Environmental Drivers of Variation in Labial Teeth Number in *Ascaphus* Tadpoles

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ABSTRACT

Anuran tadpoles exhibit extraordinary diversity in mouthpart morphology, reflecting phylogenetic relationships, life history specializations, and ecological adaptations. In this study, we investigate patterns and potential environmental drivers of variation in labial teeth counts across and within the coastal tailed frog (*Ascaphus truei*) and the Rocky Mountain tailed frog (*A. montanus*), which together constitute the basal frog family Ascaphidae. Tailed frogs occupy cold, fast-flowing, high gradient streams in mesic forests of western North America, with differences in environmental conditions that reflect their coastal and continental distributions. Despite a very strong relationship between the number of labial teeth and developmental (Gosner) stage, we found significant relationships between tooth counts and site-specific environmental predictors after controlling for stage. The most important environmental predictor varied between the species, with percent canopy cover for *A. truei* and average benthic substrate size for *A. montanus*. There were also differences in the overall tooth counts between species, with *A. truei* having more teeth in all rows compared to *A. montanus*. These findings demonstrate that differences in oral morphology in response to local environmental conditions can override the homogenizing effects of gene flow in both *Ascaphus* species, likely through a combination of local adaptation and phenotypic plasticity. Follow-up investigations should include the assessment of community level variability across sites, and experimental tests to confirm functional relationships between environmental variation and oral morphology, in addition to partitioning genetic from plastic mechanisms.

INTRODUCTION

Most anuran tadpoles have complex, keratinized mouthparts, with an oral disc containing multiple rows of labial teeth surrounding a jaw sheath. These mouthparts are highly flexible, allowing tadpoles to graze irregular surfaces efficiently (Wassersug and Yamashita, 2001; Annibale et al., 2019). The keratinized labial teeth assist in feeding by both anchoring the oral disc to the substrate and scraping material off of it (Wassersug and Yamashita, 2001; Venesky et al., 2010; 2013). Secure attachment to the substrate may be particularly important for species inhabiting fast-flowing streams, since the claw-like labial teeth can prevent tadpoles from being swept downstream (Vera Candioti and Altig, 2010). The number, size, shape, and arrangement of labial tooth rows varies interspecifically, indicating that tadpole mouth morphology reflects both life history and ecological adaptations (Altig and McDiarmid, 1999; Altig, 2006). Mouth morphology is also known to vary within species during development (Altig and McDiarmid, 1999; Conradie and Conradie, 2015), though less is known about how intraspecific variation may be driven by adaptation to environmental differences across sites.

The objective of this study is to assess patterns and potential drivers of variation in labial teeth in the two species of the genus *Ascaphus*: *A. truei* (the coastal tailed frog, Fig. 1) and *A. montanus* (the Rocky Mountain tailed frog). *Ascaphus* represents one of the oldest extant lineages of frogs on Earth (Frost et al., 2006). The two species diverged over 5 million years ago, following the rise of the Cascade Mountain range in North America and associated climatic changes (Nielson et al., 2001). Today, tailed frogs occupy cold, fast-flowing, high gradient streams in mesic, forested landscapes of the Coast Ranges and Cascade Mountains (*A. truei*), and northern Rocky Mountains (*A. montanus*). Both species have an extended tadpole stage of one to four years, with greater variation found across the more geographically and climatically diverse range of *A. truei* (Hayes and Quinn, 2015). In this study, we focus on variation in the number of labial teeth in the labial tooth rows (Fig. 2), a trait that has not yet been investigated in detail across multiple populations of anuran tadpoles.

Figure 1. *Ascaphus truei* tadpole in Lookout Creek, OR. Photo: A.S. Cicchino.

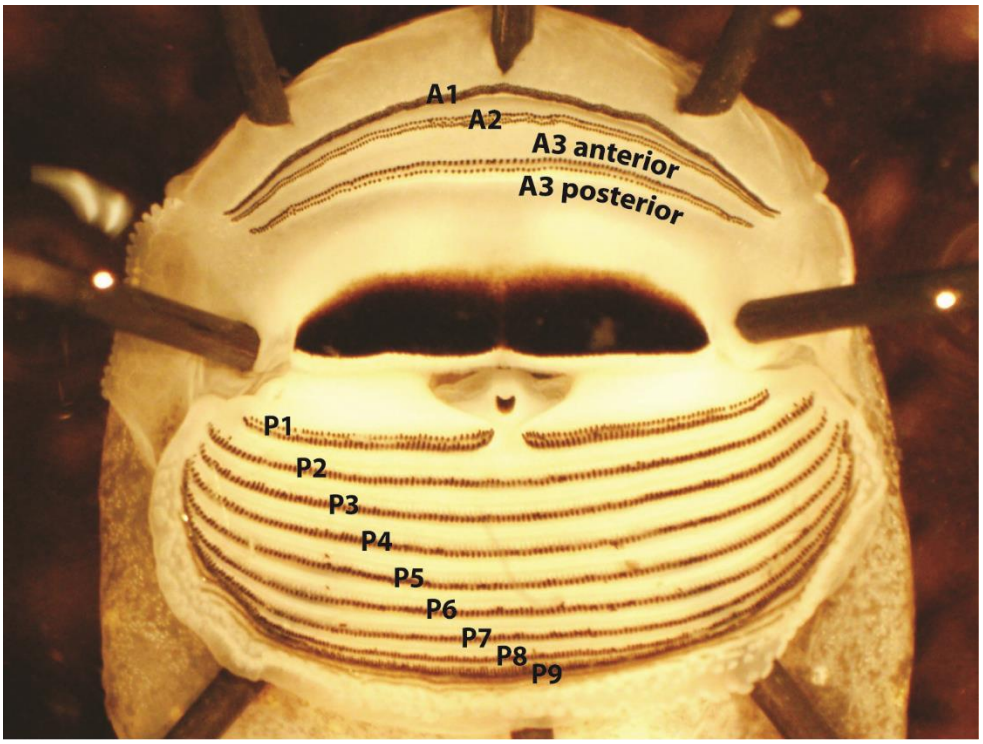

Figure 2. Pinned specimen of *Ascaphus montanus* (specimen ID WCF08228 from Lost Horse Creek, MT) showing the jaw sheath (black feature at center) and labial tooth rows (labelled). The A2, A3, and P1 rows are biserial (labelled for row A3 in this example). The labial tooth row formula (see methods) for this specimen is 3/9(1), where (1) indicates the medial gap in the P1 row.

Despite occupying superficially similar habitats, these species experience different climatic conditions that reflect their coastal (milder, more stable temperatures, and more mesic on average for *A. truei*) and continental (more seasonal variation in temperatures and drier on average for *A. montanus*) climate regimes. Due to these environmental differences and their long period of divergence, we expected to see variation in labial teeth counts between species, although we had no *a priori* hypothesis regarding the direction or magnitude of difference.

