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Spatiotemporal patterns of dissolved free amino acids in New England rivers could be unique and stable odor signatures for imprinting and homing by Atlantic salmon

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Abstract

The phenomenon of homing by salmonid fishes to natal sites for breeding is well-established, but the chemicals in rivers that they learn as juveniles and identify as adults are not fully determined. Dissolved free amino acid (DFAA) profiles have been hypothesized to allow salmonids to distinguish their natal river from others nearby. To evaluate this hypothesis, we sampled DFAAs in spring and fall (when juveniles learn and adults return, respectively) from three rivers in New England, USA that support landlocked Atlantic salmon, *Salmo salar*. We used three approaches to determine the level of consistency between seasons and difference between rivers that would be needed for DFAA to support imprinting and subsequent homing for reproduction: hierarchical cluster analysis, statistical difference tests, and equivalence tests. DFAAs were not detected in the water column of the study rivers, but sediment porewater samples yielded DFAAs at measurable concentrations. Hierarchical cluster analysis, difference testing, and equivalence testing all indicated that some combinations of sediment porewater DFAA concentrations differed among rivers and were similar between spring and fall within a river. Specifically, equivalence tests revealed subsets of sediment porewater DFAAs that were seasonally equivalent within each river and none of the seasonally equivalent DFAAs were common among all three rivers (i.e., each river had a unique DFAA profile). However, exceptions detected in the cluster analysis and equivalence testing raise questions regarding the extent to which DFAAs might be sufficient for salmon imprinting and homing. Thus, DFAAs may fulfill some of the hypothesized prerequisites as salmon imprinting and homing odor cues, but our lack of understanding of salmon discriminatory abilities and limited DFAA data preclude definitive conclusions about the sufficiency of DFAAs alone as homing cues.

Keywords: natal, migratory, diadromous, pheromone, olfactory, pore water, hyporheic, odorant

Introduction

Salmonid fishes (here, for simplicity, referred to as salmon) undertake long-distance foraging migrations and return, with great fidelity, to natal rivers to spawn (Thorstad et al. 2010, Quinn 2018). In a process that has been documented throughout the many salmonid species, juveniles imprint on odors unique to their home river during critical stages of development, store this olfactory information in long-term memory, and during the riverine portion of their spawning migration, maturing salmon then return to their natal tributaries using olfactory signals (Hasler and Scholz 1983). According to the olfactory imprinting hypothesis, each salmon-bearing river must have an odor profile that is both spatially unique (i.e., distinguishable from other rivers in the same system), and temporally stable, across seasons and years (Hasler and Wisby 1951, Hasler and Scholz 1983). Despite extensive study, critical gaps in our understanding of the mechanisms of olfactory imprinting and navigation remain (reviewed by Bett and Hinch 2016). Notably, the specific odorants used by salmon for imprinting and homing have not yet been identified.

Salmonid olfactory neurons respond to several classes of chemical stimuli, including amino acids, steroids, bile acids, prostaglandins, and minerals (Bett and Hinch 2016). Several of these compounds have been hypothesized to be odorants that guide homing salmon to their natal sites. Some compounds are likely to remain very stable seasonally and inter-annually (e.g., minerals [Bodznick 1978]) while for others, the temporal dynamics of compound concentrations and stability are less well understood (e.g. bile acids associated with the presence of conspecifics; dissolved free amino acids (DFAAs) associated with lotic biotic sources). Recent studies using electrophysiology, chemical ecology, and behavioral approaches, suggest that DFAAs may be imprinting odorants for various species of Pacific salmon (*Oncorhynchus* spp.)

including masu (*O. masou*), chum (*O. keta*), and landlocked sockeye salmon (*O. nerka*) (Sato et al. 2000, Shoji et al. 2000, Shoji et al. 2003, Ueda et al. 2007, Yamamoto and Ueda 2009, Ueda 2011, Havey et al. 2017). Further, many DFAAs are derived from sources (e.g., terrestrial plant material, algae, and bacteria, and fungi in aquatic and soil environments (Ishizawa et al. 2010; Thorp and Bowes 2017) that could be correlated with resource or habitat quality for salmonids (e.g., forested, forest leaf inputs, algae, bacteria, fungi) and thus a possible target of selection. These diverse biotic sources of DFAAs present a paradox with respect to salmon imprinting and homing. Given the number of DFAAs, their proportions and concentrations provide considerable scope for variation among streams. However, they are likely to vary seasonally and perhaps among years as well, presenting challenges for salmon exposed to them in the spring of one year and returning in the fall, for example, several years later.

Anadromous and landlocked forms of Atlantic salmon (*Salmo salar*) display high homing fidelity (Youngson et al. 1994, Harbicht et al. 2020) and rich population genetic structure that depends on homing (Tessier and Bernatchez 1999, Garcia de Leaniz et al. 2007). Few studies have investigated DFAAs as possible imprinting odorants for Atlantic salmon (Morin et al. 1989, Armstrong et al. 2022), and no comprehensive DFAA profile for any Atlantic salmon river has been established. Therefore, the unique concentrations and combinations of amino acids upon which Atlantic salmon may imprint remain unknown.

We conducted a spatiotemporal study to test whether DFAAs in three landlocked Atlantic salmon-bearing rivers in New England, USA were consistent with the requirements for imprinting and later homing for reproduction. These populations are similar in life history to anadromous populations other than their use of large lakes rather than the ocean for feeding (Hutchings et al. 2019). Specifically, they commonly spend one or two years feeding in streams

prior to downstream migration (e.g., Hutchings 1986), and thus would experience the odorants of the natal stream in all seasons before leaving (Regish et al. 2021). Previous studies have suggested that salmon may use the molar percentage (mol%) of DFAAs to discriminate among natural stream waters (Yamamoto and Ueda 2009, Yamamoto et al. 2013). Reasoning that the fish are more likely responding to the proportions of different DFAAs rather than absolute concentrations, we tested the hypothesis that the relative abundance, or mol%, of DFAA concentrations from the water column and sediment pore water in these rivers is stable across spring and fall seasons, and unique to each river and may therefore serve as an effective imprinting and homing signal. Our adoption of the mol% approach also facilitates comparison with the work by Yamamoto and Ueda (2009) and Yamamoto et al. (2013).

Methods

Study System and Focal Species

Sexually mature landlocked Atlantic salmon migrate from Sebago Lake and Lake Champlain into rivers in the fall (September – October) and reproduce (DeRoche 1976, Harbicht et al. 2020) in late October through November. Embryos are buried in gravel nests called redds (Hill et al. 2019). Embryos overwinter in the redds where they hatch and become alevins with attached yolk-sacs. Alevins rapidly develop, and once the yolk-sac is absorbed they emerge as fry in spring from the redd into the river (Prévost et al. 2020), where they feed until they migrate to the lake in the spring (May – June) as smolts that are one to three years old (Regish et al 2021). Once in the lake, smolts feed and grow to sexual maturity. Lake residence time can range from less than a year to three years in Lake Champlain and up to 9 years in Sebago Lake (Kalejs 2022). However, most fish reside in the lake for approximately 17 months. The average age at

127 reproduction for natural-origin salmon in Sebago Lake is 4.1 years (Hutchings et al. 2019) and
128 likely similar for natural-origin salmon in Lake Champlain.
129 High homing fidelity of landlocked Atlantic salmon to rivers in the Lake Champlain and
130 Lake Sebago watersheds was reported by (Harbicht et al. 2020) and (DeRoche 1976). Harbicht et
131 al. (2020) evaluated adult return rates of one year old smolts stocked into the Winooski River
132 and Bouquet River. These authors provided numbers of homing and straying fish observed
133 during the study and we calculated average homing and stray rates of 96% and 4%, respectively
134 (Table S1). DeRoche (1976) reported a multi-year marking study on landlocked Atlantic salmon
135 in Sebago Lake, Maine and concluded for the Crooked River that it, "... provided unquestionable
136 evidence that salmon have strong tendencies to home back to 'parent' streams at spawning time
137 whether they were produced naturally there or stocked directly into the streams as yearlings." [p.
138 31]. Given that the juveniles in these populations spend one or more years in the stream, migrate
139 to the lake in the spring, and return in the fall after one or more years in the lake, the odorant
140 signals must be consistent across seasons and years, and different among streams, to explain the
141 observed homing behavior.
142
143 *Sediment and water column sample collection for DFAAs*
144 We collected DFAA samples from the water column at eight sites on five different rivers
145 in New England used for spawning by landlocked Atlantic salmon. We sampled one site each in
146 the Huntington and Winooski rivers in Vermont and two sites in the Boquet River in New York,
147 all tributaries of Lake Champlain. We also sampled one site in Panther Run and three sites in the
148 Crooked River, tributaries of Lake Sebago in Maine (Fig. 1; Table 1). Although both watersheds
149 had a history of agricultural land use, they are currently dominated by forest, have been

150 glaciated, and in these broad contexts the rivers and their DFAA profiles should resemble other
151 salmon rivers, with the possible exception of regions with less agriculture.
152 We collected preliminary samples during the fall months of September, October, and
153 November of 2015 from the water column of the Boquet, Crooked, Huntington, Winooski, and
154 Panther Run (Table S2). At each site and each month, we collected triplicate samples from
155 approximately 5 cm below the water surface using a new, sterile 20 mL plastic Luer-Lock
156 syringe that was rinsed several times with river water at each site. Analyses from this first set of
157 samples indicated an absence of DFAA in the water column at or above our threshold of
158 detection (~1.0nM), prompting us to expand the parameters of our future sampling methods as
159 follows. In June (spring) and October (fall) of 2016, we returned to just the Huntington River and
160 collected triplicate water column samples from 5 cm below the water surface to assess seasonal
161 variation and for comparison with the October 2015 samples in which DFAA in the water were
162 not detected (Table X). Additionally, during June and October of 2016, we collected 6 water
163 samples from 5 cm below the surface of the riverbed sediments (hereafter, "sediment porewater"
164 samples) from the Huntington River (Table X). The 2016 sediment porewater samples were the
165 only samples containing DFAA above our 1.0 nM threshold of detection. Therefore, in both June
166 (spring) and October (fall) 2017, we proceeded with the primary sampling for this study by
167 collecting both water column and sediment porewater samples from three sites: the Huntington
168 River, one of the sites on the Crooked River, and a site on the North Branch of the Boquet River
169 (Fig. 1; Table S2). Each sediment porewater sampling site was in a pool just upriver of a riffle
170 where Atlantic salmon redds had been observed in the previous years. On each sampling date
171 and location, sediment porewater samples were collected from six replicate sites (spatially
172 independent), randomly determined along a transect perpendicular to streamflow, using a

modified PVC pipe we designed (Supplementary Fig. S1). For each site, the sampler was pushed ~5 cm deep into the sediments and the sterile syringe inside the PVC pipe collected 20 mL of water from within the interstitial spaces of fine sediments. Triplicate samples from the water column were collected as stated above.

For each sample, an individual syringe was removed from the benthic sampler for filtering and storage. To avoid contamination, nitrile gloves were worn during collection and equipment handling, and fresh gloves were exchanged between samples and before transfer of vials to dry ice. For all samples, approximately 0.75 mL of water was filtered through a 0.45 µm (nominal pore size) Millipore filter directly into pre-combusted (500 °C), pre-labeled 1.5 mL glass autosampler vials and immediately placed on dry ice. Frozen samples were stored in a -80°C freezer and analyzed within several weeks of collection. Some replicate samples were lost due to cracking of glass vials during freezing or transport resulting in 3 – 6 replicates per sampling unit (e.g., fall and spring in each river). These replicate samples (n=3-6) were used to calculate the seasonal means for each river and year.

DFAA analysis

Fluorescent derivatives of free amino acids with o-phthaldialdehyde (OPA) and mercaptopropionic acid (MPA) were analyzed on an Agilent 1260 liquid chromatography system following a modified Agilent Technologies procedure (Agilent Technologies 2017). A total of 5 µL of sample, 3 µL of 0.5 M borate buffer (pH = 9.50) and 2 µL of OPA/MPA reagent (pH = 9.50) were mixed in-line with a programmable autosampler for 2 min. After reaction, the pH of the solution was adjusted in-line to ~6 with a diluent. For detailed information on the preparation of reagents, see the supplementary information.

