraulic habitat types is growing (Yarnell et al. 2019). Mountain systems in particular face high stressors and are sensitive to environmental changes (Moser et al. 2019). Many of the aquatic habitats in the Sierra Nevada are dependent on snowmelt, yet California's increasingly common drought years and resulting low snowpack are anticipated to decrease snowmelt feeding into aquatic systems (Smits et al. 2020). With deteriorating snowpack and warming lakes, the adaptability of aquatic fauna to find refugia is expected to be a tremendous benefit to their survival (Birrell et al. 2020, Frakes et al. 2021). With this study, we hope to contribute to a larger body of knowledge and facilitate directions for future mountain aquatic research.

AUTHOR CONTRIBUTION STATEMENT

CAP led study design, field and lab work, data analyses, and wrote the manuscript. MRC helped design the study, provided methods guidance, helped interpret data, and assisted in manuscript revisions. MPM provided feedback on field and lab procedures and helped interpret data. All authors revised the article critically for important intellectual content and approved the final version to be published.

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646

FIGURES

Figure 1. Lateral view of *Limnephilus externus* and its case shown overlaying a metric ruler.

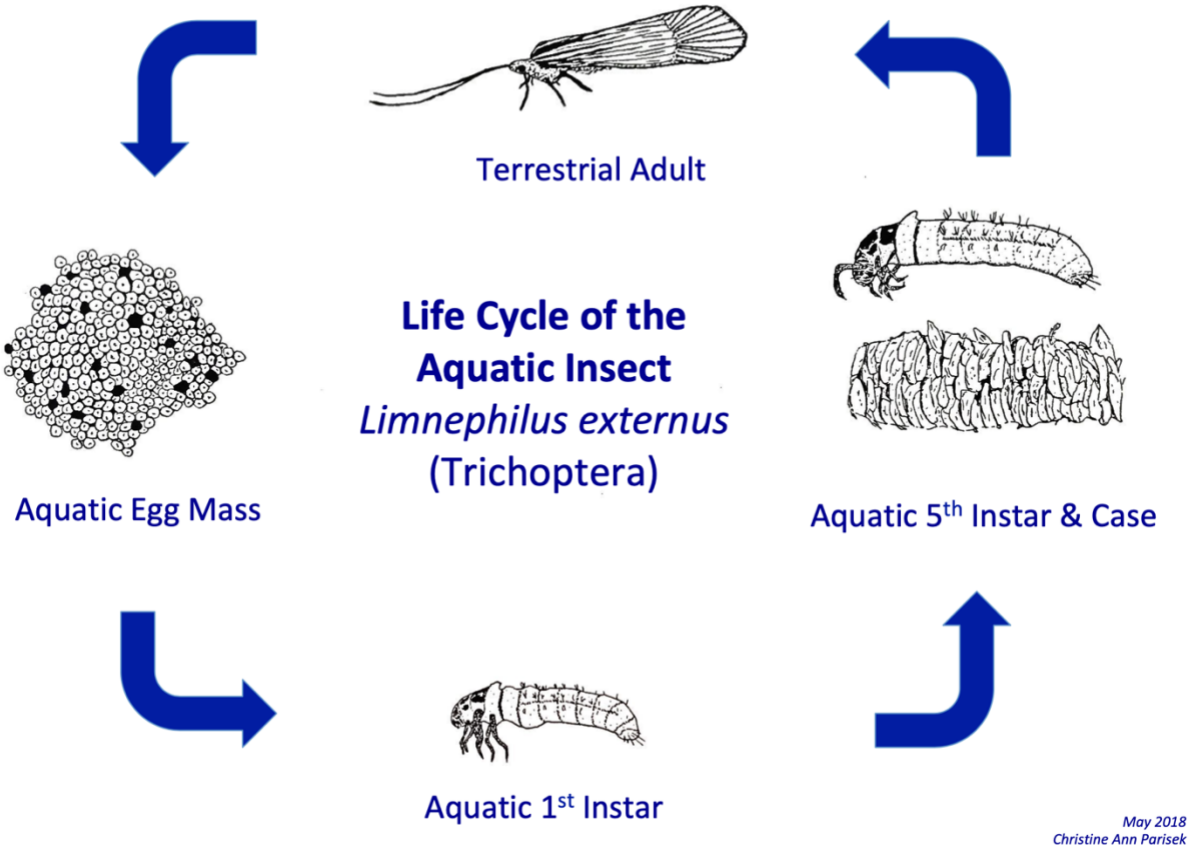


Figure 2. Illustration summarizing key aspects of the life cycle of the aquatic insect, *Limnephilus externus*. Illustration by Christine Parisek.