At the intraspecific level, genetic studies of *Ascaphus* indicate that gene flow is common in spite of the rough terrain they occupy, both overland (more common in *A. truei*, Spear & Storfer, 2008) and along river corridors (more common in *A. montanus*, Spear & Storfer, 2010). We would therefore expect either minimal variation in labial teeth counts across sites in the absence of selection based on local site conditions, or a latitudinal signature of spatial autocorrelation in tooth counts (i.e., due to random fixation of phenotypes during range expansion after the Last Glacial Maximum). However, given the importance of labial teeth for both feeding and the ability of tadpoles to maintain their position in the stream, we expect that adaptation will overcome the homogenizing effects of gene flow across sites where environmental conditions vary. Additionally, environmentally-driven phenotypic plasticity may play a role in maintaining differences in oral morphology across sites.

Local site conditions that may impact labial teeth number include canopy cover, substrate size, and stream temperature. Because canopy cover determines how much light reaches the stream, we expect that higher canopy cover will reduce in-stream production, selecting for the greater feeding efficiency associated with an increased number of labial teeth. Benthic substrate size can influence labial teeth through a variety of mechanisms, including feeding kinematics and substrate attachment. For example, sites with larger substrate sizes may be associated with tadpoles with fewer teeth if large rocks provide refuge from high flows, reducing the need for increased numbers of teeth for substrate attachment (Wahbe and Bunnell, 2003). Finally, given that temperature is known to affect stream diatom community composition, we expect that temperature differences among sites will drive selection on feeding efficiency and tooth number based on the quality and composition of the diatom community assemblage (Potapova and Charles, 2002). However, although we expect tooth number to vary in streams with different temperatures and diatom communities, we have no directional hypothesis for how teeth counts will relate to stream temperature.

We addressed these hypotheses by sampling tadpoles and environmental data at 16 sites across the range of *A. truei* and eight sites across the range of *A. montanus* (Fig. 3). We then analyzed these data using linear models both across and within species, taking into account tadpole developmental stage and site–specific environmental conditions. Our findings demonstrate that differences in oral morphology in response to local environmental conditions can override the homogenizing effects of gene flow in both *Ascaphus* species, likely through a combination of genetically-based local adaptation and phenotypic plasticity.

MATERIALS AND METHODS

Tadpole collection

We sampled stream reaches (~100 m in length) in the summers of 2016 and 2017 across British Columbia (n=3), Oregon (n=10), California (n=3), and Montana (n=8, site coordinates available in Table S1). We captured tadpoles by shifting and brushing rocks and benthic substrate while holding an aquarium net downstream. Tadpoles were held in thermoses in the field, and were later euthanized, photographed laterally on a grid, fixed in formalin, and stored in 95% ethanol. All tadpole collection and euthanasia was performed under the following permits: California Department of Fish and Wildlife permit number SC-12131; Oregon Department of Fish and Wildlife permit numbers 117-16 and 110-17; Montana Department of Fish, Wildlife, and Parks permit numbers 2016-100 and 2017-060; Simon Fraser University Animal Care Committee protocol 1130B-14; and Institutional Animal Care and Use Committee protocol 16-6667AA.

Labial teeth data collection

We selected ten specimens from each site for a total of 240 individuals (160 *A. truei* and 80 *A. montanus*). We selected specimens that were large enough to pin and photograph without destroying the specimen (Gosner stage > 28), and whose Gosner stage was not so advanced that the suctorial mouth was reduced (Gosner stage < 41). Specimens were pinned with the mouth open to show a full view of the labial teeth rows. We took at least three photographs of each specimen using an Olympus SZX10 scope: full view of mouth (e.g., Fig. 2), anterior rows only, and posterior rows only.

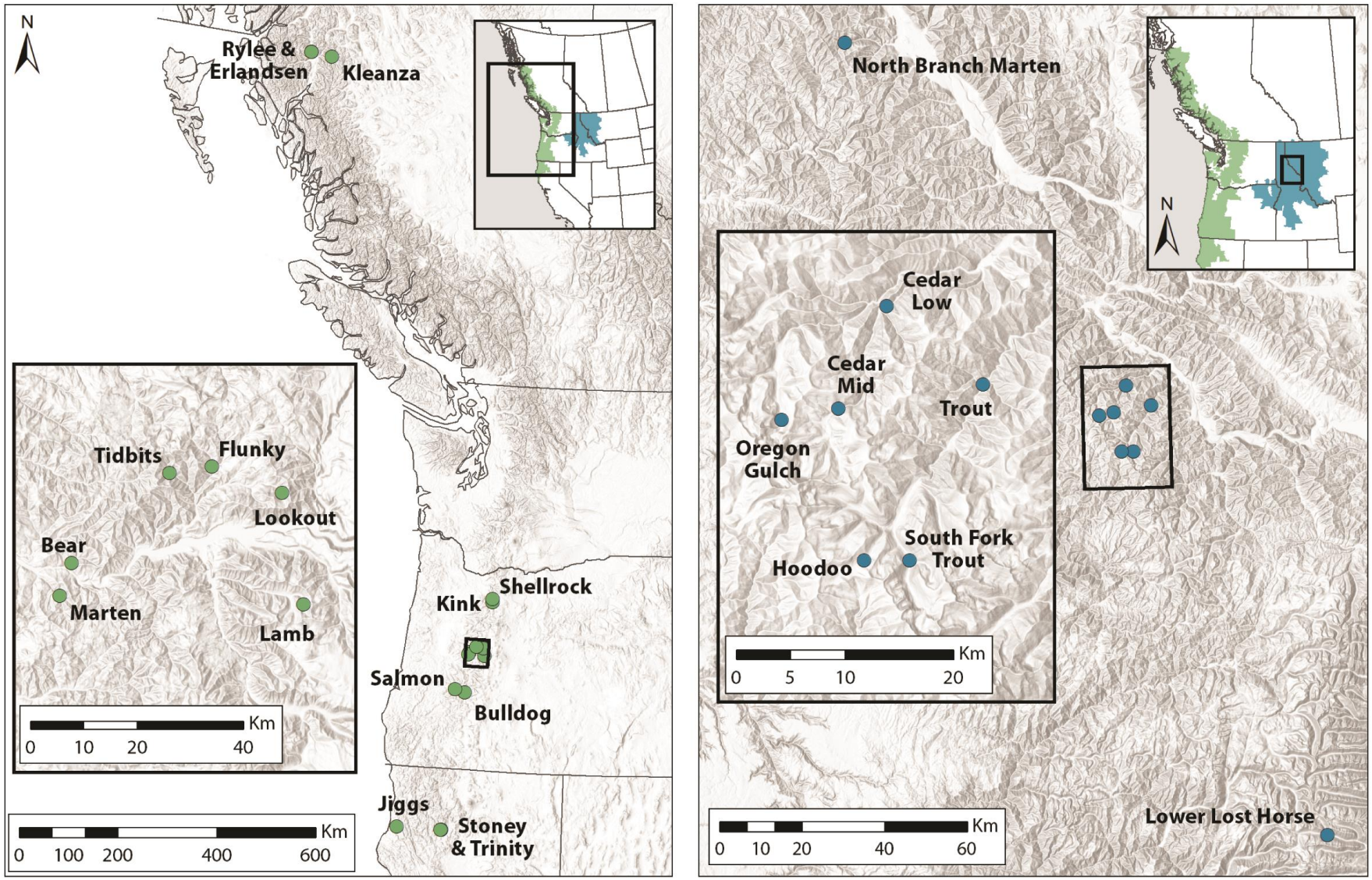

Figure 3. Sampling locations for *Ascaphus truei* (left) and *Ascaphus montanus* (right). Inset maps show estimated range boundaries for *A. truei* (in green) and *A. montanus* (in blue). Range maps from the IUCN SSC Amphibian Specialist Group (2015).