196 Two solvents, referred to here as A and B, were used to achieve the separation of
197 derivatized amino acids. Solvent A was 9.6 mM anhydrous sodium di-hydrogen phosphate and
198 9.7 mM boric acid adjusted to pH 8.15, then filtered through combusted (450 °C for 3 h) 47 mm
199 Whatman GF/F filters directly into combusted 1 L amber-glass solvent bottles. Solvent B was
200 methanol:acetonitrile:water (45:45:10 v/v). The column was a Rapid Resolution HT Eclipse Plus
201 C18 (4.6 x 50 mm, 1.8-µm) column with guard. Amino acid derivatives were separated with a
202 linear binary gradient starting with 98% A for 1 min, then 43% for 7 min, and 0% for 7.1 min.
203 After 7.4 min, the system was returned to 98% A and equilibrated for 0.2 min. Equilibration of
204 the system continued during the automated derivatization procedure preceding chromatography.
205 The flow rate was 2 mL min^{-1} at 40°C, and total run time was 9.6 min. Excitation for
206 fluorescence detection was set at 350 nm, and emission was recorded at 420 nm.
207 For quantification, varying concentrations of standards (n = 5) were measured and
208 response factors calculated excluding the origin. The procedural blank (Milli-Q Plus UV water)
209 was analyzed along with samples and subtracted from concentrations of amino acids. Identity of
210 amino acids was verified in selected samples (n = 2) during batch analysis by spiking a standard
211 amino acid mix at final concentrations of 20 nM. The limit of quantification was 0.8-1.1 nM for
212 individual amino acids. Methods using fluorogenic derivatives of free amino acids are among the
213 most sensitive methods available (compared to UV-absorbance, reflective light scattering or
214 mass spectrometry), and were also optimized for river environments.
215 Additional method-testing was performed to investigate blank contributions from the
216 filtration procedure, potential retention of amino acids on 0.45 µm Millipore syringe filters, and
217 matrix interference. Filtering was performed with Milli-Q Plus UV water or amino acid solutions
218 at 20 nM following the sample collection protocol described above, and there was no

219 contamination from filtering. Matrix interference was tested by spiking amino acid solutions to
220 samples (n = 6) at 20 nM final concentrations. The recovery of amino acids after filtration was
221 99.3 ± 2.5 % (n = 6). Spikes of amino acids in samples were recovered at 102.2 ± 3.1 % (n = 3),
222 indicating negligible matrix interference.
223
224 *Data analysis*
225 We calculated the relative abundance (mol%) for individual amino acids in each
226 replicate sample by dividing the concentration of an individual amino acid by the sum of all
227 amino acid concentrations in that replicate sample. Our primary analyses used relative
228 abundances of DFAAs rather than absolute concentrations because 1) behavioral responses of
229 aquatic animals are stimulated by DFAA mixture composition (e.g. Borroni et al. 1986; Bryant
230 and Atema 1987), 2) animals generally use concentration-invariant odor recognition of specific
231 compounds or specific ratios of ubiquitous compounds (Hildebrand and Shepherd 1997), 3)
232 variation in stream discharge could cause an odorant to vary greatly in intensity, or absolute
233 concentration, from day-to-day or year-to-year, whereas relative abundances may remain stable
234 and recognizable so long as the intensity is sufficient to stimulate receptor neurons, and 4)
235 absolute concentrations may indicate only the magnitude of olfactory signals while relative
236 abundances are more indicative of the suite of chemicals (e.g., odor profile) triggering receptors
237 for a corresponding odor (e.g., the smell of a skunk varies in intensity but the relative
238 abundances of chemicals create the recognizable odor). DFAA concentrations below the
239 threshold of 1.0 nM essentially had a concentration of zero, resulting in left-censored and zero-
240 inflated data distributions. To explore variation in relative concentrations (i.e., mol%), we
241 converted the 1 nM lower detection threshold to a mol% lower detection threshold for each

individual replicate as: sample-specific mol% lower detection threshold = 100 · 1.0 nM/total DFAA concentration in nM per replicate sample. We accounted for the effects of left censoring and zero inflated distributions (i.e., violating assumption of normality, bias in the mean, underestimating the standard error) using a tobit model (Tobin 1958) that considers values below 1 nM, or sample-specific mol% lower detection threshold, as censored data lying somewhere between zero and this threshold, and adjusts the variance accordingly (Lubin et al. 2004). All data were natural-log transformed, which stabilized the variance better than the logit or square-root transformations.

Spatiotemporal patterns of amino acids

To analyze spatiotemporal patterns of DFAAs in benthic samples of salmon-bearing rivers, we used three different approaches that either tested for similarities, differences, or equivalences in mol% of DFAAs among seasons and rivers. This three-pronged approach was developed to address the inherent complexities of the task faced by salmon, and the nature of the data. That is, from the perspective of salmon the mol% of DFAAs in a river need not be identical across seasons and years to be recognizable as the same river. However, mol% of DFAAs must be sufficiently similar to be recognized against the spatial variation among rivers, and we cannot be certain how much similarity is sufficient. Therefore, we used the three statistical approaches described below to evaluate these different aspects of the mol% of DFAAs salmon may use during imprinting and spawning.

First, we used a hierarchical cluster analysis to characterize the multivariate spatiotemporal patterns of mol% of DFAAs and thus test patterns of similarity among rivers and seasons. Hierarchical cluster analysis of amino acid content has been used for conceptually

similar analyses such as identifying the floral and geographical origin of honey (Cometto et al. 2003, Iglesias et al. 2004, Mohammed and Babiker 2010), coffee varieties (Casal et al. 2003), cheese ripeness (García-Palmer et al. 1997), and classifying wine (Hernández-Orte et al. 2002, Héberger et al. 2003). We calculated the mean of natural-log (X + sample-specific mol% lower threshold, as described above and applied consistently among the three analyses) for each DFAA within each river in each season, then conducted a hierarchical cluster analysis (Ward's minimum variance) with no additional standardizations to test for general similarity patterns among rivers and seasons (Romesburg 2004). We used inferences from multivariate analyses if concentrations of all replicate samples for a DFAA were zero (i.e., below 1 nM) for one or both seasons, because a value of zero for an individual DFAA is useful in a multivariate context and this multivariate analysis does not assume normality. We used a dendrogram to display the patterns and test the hypothesis that clusters would reflect temporal variation within rivers, and each river would be separate from the others.

Second, we used traditional difference testing to assess differences in the mol% of individual DFAAs between seasons within rivers (e.g., Boquet Fall vs Spring) as well as within seasons among rivers for each year sampled (Boquet Fall vs Huntington Fall; e.g., Fig. 2). This test is similar to analysis of variance (ANOVA) that has been used in other studies exploring spatiotemporal patterns of DFAAs in salmon-bearing rivers (e.g., Yamamoto et al. 2013). We conducted univariate two-sided t tests using the tobit model for left-censored, zero-inflated data using proc lifereg in SAS/STAT 15.1(SAS Institute Inc. 2014). Here, a two-sided t test was used to compare two means and test the null hypothesis that the difference between means is zero, or no difference between means ($\alpha = 0.05$). We used this difference testing approach in a comparative context to what has been done in prior studies, which in some cases was not valid

(e.g., inferences that means are similar based on no difference, $P > 0.05$). Difference tests can only be used to infer significant differences between means or reject the null hypothesis that the difference between means equals zero (e.g., $p < 0.05$), and do not provide support for the null being true. An advantage of difference tests is that they can be used for inferences of very small differences in means (Fig. 2a). However, if the hypothesis of interest is one of biological equivalence between means, which was our focus, traditional difference-based null hypothesis significance testing cannot provide such evidence. Data were treated similar to the multivariate analysis with two exceptions. If a DFAA was not detectable in all replicate samples within one season or river, we assumed the concentration of this DFAA was zero and performed a one-sample t test to determine whether the corresponding season or river-specific mean with detectable DFAA concentrations differed from zero. Whereas, if concentrations of all replicate samples of a DFAA were zero, or below 1 nM, for both seasons, between rivers, or were detected in < 3 samples from one season or river, then we did not apply univariate tests.

Difference tests are powerful and commonly used for evaluating the traditional "difference between means equals zero" null hypothesis significance test, but equivalence testing, our third approach, can distinguish between statistically significant and biologically relevant differences between means (Limentani et al. 2005, Davit et al. 2009, Rose et al. 2018). Moreover, it is unlikely that the true effect is a zero difference in DFAAs between seasons or rivers, but rather that the difference is too small to be biologically relevant. The null hypothesis of an equivalence test is that the means are different by at least some researcher-defined amount termed an equivalence limit (Fig. 2a). Thus, if an equivalence test rejects the null hypothesis that two means are different by at least that amount, then we infer that the two means are equivalent within that limit (the smallest effect size of interest). In contrast to difference testing that can

311 detect small differences between means that may not be functionally important, equivalence
312 testing provides some flexibility in how large a difference between means is deemed important.
313 For example, if Atlantic salmon cannot detect a 0.5 mol% difference in a particular amino acid in
314 river water, then differences within 0.5 mol% can be ignored or considered equivalent,
315 depending on the question. Equivalence testing is widely used in pharmaceutical studies (Davit
316 et al. 2009) to compare physiological responses to innovator and generic drugs, and is becoming
317 more common in ecological studies, such as evaluating equivalence of male and female bird
318 songs (Rose et al. 2018).
319 Here, we assessed whether the mol% for each DFAA were equivalent between spring and
320 fall and across rivers. Seasonal equivalence of mol% estimates for DFAAs was assessed by
321 specifying equivalence limits, or a difference in means between fall and spring that migrating
322 salmon likely perceive as different or, alternatively, within at least these limits that salmon
323 perceive as equivalent. Briefly, the equivalence test calculated an upper and lower confidence
324 interval for the difference in each DFAA mol% between seasons (i.e., fall minus spring). If
325 means and confidence intervals were fully contained within the region of equivalence, then
326 equivalence between means was concluded (i.e., the two one-sided null hypotheses are rejected)
327 (Fig. 2a). Whereas, if means and confidence intervals extended outside the region of
328 equivalence, then equivalence between means can be rejected (i.e., one or both of the one-sided
329 null hypotheses are not rejected) (Fig. 2a). We conducted univariate equivalence tests of the
330 mol% DFAAs between seasons and among rivers using the tobit model for left-censored, zero-
331 inflated data using proc lifereg in SAS/STAT 15.1 (SAS Institute Inc. 2018). For comparisons
332 between seasons or rivers in which concentrations of all replicate samples of a DFAA were zero
333 or were detected in < 3 samples for one season or river, we set one of the equivalence limits to

zero and tested for equivalence between zero and the non-zero equivalence limit that was determined as described below. Similar to difference tests, if concentrations of all replicate samples of a DFAA were zero, or below 1 nM, for both seasons, between rivers, or were detected in < 3 samples from one season or river, then we did not apply equivalence tests.

It is uncertain what concentration or composition of DFAAs is needed to convey the essential information about rivers for salmon homing, and how much variation can occur before water sources are perceived as not equivalent. To estimate an equivalence threshold for our analyses, we used data from Yamamoto et al. (2013) on DFAA composition in the Teshio River in Hokkaido, Japan, where chum salmon (*O. keta*) migrate to the ocean as juveniles in the spring, and then return to spawn in the fall several years later. The authors collected river water in the spring of 2005 and 2006, and the fall of 2009 and 2010, when salmon leaving in 2005 and 2006 would typically return. The authors created artificial stream water using the mean concentrations of 15 detectable DFAAs, representing each season and year. Adult Teshio River chum salmon preferred artificial stream water comprised of the DFAAs measured in the Teshio River in the spring and fall over an unfamiliar water source, but did not appear to distinguish between the artificial stream waters based on the spring or fall DFAAs (Yamamoto et al. 2013).

We used these DFAA data reported by Yamamoto et al. (2013) to establish equivalence thresholds for our study as they represent the only data we are aware of that represent seasonal and interannual DFAA differences in a single river where homing adult salmon return with high fidelity. The authors suggested that salmon treat these temporally different DFAA profiles as equivalent for homing. Therefore, we calculated the maximum proportional difference between the minimum and maximum mol% of each DFAA across all four collections (spring and fall of two different years) and used the median of the maximum proportional difference across all 15

DFAAs as our equivalence limits. The median of the maximum proportional difference was ± 38.8 %. Numerically, in the two one-sided tests this is expressed as a lower limit of 61.2 % for the spring mean < fall mean × natural-log of 0.612 and its reciprocal in the second test as an upper limit of 163 % for spring mean > fall mean × natural-log of 1.63. By convention the reciprocal is used because all data are expressed as test/reference ratio (Davit et al. 2009). One amino acid detected by Yamamoto et al. (2013), proline, was not measured in our sampling. Differences between means within these limits were considered equivalent (i.e., rejecting the two one-sided null hypotheses, Fig 2), and thus mean differences within 38.8% between seasons or among rivers were deemed equivalent to migratory salmon. These equivalence limits are larger than those typically used in pharmaceutical studies (20 %) testing equivalence of different drugs, but those studies analyze physiological blood levels of each drug and not abundances of compounds in the environment that are sensed by smell and taste as in this study. We also explored how sensitive our results were to the equivalence limits derived above (38.8 %, 0.612-1.63), by using a 20 % narrower limit (20 %, 0.80-1.25), common in pharmacology (e.g., Davit et al. 2009), and a 20 % wider limit (60 %, or 0.40-2.50).

Given the multiple comparisons of the 14 DFAAs within and among seasons and rivers, we controlled for the familywise error rate (false positive, or Type I error) of the difference-based t tests using a method similar to the Bonferroni developed by Holm (1979). Families for the error rate were grouped by paired comparisons between seasons within rivers and between rivers within seasons. For equivalence-based tests, the familywise error rate correction is based on the number comparisons for which a Type I error is most likely (Davidson and Cribbie 2020). In this case, a Type I error is when the means are deemed equivalent (i.e., difference in means < equivalence limits) when in fact they are not equivalent (i.e., difference in means > equivalence

limits). Mean differences that fall just outside the equivalence limits to within twice the equivalence limits are the most susceptible to Type 1 error (Fig. 2b). Mean differences greater than twice the equivalence limits are unlikely to be mistaken as equivalent and Type I error is not a concern. Error rate families were grouped the same as difference-based tests. Within each family the alpha value was divided by $k^2/4$, where k was the number of problematic cases in the family (Caffo et al. 2013). However, the number of equivalence tests susceptible to Type I error did not exceed two across all families of tests, which resulted in the corrected alpha equal to the original alpha of 0.05 ($k^2/4=1$). Further, we did not use omnibus multivariate (e.g., MANOVA) approaches because the sample size (maximum of six replicates per river per season) was less than the number of dependent variables (e.g., 14 DFAAs). However, the use of molar percentages as the response variable is multivariate in the sense that changes in concentration of one DFAA affect the proportions of the others.