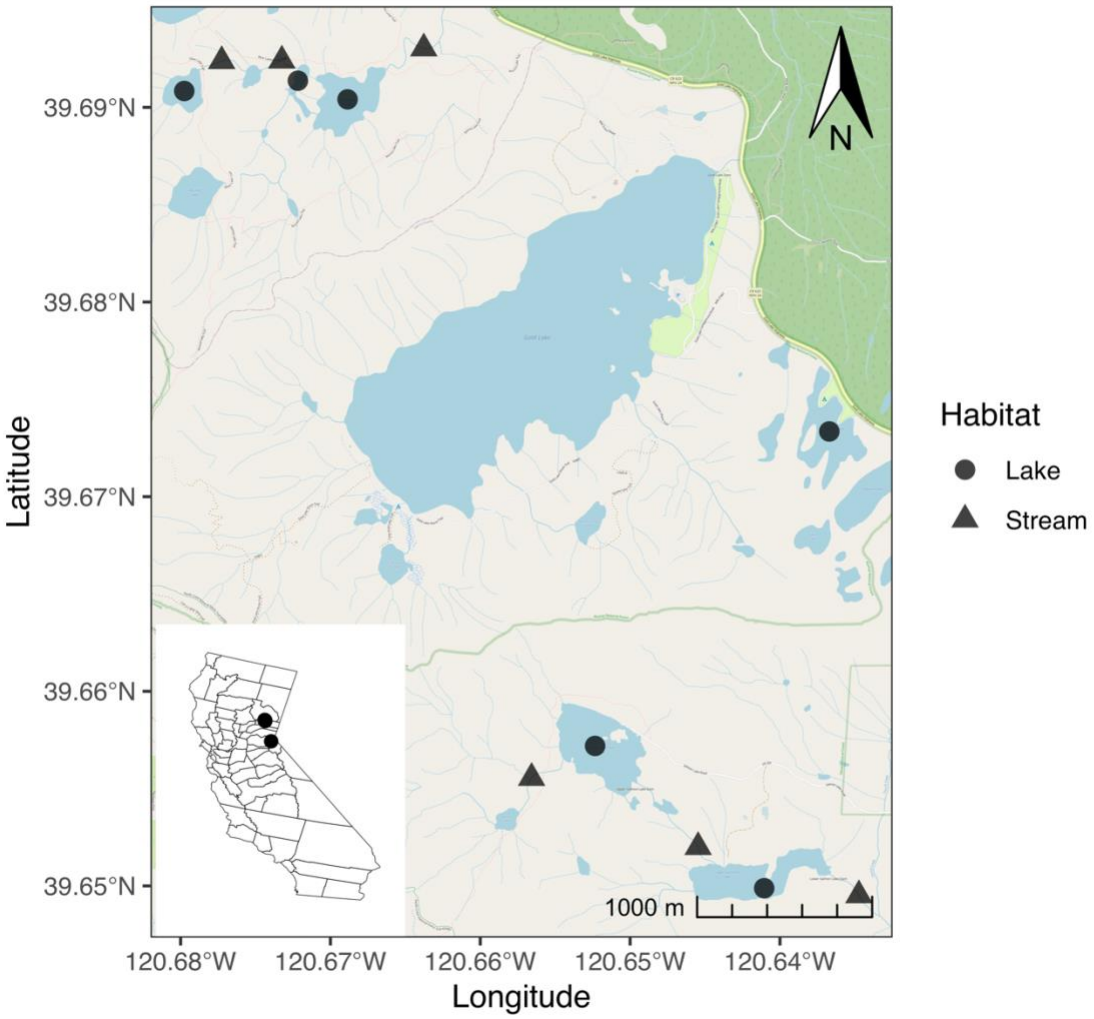


Figure 3. Lotic (triangle) and lentic (circle) sampling sites in Lakes Basin, northern Sierra Nevada, CA. Silver, Little and Big Bear Lakes share connectivity. Upper and Lower Salmon Lakes share connectivity. Goose Lake has no inlet or outlet stream. Inset map of California, USA displays primary field location (Lakes Basin) and contextual site (Tamarack Lake) in the Sierra Nevada mountain range.