For each specimen, we first calculated the labial tooth row formula (LTRF), designated as: the number of rows in the anterior labium (A) / the number of rows in the posterior labium (P). Anterior and posterior rows were numbered from anterior to posterior within the labium (e.g., A1, A2 and P1, P2, see Fig. 2 for an example; Altig and McDiarmid, 1999). The most posterior row was defined as requiring distinct, discernible teeth throughout the row. If upon zooming in on a row no distinct teeth were discernible, the row was not included in the LTRF. "A" and "P" were followed by a row number in parentheses if a medial gap was present in the row. Rows consisting of two sub-rows on each tooth ridge were recorded as biserial and referred to as anterior or posterior.

We then used Adobe Photoshop and ImageJ (Rasband, 2018) to count individual teeth in the following rows: A3 anterior, A3 posterior, P1 posterior, P2, and P3. In specimens where the P2 row was biserial, we counted the P2 posterior row. These five rows were selected because their ends were consistently discernible from the center to the outer edges of the oral disc. Each tooth was individually marked with a red dot in ImageJ and counted moving from left to right. The same count was then performed with a dot of a different color moving from right to left. If the two counts matched, the number of teeth was recorded for the row. If the two counts were not the same, a third count was performed to serve as a tiebreaker. In a small number of cases, counts were initially listed as "NA" for one of the following reasons: a photograph was too blurry for all teeth to be discernible, there were teeth missing, there was damage to the tooth row, or the view of the teeth was obstructed. In these cases, we filled in NA values using one or more of the following approaches: (1) we used ImageJ to sharpen blurry edges to improve count accuracy; (2) we re-photographed the specimen; and/or (3) we estimated tooth counts based on the spacing in the rest of the row in areas of glare or missing teeth.

We counted individual teeth in all five rows for 114 individuals. Correlations among row counts were then used to reduce the number of rows counted to three (A3 anterior, A3 posterior, and P2) for the remainder of the specimens, as counts for these rows were not as highly correlated (see results). Counts of these three rows were used in all subsequent analyses.

Gosner staging and length

Each tadpole was assigned a developmental stage based on a (modified) staging system developed by Gosner (1960). The unique and slow development of *Ascaphus* tadpoles (Brown, 1990; Brown, 1975) required that we modify the original Gosner staging system, accommodating distinctive developmental features in *Ascaphus*, such as the late reduction of the suctorial disc and oral labia (A. Cicchino, unpubl.). We used ImageJ to measure the length of each tadpole (tip of snout to tip of tail) from the lateral photographs; each tadpole was measured twice and the values were averaged.

Environmental predictor variables

Benthic substrate structure.—At each stream reach we collected data on benthic substrate across three transects where *Ascaphus* tadpoles were found (i.e., in *Ascaphus* habitat). At each transect, we used a gravelometer to classify 30 randomly sampled pieces of substrate into one of 15 classes (ranging from 2 mm to greater than 180 mm) for a total sample size of 90 pieces of substrate per site. We calculated two metrics from these data: average of substrate sizes, and standard deviation of substrate sizes. For both metrics we treated the >180 mm size class as 180 mm to maintain equal sample sizes.

Canopy cover.—We measured canopy cover using a convex spherical crown densiometer from the center of each of the three transects established for benthic substrate structure. We counted the number of squares out of 24 that were not occupied by canopy while facing upstream, downstream, to the right bank, and to the left bank. Counts were averaged to provide a measure of canopy openness for the site, which we then converted to percent canopy cover using a linear model provided by the densiometer manufacturer (Forestry Suppliers Densiometer No. 43887).

Annual temperature.—Annual temperatures were calculated from continuous temperature loggers (Onset Hobo Pendants UA-001-064) installed at each site. Data loggers were installed at the upstream and downstream ends of the stream reach using rebar pounded into the substrate. Data loggers were protected from debris and sunlight using a PVC housing drilled with holes to allow water flow. Temperature was recorded every 4 hours. Annual temperature for most sites was calculated on either a 2016-2017 or 2017-2018 water year (October 1 - September 30), although some sites used a different time frame or were missing small amounts of data (see Table S1 for dates and loggers used in calculations for each site).

Analysis

Gosner stage.—We first determined if we could treat Gosner stage as continuous in our models, rather than as an ordered factor. For each of the three labial tooth rows, we ran two GLMs treating Gosner stage as either a continuous or ordinal variable. We checked the goodness of fit of the GLM with a Poisson error distribution (suitable for count data) using a chi-square test based on the residual deviance and degrees of freedom. If the Poisson distribution did not fit the data, we used a negative binomial distribution fit with the R package *MASS* (Venables and Ripley, 2002). We then compared the models using a likelihood ratio test, AIC, and BIC. We ran this analysis separately for each species on each labial tooth row.

Relationship between number of teeth and Gosner stage.—We used GLMs (again fit as either Poisson or negative binomial based on goodness of fit tests) to investigate the relationship between the number of teeth in each row and Gosner stage for each species. We then calculated a pseudo-R^2 for each model (Zuur et al., 2009).

Relationship between number of teeth, species, site, and environmental variation.—From the set of three environmental predictor variables, we selected predictors for each species that minimized pairwise correlations ($|r| < 0.75$). All correlations and subsequent statistical analyses were performed using R v. 3.5.3 (R Core Team, 2020). We investigated variation in tooth counts by species, site, latitude (as UTM Northing), and environmental predictors using GLMM models fit with the *lme4* R package v. 1.1-20 (Bates et al., 2015). We tested for overdispersion of Poisson models (using a function provided by B. Bolker: https://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#overdispersion) and fit negative binomial models to overdispersed counts. In both cases, we fit models using a Laplace Approximation with two random effects fit as intercepts: Gosner stage (all models) and site (all models except site effect models). For site effect models, we conducted post-hoc tests of pairwise site comparisons using the R package *emmeans* v. 1.4.5 (Lenth, 2020), using the Tukey method for p-value adjustment. For the environmental models, we first fit a full model to the scaled environmental predictors, then iteratively removed uninformative predictors based on a p-value cutoff of 0.05. We compared these models using AIC and calculated marginal R^2 values (with the trigamma estimate) using

the *MuMIn* R package v. 1.43.15 (Barton, 2019). We also checked for a relationship between tadpole size (measured as length) and Gosner stage across species using a linear model.