Results

On all dates and at all eight sites in the five rivers surveyed during fall 2015, DFAA concentrations in water column samples were below our threshold of detection (~1.0 nM). Similarly, DFAA concentrations were below 1 nM in water column samples collected 5 cm below the water surface and 5 cm above the river bottom in June and October, 2016 at the Huntington River site. However, benthic samples contained all 14 analyzed DFAAs at detectable concentrations, and 7 of the 14 DFAAs were detected in samples at all sites. Most (70%) of the benthic DFAA sample concentrations were < 10 nM and 28 % were below the 1 nM threshold. Absolute concentrations (nM) of benthic DFAAs varied between seasons, years and rivers (Fig. 3), with mean seasonal values ranging from below our 1 nM threshold to as high as 132.5 nM

(glutamic acid in the Boquet River in fall, 2017). Despite this variation, several amino acids had consistently high concentrations. For example, glutamic acid had the highest concentration (54.5 ± 65.8 nM, mean ± 1 standard deviation) across seasons, rivers and years, except the Crooked River in Fall 2017. Alanine, arginine, and aspartic acid concentrations were also consistently high. In contrast, valine was only measurable in the Huntington River, and phenylalanine had the lowest mean concentration (3.2 ± 9.7 nM ± 1 SD) across seasons, rivers and years.

Absolute concentrations varied considerably (Fig. 3), but the relative abundances (mol%) of DFAAs varied less across seasons, within each river (Fig. 4). Mean seasonal values ranged from undetected to 41.4 mol% (glutamic acid in the Boquet River in fall, 2017). More than half (54 %) of the benthic DFAA relative abundances were < 5.0 mol% and 28 % were < 1 mol%. Glutamic acid had the highest relative abundance (29.3 ± 12.3 mol%, mean ± 1 SD) and the DFAAs with the lowest abundances were isoleucine (0.9 ± 1.5 mol%, mean ± 1 SD) and phenylalanine (0.5 ± 1.1 mol%, mean ± 1 SD), which were only detected in two rivers. Based on DFAA mol% data, hierarchical cluster analysis indicated a general grouping of benthic DFAAs by river, but also a number of exceptions (Fig. 5). Specifically, three of the Huntington River samples clustered together but the fall 2017 sample was within the other main cluster, including the samples from the Boquet and Crooked rivers. Within this latter cluster, in several cases samples from the same river in different seasons were not closely clustered (Fig. 5).

Difference tests revealed that 2 to 7 of the 14 DFAAs were significantly different ($p < 0.05$) in mol% across seasons within each river and year (Table S3; Fig. 4). Across all between-season comparisons 30 % (17 of 56 potential comparisons) were different; the specific DFAAs that differed seasonally varied among rivers (Fig. 4). In the Boquet River, 5 DFAAs were significantly different across seasons (aspartic acid, histidine, leucine, threonine, and tyrosine).

In the Crooked River, 2 differed (aspartic acid and tyrosine). In the Huntington River, 3 were different in 2016 (arginine, glutamic acid, and threonine), and 7 were different in 2017 (arginine, glutamic acid, histidine, leucine, lysine, phenylalanine, and tyrosine). No DFAAs were consistently different between seasons across all four river-year comparisons.

Tests for differences in DFAAs between rivers within a season (e.g., Boquet 2017 fall vs Huntington 2017 fall) indicated significant differences for 1 - 7 DFAAs (Table 2). For fall vs fall comparisons 33 % (14 of 42 potential comparisons) were significantly different, and 29 % of spring vs spring comparisons (12 of 42 potential comparisons) were significantly different (Table 2). Three DFAAs (glycine, lysine, and threonine) were significantly different across all three between-river fall vs fall comparisons, whereas no DFAAs were significantly different across all three between-river spring vs spring comparisons (Table 2). No DFAAs were consistently different across both between-river fall vs fall and spring vs spring comparisons (Table 2).

Equivalence tests revealed seasonal equivalence in DFAA mol% within rivers between seasons in many but not all cases (Table S4; Fig. 4). Across all between-season within-river comparisons 21 % (12 of 56 potential comparisons) were equivalent. In the Boquet River, four DFAAs were equivalent across seasons (alanine, aspartic acid, glutamic acid, threonine). In the Crooked River, two DFAAs were equivalent (arginine, threonine). In the Huntington River in 2016, four DFAAs were equivalent (alanine, arginine, isoleucine, valine) and in 2017, two DFAAs (aspartic acid and threonine) were equivalent across seasons. Each river contained a unique subset of seasonally equivalent, or stable, DFAAs, and none of the seasonally equivalent DFAAs were common among all three river-years.

Equivalence was also assessed between rivers within a season (i.e., Boquet fall vs Huntington fall; Boquet spring vs Huntington spring) because the imprinting and homing process

449 requires that odorants allow spawning salmon to differentiate between rivers in the fall as well as
450 allow river-specific imprinting by juveniles in the spring and homing in the fall. Equivalence
451 tests revealed that the mean mol% of 1 to 5 DFAAs were equivalent between rivers within a
452 season (Table 3). For fall vs fall comparisons 17 % (7 of 42 potential comparisons) of DFAAs
453 were equivalent, and for spring vs spring comparisons 26 % (11 of 42 potential comparisons) of
454 DFAAs were equivalent. Aspartic acid was equivalent for all between-river fall comparisons,
455 and threonine was equivalent for all between-river spring comparisons (Table 3). No individual
456 DFAA was equivalent between rivers for both spring vs spring and fall vs fall comparisons
457 (Table 3).
458 We also compared equivalence patterns of DFAAs between seasons and between rivers
459 (Tables S3 and 4; summarized in Table 4) and found that no DFAA was equivalent for all
460 between seasons comparisons (see Table S4) as well as equivalent for all between river
461 comparisons (Table 3). The percentage of DFAAs equivalent between seasons was 29 % (12 of
462 42 potential comparisons) and between rivers was 21 % (18 of 84 potential comparisons; Table
463 4). However, the subsets DFAAs equivalent between seasons (Table S4) versus the subsets
464 equivalent between rivers (e.g., fall vs fall) were different, or unique (Table 4).
465 The two univariate methods to evaluate seasonal stability of DFAAs (i.e., equivalence
466 test vs difference test with $p > 0.05$) differed in overall number of DFAAs identified as
467 seasonally stable. Difference tests identified more (62 %) seasonal DFAA comparisons as not
468 different than equivalence tests identified as equivalent (26 %). Similarly, for between-river
469 within-season comparisons, difference tests indicated 59 % of comparisons were not different
470 compared to 26 % equivalent based on equivalence tests, a 2.3x difference. Correspondence in
471 the number of between-season within-river DFAAs comparisons identified as having similar

means based on equivalence and difference tests ($p > 0.05$) was 20 % (7 of 35 comparisons that were either equivalent or not different) (Table 4; Fig. 4).

Using narrower equivalence limits, such as those used in pharmacology (e.g., 20 %), 3 DFAAs were equivalent between seasons across all river comparisons, or four times fewer seasonally equivalent DFAAs than using the equivalence limits (39 %) derived from salmon-bearing rivers. Using arbitrarily wider equivalence limits (60 %) indicated that 28 DFAAs were equivalent between seasons across all river comparisons, or 2.3x more seasonally equivalent DFAAs compared to limits derived from salmon-bearing rivers (i.e., 12 DFAAs).

Discussion

The salient contributions of this study were first, the lack of detection of DFAAs in the water column from salmon-bearing rivers. Second, we detected DFAAs in benthic water from these rivers and compared fall and spring samples in a given river, and between rivers in the same season. Such comparisons are needed to assess whether DFAAs can provide chemical signatures that are sufficiently stable, temporally, and sufficiently different, spatially, to support olfactory imprinting and homing by salmon. Equivalence tests indicated that only the Crooked and Boquet rivers contained a unique subset of DFAAs that were similar between spring vs fall within the same year (i.e., the temporal stability hypothesis) and that no rivers shared the same exact subset of DFAAs that were similar across the seasons (i.e., the river specific hypothesis). However, the Huntington River had between year differences in DFAA patterns. These results suggest that there are unique subsets of DFAAs within each river system that might provide identifying information but if these DFAA mol% data from a few rivers and two years are representative of other rivers, then the complex seasonal and river-specific patterns make the

orientation challenges using just DFAA odorants daunting. The multivariate cluster analyses were more ambiguous, as clear clustering by season and river-specific DFAA concentrations was apparent for only the Crooked River and Huntington in 2016. Finally, difference tests (e.g., traditional t test) were deemed inconclusive and invalid for the hypotheses being tested. Thus, our three approaches to the analyses of DFAA stability (cluster analysis, difference tests, and equivalence tests) each provide a unique framework for future studies that have varying degrees of robustness and biological relevance to salmonid olfaction and behavior. The overall patterns and exceptions revealed by each approach stem from the complexity of stream biochemistry, and by extension they challenge our thinking about how salmon learn and use odorants in streams for homing.

Given previous reports of DFAAs in the water column as possible odorants for salmon homing, we were surprised that DFAAs in all the water column samples were below the ~1 nM quantification threshold of the analysis. Studies of other freshwater systems, including some salmon rivers, consistently found concentrations of individual amino acids > 1 nM in the water column and at similar concentrations to those found in the sediment pore water in this study (rivers in Japan: Shoji et al. 2000, Shoji et al. 2003, Yamamoto and Ueda 2009, Chen et al. 2017, Alaskan rivers: Dittman et al. 2022), but where measured sediment porewater DFAA concentrations were orders of magnitude higher than concentrations in the water column (Thomas and Eaton 1996, Thomas 1997). Hence, it is possible that overall DFAAs were lower in these New England rivers, but we note that the DFAA composition detected is similar to the aforementioned rivers. Variation in DFAA concentrations among water bodies may result from differences in biological productivity and dissolved organic matter (Thomas 1997). Alternatively, DFAAs could be present in the water column of the rivers we studied at

518 concentrations detectable by salmon but below our limit of ~1 nM. Electro-olfactogram (EOG)
519 studies, used to measure olfactory sensitivity, demonstrated (with few exceptions) salmonid
520 DFAA detection thresholds of ~1 nM or greater (reviewed by Bett and Hinch 2016). However,
521 EOG responses are a measure of summed responses of many neurons in the olfactory epithelium
522 and therefore may not reflect true detection limits of the salmon olfactory system. Conversely,
523 our results could be interpreted to indicate that DFAAs in the water column are not the only
524 imprinting and homing odorants for these Atlantic salmon.

525 Although studies of Atlantic salmon (Armstrong et al. 2022) and other salmonids have
526 implicated DFAAs in homing (reviewed by Ueda 2011, Bett and Hinch 2016), salmonids may
527 use other odors or odors in combination with DFAAs for imprinting and homing (Rajakaruna et
528 al. 2006, Hinz et al. 2013, Bett and Hinch 2015). For example, based on analyses of DFAAs pre-
529 and post-arrival of spawning sockeye salmon in the Wood River of Alaska, Dittman et al. (2022)
530 concluded that the DFAAs could not be the sole homing odorant because they did not differ
531 sufficiently among rivers (i.e., odorants were not unique to each river) and also because DFAAs
532 changed after adult salmon arrived (i.e., odorants were not seasonally stable). However, these
533 inferences were based on the statistical criteria of multivariate distance measures and difference-
534 based testing with low power (i.e., 2 replicate DFAA samples from each river pre- and post-
535 arrival of salmon). This design and analyses also precluded equivalence testing to assess whether
536 there were unique subsets among rivers that were not biologically equivalent (i.e., DFAAs
537 concentrations unique to each river) as well as the testing of subsets of DFAAs within rivers that
538 may have been unaffected by the odors from migrating salmon (i.e., seasonally stable).
539 Therefore, spatiotemporal patterns of DFAAs and their role in salmon homing and imprinting

remain unresolved, and given the interaction with sediments at multiple life stages, we now discuss the possible role of sediment porewater DFAAs.

That these New England rivers did not contain measurable DFAAs in the water column raises the question of how juvenile salmon might utilize sediment porewater odors for imprinting and how adult salmon use sediment porewater odors for homing. As suggested earlier, perhaps salmon can detect and utilize DFAAs in the water column at concentrations below our levels of detection. Moreover, we known that once in the water column, DFAAs are subject to photo-degradation (Lundeen et al. 2014) and microbial uptake and transformation (Kirchman 2003, Hobbie and Hobbie 2012) and are therefore highly labile, which could limit the spatial scale of a porewater DFAA odor plume into the water column. Indeed, many important hyporheic effects in streams are highly localized and not measurable in the water column (e.g., coldwater and nutrient-rich upwellings) but can be detected by fish and other organisms (Jones and Mulholland 2000, see Fig. 3 in Geist et al. 2002). Additionally, we know sediments are the major source of DFAAs in both fresh and marine waters (Thomas and Eaton 1996), and juvenile Atlantic salmon regularly interact with the benthic environment and, therefore, may be exposed to sediment DFAAs at several life history stages, many of which would be common to other salmonids. All salmonids could experience sediment DFAAs during their embryonic incubation in their natal gravel. Salmon can learn odors between hatching and emergence (Quinn et al. 2006, Dittman et al. 2015, Bett et al. 2016, Havey et al. 2017, Armstrong et al. 2022), when they are incubating in benthic gravel nests, surrounded by interstitial water. Indeed, Atlantic salmon often select areas with hyporheic upwelling for their redds (Heggenes et al. 2010, Saltveit and Brabrand 2013), as do other salmonids, and redd morphology and sediment texture promote hyporheic exchange (Cardenas et al. 2016). Juvenile Atlantic salmon also enter the interstitial spaces in the hyporheic

563 zone (Heggenes et al. 2013) and commonly rest near the river bottom (Thorstad et al. 2012),
564 especially in winter (Whalen and Parrish 1999). Hence, we speculate that hyporheic upwellings,
565 which occur at spatial scales relevant to salmon, may create discrete benthic areas of detectable
566 concentrations of DFAAs that salmon could use for imprinting and homing. Indeed, salmon are
567 known to return to the specific stream areas where they emerged as fry years before (Quinn et al.
568 2006). Also, the use of discrete areas with odors, or odor signposts, by salmon during imprinting
569 and homing would mitigate the possibility of olfactory "fatigue" – an adaptive response in which
570 sustained exposure to a constant concentration of odor will cease to stimulate olfactory neurons
571 (Atema 2012). Therefore, relative to the more homogenous chemical cues in the well mixed
572 water column, we propose that sediment odors could provide juvenile salmon with locally
573 specific olfactory information and adult salmon with spatially relevant odors that could preclude
574 olfactory fatigue during homing.