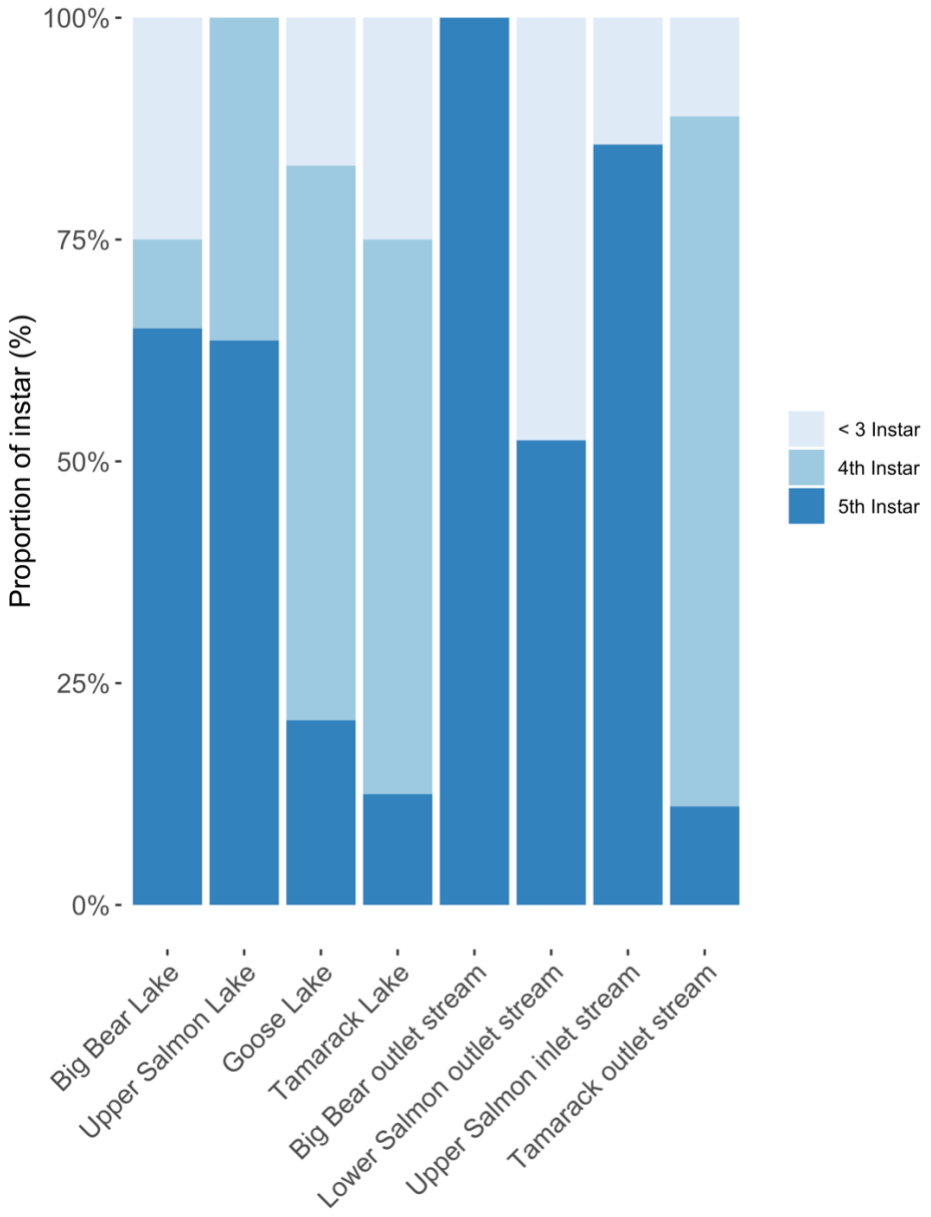


Figure 4. Proportion of *Limnephilus externus* individuals of each instar per site in July 2017.