Visualizations of tooth counts.—Because Gosner stage accounts for a significant amount of the variation in tooth counts in all rows (see results), we corrected tooth counts for Gosner stage in order to visualize relationships between species and predictor variables. Treating each species separately, we adjusted each count to the median Gosner stage using species-specific slopes (e.g., Lemmon, 2009). Note that these visualizations do not replicate GLMM modeling results, but instead provide a more simplified visualization of the major relationships.

RESULTS

Labial teeth data collection

All specimens had identical LTRF values for the number of anterior rows (3), but varied in the number of posterior rows (7-9). The number of posterior rows was significantly correlated with Gosner stage in both *A. truei* (Spearman rho = 0.48, p-value < 0.0001) and *A. montanus* (Spearman rho = 0.71, p-value < 0.0001). All specimens had a medial gap in the P1 row and were biserial in rows A2, A3, and P1. All 30 individuals from the three British Columbia sites also had a biserial P2 row (Table S2).

Based on correlations in counts across the five rows of 114 individuals, we reduced the number of rows counted to A3 anterior, A3 posterior, and P2 for the remainder of the specimens. Rows P1 and P3 were dropped since they were highly correlated with P2 ($r > 0.95$ in both cases). P2 was retained over P1 because it was not biserial; P2 was retained over P3 because the P2 row photo quality was generally higher than P3 (e.g. depth of field was superior/least blurry at the edges of the oral disc).

Environmental predictor variables

Annual temperatures and canopy cover reflected the coastal and continental climates of *A. truei* and *A. montanus*, respectively, with substrate size showing less variation between regions (Fig. 4). For both species, we retained predictor variables that were correlated at $|r| < 0.75$ (Table S3). For *A. truei*, we retained canopy cover, average substrate size, and the standard deviation of substrate size. We selected canopy cover instead of average temperature ($r = 0.80$) because our hypothesis for canopy cover was more mechanistic. For *A. montanus*, we retained average substrate size, canopy cover, and annual temperature.

Analysis

Gosner stage.—In both species, a Poisson GLM fit the A3 anterior and P2 data, while a negative binomial GLM was the best fit for the A3 posterior data. For the A3 anterior data, treating Gosner stage as a continuous variable was the better model according to AIC and BIC, with the likelihood ratio test showing no significant difference between the models. For the A3 posterior row, treating Gosner stage as a continuous variable was the better model according to BIC, with strong support for both models based on AIC. The likelihood ratio tests for A3 posterior showed a significant difference between the models for both *A. truei* ($p = 0.01$) and *A. montanus* ($p = 0.04$). In both species, the P2 data had the least support for a continuous treatment, with BICs in

support of continuous treatment, but AIC and likelihood ratio tests with little to no support (full results in Table S4). Given these mixed results, we chose to treat Gosner stage as continuous for the remainder of analyses to simplify model fitting and interpretation.

Relationship between number of teeth and Gosner stage.—In both species and across all three rows, Gosner stage explained a significant proportion of the variance in tooth counts, with number of teeth increasing as Gosner stage increased. Gosner stage explained between 48-73% of the variation in labial teeth counts in *A. truei*, and 53-65% of the variation in *A. montanus*. For both species, the most variance was explained in row P2 and the least variance in row A3 posterior (Table 1).

Relationship between number of teeth, species, site, and environmental variation.—There were significant differences in tooth counts across species, with *A. truei* having higher counts in every row compared with *A. montanus* after accounting for Gosner stage and site (Fig. 5, Table S5). We checked to see if these differences were due to overall larger *A. truei* tadpoles for a given Gosner stage. We found that, among tadpoles of the same Gosner stage, *A. truei* and *A. montanus* tadpoles did not significantly differ in length ($p = 0.056$, Fig. S1, Table S5); in fact, *A. montanus* were, on average, slightly (though not significantly) larger than *A. truei* tadpoles.

In both species, there were at least three (and up to eight) significant ($p < 0.05$) pairwise site-specific differences in tooth counts among sites and across tooth rows after accounting for Gosner stage (Tables S6-S8, Figs. S2 and S3). One model (*A. montanus* A3 posterior negative binomial GLMM) failed to converge due to overparameterization, so parameter estimates should be viewed with caution (Table S6). More variation was explained at the site level in *A. montanus* (37% of the variance averaged across rows) compared to *A. truei* (13% of the variance on average, Table S6). We found weak signatures of a latitudinal effect in both species. In *A. montanus*, which has a relatively small latitudinal distribution (our sampling covers less than two decimal degrees in latitude: 46.135564° to 47.885787°) only the P2 row showed a significant relationship with latitude ($p = 0.022$) with 11.5% of the variance explained (Table S9, Fig. S2). In *A. truei*, which covers a much larger latitudinal distribution (almost 14 decimal degrees across our sampling distribution: 40.887692° to 54.604844°), only the A3 posterior row showed a significant relationship with latitude ($p = 0.005$), with only 3.6% of the variance explained (Table S9, Fig. S3).

In *A. truei*, canopy cover was always the most important (and only significant) predictor variable for all teeth rows (Table 2 and S10). Coefficients for canopy cover were always positive (Table 3 and S10). Marginal R^2 values were low for *A. truei* and ranged from 3-4%. In *A. montanus*, average benthic substrate size was always the most important (and always significant) predictor variable for all teeth rows, with canopy cover also significant in explaining P2 counts (Table 2 and S10). Coefficients for substrate size were always positive, while the canopy cover coefficient in the P2 model was negative (Table 3 and S10). Marginal R^2 values for the final models in *A. montanus* ranged from 19-24%. The "final models" (Table 3) were selected as the most parsimonious model where all predictors had significant coefficients ($p < 0.05$); model parameters for all candidate models are provided in Table S10. A visualization of the relationship between the Gosner-corrected A3 anterior tooth counts and most important predictor in each species is provided in Fig. 6.