575 In addition to their temporal stability, Hasler and Wisby (1951) noted that imprinting and
576 homing odors must also be unique to each river. Our first approach, hierarchical cluster analysis,
577 revealed clustering both by season (e.g., Boquet Spring 2017 with Crooked Spring 2017, Boquet
578 Fall 2017 with Huntington Fall 2017) indicating seasonal similarities of DFAA patterns
579 among rivers and within rivers (e.g., Huntington Fall, Spring, 2016, Spring 2017; Crooked Fall,
580 Spring 2017) suggesting river-specific patterns (Fig. 5). The lack of clustering of the Huntington
581 River Fall 2017 with other Huntington River DFAA samples may have been due to changes to
582 the riverbed morphology we observed, likely resulting from flooding in spring 2017, when the
583 7th highest precipitation totals on record for the region occurred (National Weather Service
584 2019). This event may have been responsible, in some way, for the between-year changes in
585 DFAAs, which resulted in overall lower concentrations and non-detectable concentrations of

586 isoleucine and valine in 2017. However, the Boquet and Crooked River samples from fall and
587 spring also did not form a cluster pair, although all the samples from those two rivers formed a
588 group. Interestingly, these two rivers flow into different lakes (the Boquet into Lake Champlain,
589 and the Crooked into Sebago Lake). These results further illustrate the complexity of the
590 seasonal and tributary-specific patterns of DFAAs and the orientation challenges this complexity
591 represents for salmon utilizing these cues to guide homing. In these analyses, as with other
592 aspects of the interpretation of the DFAA data, amino acids that were not detected influenced the
593 overall results, and it remains unclear how salmon might perceive their absence or scarcity
594 relative to other, more concentrated ones.

595 The need to develop a functional definition of "stability" and "similarity" or
596 "equivalence" in the context of imprinting and homing odors is a challenge for this and similar
597 future studies. Current knowledge of salmonid imprinting has been gained largely through
598 experiments with artificial odorants, typically by exposing hatchery-reared, juvenile fish to either
599 morpholine or phenethyl alcohol, releasing the fish, and then tracking returning adults to a river
600 site or experimental enclosure where the odorant was added (e.g., Scholz et al. 1976). These
601 artificial odorants were useful as a tool for studying imprinting and homing because they could
602 be carefully titrated to maintain pre-determined, fixed concentrations at specific times of the year
603 and developmental periods of the fish, thereby fulfilling the requirements that imprinting and
604 homing odors must be both unique to a river and stable over time (Hasler and Scholz 1983).
605 However, odorants in rivers are likely much more complex, and may contain multiple
606 components that fluctuate in concentration and composition with seasonal and annual changes in
607 precipitation, forest composition and leaf litter inputs, anthropogenic influence, or other
608 ecological processes (Dittman and Quinn 1996). The odors of conspecifics may also be an

609 important part of the overall chemical bouquet, as has been hypothesized for many years
610 (Nordeng 1977, Stabell 1984), and ultimately these odors may also be associated with amino
611 acids (Rajakaruna et al. 2006, Hinz et al. 2013).

612 To understand whether the relative similarities identified by the cluster analysis may in
613 fact constitute "stability", we utilized the traditional t-test NHST approach based on rejecting the
614 null hypothesis of no difference between individual DFAA seasonal means. However, the
615 biological inferences one may draw from this NHST approach remain unclear. Specifically,
616 difference-based tests, such as the t test, ANOVA, PERMANOVA and many others, rely on
617 rejecting a null hypothesis that the difference between means is a point value, almost invariably
618 that value is zero, and rejecting the null assumes the true value is any value other than the null
619 value of zero. Hence, NHST with point values of zero can detect differences that may be
620 biologically trivial (e.g., Fig. 2 aspartic acid). A further limitation is that a continuous variable
621 (such as a mean difference in DFAAs) can take on a value that is close to the null value of zero
622 but still not equal and thus the null is rejected. We can also never prove that the true value is any
623 particular point value; we can only disprove a point value in NHST. Hence, a traditional null
624 hypothesis cannot be proven and therein lies the problem with using the NHST approach to make
625 any inferences about similarity, or "stability", of DFAA means when the null hypothesis is not
626 rejected (e.g., Yamamoto et al. 2013). The difference tests indicated that more than twice as
627 many DFAA comparisons were not different (both between seasons and between rivers)
628 compared to the same DFAA comparisons using equivalence tests, regardless of whether we
629 controlled for multiple comparisons. Also, the overlap in difference-based and equivalence-based
630 tests indicating stability, by their respective criteria, was low (20 %), further demonstrating that
631 using the NHST approach to assess DFAA "stability" is invalid and ambiguous. Moreover, fish

may perceive a difference (i.e., reject the null hypothesis of no difference), and yet not perceive the waters as different in essence.

We therefore employed an alternative method for assessing similarity based on the ability of salmon to discriminate between amino acid mixtures representing natural levels of DFAAs in rivers. The equivalence tests, using data from a study of Japanese chum salmon to define equivalence limits, indicated that each river contained a unique subset of stable DFAAs across seasons (spring vs fall), and none of the between season (spring vs fall) equivalent DFAAs were common among all three rivers. Rivers shared some but not all of the same seasonally equivalent DFAAs. An important caveat here is the unexplained interannual shift in the subsets of equivalent DFAAs in the Huntington River, as consistency among imprinting and homing years is a necessary component of the imprinting and homing as hypothesized by Ueda (2011), and the original olfactory imprinting hypothesis (Hasler and Scholz 1983). However, these two years of data are insufficient to fully evaluate the hypothesis that stability in the relative concentrations of DFAAs across rivers could produce a river-specific amino acid odor signature for imprinting and homing. Another important caveat regarding equivalence testing is that the validity of the conclusions depends on accurate and appropriate biological information about salmon olfactory discrimination during homing to establish the equivalence limits, i.e., the difference in means considered biologically zero. Setting the equivalence limits is challenging because temporal patterns of amino acid composition within a river are so rarely measured and paired with homing behavior experiments. We relied on a unique dataset of multi-year seasonal data on river DFAA profiles and accompanying behavioral discrimination data from chum salmon in Japan (Yamamoto et al. 2013).

The extraordinary complexity of salmon life history patterns, including multiple year classes of seaward emigrants, multiple year classes of returning adults, and sometimes broad seasonal migratory timing, suggests that the unique distinguishing features of a river's chemical profile must be consistent across multiple years and seasons. For example, the chum salmon studied in Yamamoto et al. (2013), mature and return to their natal rivers at multiple ages (Morita et al. 2005). Consequently, fish migrating to sea and imprinting to spring 2006 river water would need to distinguish Fall 2009 water (3-year-old adults) and Fall 2010 water (4-year-old adults) as equivalent. If multiple return years of salmon were considered in the analysis, equivalence test limits derived from the Yamamato et al. (2013) DFAA data could be broader, making the distinction between tributaries less obvious. However, when we increased the current equivalence limits based on Yamamato et al. (2013) by +/- 60 % (or 100% greater than the typical 80-125 % equivalence thresholds) the number of equivalent DFAAs increased but still none of the rivers had the exact same subset of equivalent DFAAs in common, suggesting unique river-specific patterns exist even with this much additional variation. Whether this arbitrarily wider threshold is truly representative of potentially greater DFAA variation is unknown. Further, an individual salmon would not experience all the interannual variation, just the variation during their imprinting and homing. A more complete understanding of the ability of salmonids to discriminate and generalize tributary specific odor characteristics from complex and dynamic odor mixtures will be critical for future studies. For example, electrophysiological experiments (Sorensen et al. 1990, Laberge and Hara 2003) and behavioral choice experiments (e.g., Y-maze: Havey et al. 2017) are needed to clarify the discriminatory abilities of salmon to DFAAs. However, equivalence testing offers a useful new approach to explore the chemical ecology of rivers and salmon homing behavior in natural settings.

It is important to consider that, while our data collection took place in a series of streams in the northeastern United States, the processes of olfactory imprinting and homing by salmonids (and likely other fishes as well) are exceptionally flexible. Salmonid home to small and large streams, fed by springs, surface runoff from rain and snow, lakes, and glacial meltwaters, as well as a variety of spawning habitats in lakes themselves rather than rivers. Thus the organic and inorganic chemistry of these natal waters, and their patterns of seasonal and interannual variation, are likely very great. Salmonid species and populations include ones that migrate home and breed over the entire year, and the juveniles are similarly variable in the spatial and temporal patterns of movement prior to and during migration (Quinn 2018). Despite experiencing a wide range of photoperiods, thermal regimes, and chemo-scapes, the vast majority of juveniles that survive to maturity return to spawn at natal sites.

In conclusion, our results indicate that profiles of the relative abundance (mol%) of DFAAs from benthic water in salmon rivers of the northeastern USA were in many instances stable, though not constant, across seasons and differ among rivers. However, the exceptions that were detected raise questions regarding the extent to which they might be sufficient for salmon imprinting and homing, though they might contribute to a broader suite of odorants. Further, we have also identified many outstanding questions, notably the differences in DFAA concentrations in sediment pore water vs. the water column, the need for data to determine equivalence of odor cues, and hence, rigorously test the discriminatory ability of salmon. Finally, but importantly, the strengths and weaknesses of the three analytical approaches we used (cluster analysis, difference tests, and equivalence tests) highlight the difficulties in deciding how to assess seasonal stability and river-specific odors used by salmon. Perhaps the most important conclusion from our results is that we need greater understanding of the salmons' ability to discern complex, dynamic odor

mixtures as inherently similar or different to interpret seasonal and annual chemical changes as equivalent for identifying their natal rivers. To this end, we also need more comprehensive sampling of DFAAs, especially replication within rivers, samples from different habitats (e.g., water column, sediment pore water, hyporheic, etc.) between imprinting and homing periods, and samples from a sufficient number of years to include the imprinting period and later homing of those same individuals for rigorous analyses of individual DFAAs and combined DFAA metrics. This further reminds us how little we truly understand salmon homing, despite decades of research.

Author contributions

Initial project conceptualization: Minkoff, Ardren, Kaiser, Dittman, and Atema

Data collection: Minkoff and Ardren with guidance from Kaiser, Dittman and Taylor

Chemical analyses: Kaiser

Statistical analyses and interpretation: led by Taylor, with input from all authors

MS drafting and interpretation of results: Minkoff, Quinn, Dittman, Ardren and Taylor

MS editing: all authors

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Literature Cited

Agilent Technologies 2017. High-Speed Amino Acid Analysis (AAA) on 1.8 µm Reversed-Phase (RP) Columns, Application No. 5989-6297.

Armstrong, M. E., D. Minkoff, A. H. Dittman, D. May, E. K. Moody, T. P. Quinn, J. Atema, and W. R. Ardren. 2022. Evidence of an olfactory imprinting window in embryonic Atlantic salmon. Ecology of Freshwater Fish 31:270-279.

Atema, J. 2012. Aquatic odour dispersal fields: opportunities and limits of detection, communication, and navigation. Pages 1-18 in C. Brönmark and L.-A. Hansson (editors). Chemical Ecology in Aquatic Systems. Oxford University Press.

Bett, N. N., and S. G. Hinch. 2015. Attraction of migrating adult sockeye salmon to conspecifics in the absence of natal chemical cues. Behavioral Ecology 26:1180-1187.

Bett, N. N., and S. G. Hinch. 2016. Olfactory navigation during spawning migrations: a review and introduction of the Hierarchical Navigation Hypothesis. Biological reviews of the Cambridge Philosophical Society 91:728-59.

Bett, N. N., S. G. Hinch, A. H. Dittman, and S.-S. Yun. 2016. Evidence of olfactory imprinting at an early life stage in Pink salmon (*Oncorhynchus gorbuscha*). Scientific Reports 6:36393.

Bodznick, D. 1978. Water source preference and lakeward migration of sockeye salmon fry (*Oncorhynchus nerka*). Journal of Comparative Physiology 127:139-146.

Borroni, P. F., L. S. Handrich, and J. Atema. 1986. The role of narrowly tuned taste cell populations in lobster (*Homarus americanus*) feeding behavior. Behavioral Neuroscience 100:206.

Bryant, B. P., and J. Atema. 1987. Diet manipulation affects social behavior of catfish. Journal of Chemical Ecology 13:1645-1661.

Caffo, B., C. Lauzon, and J. Röhmel. 2013. Correction to "Easy Multiplicity Control in Equivalence Testing Using Two One-Sided Tests". The American Statistician 67:115-116.

Cardenas, M. B., A. E. Ford, M. H. Kaufman, A. J. Kessler, and P. L. M. Cook. 2016. Hyporheic flow and dissolved oxygen distribution in fish nests: The effects of open channel velocity, permeability patterns, and groundwater upwelling. Journal of Geophysical Research: Biogeosciences 121:3113-3130.

Casal, S., M. R. Alves, E. Mendes, M. B. P. P. Oliveira, and M. A. Ferreira. 2003. Discrimination between Arabica and Robusta coffee species on the basis of their amino acid enantiomers. Journal of Agricultural and Food Chemistry 51:6495-6501.

Chen, E. Y., J. B. K. Leonard, and H. Ueda. 2017. The behavioural homing response of adult chum salmon *Oncorhynchus keta* to amino-acid profiles. Journal of Fish Biology 90:1257-1264.

Cometto, P. M., P. F. Faye, R. D. Di Paola Naranjo, M. A. Rubio, and M. A. J. Aldao. 2003. Comparison of free amino acids profile in honey from three Argentinian regions. Journal of Agricultural and Food Chemistry 51:5079-5087.

Davidson, H., and R. A. Cribbie. 2020. A more powerful familywise error control procedure for evaluating mean equivalence. Communications in Statistics - Simulation and Computation 49:2914-2929.

