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 different flow types, which raises questions about how co-occurrence may influence ability to adapt to changing climatic conditions. One such co-occurring species is the aquatic insect *Limnephilus externus* Hagen, 1861 (Trichoptera:Limnephilidae), a species known to be widely distributed in lakes of the Nearctic and Palearctic regions. Here, we test whether lake–stream populations of the caddisfly *L. externus* are evolutionarily or ecologically distinct. We examined larval body and case morphology, interspecies phoretic associations, and the mitochondrial DNA cytochrome *c* oxidase I gene among lake and stream populations of *L. externus*. We also explored potential morphological differences among distinct haplotypes. 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 this species. We also observed distinct regional differences in caddisfly body condition and case construction sturdiness and found distinct assemblages of microinvertebrates associated with the caddisfly's body and cases. Lake–stream *L. externus* did not show genetic divergence; however, 3 potentially distinct haplotypes were present across the research sites as well as in sequences from North America and Canada. *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. Combined life history and phylogenetic studies provide valuable insight into the ecological and evolutionary dynamics that influence the adaptability of aquatic fauna to climatic change.
Key words: lentic–lotic, lake–stream, aquatic insect, caddisfly, DNA barcoding, haplotype, phenology, morphology, phoresy, eco–evo, phylogenetic, life history

Lentic and lotic habitats are believed to differentially influence ecological and evolutionary dynamics. Indeed, the distinction between these 2 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 necessary for plant and animal species to persist (Wubs et al. 2016), and dendritic network patterns create variation in metacommunities among headwater and mainstem habitats (Brown and Swan 2010). Lakes are commonly understood to favor species with better ability to disperse, possibly because lakes are less stable than streams relevant to speciation over evolutionary timescales. For example, lentic odonate species have larger latitudinal ranges than their lotic counterparts in the Nearctic and Palearctic (Hof et al. 2006). Some studies have found that lotic species have greater genetic population differentiation and potential for cryptic diversity than lentic species (Marten et al. 2006), but 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 from work on Threespine Stickleback (*Gasterosteus aculeatus* Linnaeus, 1758). In sticklebacks, co-occurrence seems possible

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because of morphological variability, parapatric speciation, or both (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, 1819) 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 y (Rypel et al. 2006). Minnows (*Phoxinus* spp. Rafinesque, 1820) from lakes and streams also often exhibit a similar morphological pattern, though some evidence to the contrary suggests that, in minnows, these differences may be region dependent (Ramler et al. 2017, Scharnweber 2020). Overall, case studies suggest a range of possible ecological and evolutionary mechanisms for co-occurrence of a species in lake and stream habitats, such as source–sink dynamics, niche diversity, developmental plasticity, genetic variation, sympatric or parapatric speciation, and isolation by distance among other hypothesized processes.

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 aquatic organism populations that co-occur in these mountainous lake and stream habitats remain poorly understood. Aquatic invertebrates, such as caddisflies that co-occur in lentic and lotic habitats, provide an opportunity to explore population dynamics between these habitat types. 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 change in future climate change scenarios.

In this study, we sought to understand the degree to which populations of the caddisfly *Limnephilus externus* Hagen, 1861 (Trichoptera:Limnephilidae) that co-occur in lakes and streams are evolutionarily and ecologically distinct. To do so, we tested whether populations of lentic and lotic *L. externus* differed in population genetic structure, abundance, larval phenology, larval body and case morphology, and interspecies phoretic interactions. We asked if population measures, including genetic, phenological, morphological, and interspecies phoretic frequency measures, differed between lentic and lotic populations of *L. externus*. Further, we sought to examine morphological differences between distinct *L. externus* haplotypes that emerged from this analysis.

METHODS

We conducted a field study and used a combination of life history and genetic information to examine variation between lentic- and lotic-dwelling *L. externus*. To visualize population genetic structure, we constructed a haplotype network and ran phylogenetic analyses based on the mitochondrial DNA cytochrome *c* oxidase subunit 1 (COI) gene. To compare abundance, we performed population surveys scaled by time. On individual caddisflies, we quantitatively measured head capsule width, body length, body width, pronotum length, pronotum width, case length, and case width. We also collected qualitative nominal data on body features (abdominal condition, gill length, gill thickness, posterior extension of head capsule pigmentation, and abdominal mites) and case features (shape, presence of silt, primary material type, structure sturdiness, length of case material pieces, presence of lateral extensions, assembly uniformity, and microinvertebrate hitchhikers). To compare morphological differences between lake and stream populations, we performed 2-tailed *t*-tests on quantitative variables and Fisher's exact tests on qualitative nominal data. Finally, to explore the potential for morphological differences between distinct *L. externus* haplotypes, we performed 2-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc tests on quantitative variables and Fisher's exact tests for qualitative variables.

Study organism

Limnephilus externus is a caddisfly whose larvae (Fig. 1) typically inhabit lentic habitats, such as lakes, permanent to semipermanent shallow ponds, and wetlands (Berté and Pritchard 1986, Wissinger et al. 2003, Jannot et al. 2008). The 5 larval instars and the pupa are aquatic. After pupation, *L. externus* emerge as terrestrial winged adults (Fig. 2). The larvae create bulky, cylindrical, nonrigid cases (hedgehog cases; Johansson and Johansson 1992) assembled from fragments of vegetation, detritus, and other organic matter (Berté and Pritchard 1986, Wiggins 2004). *Limnephilus externus* flight duration is not well documented, but adults of this species likely live <2 mo (Berté and Pritchard 1986, Wissinger et al. 2003). The species is well documented in lake habitats throughout western North America, Canada, and the Palearctic realm (Morse 1993, Ruiter et al. 2013, Mendez et al. 2019). Although there are very few records of *Limnephilus* spp. larvae in streams, there are several documented stream site records from California, USA (Pratha 2014). Also, members of this research team have observed their presence in several streams in the northern Sierra Nevada mountain range in California, which informed our choice of this species for this study.

