INVESTIGATING OLFACTORY IMPRINTING RELATED BEHAVIORS IN JUVENILE LAKE STURGEON (*ACIPENSER FULVESCENS*) AND THE POTENTIAL ROLE OF STREAM SPECIFIC AMINO ACID PROFILES

By

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ABSTRACT

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Olfactory imprinting is one mechanism thought to guide natal stream homing and facilitate natal site fidelity, an important reproductive strategy that leads to localized adaptations in genetically distinct fish populations. My thesis investigates olfactory imprinting during early ontogeny in lake sturgeon and the potential role of stream specific amino acid profiles as the guiding odors in olfactory imprinting and stream discrimination by lake sturgeon. In Chapter 1, I test the hypothesis that olfactory memory formation occurs in early development and provide behavioral evidence of olfactory imprinting during the free-embryo and exogenous feeding life stages in lake sturgeon. In Chapter 2, I describe the temporal and spatial variability of amino acid profiles in Great Lakes tributaries and discuss the potential utility and limitations for amino acids to function as odorants guiding olfactory imprinting in lake sturgeon in the Great Lakes. In this chapter, I also provide empirical evidence for olfactory memory formation to artificial amino acid profiles during early ontogeny and suggest further studies to see unequivocal evidence on whether lake sturgeon discriminate stream specific amino acid profiles. This thesis supports the use of streamside rearing facilities for exposing lake sturgeon to natal stream odors during early life stages.

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KEY TO ABBREVIATIONS

cm	centimeter
mm	millimeter
μm	micrometer
cc	cubic centimeter
L	liter
ml	milliliter
μl	microliter
M	molar
mM	millimolar
h	hour
min	minute
s	second
mg	milligram
kV	kilovolt
°C	Celsius
SE	standard error
Δ	delta (change)
PFHA	perfluorohexanoic acid
LC-MS/MS	liquid chromatography - tandem mass spectrometry
UPLC	ultra-performance liquid chromatography
ESI-MS/MS	electrospray ionization - tandem mass spectrometry

CHAPTER 1:

Behavioral evidence of olfactory imprinting during early ontogenetic stages of lake sturgeon (*Acipenser fulvescens*)

ABSTRACT

Spawning site selection and the timing of spawning migrations affects offspring development and survival in migratory fish species. Offspring survival and advantages passed on to offspring through spawning site selection are thought to have led to the evolution of natal site fidelity and natal homing in many fish species, leading to localized adaptations for specific spawning sites in genetically distinct populations of the same species. Olfactory imprinting guided natal stream homing has been documented in Pacific salmon for decades and is suspected to occur in other natal homing species. Genetic structuring of lake sturgeon (*Acipenser fulvescens*) populations across the Great Lakes suggests natal site fidelity and natal homing in this species. The lake sturgeon management community has embraced the use of streamside rearing facilities in stocking and reintroduction programs, which raise fish in river water to expose developing lake sturgeon to natal stream odorants that guide olfactory imprinting. Evidence for olfactory imprinting in lake sturgeon is limited to studies of gene expression and development of the olfactory system, and the timing of olfactory imprinting in lake sturgeon is unknown. This study investigates olfactory memory formation during early ontogenetic stages in lake sturgeon to two artificial odorants, phenethyl alcohol and morpholine, by measuring behavioral responses to the odorants in juvenile lake sturgeon. Lake sturgeon raised in artificial odorants during the free-embryo and exogenous feeding larval stages displayed larger behavioral responses to the artificial odorants as juveniles than fish not raised in the artificial odorants. This study provides the first behavioral evidence of olfactory imprinting in lake sturgeon. Findings from this study support the use of streamside rearing facilities and the importance of exposing developing lake sturgeon to natal stream odorants to ensure olfactory imprinting guided natal stream homing is successful in stocked lake sturgeon.

INTRODUCTION

Migrations from feeding to specific spawning locations are common in many fish species and provide mechanisms by which adults pass along advantages to offspring based on the indirect benefits of spawning timing and site selection (Jørgensen et al., 2008; Leggett, 1977). Both the timing of migration and the site selected have implications on the rate of early development and offspring survival (Forsythe et al., 2012; Reznick et al., 2006). Benefits inherited by offspring based on optimal spawning choices by parents selects for repeated spawning at the optimal location, or spawning site fidelity, which leads to localized adaptions and genetically distinct populations of different spawning sites (Leggett, 1977). Natal site fidelity creates a functional barrier between genetically distinct population with beneficial site-specific adaptations. However, interbreeding caused by straying of adults into other spawning sites may lead to outbreeding depression, which is the reduction in fitness because of the breakdown of coadapted genotypes that are adapted to species and (largely natal) environments (Edmands, 2007).