Davit, B. M., P. E. Nwakama, G. J. Buehler, D. P. Conner, S. H. Haidar, D. T. Patel, Y. Yang, L. X. Yu, and J. Woodcock. 2009. Comparing generic and innovator drugs: A review of 12

779 years of bioequivalence data from the United States Food and Drug Administration.
780 Annals of Pharmacotherapy 43:1583-1597.
781 DeRoche, S. E. 1976. The Sebago Lake study. Fisheries Research Bulletin 9. Maine Department
782 of Inland Fisheries and Wildlife, Augusta, ME 04330, USA.
783 Dittman, A., and T. Quinn. 1996. Homing in Pacific salmon: mechanisms and ecological basis.
784 Journal of Experimental Biology 199:83-91.
785 Dittman, A. H., C. J. Cunningham, and T. P. Quinn. 2022. Can unique amino acid profiles guide
786 adult salmon to natal streams? A comparison of streams sampled prior to and after the
787 arrival of adult Pacific salmon. Hydrobiologia 849:3501-3513.
788 Dittman, A. H., T. N. Pearsons, D. May, R. B. Couture, and D. L. G. Noakes. 2015. Imprinting
789 of Hatchery-Reared Salmon to Targeted Spawning Locations: A New Embryonic
790 Imprinting Paradigm for Hatchery Programs. Fisheries 40:114-123.
791 Garcia de Leaniz, C., I. A. Fleming, S. Einum, E. Verspoor, W. C. Jordan, S. Consuegra, N.
792 Aubin-Horth, D. Lajus, B. H. Letcher, A. F. Youngson, J. H. Webb, L. A. Vøllestad, B.
793 Villanueva, A. Ferguson, and T. P. Quinn. 2007. A critical review of adaptive genetic
794 variation in Atlantic salmon: implications for conservation. Biol Rev Camb Philos Soc
795 82:173-211.
796 García-Palmer, F. J., N. Serra, A. Palou, and M. Gianotti. 1997. Free amino acids as indices of
797 Mahón cheese ripening. Journal of Dairy Science 80:1908-1917.
798 Geist, D. R., T. P. Hanrahan, E. V. Arntzen, G. A. McMichael, C. J. Murray, and Y.-J. Chien.
799 2002. Physicochemical characteristics of the hyporheic zone affect redd site selection by
800 chum salmon and fall Chinook salmon in the Columbia River. North American Journal of
801 Fisheries Management 22:1077-1085.

802 Harbicht, A. B., D. J. Fraser, and W. R. Ardren. 2020. Minor shifts towards more natural
803 conditions in captivity improve long-term survival among reintroduced Atlantic salmon.
804 Canadian Journal of Fisheries and Aquatic Sciences 77:931-942.
805 Hasler, A. D., and W. J. Wisby. 1951. Discrimination of stream odors by fishes and its relation to
806 parent stream behavior. The American Naturalist 85:223-238.
807 Hasler, A. D., and A. T. Scholz. 1983. Olfactory Imprinting and Homing In Salmon. Springer-
808 Verlag, Berlin.
809 Havey, M. A., A. H. Dittman, T. P. Quinn, S. C. Lema, and D. May. 2017. Experimental
810 evidence for olfactory imprinting by Sockeye Salmon at embryonic and smolt stages.
811 Transactions of the American Fisheries Society 146:74-83.
812 Héberger, K., E. Csomós, and L. Simon-Sarkadi. 2003. Principal component and linear
813 discriminant analyses of free amino acids and biogenic amines in Hungarian wines.
814 Journal of Agricultural and Food Chemistry 51:8055-8060.
815 Heggenes, J., G. Bremset, and Å. Brabrand 2010. Groundwater, critical habitats, and behaviour
816 of Atlantic salmon, brown trout and Arctic char in streams. NINA Report 654, 28 pp.
817 Trondheim, Norway.
818 Heggenes, J., G. Bremset, and Å. Brabrand. 2013. Visiting the hyporheic zone: young Atlantic
819 salmon move through the substratum. Freshwater Biology 58:1720-1728.
820 Hernández-Orte, P., J. F. Cacho, and V. Ferreira. 2002. Relationship between varietal amino acid
821 profile of grapes and wine aromatic composition. Experiments with model solutions and
822 chemometric study. Journal of Agricultural and Food Chemistry 50:2891-2899.

Hildebrand, J. G., and G. M. Shepherd. 1997. Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. Annual Review of Neuroscience 20:595-631.

Hill, N. L., J. R. Trueman, A. D. Prévost, D. J. Fraser, W. R. Ardren, and J. W. Grant. 2019. Effect of dam removal on habitat use by spawning Atlantic salmon. Journal of Great Lakes Research 45:394-399.

Hinz, C., I. Namekawa, J. Behrmann-Godel, C. Oppelt, A. Jaeschke, A. Müller, R. W. Friedrich, and G. Gerlach. 2013. Olfactory imprinting is triggered by MHC peptide ligands. Scientific Reports 3:2800.

Hobbie, J. E., and E. A. Hobbie. 2012. Amino acid cycling in plankton and soil microbes studied with radioisotopes: measured amino acids in soil do not reflect bioavailability. Biogeochemistry 107:339-360.

Holm, S. 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6:65-70.

Hutchings, J. A. 1986. Lakeward migrations by juvenile Atlantic salmon, *Salmo salar*. Canadian Journal of Fisheries and Aquatic Sciences 43:732-741.

Hutchings, J. A., W. R. Ardren, B. T. Barlaup, E. Bergman, K. D. Clarke, L. A. Greenberg, C. Lake, J. Piironen, P. Sirois, L. E. Sundt-Hansen, and D. J. Fraser. 2019. Life-history variability and conservation status of landlocked Atlantic salmon: an overview. Canadian Journal of Fisheries and Aquatic Sciences 76:1697-1708.

Iglesias, M. T., C. de Lorenzo, M. d. C. Polo, P. J. Martín-Álvarez, and E. Pueyo. 2004. Usefulness of amino acid composition to discriminate between honeydew and floral

honeys. Application to honeys from a small geographic area. Journal of Agricultural and Food Chemistry 52:84-89.

Jones, J. B., and P. J. Mulholland 2000. Streams and Groundwater. Pages 425. Academic Press, Cambridge, MA.

Kalejs, N. 2022. Use of a picket weir and passive integrated transponder tags to assess the landlocked Atlantic salmon spawning run in the Crooked. Pages 47. Wildlife and Sport Fish Restoration Report, 353. Maine Department of Inland Fisheries & Wildlife Fisheries and Hatcheries Division, Augusta, ME.

Kirchman, D. L. 2003. The contribution of monomers and other low-molecular weight compounds to the flux of dissolved organic material in aquatic ecosystems. Pages 217-241 in S. E. G. Findlay and R. L. Sinsabaugh (editors). Aquatic Ecosystems. Academic Press, Burlington.

Laberge, F., and T. J. Hara. 2003. Behavioural and electrophysiological responses to F-prostaglandins, putative spawning pheromones, in three salmonid fishes. Journal of Fish Biology 62:206-221.

Limentani, G. B., M. C. Ringo, F. Ye, M. L. Bergquist, and E. O. McSorley. 2005. Beyond the t-test: Statistical equivalence testing. Analytical Chemistry 77:221-226.

Lombard, P. J., and G. A. Hodgkins 2020. Estimating flood magnitude and frequency on gaged and ungaged streams in Maine. Pages 56. Scientific Investigations Report. Reston, VA.

Lubin, J. H., J. S. Colt, D. Camann, S. Davis, J. R. Cerhan, R. K. Severson, L. Bernstein, and P. Hartge. 2004. Epidemiologic evaluation of measurement data in the presence of detection limits. Environmental Health Perspectives 112:1691-6.

867 Lumia, R., D. A. Freehafer, and M. J. Smith 2006. Magnitude and frequency of floods in New
868 York. Scientific Investigations Report 2006-5112, United State Geological Survey.
869 Lundeen, R. A., E. M. Janssen, C. Chu, and K. McNeill. 2014. Environmental photochemistry of
870 amino acids, peptides and proteins. Chimia 68:812-7.
871 Mohammed, S. E., and E. Babiker. 2010. Identification of the floral origin of honey by amino
872 acids composition. Australian Journal of Basic and Applied Sciences 4:552-556.
873 Morin, P.-P., J. J. Dodson, and F. Y. Doré. 1989. Cardiac responses to a natural odorant as
874 evidence of a sensitive period for olfactory imprinting in young Atlantic salmon, *Salmo*
875 *salar*. Canadian Journal of Fisheries and Aquatic Sciences 46:122-130.
876 Morita, K., S. H. Morita, M.-a. Fukuwaka, and H. Matsuda. 2005. Rule of age and size at
877 maturity of chum salmon (*Oncorhynchus keta*): implications of recent trends among
878 *Oncorhynchus* spp. Canadian Journal of Fisheries and Aquatic Sciences 62:2752-2759.
879 National Weather Service 2019. National Weather Service: Burlington, Vermont seasonal
880 precipitation totals.
881 https://www.weather.gov/media/btv/climo/extremes/topmonthlypcpn.pdf/. U.S. National
882 Weather Service.
883 Nordeng, H. 1977. A pheromone hypothesis for homeward migration in anadromous salmonids.
884 Oikos 28:155-159.
885 Olson, S. A., and A. G. Veilleux 2014. Estimation of flood discharges at selected annual
886 exceedance probabilities for unregulated, rural streams in Vermont, with a section on
887 Vermont regional skew regression. Pages 37. Scientific Investigations Report. Reston,
888 VA.

889 Prévost, A. D., N. L. Hill, J. W. A. Grant, W. R. Ardren, and D. J. Fraser. 2020. Patterns of
890 reproductive success among reintroduced Atlantic salmon in two Lake Champlain
891 tributaries. Conservation Genetics 21:149-159.
892 Quinn, T. P. 2018. The Behavior and Ecology of Pacific Salmon and Trout. 2nd edition.
893 University of Washington Press, Seattle.
894 Quinn, T. P., I. J. Stewart, and C. P. Boatright. 2006. Experimental evidence of homing to site of
895 incubation by mature sockeye salmon, *Oncorhynchus nerka*. Animal Behaviour 72:941-
896 949.
897 Rajakaruna, R. S., J. A. Brown, K. H. Kaukinen, and K. M. Miller. 2006. Major
898 histocompatibility complex and kin discrimination in Atlantic salmon and brook trout.
899 Molecular Ecology 15:4569-4575.
900 Regish, A. M., W. R. Ardren, N. R. Staats, H. Bouchard, J. L. Withers, T. Castro-Santos, and S.
901 D. McCormick. 2021. Surface water with more natural temperatures promotes
902 physiological and endocrine changes in landlocked Atlantic salmon smolts. Canadian
903 Journal of Fisheries and Aquatic Sciences 78:775-786.
904 Romesburg, H. C. 2004. Cluster analysis for researchers. 2nd edition. Lulu.com, Morrisville,
905 NC.
906 Rose, E. M., T. Mathew, D. A. Coss, B. Lohr, and K. E. Omland. 2018. A new statistical method
907 to test equivalence: An application in male and female eastern bluebird song. Animal
908 Behaviour 145:77-85.
909 Saltveit, S. J., and Å. Brabrand. 2013. Incubation, hatching and survival of eggs of Atlantic
910 salmon (*Salmo salar*) in spawning redds influenced by groundwater. Limnologica
911 43:325-331.

912 SAS Institute Inc. 2014. SAS OnDemand for Academics: User's Guide. SAS Institute Inc., Cary,
913 NC.
914 SAS Institute Inc. 2018. JMP Pro. SAS Institute, Inc., Cary, North Carolina.
915 Sato, K., T. Shoji, and H. Ueda. 2000. Olfactory discriminating ability of lacustrine Sockeye and
916 Masu salmon in various freshwaters. Zoological Science 17:313-317.
917 Scholz, A. T., R. M. Horrall, J. C. Cooper, and A. D. Hasler. 1976. Imprinting to chemical cues:
918 The basis for home stream selection in salmon. Science 192:1247-1249.
919 Shoji, T., Y. Yamamoto, D. Nishikawa, K. Kurihara, and H. Ueda. 2003. Amino acids in stream
920 water are essential for salmon homing migration. Fish Physiology and Biochemistry
921 28:249-251.
922 Shoji, T., H. Ueda, T. Ohgami, T. Sakamoto, Y. Katsuragi, K. Yamauchi, and K. Kurihara. 2000.
923 Amino acids dissolved in stream water as possible home stream odorants for Masu
924 salmon. Chemical Senses 25:533-540.
925 Sorensen, P. W., T. J. Hara, N. E. Stacey, and J. G. Dulka. 1990. Extreme olfactory specificity of
926 male goldfish to the preovulatory steroidal pheromone 17α,20β-dihydroxy-4-pregnen-3-
927 one. Journal of Comparative Physiology A 166:373-383.
928 Stabell, O. B. 1984. Homing and olfaction in salmonids: A critical review with special reference
929 to the Atlantic salmon. Biological Reviews 59:333-388.
930 Tessier, N., and L. Bernatchez. 1999. Stability of population structure and genetic diversity
931 across generations assessed by microsatellites among sympatric populations of
932 landlocked Atlantic salmon (*Salmo salar* L.). Molecular Ecology 8:169-179.
933 Thomas, J. 1997. The role of dissolved organic matter, particularly free amino acids and humic
934 substances, in freshwater ecosystems. Freshwater Biology 38:1-36.