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

Study area

We sampled for *L. externus* in 2 regions of the northern Sierra Nevada mountain range, California. The primary research location was in the Lakes Basin, which is a high-elevation (2000 m) mountainous region featuring a dendritic network of headwater streams and oligotrophic lakes. We selected 6 closely situated lakes and 6 of their connecting streams in the headwaters of 2 adjoining watersheds: the Feather River watershed (Silver, Little Bear, Big Bear, and Goose lakes) and the Yuba River watershed (Upper and Lower Salmon lakes) (Fig. 3). To add context to the study, we sampled additional *L. externus* populations collected from 2 additional lakes in the Feather River watershed (Haven Lake, without inlet or outlet stream, and Long Lake, only used for genetic analyses) as well as from a lake–stream pair in the Upper Truckee River watershed ~100 km south of the Lakes Basin (Tamarack Lake and its outlet stream, only used in phenology assessment, genetic analyses, and haplotype morphology comparison).

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 1st sampling event in late June 2017 occurred during peak snowmelt and streamflow. A 2nd sampling event in July 2017 occurred after peak water levels had subsided. For this reason, we used only July specimens for lake–stream comparisons and sequencing. Water-quality parameters, measured as spot samples during population and habitat surveys, were similar across all lake and stream study sites and were typical of water quality in the higher elevations of the Sierra Nevada mountains. Conductivity was consistently <25 µS/cm, and 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

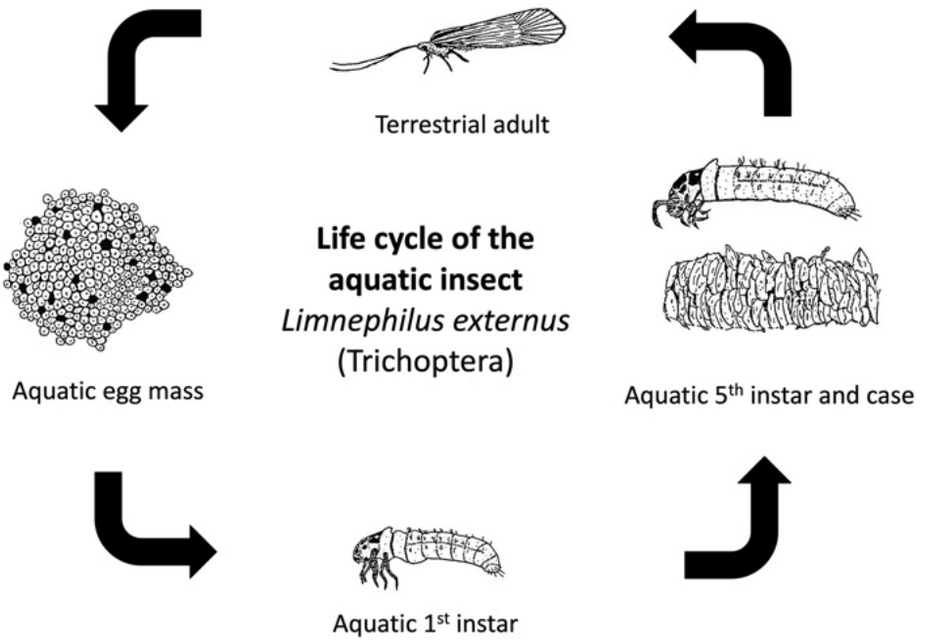

Figure 2. Illustration summarizing key aspects of the life cycle of the aquatic insect *Limnephilus externus*.

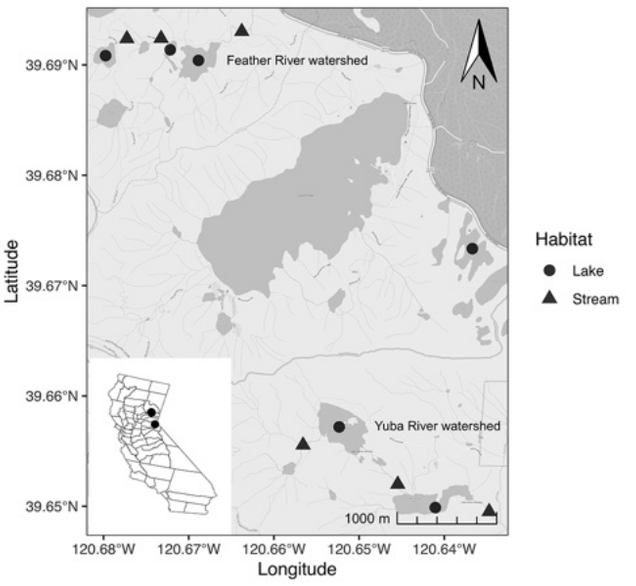

Figure 3. Lotic (triangle) and lentic (circle) sampling sites in the Lakes Basin, northern Sierra Nevada mountains (California, USA). In the Feather River watershed, Silver, Little, and Big Bear lakes share connectivity. In the Yuba River watershed, Upper and Lower Salmon lakes share connectivity. Goose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

streams and were higher, on average, in July (mean = 20.5°C) than June (mean = 18.0°C).

Sampling

We sampled *L. externus* in both lentic (lake habitats ≥100 m from the nearest inlet or outlet) and lotic (stream habitats within 100 m of lake outlets or inlets) habitats. At each lake and stream, we selected 5 sampling areas (1 m^2) along the littoral zone (lakes) or benthic zone (streams) in water depths of 5 to 50 cm. Sampling areas were spaced ≥1 m apart. We performed population surveys for a timed interval (12 min/1-m^2 area) by sampling a combination of cobble, boulder, and bedrock. At each site we examined and picked up 100 to 125 cobble-sized rocks to document the abundance of *L. externus*. We collected all found *L. externus* individuals, which we preserved in 70% ethanol and transported to the lab for further analysis.

In the lab we visually sorted *L. externus* larvae into 5 instars based on case size. We performed all subsequent analyses on only individuals of the largest size class (presumed 5^{th} instar). A posteriori measurements of head capsule width of the largest size class (mean = 1.61 mm, range = 1.40–1.82 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, range = 1.51–1.78 mm, Wissinger et al. 2003).

Population genetics

We examined genetic variation among sampled *L. externus* populations through sequencing and analysis of the COI gene. To obtain genetic material, we randomly selected 29 individuals across 13 sites, removed a single leg from each individual, and placed each leg in a unique microplate well with 1 to 2 drops of 70% ethanol. Samples were sent to the Canadian Center for DNA Barcoding at the University of Guelph in Ontario, Canada, for standard DNA extraction, COI gene isolation, and gene amplification with established quality assurance/quality control standards. Forward primer C_LepFolF and reverse primer C_LepFolR were used to conduct polymerase chain reaction amplification on the marker COI-5P, a standard insect barcoding marker.