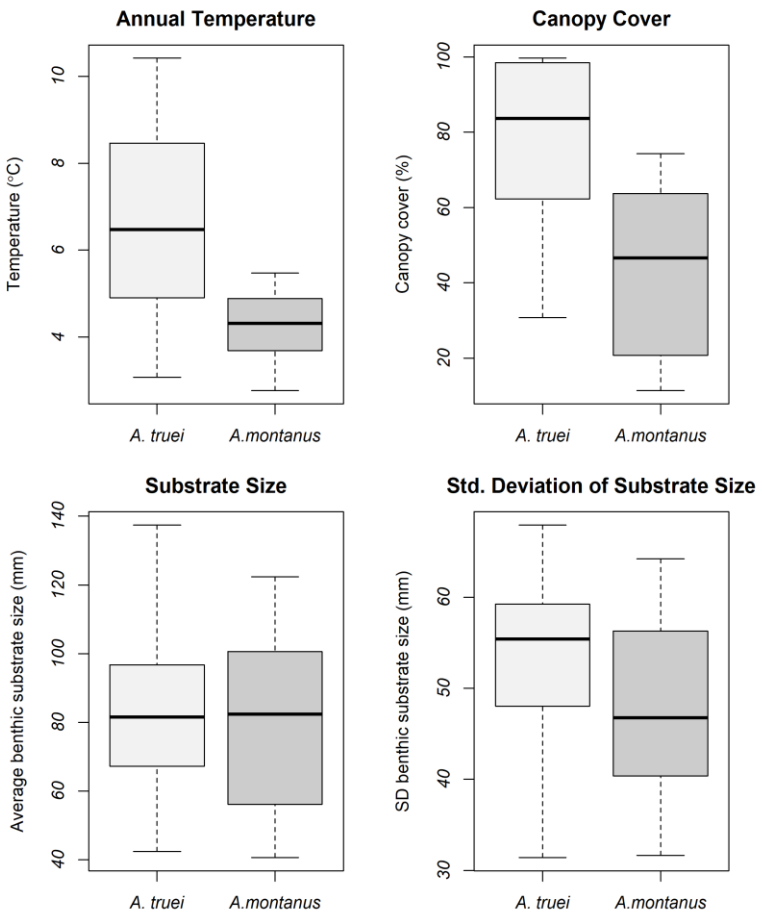

Figure 4. Boxplots of predictor variables for *Ascaphus truei* and *A. montanus*. The whiskers represent the maximum and minimum values.

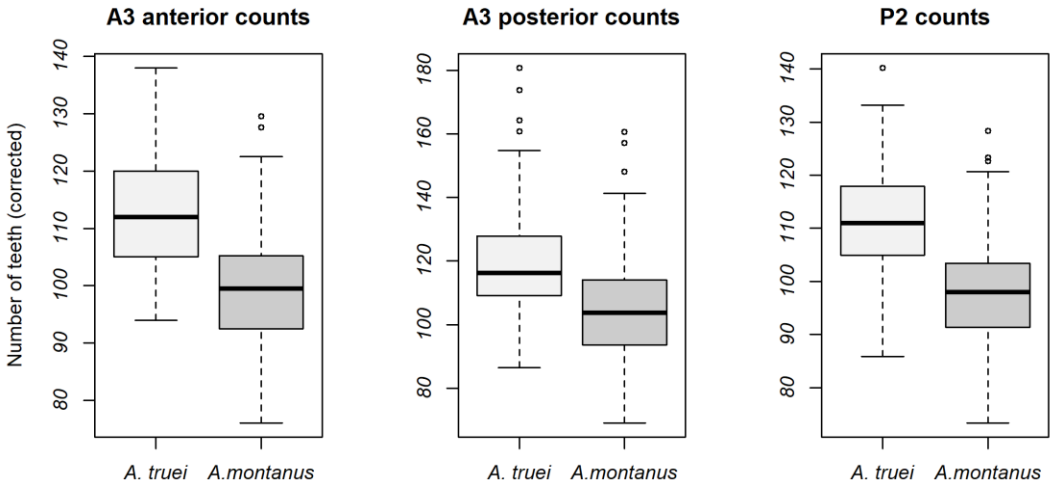

Figure 5. Boxplots of Gosner-corrected tooth counts for *Ascaphus truei* and *A. montanus*. The whiskers extend to 1.5 times the interquartile range from the first and third quartiles; open circles are beyond this range.

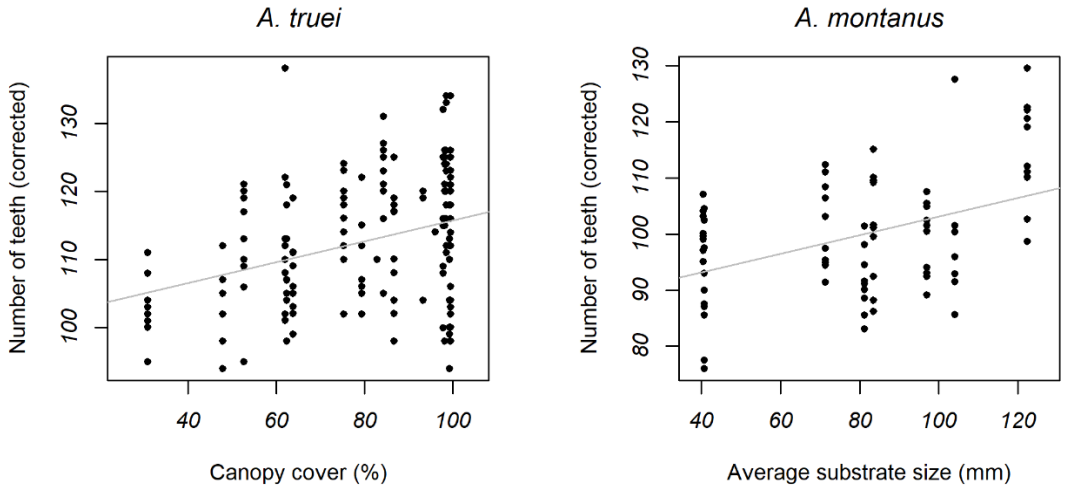

Figure 6. Scatterplots between Gosner-corrected A3 anterior tooth counts for *Ascaphus truei* and canopy cover (left), and *A. montanus* and average benthic substrate size (right). Gray lines represent linear model fit.

Table 1. GLM model results for tooth row counts and Gosner stage.

Species	Tooth Row	Model fit	Parameter	Coefficient estimate	Coefficient std. error	z-value	p-value	Model pseudo R^2
Ascaphus truei	A3 anterior	poisson	Intercept	3.411	0.094	36.20	< 0.0001	0.606
			A3 anterior	0.037	0.003	13.80	< 0.0001	
	A3 posterior	negative binomial	Intercept	3.160	0.132	23.96	< 0.0001	0.483
			A3 posterior	0.046	0.004	12.15	< 0.0001	
	P2	poisson	Intercept	2.770	0.097	28.70	< 0.0001	0.728
			P2	0.055	0.003	20.04	< 0.0001	
Ascaphus montanus	A3 anterior	poisson	Intercept	3.030	0.155	19.56	< 0.0001	0.556
			A3 anterior	0.046	0.005	10.15	< 0.0001	
	A3 posterior	negative binomial	Intercept	2.354	0.245	9.62	< 0.0001	0.532
			A3 posterior	0.067	0.007	9.34	< 0.0001	
	P2	poisson	Intercept	2.522	0.158	15.93	< 0.0001	0.651
			P2	0.060	0.005	13.05	< 0.0001	

Table 2. Mixed effects candidate models tested for each species; all models include Gosner stage and site as random effects.