935 Thomas, J., and P. Eaton. 1996. The spatio-temporal patterns and ecological significance of free
936 amino acids and humic substances in contrasting oligotrophic and eutrophic freshwater
937 ecosystems. Hydrobiologia 332:183-211.
938 Thorstad, E. B., F. Whoriskey, A. H. Rikardsen, and K. Aarestrup. 2010. Aquatic nomads: The
939 life and migrations of the Atlantic salmon. Pages 1-32. Atlantic Salmon Ecology. Wiley-
940 Blackwell, New York.
941 Thorstad, E. B., F. Whoriskey, I. Uglem, A. Moore, A. H. Rikardsen, and B. Finstad. 2012. A
942 critical life stage of the Atlantic salmon *Salmo salar*: behaviour and survival during the
943 smolt and initial post-smolt migration. Journal of Fish Biology 81:500-42.
944 Ueda, H. 2011. Physiological mechanism of homing migration in Pacific salmon from behavioral
945 to molecular biological approaches. General and Comparative Endocrinology 170:222-
946 232.
947 Ueda, H., Y. Yamamoto, and H. Hino. 2007. Physiological mechanisms of homing ability in
948 Sockeye Salmon: From behavior to molecules using a lacustrine model. PAmerican
949 Fisheries Society Symposium 54: 5-16.
950 Whalen, K. G., and D. L. Parrish. 1999. Nocturnal habitat use of Atlantic salmon parr in winter.
951 Canadian Journal of Fisheries and Aquatic Sciences 56:1543-1550.
952 Yamamoto, Y., and H. Ueda. 2009. Behavioral responses by migratory chum salmon to amino
953 acids in natal stream water. Zoological Science 26:778-82.
954 Yamamoto, Y., H. Shibata, and H. Ueda. 2013. Olfactory homing of chum salmon to stable
955 compositions of amino acids in natal stream water. Zoological Science 30:607-612.
956 Youngson, A. F., W. C. Jordan, and D. W. Hay. 1994. Homing of Atlantic salmon (*Salmo salar*
957 L.) to a tributary spawning stream in a major river catchment. Aquaculture 121:259-267.

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Table 1. Characteristics of the five rivers sampled in New England, USA.

Watershed	River	Catchment area (km^2)	Mean annual discharge (m^3/s)	Percent forested	Mean annual precipitation (m)	Reference
Lake Champlain, New York	Boquet River	700	9.17	90	0.87	Lumia et al. (2006)
Lake Champlain, Vermont	Huntington	172	3.2	90	1.16	Olson and Veilleux (2014)
Lake Champlain, Vermont	Winooski	2745	51.5	71	1.16	Olson and Veilleux (2014)
Lake Sebago, Maine	Crooked	389	9.5	85	1.14	Lombard and Hodgkins (2020)
Lake Sebago, Maine	Panther Run	80.1	2.0	78	1.14	Lombard and Hodgkins (2020)

Table 2. Difference tests of comparisons between rivers within seasons (i.e., fall versus fall; spring versus spring) of mean molar percentage of benthic dissolved free amino acids (DFAA) in two rivers flowing into Lake Champlain, USA. The Crooked River in Maine is excluded here because it is not accessible by Atlantic salmon in Lake Champlain. The two-sided t tests used a Tobit model for left-censored data (proc lifereg procedure in SAS Software). Molar percentages were in the natural-log scale for the t tests. Italics indicates a one-sided t test was used with fall or spring mean equal to zero because the DFAA was not detected in either the fall or spring samples. Except where noted there are n=6 independent replicates for spring and fall. § indicates spring or fall had only two replicates in which the DFAA was detectable. † indicates spring or fall had only one replicate in which the DFAA was detectable. ND indicates that a valid t test could not be conducted because the DFAA was not detectable in any of the samples for both rivers, or the DFAA was not detected in any samples from one river and in less than three samples from the other river. * indicates the null hypothesis was not rejected based on the step-down Holm method controlling the type I familywise error rate (family = each unique stream-year pair), and the unadjusted p value is reported here. Bold indicates tests significantly different ($p < 0.05$) after controlling the type I error rate using the Holm method.

	Difference fall vs. fall			Difference spring vs spring		
Amino acid	Boquet 2017 Huntington 2016	Boquet 2017 Huntington 2017	Huntington 2016 Huntington 2017	Boquet 2017 Huntington 2016	Boquet 2017 Huntington 2017	Huntington 2016 Huntington 2017
Alanine	**<0.001**	0.504	**<0.001**	**<0.001**	0.083	0.037*
Arginine	0.190	0.274	0.929	**<0.001**	0.073	0.042*
Aspartic acid	0.156	**<0.001**	0.240	0.535	0.022*	0.174
Glutamic acid	0.050*	0.013*	0.259	**0.001**	**<0.001**	0.515
Glycine	**0.004**†	0.013*	**<0.001**†	0.081	0.519	0.140
Histidine	**<0.001**§	ND	**<0.001**	**<0.001**	0.008*	0.690
Isoleucine	ND	ND	ND	**<0.001**§	0.735§	**<0.001**§
Leucine	ND	ND	ND	**<0.001**	**<0.001**	0.034*
Lysine	**<0.001**†	**0.001**	**<0.001**†	0.315	**0.001**	0.025*
Phenylalanine	ND	ND	ND	ND	ND	0.195
Serine	0.672	0.075	0.353	0.046*	0.043*	0.592
Threonine	**<0.001**	**0.001**	**<0.001**	0.080	**0.001**	0.992
Tyrosine	0.010*§	ND	ND	0.067	0.022*	0.331
Valine	*0.999*	ND	***<0.001***	*<0.001*§	ND	0.127§

Table 3. Equivalence tests of comparisons between rivers within seasons (i.e., fall versus fall; spring versus spring) of mean molar percentage of benthic dissolved free amino acids (DFAA) in two rivers flowing into Lake Champlain, USA. The Crooked River in Maine is excluded here because it is not accessible by Atlantic salmon in Lake Champlain. Equivalence is based on two one-sided tests testing the null hypothesis that the means are different by at least some specified limits, or equivalence limits, rejecting the null hypothesis of both one-sided tests indicates that the means are equivalent at least within the specified limits (0.612 – 1.634), derived from Table 1 of Yamamoto et al. (2013). Molar percentages and equivalence limits were in the natural-log scale for the two one-sided tests . Shading indicates seasonal equivalent DFAA means that was determined if both the lower and upper one-sided test p <0.05. Italics indicates the lower equivalence limit was set to zero because the DFAA was not detectable in either the fall or spring samples. [§] indicates spring or fall had only two replicates in which the DFAA was detectable. [†] indicates spring or fall had only one replicate in which the DFAA was detectable. ND indicates that the two one-sided tests could not be conducted because the DFAA was not detectable in any of the samples for both seasons, or the DFAA was not detected in any samples from one season and in less than three samples from the other season.

	Equivalence fall vs fall						Equivalence spring vs spring					
	Boquet 2017-Huntington 2016		Boquet 2017-Huntington 2017		Huntington 2016 Huntington 2017		Boquet 2017-Huntington 2016		Boquet 2017-Huntington 2017		Huntington 2016 Huntington 2017	
Amino acid	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
Alanine	0.730	<0.001	<0.001	<0.001	<0.001	0.857	0.975	<0.001	0.112	<0.001	0.254	<0.001
Arginine	0.001	0.214	0.002	0.238	<0.001	<0.001	<0.001	0.314	<0.001	0.003	<0.001	0.004
Aspartic acid	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.093	0.005	<0.001	0.003	0.262	<0.001
Glutamic acid	0.022	<0.001	0.374	<0.001	0.040	<0.001	0.829	<0.001	0.984	<0.001	0.001	0.043
Glycine	0.871[†]	<0.001[†]	<0.001	0.658	<0.001[†]	0.998[†]	0.568	<0.001	0.022	<0.001	0.309	<0.001
Histidine	<0.001[§]	0.996[§]	ND	ND	0.999	<0.001	<0.001	0.746	<0.001	0.509	0.002	0.020
Isoleucine	ND	ND	ND	ND	ND	ND	<0.001[§]	1.000[§]	0.082[§]	0.238[§]	<0.001	0.994
Leucine	ND	ND	ND	ND	ND	ND	<0.001	0.777	<0.001	0.999	0.180	<0.001
Lysine	0.993[†]	<0.001[†]	<0.001	0.913	<0.001[†]	1.000[†]	0.256	0.004	<0.001	0.318	0.698	<0.001
Phenylalanine	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.717	0.022
Serine	0.031	0.155	<0.001	0.465	0.012	0.348	0.002	0.857	0.002	0.883	0.015	<0.001
Threonine	<0.001	0.997	<0.001	0.315	0.994	<0.001	<0.001	0.047	<0.001	0.001	<0.001	<0.001
Tyrosine	<0.001[§]	0.715[§]	ND	ND	ND	ND	<0.001	0.602	<0.001	0.042	0.007	0.295
Valine	*0.999*	*<0.001*	ND	ND	*0.999*	*<0.001*	*0.999*[§]	*<0.001*[§]	ND	ND	0.018	0.830

Table 4. Summary of equivalence and difference tests for comparisons within and between rivers from tables 1- 4 for benthic porewater dissolved free amino acids. Shading indicates means are equivalent at $p < 0.05$ and unshaded indicates not equivalent based on two one-sided equivalence tests. No difference indicates means are not different at $p > 0.05$ based on difference-based t tests. Different indicates means are different at $p < 0.05$ based on difference-based t tests. ND indicates that difference (two-sided t test) and equivalence tests (two one-sided tests) could not be conducted because the DFAA was not detectable in any of the samples for both seasons, or the DFAA was not detected in any samples from one season and in less than three samples from the other season. The Crooked River is excluded in between river comparisons because it is not accessible by Atlantic salmon in Lake Champlain, which the Boquet and Huntington flow into.

	Equivalence between seasons spring vs fall			Equivalence between rivers fall vs. fall			Equivalence between rivers spring vs spring		
Amino acid	Boquet 2017	Huntington 2016	Huntington 2017	Boquet 2017 Huntington 2016	Boquet 2017 Huntington 2017	Huntington 2016 Huntington 2017	Boquet 2017 Huntington 2016	Boquet 2017 Huntington 2017	Huntington 2016 Huntington 2017
Alanine	no difference	no difference	no difference	different		different	different	no difference	no difference
Arginine	no difference	different	different	no difference	no difference	no difference	different		no difference
Aspartic acid	different	no difference	no difference	no difference	different	no difference	no difference	no difference	no difference
Glutamic acid	no difference	different	different	no difference	no difference	no difference	different	different	no difference
Glycine	no difference	no difference	no difference	different	no difference	different	no difference	no difference	no difference
Histidine	different	no difference	different	different	ND	different	different	no difference	no difference
Isoleucine	ND	no difference	ND	ND	ND	ND	different	no difference	no difference
Leucine	different	no difference	different	ND	ND	ND	different	different	no difference
Lysine	no difference	no difference	different	different	different	different	no difference	different	different
Phenylalanine	ND	ND	different	ND	ND	ND	ND	ND	no difference
Serine	no difference	no difference	no difference	no difference	no difference	no difference	no difference	no difference	no difference
Threonine	different	different	no difference	different	different	different	no difference	different	no difference
Tyrosine	different	no difference	different	no difference	ND	ND	no difference	no difference	no difference
Valine	ND	no difference	ND	no difference	ND	different	different	ND	no difference

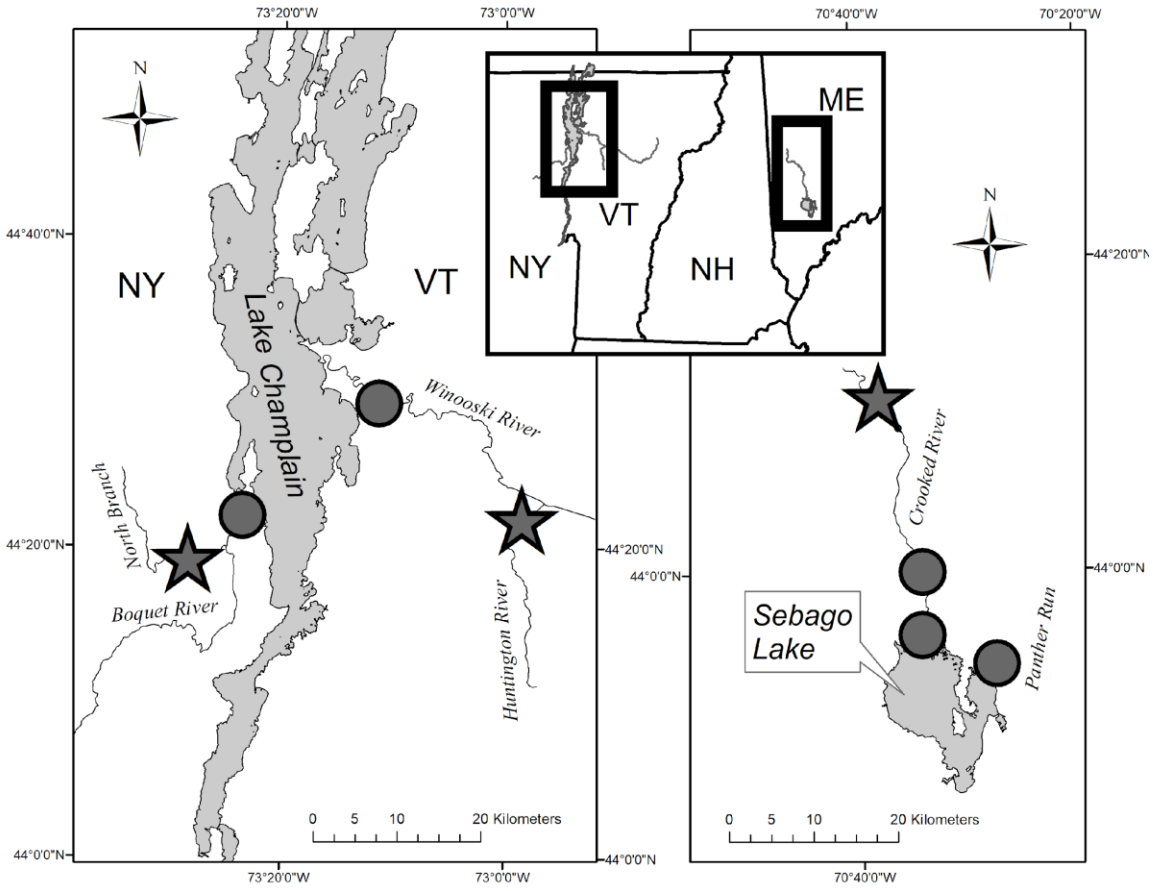

Figure 1: Rivers in New England where water column and sediment porewater samples were collected for dissolved free amino acids. Circles indicate locations where only water column samples were collected; stars indicate locations where both water column and sediment porewater samples were collected.