We visualized haplotype relationships and their spatial distribution for the Lakes Basin and Tamarack specimens via a haplotype network. To do so, we created a haplotype network with PopART software (version 1.7 for Mac OSX version ≥10.6; Leigh and Bryant 2015). We used the minimum spanning network algorithm on 28 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 gene sequences with those found in the Barcode of Life Data (BOLD) Systems (Ratnasingham and Hebert 2007). We searched the BOLD Systems Public Data Portal for nucleotide sequences belonging to "*Limnephilus externus*" and exported all 252 matching records and their metadata. Data came from 10 institutions, spanned 3 countries, and broke into 3 barcode index number (BIN) clusters (i.e., algorithm-generated operational taxonomic units that are performed once/mo based on diverging sequences) (Ratnasingham and Hebert 2013). We performed initial sequence metadata review in R (version 4.2.0; R Project for Statistical Computing, Vienna, Austria) using base R and the packages *readr* (version 2.1.3; Wickham et al. 2022) and *tidyverse* (version 1.3.2; Wickham et al. 2019). We removed 24 sequences without BIN information and 40 sequences with invalid residues. We used Geneious software (version 10.2.3; Boston, Massachusetts; https://www.geneious.com; Kearse et al. 2012) to align all sequences with a global alignment with free end gaps and a 65% similarity cost matrix. We removed additional sequences 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 haplotype branch. The final nucleotide alignment comprised 29 original sequences and 25 unique BOLD Systems sequences (Appendix S1).

We used Geneious software to construct phylogenetic trees of the 54 COI gene sequences with both a distance-matrix method (unweighted pair group method with arithmetic mean [UPGMA]; Sokal and Michener 1958) and a

Bayesian inference method (MrBayes; version 3.2.6; Felsenstein 1992, Tuffley and Steel 1997, Huelsenbeck and Ronquist 2001). For the UPGMA analysis, we used a bootstrap resampling method (100 replicates) to build trees for 3 different pairwise genetic distance models (i.e., Jukes–Cantor, HKY, Tamura-Nei). For Bayesian analyses, we used both the JC69 (nst = 1) and HKY85 (nst = 2) substitution models (Huelsenbeck and Ronquist 2001). We selected the only 2 imported BOLD Systems sequences available from the Palearctic (Finland) as outgroups. All trees produced with the UPGMA and Bayesian analyses contained similar distinct branching and haplotypes; thus, we only present results from the UPGMA Jukes–Cantor model, which assumes equal rates of nucleotide substitutions as an inferred phylogenetic relationship. Algorithm-generated BIN assignments from BOLD Systems are included in the branch label of exported BOLD sequences in the tree. To identify distinct groups, we used a criterion of ≥0.01 (1%) dissimilarity between parallel branches, which resulted in substantially larger variation between groups than within groups.

Morphology and phoretic associations

We processed and measured *L. externus* 5th-instar larvae collected in July (n = 44; 27 lake, 17 stream) and their associated cases for body and case morphology. We individually photographed the individuals and their cases, assigned them unique identification codes, and examined them under a dissection microscope at 10 to 20× magnification. We then measured each individual for head-capsule width, body length, pronotum length, body width at both the pronotum and 2nd abdominal segment, case length, and case width at its widest point. We used a micrometer (±0.01 mm) to measure body morphology and calipers (±0.1 mm) to measure case morphology.

We also qualitatively documented body and case morphological 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). We also observed 2 distinct conditions of the ventral abdomen: even color tone with robust appearance and black spotting with a transparent cuticle.

Finally, we identified phoretic associations of other organisms with *L. externus*. We found a variety of microinvertebrates (<500 µm; e.g., Chironomidae, Acari, Oligochaete, *Hydra*) attached to or embedded in caddisfly cases or clinging to abdominal gills from within the case. We identified these associated microinvertebrate taxa to the lowest possible level using a combination of expert knowledge and dichotomous key (Merritt and Cummins 1996), enumerated them, and preserved them in 70% ethanol.

To examine possible differences between lake and stream populations for samples collected in July, we performed 2-tailed t-tests for the 7 quantitative variables and Fisher's exact tests of independence on the 13 qualitative nominal variables. We checked normality via scatterplots and variance with F-tests to inform model structure. To determine differences among the 3 primary haplotypes identified in phylogenetic analyses, accounting for differences between lake and stream habitats, we performed 2-way ANOVA with Tukey's HSD post hoc tests when ANOVA results had

Table 1. Qualitative data collected on body and case morphology of *Limnephilus externus* collected in the northern Sierra Nevada mountains (California, USA). Qualitative data were assigned a nominal code (0 or 1).

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

Table 2. Number of *Limnephilus externus* ind./mo collected at each of 14 lake and stream sites in the northern Sierra Nevada mountains (California, USA) during timed sampling. NA = not applicable.

Site	June 2017 (no. ind.)	July 2017 (no. ind.)
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

p-values ≤ 0.05 for the quantitative variables and performed Fisher's exact tests for the qualitative variables ($n = 26$; 10 in H1, 6 in H2, 10 in H3). Six sequenced specimens did not have abdominal condition criteria available. We performed all analyses and created maps in R using the packages *stats* (version 4.2.3), *tidyverse*, *fBasics* (version 4021.93; Wuertz et al 2022), *sf* (version 1.0-9; Pebesma 2018), *sp* (version 1.5-1; Pebesma and Bivand 2005, Bivand et al. 2013), *ggspatial* (version 1.1.7; Dunnington 2022), *grid* (version 4.2.3), and *patchwork* (version 1.1.2; Pedersen 2022). Quantitative and qualitative data are provided in Appendix S2.