Species	Tooth Row	Model fit	Model	AIC	Δ AIC	Marginal R^2 (trigamma)
Ascaphus truei	A3 anterior	poisson	percent canopy	1198.9		0.0408
			percent canopy + average substrate	1200.8	1.9	0.0416
			percent canopy + average substrate + SD substrate	1202.7	3.8	0.0417
	A3 posterior	negative binomial	percent canopy	1353.5		0.0294
			percent canopy + SD substrate	1355.1	1.6	0.0319
			percent canopy + average substrate + SD substrate	1357.1	3.6	0.0319
	P2	poisson	percent canopy	1191.1		0.0327
			percent canopy + average substrate	1192.1	1.0	0.0356
			percent canopy + average substrate + SD substrate	1193.5	2.4	0.0381
Ascaphus montanus	A3 anterior	poisson	average substrate	600.67		0.1910
			percent canopy + average substrate	601.61	0.9	0.2028
			percent canopy + average temperature + average substrate	603.57	2.9	0.2067
	A3 posterior	negative binomial	average substrate	675.64		0.1913
			average temperature + average substrate	677.55	1.9	0.1975
			percent canopy + average temperature + average substrate	679.49	3.9	0.1974
	P2	poisson	percent canopy + average temperature + average substrate	590.71		0.2376
			percent canopy + average substrate	591.33	0.6	0.2385

Table 3. Final models selected from candidate lists (Table 2).

Species	Tooth Row	Final model	Parameter	Fixed effect estimate	Fixed effect std. error	Fixed effect z-value	Fixed effect p-value	Marginal R^2 (trigamma)
Ascaphus truei	A3 anterior	percent canopy	intercept	4.683	0.035	133.11	< 0.0001	0.0408
			percent canopy	0.031	0.010	2.99	0.003	
	A3 posterior	percent canopy	intercept	4.731	0.047	100.51	< 0.0001	0.0294
			percent canopy	0.035	0.014	2.42	0.016	
	P2	percent canopy	intercept	4.655	0.051	90.67	< 0.0001	0.0327
			percent canopy	0.037	0.013	2.82	0.005	
Ascaphus montanus	A3 anterior	average substrate	intercept	4.580	0.034	134.62	< 0.0001	0.1910
			average substrate	0.067	0.021	3.23	0.001	
	A3 posterior	average substrate	intercept	4.617	0.053	87.67	< 0.0001	0.1913
			average substrate	0.097	0.028	3.51	0.0004	
	P2	average susbstrate + percent canopy	intercept	4.555	0.043	106.66	< 0.0001	0.2385
			average substrate	0.055	0.022	2.53	0.011	
			percent canopy	-0.050	0.020	-2.45	0.014	

DISCUSSION

Research into the extraordinary diversity of anuran tadpole mouthparts has played an important role in clarifying the phylogenetic relationships of this large group (Altig, 2006; Vera Candioti and Altig, 2010), as well as the kinematics of the complex oral apparatus (e.g., Venesky et al., 2013; Venesky, Wassersug, & Parris, 2010; Wassersug & Yamashita, 2001). There has been less investigation of variation within species, however, especially in the context of environmental selection. In this study, we investigate intraspecific variation in labial tooth counts in both species of tailed frogs across multiple populations. Overall, we find significant relationships between tooth counts and site-specific environmental predictors despite significant relationships with Gosner stage. We also find differences both in overall counts and the environmental predictors that are most important between species.

Despite the broad geographic sampling across the two species, the labial tooth row formula (LTRF), was relatively consistent, with variation only in the number of posterior rows. As in many other species (Bresler and Bragg, 1954; Conradie and Conradie, 2015), much of this variation in row number was explained by size/developmental stage, with the number of posterior rows increasing as Gosner stage increased. Interestingly, all 30 tadpoles from British Columbia had a biserial P2 row, which was not found in any of the other 210 *Ascaphus* individuals, and may reflect either adaptation to local conditions or fixation of morphological variation at the northern range boundary of the species. Notably, although sample sizes were small at the *A. truei* range edges (n=3 sites in each of British Columbia and California), two of the three streams sampled in British Columbia were wider, deeper, and had more large benthic

substrate than other streams sampled across the *A. truei* range (Fig. S4), indicating that this variation in biserial rows may be functionally relevant.

Ascaphus truei had significantly more teeth in all three labial tooth rows than *A. montanus*, perhaps in response to the overall higher canopy cover, and presumed lower in-stream productivity, at sites across British Columbia, Oregon, and California. Indeed, across *A. truei* sites, canopy cover was the most important predictor of labial tooth counts in all rows. Marginal R^2 values were low (3-4%), however, indicating that canopy cover explained only a small amount of the variation in labial tooth counts after accounting for Gosner stage and site. Additionally, canopy cover was highly correlated with annual temperature at *A. truei* sites ($r = 0.80$), so temperature differences, diatom community composition, or some unmeasured environmental characteristic that is correlated with temperature and canopy cover may also contribute to differences in counts across sites. The alternative hypothesis of latitude as a driver of site-specific variation was not well supported, with only one row (A3 posterior) showing a significant relationship.

By contrast, environmental predictors explained much more variation in *A. montanus*, with marginal R^2 values between 19-24%. For this species, average benthic substrate size was the most important predictor of labial tooth counts in all rows, with tooth counts and benthic substrate size showing a positive relationship. Uncovering the functional mechanism behind this relationship will require further study. For example, if variation in oral morphology, such as the presence of medial gaps (Annibale et al., 2019) or fewer teeth per row, can increase mobility and flexibility in the tooth rows or oral disc, reductions in tooth numbers could improve feeding efficiency on smaller substrate sizes. Similarly, if streams with smaller substrate sizes are associated with lower stream flow, it is possible that fewer teeth would be needed to anchor tadpoles to the substrate. Though this simple substrate-flow relationship is not generalizable for all stream systems, across our Montana sites we found a strong correlation between substrate size and modelled average stream flow (Wenger et al., 2010), providing some support for this mechanism (Pearson $r = 0.62$, $p < 0.0001$). Interestingly, the P2 row model also includes a significant negative relationship with canopy cover, in contrast to the positive relationships with canopy cover in *A. truei*. As was seen in *A. truei*, latitude as an alternative hypothesis for site-specific variation was not well supported, with only one row (A3 posterior) showing a significant relationship.

There are other biotic environmental variables, such as disease, competition, and predation, that could explain variation in both labial tooth counts and overall mouthpart morphology for which we do not have data. For example, infection with *Batrachochytrium dendrobatidis,* the fungus that causes the disease chytridiomycosis, can cause damage to tadpole mouthparts (Hanlon et al., 2015). The intraspecific variation we found here is unlikely to be explained by *Bd* infection, however, given that damage to keratinized mouthparts was not noted in this study. Additionally, previous work has found *Bd* at zero to very low prevalence in *A. montanus* (0/226 tadpoles and adults) and *A. truei* (3/226 tadpoles and adults) (Hossack et al., 2010). More likely additional causes of variation in our study are community level characteristics such as competition and predation. Studies on anuran larval morphology have uncovered complex evolutionary interactions among tadpole morphology, community assemblage/competitive interactions, and predation pressures, leading to possible niche specialization (e.g., Relyea, 2000; 2004; Van Buskirk, 2009). For example, an experimental study in wood frog tadpoles found that competition and predation cues caused direct and

interacting plastic effects on mouthpart morphology, including changing oral disc and jaw sheath size and tooth row length (Relyea and Auld, 2005). Quantifying tadpole density and site-level predator communities will be an important follow up step in this research. For example, we know that tadpole density and predator presence/absence and community composition varies across both species and sites (author field observations, unpubl.; also reviewed in Hayes and Quinn, 2015). Previous work has also demonstrated that *A. truei* tadpole behavior changes in response to predator cues (Feminella and Hawkins, 1994).