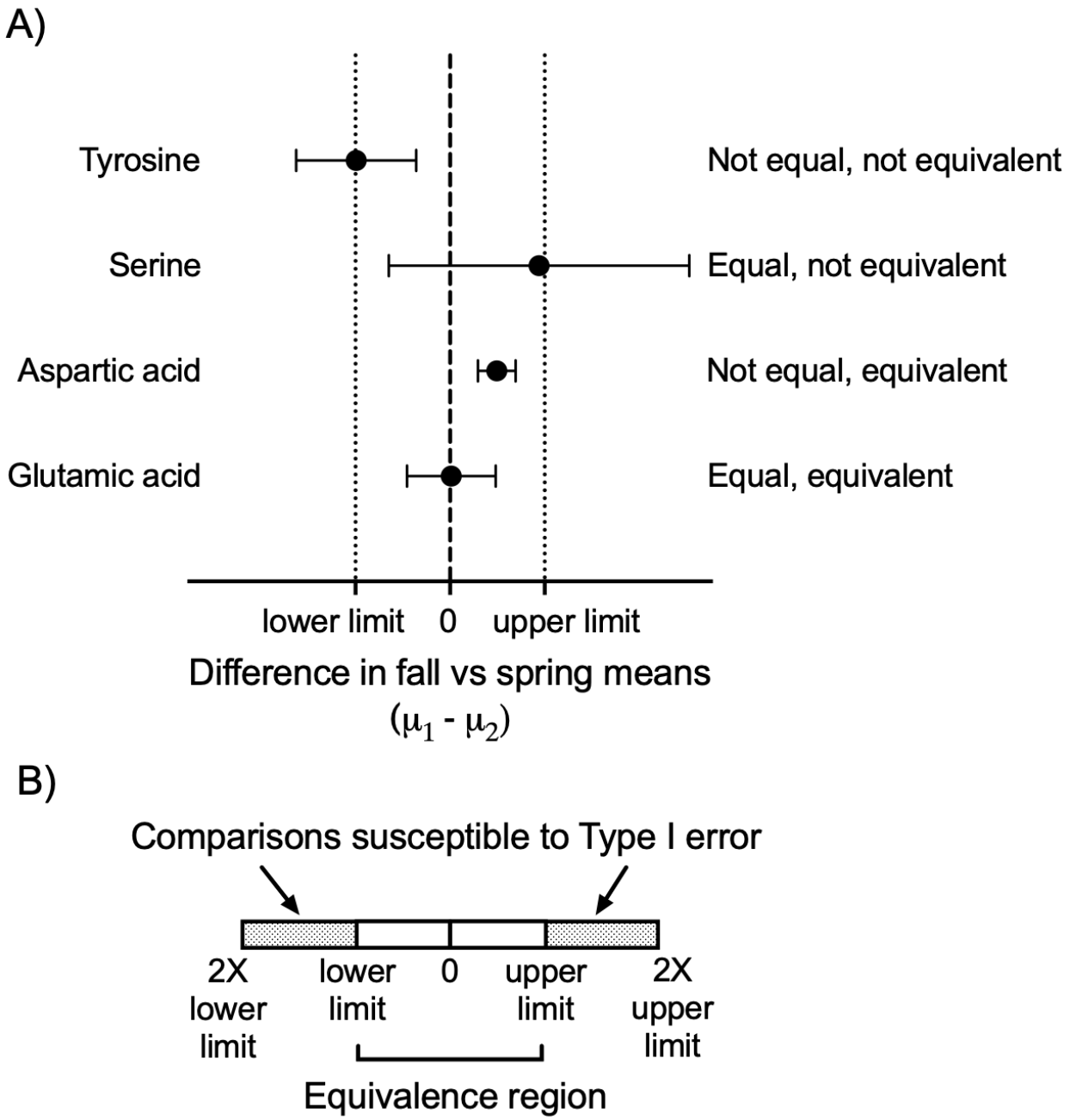

Figure 2. a) Illustrated comparison of difference and equivalence testing. Points are mean differences between fall and spring samples and bars are 90% confidence intervals (CIs) for selected amino acids from the Boquet River. Tyrosine means are not equal (i.e., they differ) because the mean difference CIs do not overlap with zero. They are also not equivalent because confidence intervals are outside the lower equivalence limit. Serine means are equal based on a two-sided t test because the confidence intervals of the mean difference include zero, and not equivalent based on two one-sided t tests because the confidence intervals of the mean difference overlap the upper equivalence limit. Aspartic acid means are different based on the two-sided t test because the confidence intervals do not overlap zero but equivalent based on two one-sided t

tests because the confidence intervals are within equivalence limits. Glutamic acid means are equal based on the two-sided t test because the confidence intervals overlap zero and equivalent by two one-sided t tests because the confidence intervals are within the equivalence limits. A 90% confidence interval instead of a 95% is used because two one-sided tests (each with an alpha of 5%) are performed. b) Illustration of the region (shaded) where mean differences are susceptible to Type I error in the equivalence testing framework.

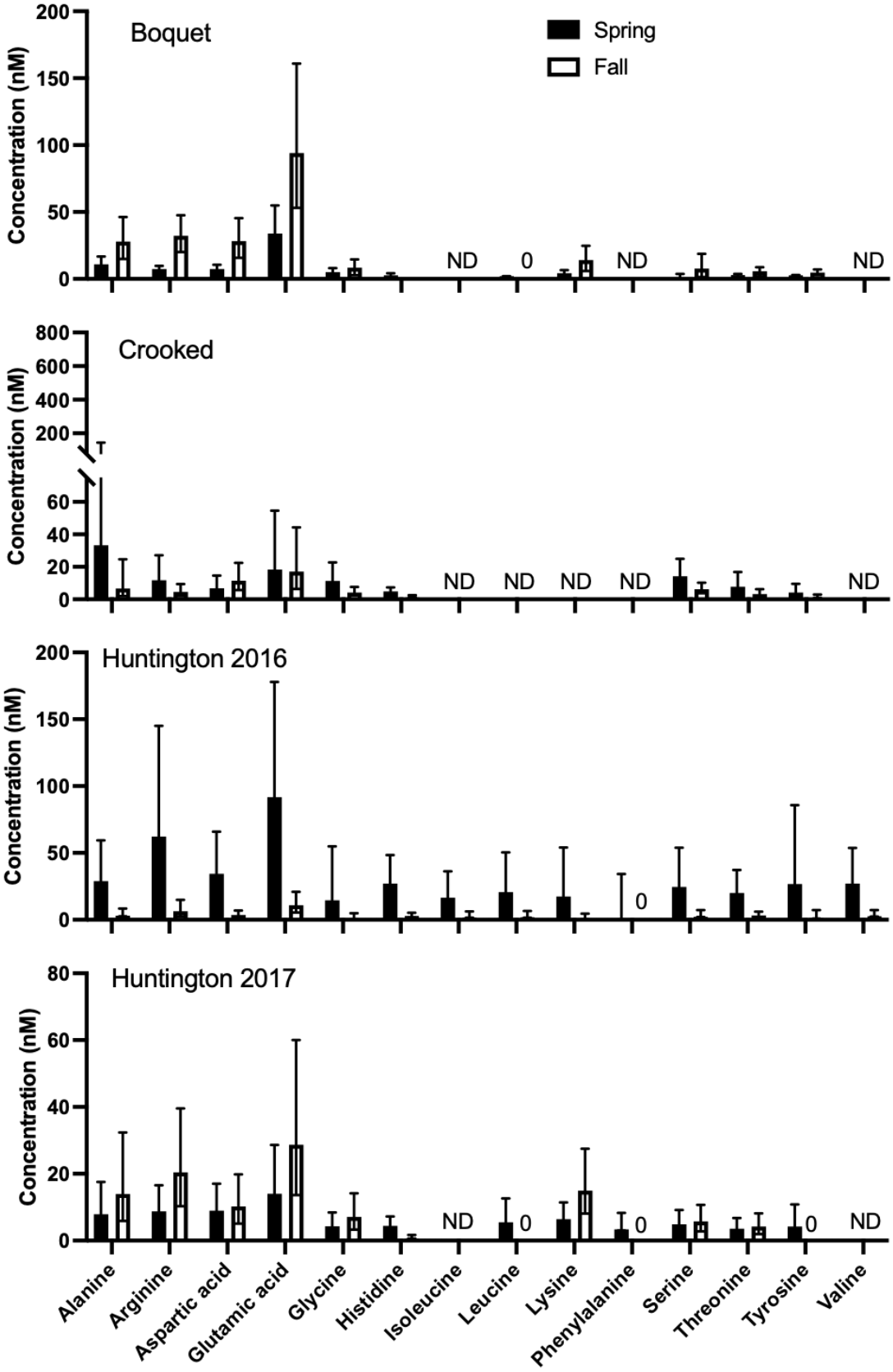
Boquet
Spring
Fall
Concentration (nM)
200
150
100
50
0
ND
0
ND
ND
Crooked
800
600
400
200
T
60
40
20
0
ND
ND
ND
ND
ND
Huntington 2016
150
100
50
0
0
Huntington 2017
80
60
40
20
0
ND
0
0
0
ND
Alanine
Arginine
Aspartic acid
Glutamic acid
Glycine
Histidine
Isoleucine
Leucine
Lysine
Phenylalanine
Serine
Threonine
Tyrosine
Valine

Figure 3. Concentrations (nM) of dissolved free amino acids (DFAA) in sediment pore water sampled in spring (black bars) and fall (white bars) from the Boquet, Crooked, and Huntington rivers. Mean and 95% confidence intervals are back-transformed from the natural-log scale. ND indicates a valid test could not be conducted because the DFAA was not detectable in any samples for both seasons or was detected in < 3 samples from one or both seasons. Zero indicates all samples were below detection limits for that season. Asterisks indicate $p < 0.05$ for two-sided t tests of the difference in means equal to zero following the step-down Holm method for controlling the familywise error rate (family = each unique stream-year pair).

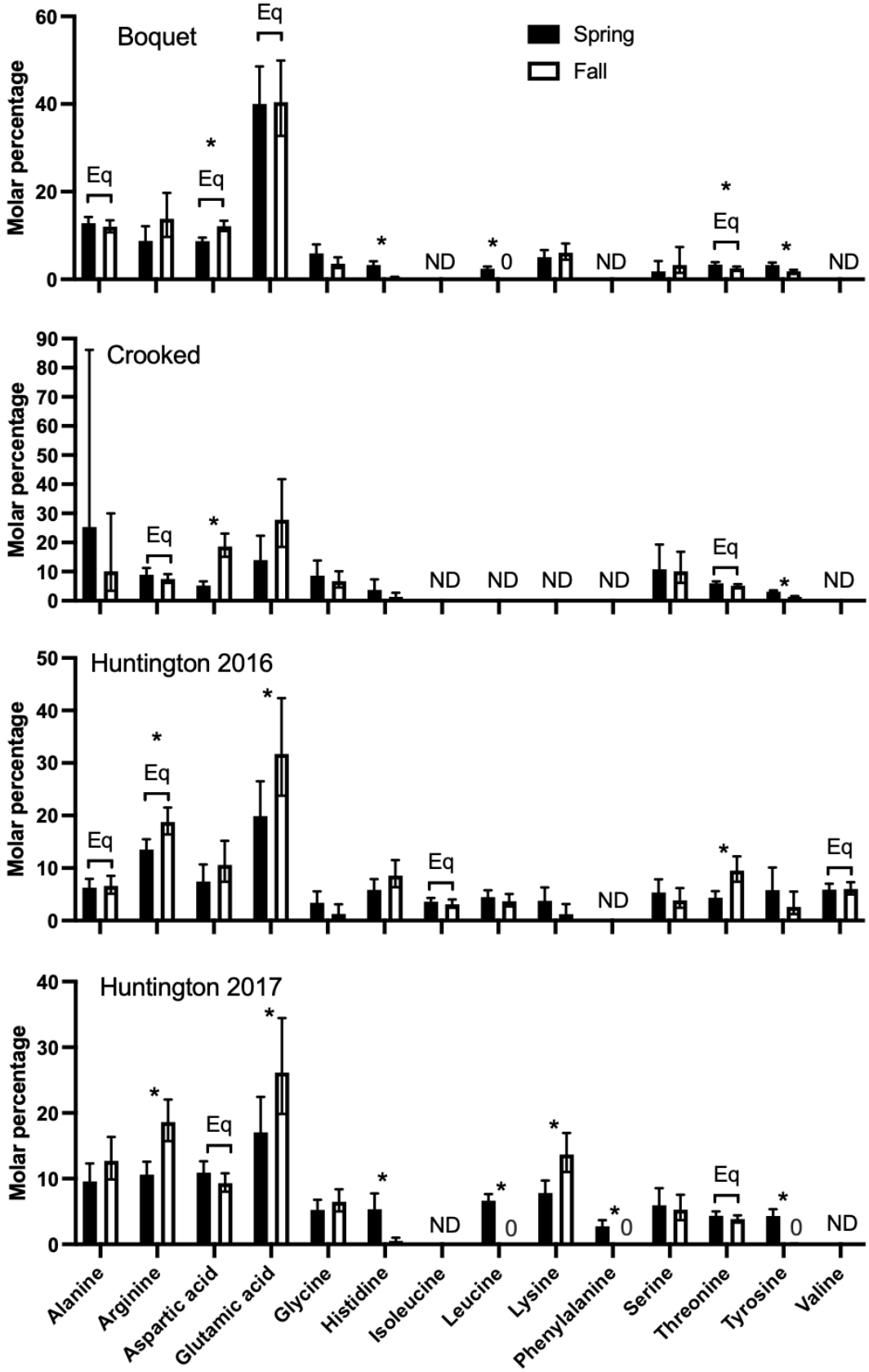
Boquet
Spring
Fall
Molar percentage
Crooked
Huntington 2016
Huntington 2017
Eq
ND
0
Alanine
Arginine
Aspartic acid
Glutamic acid
Glycine
Histidine
Isoleucine
Leucine
Lysine
Phenylalanine
Serine
Threonine
Tyrosine
Valine

Figure 4. Molar percentages of dissolved free amino acids (DFAA) in sediment pore water sampled in spring (black bars) and fall (white bars) from the Boquet, Crooked, and Huntington rivers. Mean and 95% confidence intervals are back-transformed from the natural-log scale. ND indicates a valid test could not be conducted because the DFAA was not detectable in any samples for both seasons or was detected in < 3 samples from one or both seasons. Zero indicates all samples were below 1 nM for that season. Asterisks indicate $p < 0.05$ for two-sided t tests of the difference in means is equal to zero following the step-down Holm method for controlling the familywise error rate (family = each unique stream-year pair). See table S3 and S4 for p values, including unadjusted p values, and additional information on the number of replicates for each DFAA and season comparison. "Eq" indicates that means are equivalent based on two one-sided t test, or a modification (i.e., one-sample t test) if one seasonal mean was zero.