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 7/7 lakes and 5/7 streams (Table 2). In abundance surveys scaled by time, we regularly collected 20 ind./h 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, in June, no larvae could be found in the streams, which experienced high and turbulent snowmelt flows. In July, 1 stream (Lower Salmon outlet) yielded ≥20 ind./h, whereas others had lower abundance (<10 ind./h). We observed many empty cases in both lakes and streams in July. In lakes, larvae were commonly found on or near submerged vegetation (e.g., aquatic grasses), whereas in streams larvae were found primarily attached to stable substrates (e.g., fallen logs) in pools. Although 5th-instar larvae were present among all *L. externus* populations in July 2017, the proportion of instars varied greatly among sites (Fig. 4). Fifth instars were the dominant size class at Big Bear Lake and Upper Salmon Lake. Lakes combined had roughly equal percentage of 4th and 5th instars (40.5 and 44.6%, respectively), yet streams combined had more 5th instars (59.6%) than 4th instars (14.9%). Few of the individuals we collected were 1st to 3rd instars (lakes 14.9%, streams 25.5%).

Analysis of the COI gene sequences indicated moderate intraspecies variation, low geographic structure, and wide geographic distribution of haplotypes. A haplotype network revealed 8 unique haplotypes present between the Lakes Basin and Tamarack regions (Fig. 5). Unique haplotypes were 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 3 largest haplotypes (H1, H2, H3), comprising individuals from both Lakes Basin and Tamarack, correspond with the 3 BOLD Systems algorithm-generated BINS (Figs 5, 6). The haplotype network supported findings clarified by the phylogenetic analysis, confirming 3 primary haplotypes, with the less common haplotypes nesting into 1 of these 3 primary groups on the tree. Within group dissimilarity (H1: range = 0.1–0.5%, mean = 0.2%; H2: range =

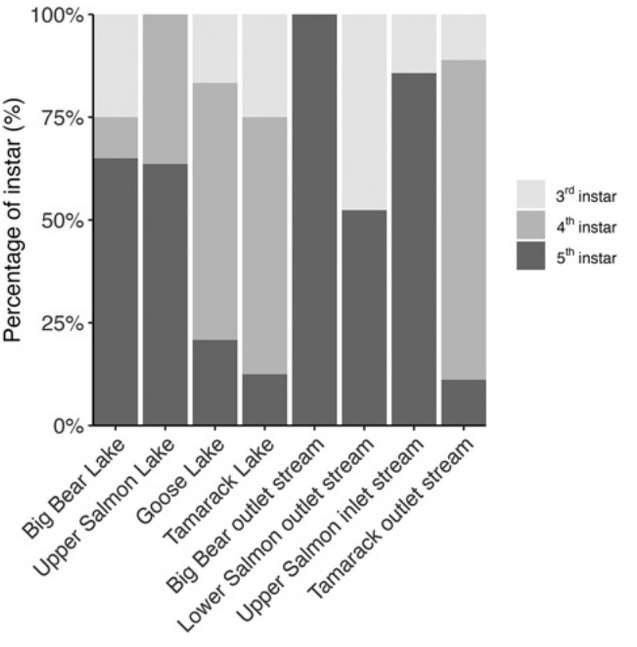

Figure 4. Percentage of instars from all *Limnephilus externus* ind./site collected in July 2017 from the 8 sites where phenology was assessed in the northern Sierra Nevada mountains (California, USA).

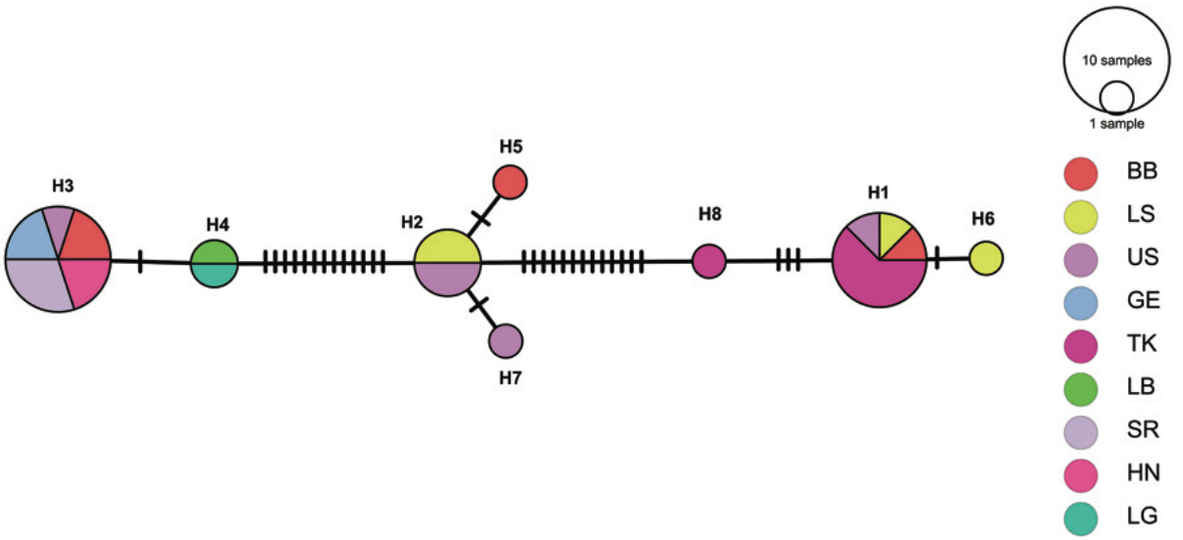

Figure 5. Haplotype (H) network showing the number of pairwise differences between groups of *Limnephilus externus* individuals collected from lakes and streams in the northern Sierra Nevada mountains (California, USA) (1 tick mark = 1 mutational difference). Eight haplotypes were present across the 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). BB = Big Bear, GE = Goose, HN = Haven, LB = Little Bear, LG = Long, LS = Lower Salmon, SR = Silver, TK = Tamarack, US = Upper Salmon.

0.1–0.5%, mean = 0.2%; H3: range = 0.1–0.4%, mean = 0.2%) was much less than between-group dissimilarity (H1 and H2: range = 0.8–1.1%, mean = 0.9%; H1 and H3: range = 0.8–1.4%, mean = 1.1%; H2 and H3: range = 0.8–1.2%, mean = 1.0%). In the phylogenic approach, all 3 haplotypes included individuals from both the United States and Canada, indicating that the 3 genetically distinct haplotypes are widely distributed. H1 included multiple individuals from Lakes Basin and 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, California), Washington State (USA), and Manitoba (Canada). H2 included individuals predominantly from the Upper and Lower Salmon Lake watershed and 1 individual from Big Bear Lake (Lakes Basin) as well as individuals from the Rocky Mountains (Colorado, USA) and individuals from across Canada (Alberta, Manitoba, New Brunswick). H3 included individuals from the hydrologically connected system that includes Silver, Little Bear, and Big Bear lakes and streams as well as nearby Goose, Long, and Haven lakes (Lakes Basin) and 1 individual from Manitoba (Canada).