Understanding the mechanisms guiding natal site homing is important for the management of migratory species and studies have shown that straying from natal sites may occur more often by stocked individuals (Quinn, 1993). Natal homing in salmon has been well studied and shown to be mediated by olfactory imprinting to stream-specific odors during early life stages (Dittman and Quinn, 1996), though the timing of spawning and ocean migrations may also be guided by geomagnetic orientation and conspecific cues (Bett and Hinch, 2016; Ueda, 2011). Natal site fidelity is widespread in fish, and it is hypothesized that olfactory imprinting may play a role in guiding natal stream migrations in many fish species, though studying

olfactory imprinting in non-salmonid species provides many challenges dependent on the ecology and life history of a species (Horrall, 1981; Cathcart, 2021).

Lake sturgeon (*Acipenser fulvescens* Rafinesque, 1817) is a potamodromous (lake to river migrating) species (Bemis and Kynard, 1997; Bruch and Binkowski, 2002), native to the Great Lakes, Mississippi River, and Hudson Bay drainages (Scott and Crossman, 1973). The species is listed as a species of concern, threatened, or endangered across much of its native range (Léonard et al., 2004) because of overfishing, habitat loss, climate change, and high juvenile mortality (Auer, 1996; Auer, 1999; Peterson et al., 2007; Pollock et al., 2015). Reduced numbers across its native range have led to the development of management plans including stocking of juvenile lake sturgeon (Hay-Chmielewski and Whelan, 1997; Hayes and Caroffino, 2012; Manny and Mohr, 2012). Conservation programs for lake sturgeon have widely embraced the importance of hatchery supplementation as a viable restoration measure (Holtgren et al., 2007).

Several life history traits of lake sturgeon pose challenges to conservation programs. Genetic differentiation among lake sturgeon populations has been documented within and between the Great Lakes and its tributaries, suggesting high natal stream fidelity (DeHaan et al, 2006; Scribner et al., 2021; Welsh et al, 2008). Homing to the spawning stream is likely an important mechanism that contributes to lake sturgeon diversity within its native range (Homola et al., 2012). Donofrio et al. (2018) demonstrated the spawning site fidelity of lake sturgeon through genetic analyses and acoustic telemetry, but also documented straying of adults between tributaries (also see Homola et al., 2012). It is therefore critical to characterize mechanisms of lake sturgeon home stream fidelity in support of current conservation programs.

Natal stream homing in lake sturgeon is believed to be guided in part by olfactory imprinting of natal stream odors during the first year of development (Cathcart, 2021). Evidence

has been limited to studies of changes in olfactory system development during early ontogeny (Dang and Zhang, 2014). One period of potential olfactory imprinting was identified following the transition from free-embryos to exogenously feeding larvae, a period of rapid forebrain development in key olfactory information centers (Dang and Zhang, 2014). These findings are consistent with strong evidence that Pacific salmon use olfactory cues learned during early life stages to home to their natal streams (Bett and Hinch, 2015; Dittman and Quinn, 1996; Hasler et al., 1978). Direct tests of the olfactory imprinting hypothesis in lake sturgeon, however, are particularly difficult because it this long-lived and late-maturing species has an average maturation age of 12-20 years for males and 14-33 years for females (Bruch and Binkowski, 2002; Dammerman et al., 2019; Thiem et al., 2013), complicating experiments to study spawning migration of adult that had been imprinted to natal stream odorants in age-0 stages.

In this study we examined one essential facet of the olfactory imprinting guided natal stream homing hypothesis, the ability to form olfactory memory of odorants during early ontogenesis (Hino et al., 2009), in lake sturgeon. Studies of olfactory imprinting in salmon have typically focused on behaviors of mature adults when migratory behaviors to spawning streams can be observed. The study of olfactory memory in juvenile fish may provide an avenue for understanding the timing of olfactory imprinting and implicate efficient and effective methods for rearing lake sturgeon in natal stream odors during critical memory forming stages.

We hypothesized that lake sturgeon imprint to odorants experienced during the free-embryo and exogenous feeding early life stages. Our hypothesis is based upon evidence that the free-embryo and exogenous feeding stages represent periods of rapid development and behavioral changes in lake sturgeon (Dang and Zhang, 2014; Eckes et al., 2015), which parallels the rapid change in physiology and behavior of salmon during the parr-smolt transformation

(PST) olfactory imprinting period (Hasler and Scholz, 1983; Morin et al., 1989). The free-embryo stage is notable for its rapid organ development and the transition from the free-embryo stage to the exogenous feeding stage marks the start of rapid forebrain development (Dang and Zhang, 2014). We further predicted that age-0 lake sturgeon change their behavior activities in response to experimental odorants following one or more exposure periods during early development. We developed methods for observing behaviors in age-0 lake sturgeon, and our experiments provide evidence for olfactory imprinting by developing lake sturgeon and identify likely periods during which imprinting occurs. Results have implications for restoration programs throughout the species range that increasingly rely on hatchery stocking to rebuild or reintroduce lake sturgeon to specific streams.