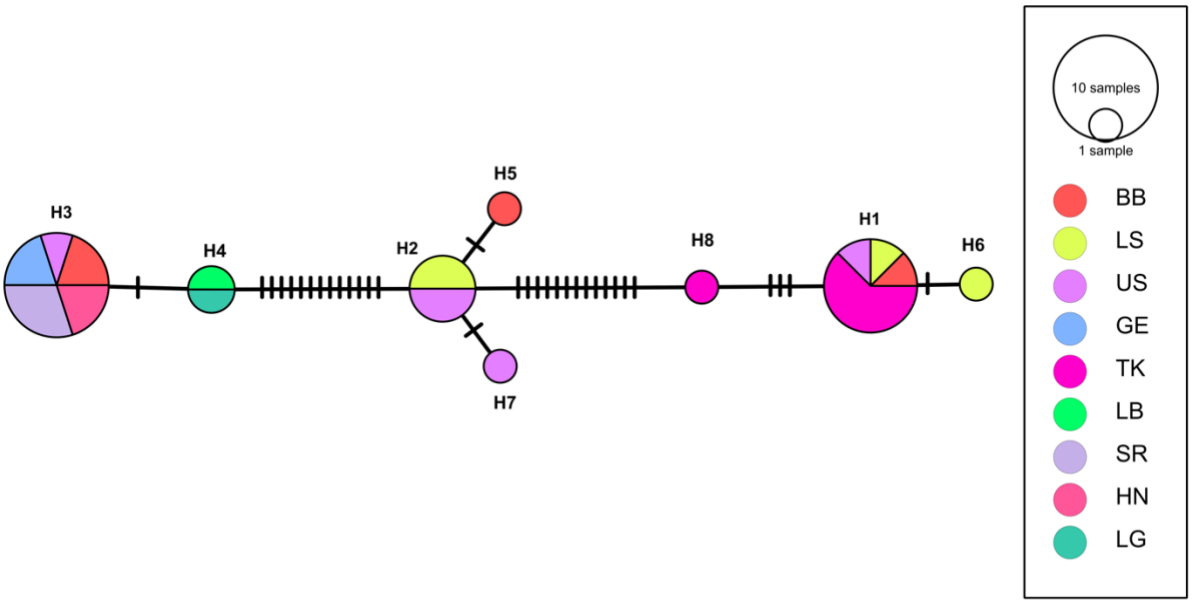


Figure 5. Haplotype network showing the number of pairwise differences between groups (1 tick mark = 1 mutational difference). Eight haplotypes were present in this study. Haplotypes are present at the following sites: **H1** (Big Bear, Lower Salmon, Upper Salmon, Tamarack), **H2** (Lower Salmon, Upper Salmon), **H3** (Big Bear, Upper Salmon, Goose, Silver, Haven), **H4** (Little Bear, Long), **H5** (Big Bear), **H6** (Lower Salmon), **H7** (Upper Salmon), and **H8** (Tamarack). Site codes are: Big Bear (BB), Lower Salmon (LS), Upper Salmon (US), Goose (GE), Tamarack (TK), Little Bear (LB), Silver (SR), Haven (HN), Long (LG).