Morphology

Limnephilus externus larvae differed in abdomen condition and gill thickness between lake and stream individuals (Fisher: $p = 0.0002$ and 0.0008, respectively; Table S1). Black-spotted, transparent abdomens with attenuated gills (Fig. 7B) were more common in lake (100%) than stream (28.6%) individuals. Thick abdominal gills (Fig. 8A) were also more common in lake (72.7%) than stream (8.3%) individuals.

Caddisfly case construction and materials varied substantially among habitats (Fig. 9A–D). Cases were substantially longer in lakes than streams in the Lakes Basin (*t*-test: $p = 0.0001$; Table S2). There were no other major differences in cases between lake and stream individuals. Although we did not statistically analyze data from the June sampling, we observed that cases included more aquatic vegetation in June, whereas in July, cases were constructed predominantly with twigs and bark. All cases from Tamarack Lake and its outlet were fragile and 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.

Caddisflies belonging to 1 of the 3 primary haplotypes differed substantially in 3 morphological variables. Among the 3 haplotypes, pronotum length (ANOVA: $F_{2,20} = 6.8$, $p = 0.005$; Table S3, Fig. 10A), body length ($F_{2,20} = 4.4$, $p = 0.026$; Fig. 10B), and case length ($F_{2,17} = 5.0$, $p = 0.02$; Fig. 10C) all differed (Table S3). Pronotum length was shorter in H1 compared with H2 and H3 (HSD: $p = 0.05$ and 0.009, respectively; Cohen's $d = -1.30$ and -1.40, respectively), and body length was shorter in H1 than H2 (HSD: $p = 0.03$; Cohen's $d = -1.15$). Head pigmentation and case structure sturdiness were also nearly or substantially different across haplotypes (Fisher: $p = 0.06$ and 0.03,

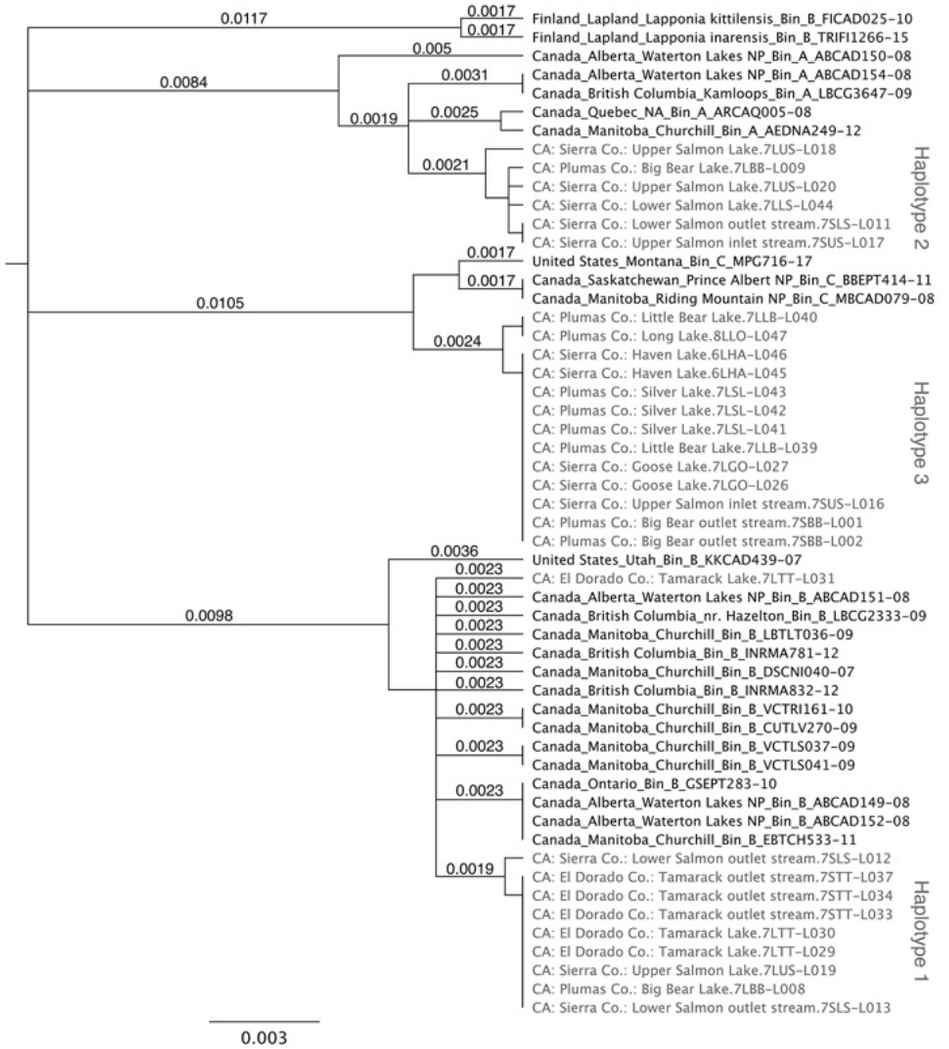

Figure 6. Phylogenetic tree of *Limnephilus externus* in the Sierra Nevada mountain range (light gray) from mitochondrial cytochrome *c* oxidase subunit 1 gene data. Additional individuals throughout the United States, Canada, and Finland (black) are included from publicly available data on the Barcode of Life Data Systems for contextual support. Each imported specimen name includes: country, state or province, county (if available), Barcode of Life Data Systems BIN cluster (A, B, C), and specimen ID. Haplotypes identified in the Sierra Nevada are in the order 2, 3, and 1 from top to bottom. The number of substitutions/site (number on horizontal branch) represents the difference between 2 parallel branches. Here, 3 haplotypes exhibit a minimum 0.01 (i.e., 1%) additive difference from each other. A ~2 to 3% additive difference between 2 parallel branches would be required for 2 haplotypes to be considered a distinct species. For clarity, substitutions/site <0.0017 are not shown. Phylogenetic tree was constructed using the Jukes–Cantor genetic distance model. Geneious version 10.2 created by Biomatters. Available from https://www.geneious.com.

respectively; Cohen's d range = −0.27 to 1.42 and −1.31 to 1.35, respectively; Table S4).