In conclusion, despite a very strong relationship between labial tooth counts and Gosner stage, we find significant relationships among environmental predictors and the labial tooth variation between species and across sites. Experimental tests will be needed to confirm functional relationships between environmental variation and differences in oral morphology, in addition to partitioning genetic from plastic mechanisms.

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DATA ACCESSIBILITY

Supplemental material will be available at **Dryad Repository ##**.

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SUPPLEMENTAL FIGURES

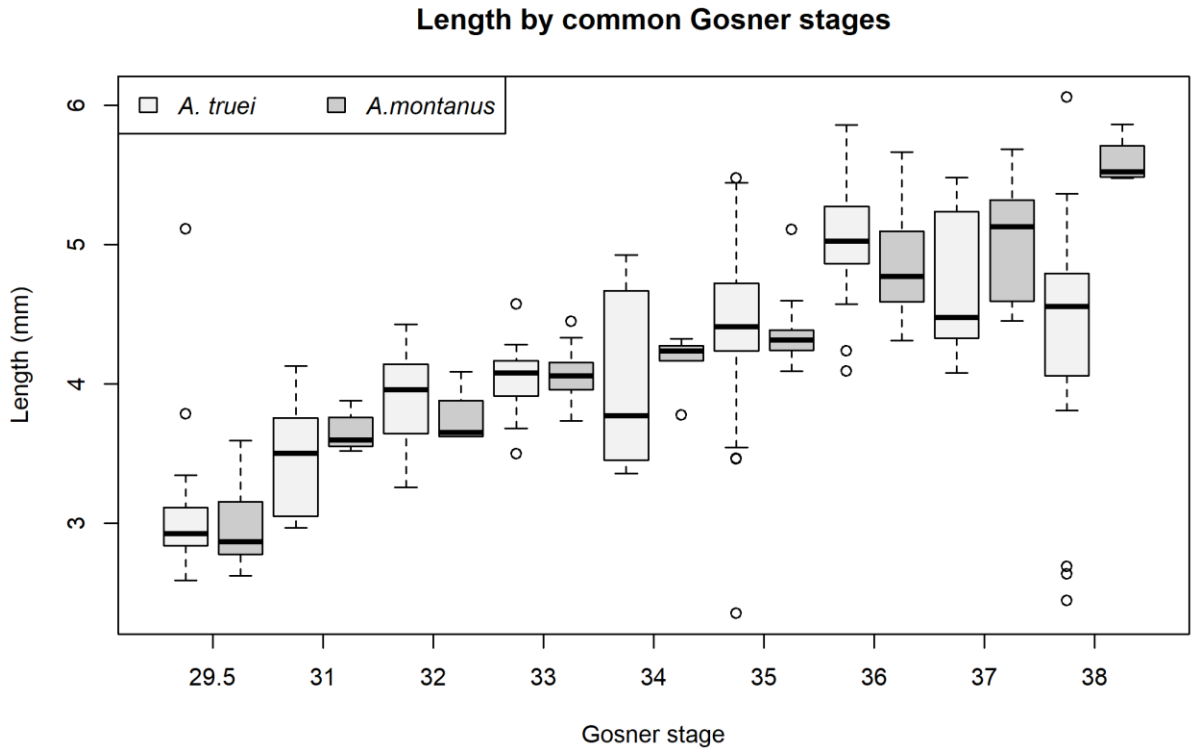

Figure S1. Paired boxplots of tadpole length by Gosner stages in common between *Ascaphus truei* (light gray) and *A. montanus* (dark gray). The whiskers extend to 1.5 times the interquartile range from the first and third quartiles; open circles are beyond this range.

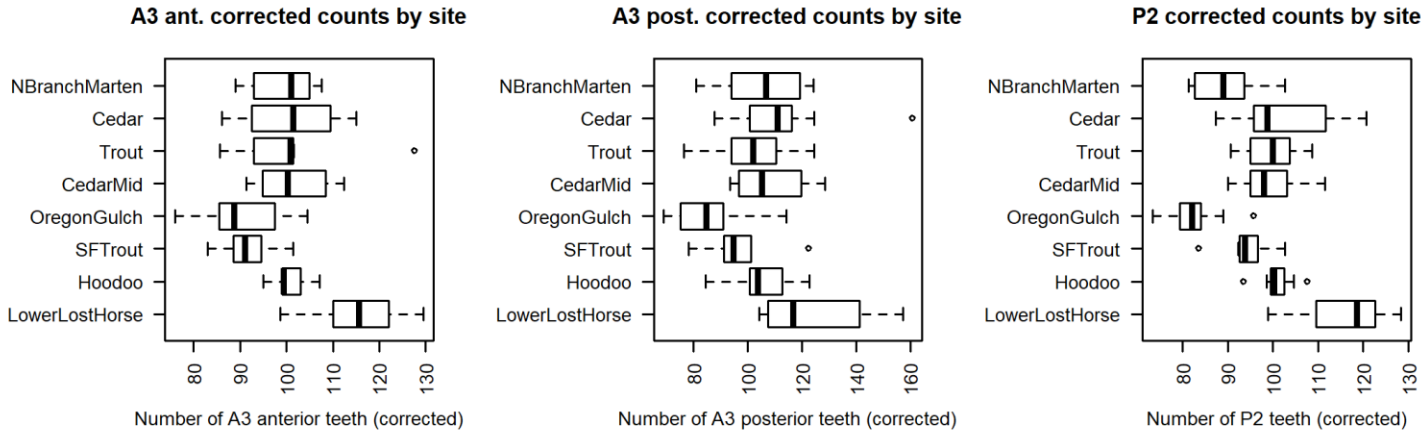

Figure S2. Boxplots of Gosner-corrected tooth counts for *Ascaphus montanus* across sites sorted from north (top of y-axis) to south (bottom of y-axis). The whiskers extend to 1.5 times the interquartile range from the first and third quartiles; open circles are beyond this range.