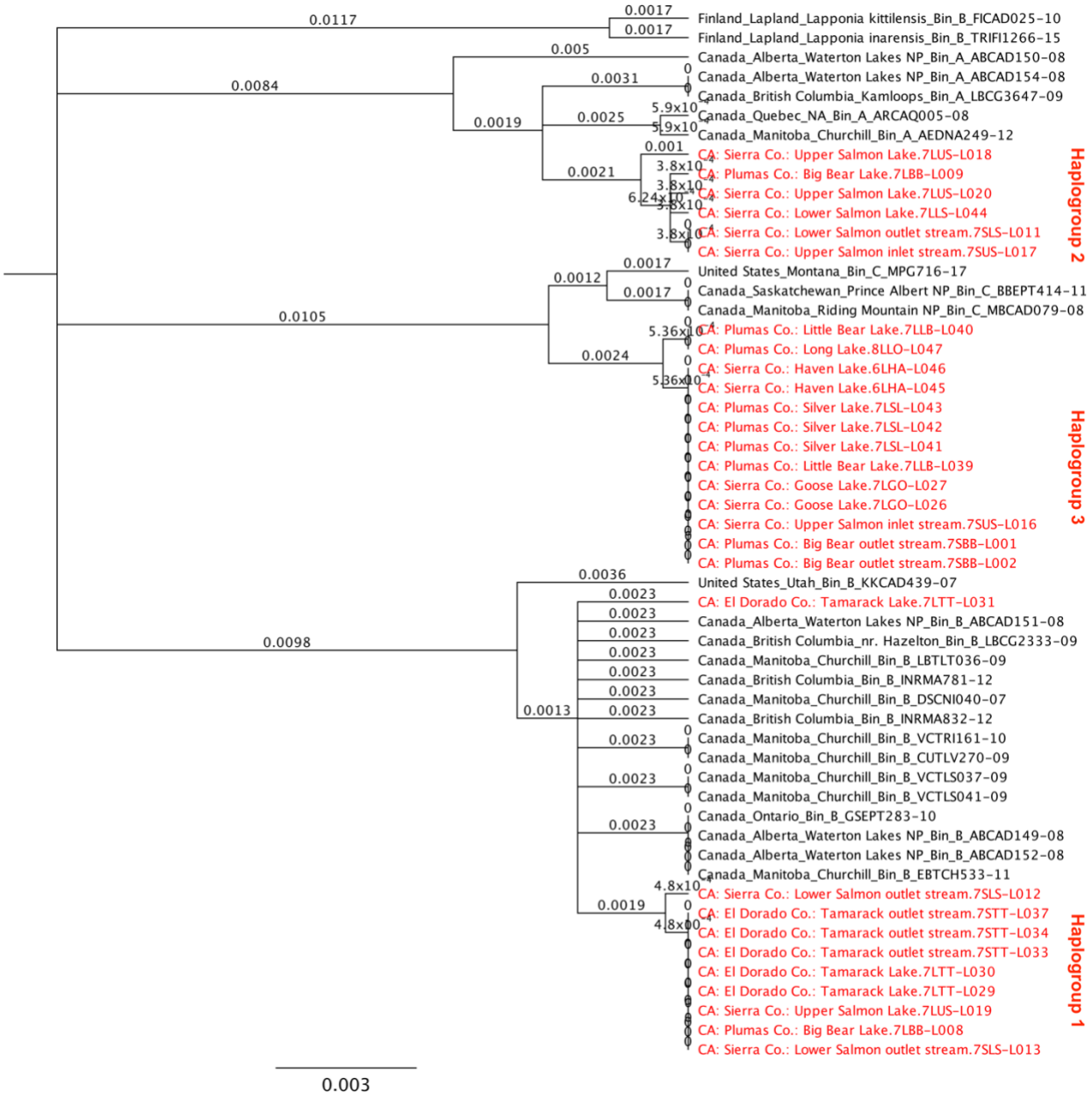


Figure 6. Phylogenetic tree of *Limnephilus externus* in the Sierra Nevada (light gray) using mitochondrial COI gene data. Additional individuals throughout the United States, Canada, and Finland (black) were included from publicly available data on BOLDSYSTEMS for contextual support. Each imported specimen name includes: country, state or province, county (if available), BOLDSYSTEMS BIN cluster (A, B, C), and specimen ID. Haplogroups identified in the Sierra

Nevada are in the order Two, Three, and One, from top to bottom. The number of substitutions per site (number on horizontal branch) represents the difference two parallel branches are from one another. Here three haplogroups exhibit a minimum 0.01 = 1% additive difference from each other; a roughly 2-3% additive difference between two parallel branches would be required for two haplogroups to be considered a distinct species. Phylogenetic tree constructed using the Jukes-Cantor genetic distance model.