Phoretic associations

Hydra, nematodes, oligochaetes, chironomid midges (3 morphospecies), and water mites were found securely fastened to many caddisfly cases, either stuck to the surface or buried into case silt (Fig. 9D, 11). These case-associated microinvertebrates were associated with both lake (36.6%) and stream (50%) caddisfly cases across the 3 primary haplotypes. We did not observe differences in the microinvertebrate assemblage composition between cases from lake and stream individuals.

Abdominal mite presence was substantially different between lake and stream habitats and among haplotypes (Fisher: $p = 0.01$ and 0.04, respectively; Tables S1, S4). 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 H1 and H2. The highest abdominal

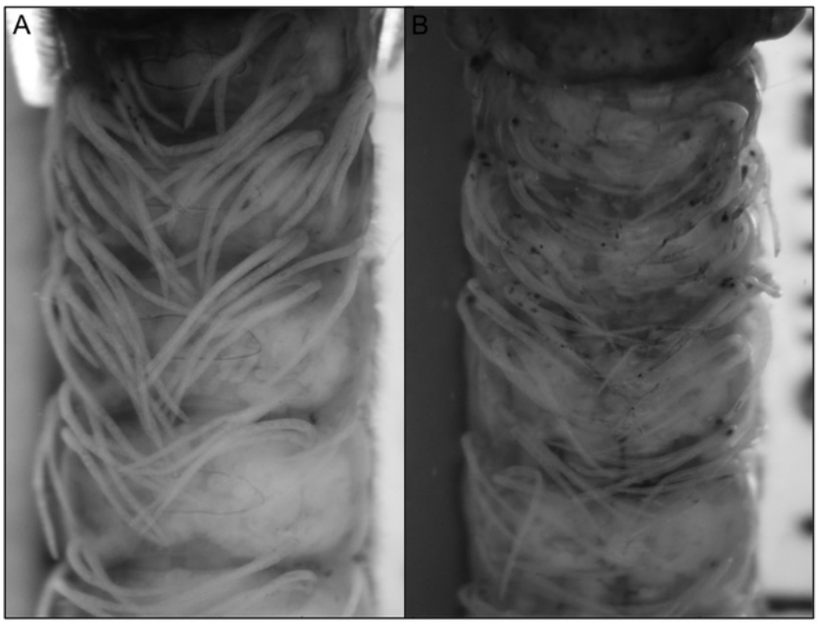

Figure 7. Ventral view of *Limnephilus externus* abdomen exhibiting no spotting, even color tone, robust appearance (A), black spotting, and increased abdomen transparency resulting in more visible tracheae, splotchy color tone, and attenuated gill appearance (B).

mite association was 31 mites on a single individual, and inflicted individuals had a mean of 4 mites. All individuals with water mites on their abdomen were observed to be less robust, to have dark and transparent abdomens, and to have attenuated black spotted gills. However, nearly ½ 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, whereas those clinging to the abdomen were identified as larval hygrobatoid water mites (Acariformes:Parasitengona:Hydrachnidiae:Hygrobatoidea), possibly in the family Hygrobatidae or Unionicolidae (Heather

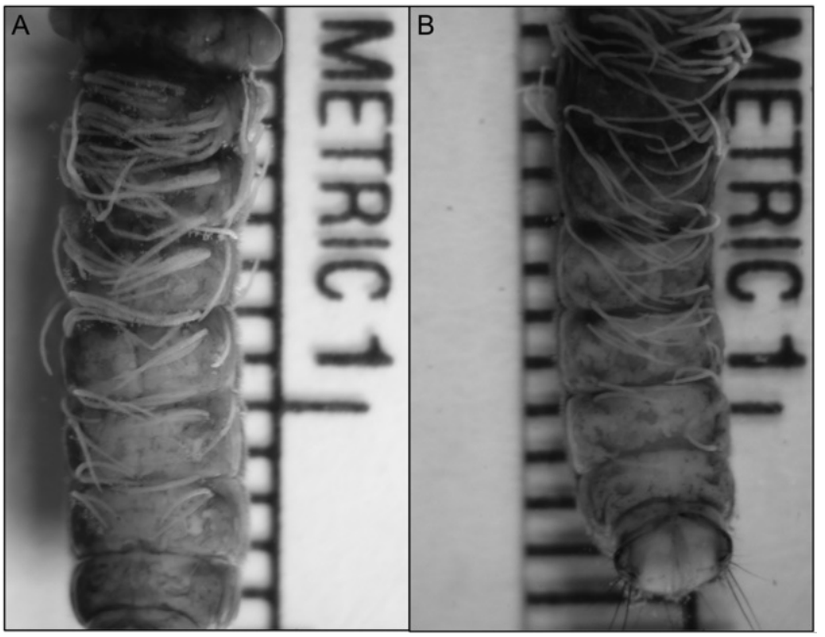

Figure 8. Ventral view of *Limnephilus externus* abdomen with thick (A) and thin (B) gills.