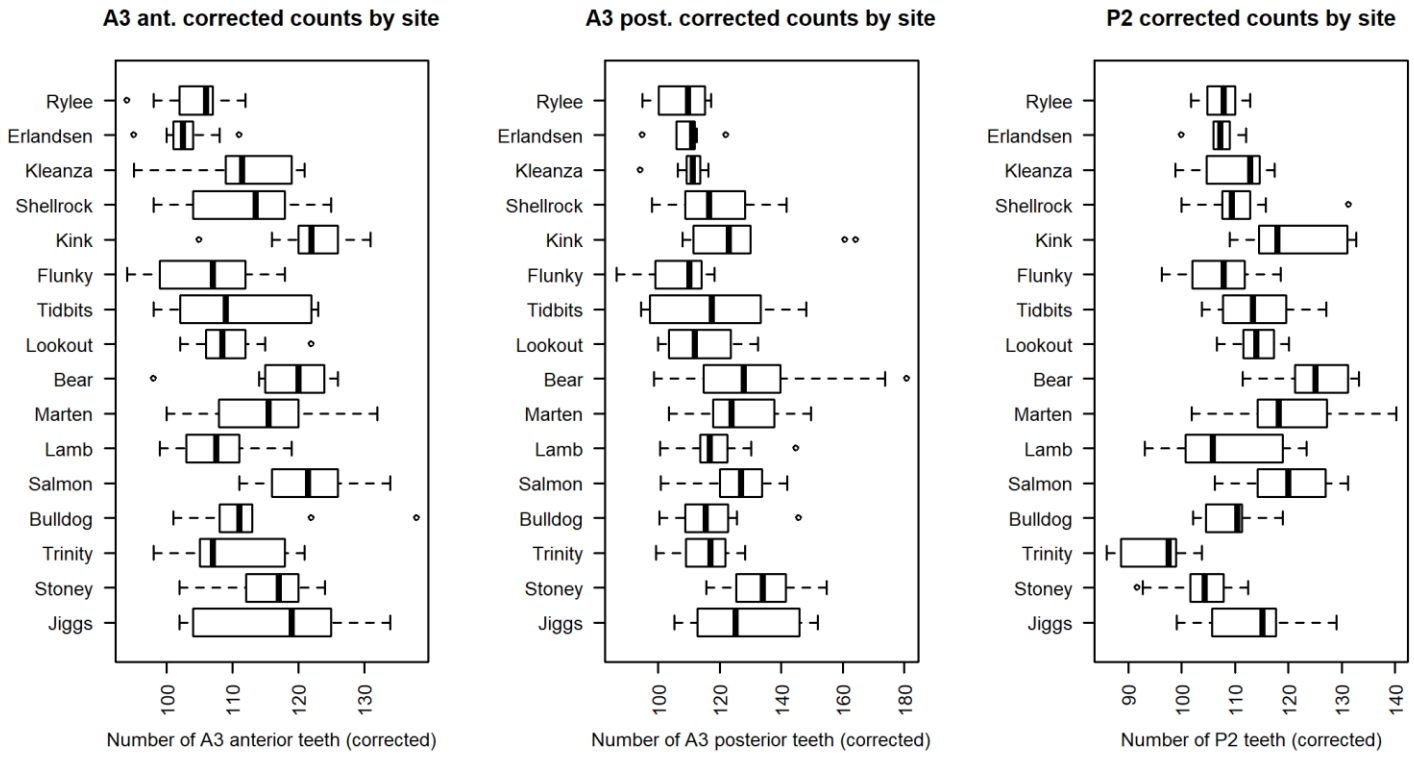

Figure S3. Boxplots of Gosner-corrected tooth counts for *Ascaphus truei* across sites sorted from north (top of y-axis) to south (bottom of y-axis). The top three sites are in British Columbia; the bottom three sites are in California; the remainder of the sites are in Oregon. The whiskers extend to 1.5 times the interquartile range from the first and third quartiles; open circles are beyond this range.

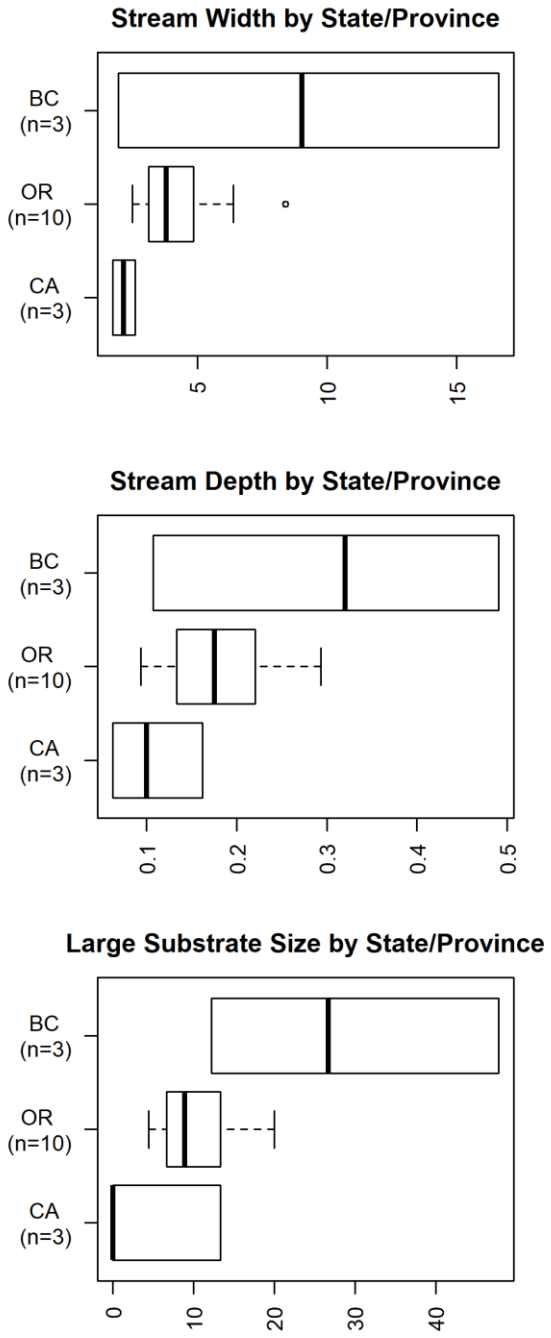

Figure S4. Boxplots of stream width (top), stream depth (middle), and percentage of substrate size in the largest size class (bottom) for *Ascaphus truei* sites distributed across the latitudinal sampling gradient: British Columbia at the northern range boundary (n=3 sites); Oregon at the center of the latitudinal distribution (n=10 sites); and California at the southern range boundary (n=3 sites). The whiskers extend to 1.5 times the interquartile range from the first and third quartiles; open circles are beyond this range. Note that two of the three sites in BC have parameter values beyond those in any stream sampled in OR or CA.

SUPPLEMENTAL TABLE CAPTIONS

Table S1. Temperature logger metadata.

Table S2. Basic count statistics for *Ascaphus truei* and *A. montanus* samples.

Table S3. Environmental predictor correlation tables for *Ascaphus truei* and *A. montanus* sites.

Table S4. AIC, BIC, and likelihood ratio test results for GLM models of tooth count by Gosner stage, fit as continuous and ordinal.

Table S5. GLMM model results for differences in tooth counts between species, including follow up test for differences in length between species for a given Gosner stage.

Table S6. GLMM model results for tooth row counts and site; p-values below 0.05 are highlighted in green.

Table S7. Post-hoc pairwise comparisons of GLMM model results for tooth row counts and site for *Ascaphus truei*; p-values below 0.05 are highlighted in green.

Table S8. Post-hoc pairwise comparisons of GLMM model results for tooth row counts and site for *Ascaphus montanus*; p-values below 0.05 are highlighted in green

Table S9. GLMM model results for tooth row counts and latitude.

Table S10. GLMM model results for tooth row counts and environmental predictors.