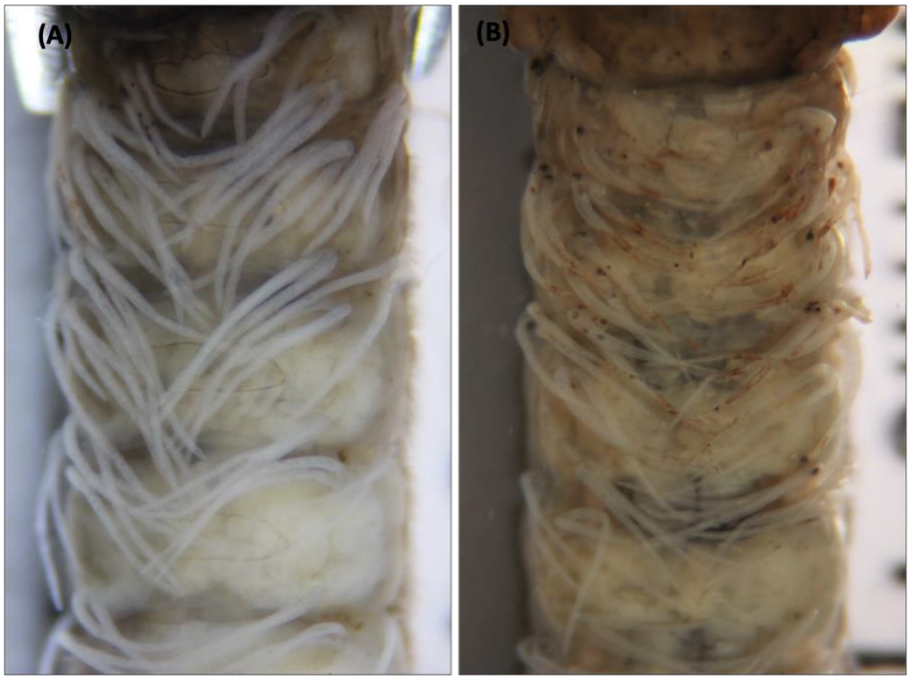


Figure 7. Ventral view of *Limnephilus externus* abdomen exhibiting (A) no spotting, even color tone, and robust appearance, and (B) black spotted, increased abdomen transparency resulting in more visible tracheae, splotchy color tone, and attenuated gill appearance.

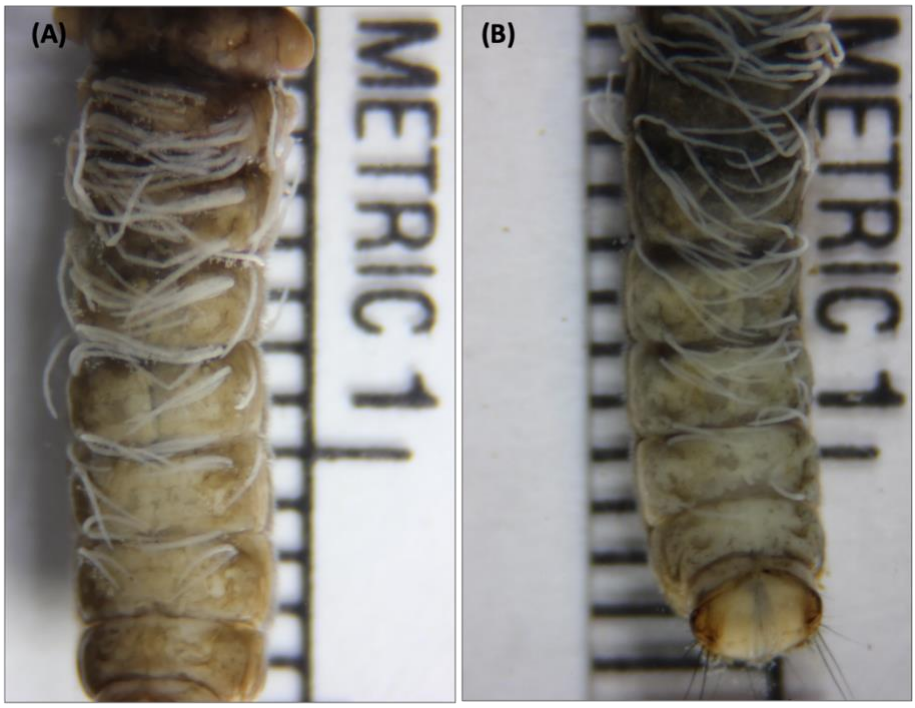


Figure 8. Ventral view of *Limnephilus externus* abdomen with (A) thick and (B) thin gills.