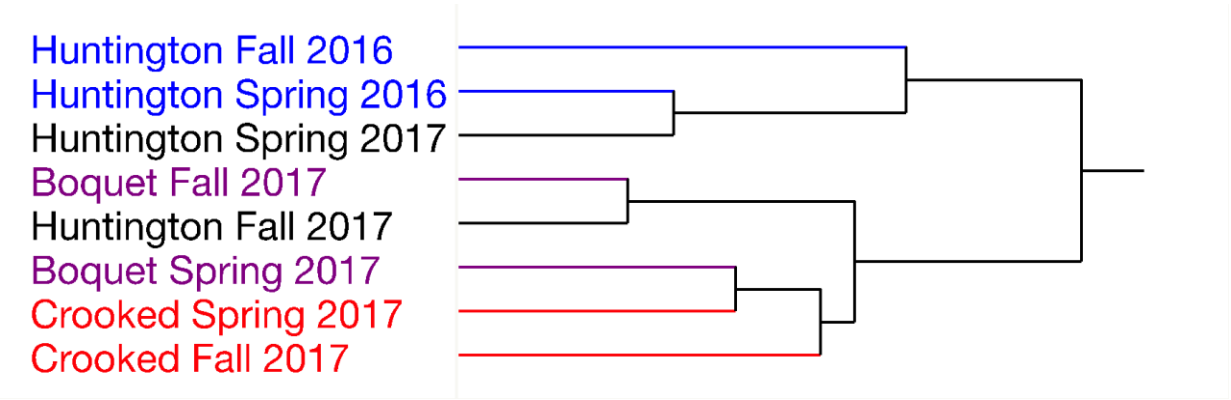

Figure 5: Hierarchical cluster analysis dendrogram of the mol% (natural-log (X + sample-specific mol% lower threshold) of benthic dissolved free amino acids in the Huntington, Boquet, and Crooked rivers in the seasons and years indicated. Season, river and year groupings that join closer to the left are more similar to each other than those that join farther to the right. For example, the Boquet and Huntington samples from the fall of 2017 are most similar to each other, and collectively they are more similar to the Crooked River samples and those from the Boquet River in spring than they are to the other Huntington River samples (i.e., Huntington fall 2016, spring 2016 and 2017). Note: the analysis included river specific seasonal means equal to zero if a DFAA was not detectable in any of the samples within a river and season, or if a DFAA was not detected in any samples from one season within a river and in less than three samples from the other season with a river.

Supplemental Information

Are spatiotemporal patterns of dissolved free amino acids among three Atlantic salmon rivers sufficient to provide unique and stable odor signatures for imprinting and homing?

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Supplementary Figure S1: Sampler used to collect sediment porewater samples.
Insets: show the scale and connection of 20 mL syringe and syringe plunger to sampler

Table S1. Adult returns of landlocked Atlantic salmon stocked as one-year old smolts to two Lake Champlain tributaries over three consecutive brood years (2010–2012). Adults were recaptured as they returned during fall spawning migrations to these two monitored rivers between 2013 and 2016. Homing adults were recaptured in the river they were stocked into as smolts. Straying adults were recaptured in the river they were not stocked into as smolts. Genetic parentage-based tagging was used to mark release groups. All data are from Harbicht et al. (2020).

River	Release Group	Smolts Stocked	Returns	Homing	Straying	Proportion Homing	Proportion Straying
Winooski	Groundwater	35,274	34	33	1	0.97	0.03
	Surface water	46,158	175	172	3	0.98	0.02
Boquet	Early Release	76,414	68	64	4	0.94	0.06
	Standard Release	87,565	129	120	9	0.93	0.07
Overall		245,411	406	389	17	0.96	0.04

Table S2. Sampling schedule for the five rivers sampled in New England, USA. N is the target number of independent replicate water column or sediment porewater samples. In some cases the number of replicates used in the analyses were less (see text) because the glass sampling vials cracked during freezing or transport. NA indicates not applicable because the stream or location was not sampled.

					Water column		Sediment porewater	
Watershed	**River**	**Years sampled**	**Fall months sampled**	**Spring months sampled**	**Sampled**	**N**	**Sampled**	**N**
Lake Champlain, Vermont	Huntington	2015	Sep. Oct. Nov.	none	Yes	3	No	NA
		2016	Oct.	Jun.	Yes	3	Yes	6
		2017	Oct.	Jun.	Yes	3	Yes	6
Lake Champlain, New York	Boquet River	2015	Sep. Oct. Nov.	none	Yes	3	No	NA
		2017	Oct.	Jun.	Yes	3	Yes	6
Lake Sebago, Maine	Crooked	2015	Sep. Oct. Nov.	none	Yes	3	No	NA
		2017	Oct.	Jun.	Yes	3	Yes	6
Lake Champlain, Vermont	Winooski	2015	Sep. Oct. Nov.	none	Yes	3	No	NA
		2017	none	none	NA	NA	NA	NA
Lake Sebago, Maine	Panther Run	2015	Sep. Oct. Nov.	none	Yes	3	No	NA
		2017	none	none	NA	NA	NA	NA

Table S3. Differences tests of comparisons between seasons within rivers of the mean molar percentage of benthic dissolved free amino acids (DFAA) in three rivers in New England, USA. The two-sided t tests used a Tobit model for left-censored data. Molar percentages were in the natural-log scale for the t tests. Italics indicates a one-sided t test was used with the fall mean equal to zero because the DFAA was not detected in the five fall samples. § indicates spring had only two replicates in which the DFAA was detectable. † indicates spring had only one replicate in which the DFAA was detectable. ND indicates that a valid t test could not be conducted because the DFAA was not detectable in any of the samples for both rivers, or the DFAA was not detected in any samples from one river and in less than three samples from the other river. * indicates the null hypothesis was not rejected based on the step-down Holm method controlling the type I familywise error rate (family = each unique stream-year), and the unadjusted p value is reported here. Bold indicates tests significantly different ($p < 0.05$) after controlling the type I error rate using the Holm method.

	Boquet	Crooked	Huntington	Huntington
Amino Acid	spring 2017-fall 2017	spring 2017-fall 2017	spring 2016-fall 2016	spring 2017-fall 2017
Alanine	0.428	0.272	0.769	0.016*
Arginine	0.063	0.257	**<0.001**	**<0.001**
Aspartic acid	**<0.001**	**<0.001**	0.176	0.958
Glutamic acid	0.949	0.030*	**0.025**	**0.004**
Glycine	0.030*	0.440	0.056†	0.056†
Histidine	**<0.001**	0.043*	0.080	**<0.001**
Isoleucine	ND	ND	0.361§	ND
Leucine	***<0.001***	ND	0.332	***<0.001***
Lysine	0.396	ND	0.039*†	**<0.001**
Phenylalanine	ND	ND	ND	***<0.001***
Serine	0.310	0.876	0.299	0.912
Threonine	**0.004**	0.069	**<0.001**	0.787
Tyrosine	**<0.001**	**<0.001**	0.091§	***<0.001***
Valine	ND	ND	0.837	ND

Table S4. Equivalence tests of comparisons between seasons within rivers of the mean molar percentage of benthic dissolved free amino acids (DFAA) in three rivers in New England, USA. Equivalence is based on two one-sided tests testing the null hypothesis that the means are different by at least some specified lower and upper limits listed here. Rejecting the null hypothesis of both one-sided tests indicates that the means are equivalent at least within the specified lower and upper equivalence limits (0.612 – 1.634, derived from Yamamoto et al. 2013). Molar percentages and equivalence limits were in the natural-log scale for the two one-sided tests . Shading indicates seasonal equivalent DFAA means, determined if both the lower and upper one-sided test p <0.05. Italics indicates the lower equivalence limit was set to zero because for these comparisons DFAA concentrations were not detectable in the fall samples. Except where noted there are n=6 independent replicates for spring and fall. § indicates spring had only two replicates in which the DFAA was detectable. † indicates spring had only one replicate in which the DFAA was detectable. ND indicates that two one-sided tests could not be conducted because the DFAA was not detectable in any of the samples for both seasons, or the DFAA was not detected in any samples from one season and in less than three samples from the other season.

	Boquet 2017 spring vs. fall		Crooked 2017 spring vs. fall		Huntington 2016 spring vs. fall		Huntington 2017 spring vs. fall	
Amino acid	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
Alanine	<0.001	<0.001	0.046	0.695	0.007	0.001	0.124	<0.001
Arginine	0.444	0.001	<0.001	0.023	0.051	<0.001	0.720	<0.001
Aspartic acid	0.009	<0.001	0.999	0.001	0.298	<0.001	<0.001	0.001
Glutamic acid	<0.001	<0.001	0.734	<0.001	0.454	<0.001	0.377	<0.001
Glycine	<0.001	0.508	0.001	0.217	0.002†	0.832†	0.067	<0.001
Histidine	<0.001	0.999	0.001	0.847	0.298	<0.001	0.001†	0.997†
Isoleucine	ND	ND	ND	ND	<0.001§	0.014§	ND	ND
Leucine	*0.916*	*<0.001*	ND	ND	<0.001	0.075	*0.999*	*<0.001*
Lysine	0.063	<0.001	ND	ND	0.002†	0.877†	0.664	<0.001
Phenylalanine	ND	ND	ND	ND	ND	ND	*0.999*	*<0.001*
Serine	0.573	0.032	0.080	0.137	0.004	0.294	0.009	0.079
Threonine	<0.001	0.039	<0.001	<0.001	0.945	<0.001	<0.001	<0.001
Tyrosine	<0.001	0.797	<0.001	0.999	0.003§	0.742§	*0.999*	*<0.001*
Valine	ND	ND	ND	ND	<0.003	0.001	ND	ND

Preparation of Reagents:
During all preparation steps, the contamination of reagents with free amino acids was minimized by using acid-washed (overnight soaking with 1M HCl), furnaced glass ware and clean pipette tips, and using nitrile gloves. Pipette tips were soaked in 0.5 M HCl (Baker Ultra grade) for 30 minutes in a clean leak-proof HDPE container. After rinsing with copious amounts of 18.2 MOhm deionized water (Millipore A10 system), pipette tips were transferred with a methanol-rinsed (Baker HPLC-grade, Teflon squire bottle) tweezer to tip boxes. Residual water was removed by gently tapping the tip box on the lab bench. The tips were finally rinsed with methanol (Baker HPLC-grade) 3-times and gently tapped on the lab bench to remove residual methanol before drying overnight in an oven at 50 C.

0.5 M borate buffer was prepared by weighing 0.65 g of boric acid (Baker Ultra reagent) in a 50 mL EPA screw-cap vial with a Teflon-backed silicone septum. Both, the HDPE cap and septum were cleaned separately by soaking in 0.5 M HCl for 30 minutes followed by rinsing with deionized water and methanol. The cap and septum were reassembled by placing the septum on the vial with a clean tweezer and pushed the HDPE cap down on the vial to position the septum. 20 mL of deionized water (Millipore A10 system) were weighed in by dispensing water from a Teflon squirt bottle. The pH was adjusted to 9.5 with 50 w/w% sodium hydroxide (Baker Ultra reagent) with a furnaced glass pipette. The final pH was checked with a pH microelectrode. The electrode was rinsed with water and methanol before immersion. The solution was stored at 4 C and made fresh every week.

The ortho-phthaldialdehyde (OPA)/mercaptopropanoic acid (MPA) derivatization reagent was made fresh before every batch analysis of samples. 36 mg of OPA was weighed into a furnaced glass vial and dissolved in methanol (Baker HPLC-grade). This stock solution was stored at 4 C and made fresh every week. The OPA/MPA reagent was made by mixing 0.65 mL of OPA stock solution with 2 mL of 0.5 M borate buffer (pH = 9.5) and 22 µL of MPA (Sigma, >99.0% HPLC).

Diluent was used to reduce the pH of the injected sample after derivatization to prolong the lifetime of the chromatography column and maintain the separation of derivatized amino acids. The diluent was prepared by mixing 33 mL of mobile phase solvent A (9.6 mM anhydrous sodium di-hydrogen phosphate and 9.7 mM boric acid adjusted to pH 8.15) and 0.5 mL of concentrated phosphoric acid (Sigma ACS reagent). Diluent was stored at 4 C and made fresh every week.