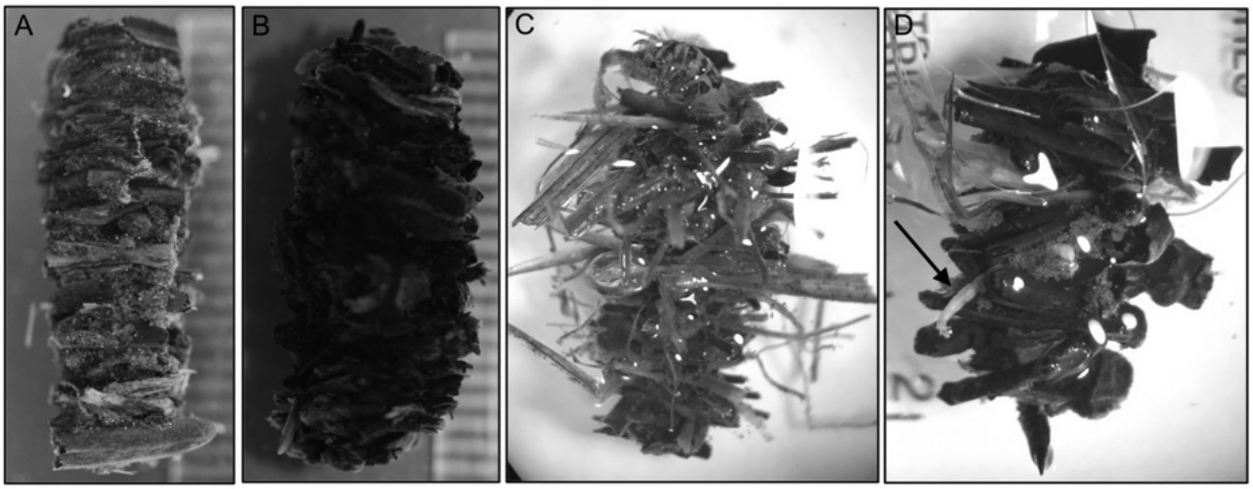

Figure 9. Variation in case types of *Limnephilus externus*: narrow and sturdy (A), bulky with twigs (B), bulky with softer vegetation (C), and fragile with lateral extensions (D). Regional differences can be seen between Basin (A–C) and Tamarack (D) lakes. A midge can be seen embedded in the case in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

Proctor, University of Alberta, Edmonton, Alberta, personal communication).

DISCUSSION

In this study, we formally documented the presence of *L. externus*, a caddisfly widely known from lentic habitats throughout North America, in both lake and stream habitats in the Sierra Nevada mountains in California. We also examined the degree to which *L. externus* co-occurring in lakes and streams are evolutionarily and ecologically distinct. Further, we briefly explored the potential for morphological differences between 3 distinct haplotypes of *L.*

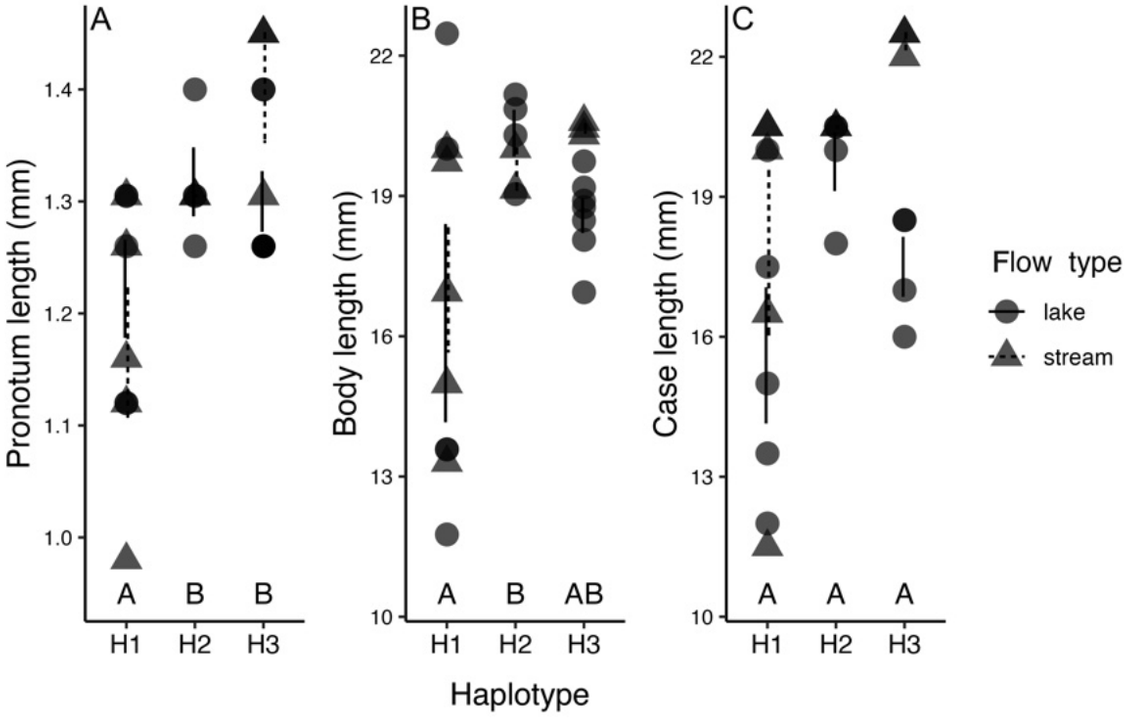

Figure 10. *Limnephilus externus* measurements of individuals for morphological variables that differed between haplotypes (H1, H2, H3): pronotum length (A), body length (B), and case length (C). Tukey's honestly significant difference post hoc test results are shown above the *x*-axis with lowercase letters. Error bars represent the SE of the mean for lake (solid) vs stream (dashed) individuals in each haplotype.

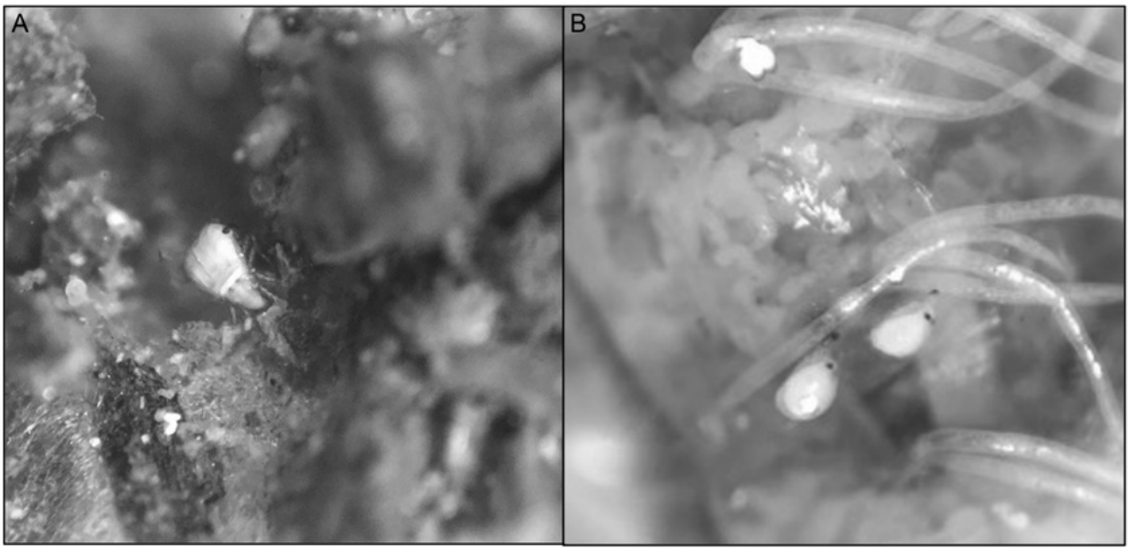

Figure 11. Acari (water mites) on *Limnephilus externus* case exterior (A) and abdomen (B).