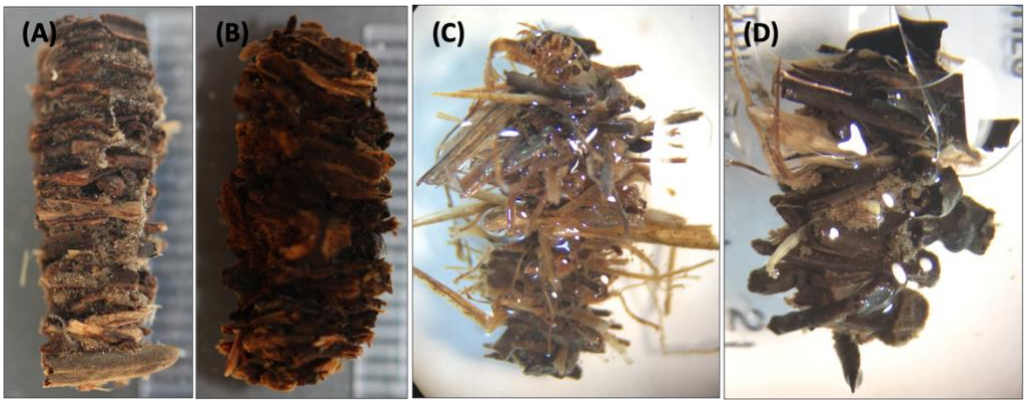
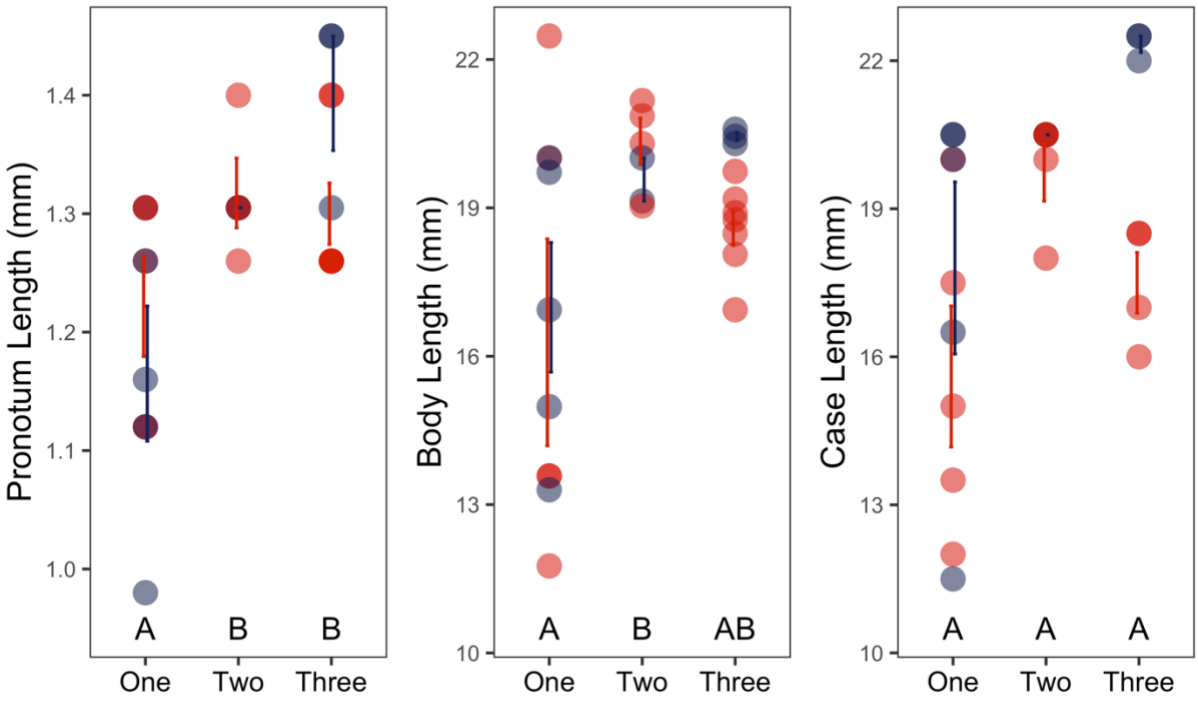


Figure 9. Variation in case types of *Limnephilus externus*: (A) narrow and sturdy, (B) bulky with twigs, (C) bulky with softer vegetation, (D) fragile with lateral extensions. Regional differences can be seen between Lakes Basin (A-C) and Tamarack (D). A midge can be seen embedded in the case in the lower left of (D).

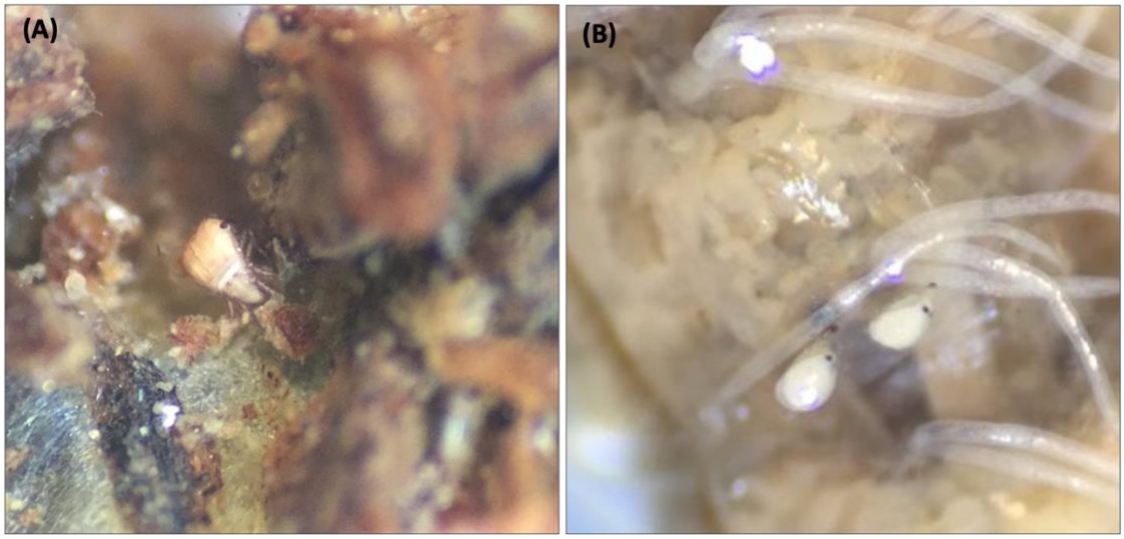


706

707 **Figure 10.** Differences among haplogroups (i.e., One, Two, Three) for each significant response
708 variable (i.e., pronotum length, body length, case length). Tukey's post-hoc test results are
709 represented above the x-axis. Lake (semi-transparent circle) and stream (semi-transparent
710 triangle) individuals are distinguished. Error bars represent the standard error of the mean for
711 lake vs stream individuals in each haplogroup.

712

713



714

715 **Figure 11.** Acari (water mites) on *Limnephilus externus'* (A) case exterior and (B) abdomen.

716 **Table 1.** Qualitative data collected on body and case morphology.

Body	0	1
Abdominal condition	Robust appearance, even color tone, no spotting	Transparent (visible tracheae), black spotted, and attenuated gills
Gill length	Does not cross midline	Crosses ventral midline
Gill thickness	Thin	Thick
Posterior extension of head capsule pigmentation	Does not extend along coronal suture	Extends along coronal suture
Abdominal mites	Absent	Present
Case	0	1
Shape	Straight	Bulged
Presence of silt	Absent	Present in crevices
Primary material type	Bark	Soft aquatic vegetation
Structure sturdiness	Breaking/Fragile	Relatively strong/sturdy
Length of case material pieces	Short	Long
Lateral Extensions	Absent	Present

Assembly uniformity	Uniform	Variable
Microinvertebrate hitchhikers	Absent	Present

717

718 **Table 2.** Number of individuals collected per month per site during timed sampling.

Site	June 2017	July 2017
Silver Lake	25	18
Little Bear Lake	0	20
Big Bear Lake	19	20
Upper Salmon Lake	1	20
Lower Salmon Lake	23	0
Goose Lake	82	20
Tamarack Lake	NA	8
Upper Salmon inlet	0	7
Salmon Creek	0	1
Lower Salmon outlet	0	20
Silver outlet	0	0
Little Bear outlet	0	0
Big Bear outlet	0	10
Tamarack outlet	NA	9

719