externus that emerged from this analysis. Lake populations had conspicuous abdominal tracheae, thicker gills, and black speckling. Lake populations also exhibited longer case construction than stream populations, and only caddisfly cases from the Tamarack region were notably more fragile in construction. Microinvertebrate hitchhikers found on the cases of the caddisflies are presumed to maintain a phoretic relationship, whereas mites on the abdomen may be demonstrating preparasitic attendance behavior. Finally, although lake populations were not genetically different from stream populations, we did find 8 unique haplotypes present. Of these 8 haplotypes, 3 are distinct and geographically widespread in the Sierra Nevada as well as throughout western North America and Canada. These haplotypes exhibited some notable morphological variation, but further research is needed to validate these results.

The use of the COI gene (i.e., DNA barcoding) for characterizing populations 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). 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).

Although this study found no genetic differences between lake and stream *L. externus*, we did find 8 unique haplotypes, at least 3 of which are geographically widespread and distinct, separated by 1 to 2% genetic difference. These putative haplotypes 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) because a minimum 2 to 3% genetic divergence is often used to distinguish haplotypes as distinct subspecies or species. We note that the 3 primary haplotypes in our analysis do match with the 3 algorithm-generated BINs identified by BOLD Systems, which are intended to nearly approximate species, suggesting further work would be valuable to explore these relationships.

Limnephilus externus' 3 primary haplotypes are widely distributed throughout the USA 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 haplotype 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 gene evolves quickly within the *Limnephilus* genus, supporting its use when exploring recent divergences (McCullagh et al. 2015, Steinke et al. 2022). This rapid evolution of the COI gene, or the widespread gene flow hypothesis, could account for the large variation in morphology we found in 3 widely distributed haplotypes and for their distribution across entire countries. Our results expand on the extensive genetic analyses and collections of *L. externus* in North America and the Manitoba province of Canada (Zhou et al. 2011, Ruiter et al. 2013). Our findings allude to hidden biodiversity patterns and the need to further identify species boundaries in aquatic insect taxa.

Lake and stream populations of *L. externus* exhibited distinct ecological phenotypes. Lake and stream populations differed in abundance, phenology, some aspects of body and case morphology, and abdominal mite presence. Fifth-instar *L. externus* were present at all lake and stream

sites, and 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 also had thicker abdominal gills than those in streams. These morphological differences could represent adaptations resulting from several possible abiotic variables 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* Keffermüller and Machel, 1967 from the lotic *Baetis bundyae* Lehmkuhl, 1973, 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).

Our findings that lake *L. externus* constructed cases using longer pieces of material than those in streams and that cases from the Tamarack region had weaker construction may provide insights into the effects of spatial variables on aquatic insect behavior. 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 variables (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). Another study reported that differences in case structure between 2 *Limnephilus* spp. *(Limnephilus pantodapus* McLachlan, 1875 and *Limnephilus rhombicus* [Linnaeus, 1758]) affected the behavior of predaceous dragonfly larvae (Johansson and Johansson 1992). Further, 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). We note that spatial dependence among this study's sampling locations is possible but was not explicitly examined in the statistical analysis due to sample size per site.

Our findings that the 3 distinct *L. externus* haplotypes vary to some degree in pronotum length, total body length, case sturdiness, and presence of abdominal mites merits further investigation. Haplotypes also exhibited a small difference in head pigmentation, which has previously been used to distinguish between *Limnephilus* species (Ruiter et al. 2013). We consider these morphological haplotype differences to suggest that real clade-level differences may exist. However, this study was designed to investigate differences between lakes and streams in a single region, and, therefore, a representative sampling of each haplotype may not have been achieved and warrants further study.

Finally, we discovered ecological associations on the body (i.e., mites) and case (i.e., chironomid midges, water mites, hydrae, oligochaetes) of *L. externus* and documented their prevalence. We observed ≥3 morphospecies of chironomid midge on the cases, suggesting that the microinvertebrate assemblage on the cases may be diverse. Water mites observed on case exteriors were identified as adult oribatids (Heather Proctor, University of Alberta, Edmonton, 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 (nonharmful) association. In contrast, mites found on the abdomen of *L. externus* larvae may pose a greater threat to their host. Abdominal water mites were identified as larval hygrobatoid water mites (Heather Proctor, University of Alberta, Edmonton, Alberta, Canada, personal communication). Hygrobatoid mites are known to engage in preparasitic 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* spp. have been documented to host water mite larvae (Hygrobatoidea), with prevalence ranging from 4 to 42% (Fairchild and Lewis 1987). Other aquatic organisms, like the fish *Ancistrus multispinis* (Regan, 1912) 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, although Grabner (2017) found testing for parasitic taxa using polymerase chain reaction might be an efficient and cost-effective method for identifying links between host feeding type and prevalence. Additional studies are needed to identify the nature of these associations and their consequences to *L. externus*.

The frequency of aquatic invertebrate species that coinhabit lentic and lotic ecosystems is unknown and reflects the paucity of studies of aquatic fauna across habitat types. 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 likely prevent genetic differentiation. Our findings also 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.

Although lotic and high elevation lake shoreline habitats have been recognized for their ecological similarities, their responses to climatic changes will be vastly different. Wissinger et al. (2016) observed coldwater 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 aquatic research.

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Author contributions: 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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Original cytochrome *c* oxidase subunit 1 sequence data from this study are publicly accessible in the BOLD Systems (https://doi.org/10.5883/DS-LIMNEPH). Code is available from https://github.com/caparisek/limnephilus_externus (https://doi.org/10.5281/zenodo.7793529). Morphological quantitative and qualitative data are provided in Appendix S2.

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