METHODS

Experimental animals

Lake sturgeon used in experiments were reared from egg fertilization conducted at the Black Lake Sturgeon Rearing Facility in Onaway, MI, USA, which operates as a flow-through streamside rearing facility (SRF) using water supplied directly from the Upper Black River at ambient temperature. Eggs and sperm were sampled from lake sturgeon in the Upper Black River on May 4, 2021 and eggs were fertilized within the day following standardized hatchery procedures (Bauman et al., 2015; Crossman et al., 2011). Offspring from one individual male and one individual female were used in the experiments. The use of full siblings was expected to reduce variation due to additive genetic effects (Dammerman et al., 2015; 2020). Experimental animals were used with approval from the Michigan State University Animal Use and Care Committee (AUF PROTO202000023/AMEND202100062).

Exposure to experimental imprinting odorants

Fish were exposed to two experimental odorants mixed in hatchery water, Phenethyl Alcohol (PEA) at 1.04×10^{-7} M and morpholine at 9.9×10^{-11} M. Odorants and odorant concentrations were selected to replicate olfactory imprinting studies in Pacific salmon as has been used to demonstrate olfactory imprinting guided natal homing behaviors (Bett and Hinch, 2016). These compounds are potent odorants for fish and allowed control of the exact concentrations and periods during which odorants were experienced without potential for confounding effects of background odorants. Experimental odorant exposure occurred during four early developmental stages: fertilized egg (0 days post-fertilization [dpf]), hatched free-embryo larvae (12 dpf), exogenously feeding larvae (19 dpf) – when individuals began feeding on brine shrimp (*Artemia* spp.), and a juvenile stage (49 dpf) – when individuals began feeding

on blood worms (Diptera: Chironomidae). Lake sturgeon exposure to odorants was organized into ten unique combinations of developmental stage treatment (Table 1.1). One group was never exposed to the experimental odorants and one group was exposed during all stages. Four groups of fish were exposed during a single stage and four groups were exposed during two to three consecutive stages including either the egg or juvenile stage.

Table 1.1. Timing and duration of the four developmental stages (egg, free-embryo [FE], exogenous feeding larvae [larvae], and juvenile [juv.]) and ten experimental odorant exposure treatments. Start time for each developmental stage refers to the number of days post-fertilization (dpf) and stage duration refers to the total length (days) of each developmental stage. Exposure stages associated with each treatment are indicated in white (developmental stages without odorant exposure) and grey (developmental stages with odorant exposure). Exposure length was measured in days. Lag time represents the range of days between the final day of odorant exposure and the start of behavior experiments for individuals in each treatment group.

	Developmental stages					
	Egg	Free-Embryo	Exogenous Feeding Larvae	Juvenile		
Start time (dpf)	0	12	19	49		
Stage duration (days)	12	7	30	14		
Treatment	**Exposure stages**				Exposure (days)	Lag time (days)
Control					0	70-71
Egg					12	59 - 61
FE					7	52 - 54
Larvae					30	22 - 24
Juv.					14	8 - 10
Egg-FE					19	52 - 54
Larvae-Juv.					44	8 - 10
Egg-FE-Larvae					49	22- 24
FE-Larvae-Juv.					51	8 - 10
Egg-FE-Larvae-Juv.					63	7 - 8

The selected developmental stages represent four distinct periods of development, behavior, and location/habitats lake sturgeon occupy in natural stream environments when olfactory imprinting may occur. The beginning and end of each stage were determined by critical

thermal units and physiology (Eckes et al., 2015). The egg stage began immediately with fertilization in water column and eggs adhere to the stream substrate. The free-embryo stage begins at hatch and is the period when lake sturgeon burrow into the substrate to avoid predators and consume their yolk-sac (Detlaff et al., 1993; Kempinger, 1988). The exogenous feeding larvae stage represents the period when fish have depleted their yolk-sac, begun feeding from the external environment, and emerged from the gravel to drift downstream in river currents (Auer and Baker, 2002). The juvenile stage for our experiment occurred after larval drift has been completed and lake sturgeon are typically foraging for food in the natal river system. Exposure to the experimental odorants during multiple consecutive stages allowed us to compare the effects of exposure stage and exposure duration on olfactory imprinting. Although olfactory imprinting likely occurs during a short developmental window (Gerlach et al., 2019), other forms of olfactory memory may have shorter retention times and exposure during long periods of time may allow reinforcement of olfactory memory (Triki and Bshary, 2019).

Rearing conditions

Each treatment group was raised in a separate 18-gallon tank in a flow-through system with 50 micron filtered stream water from the Black River from fertilization through the start of experiments. Fertilized eggs were placed in McDonald hatching jars (Pentair, Apopka, FL). Hatched fish were held in 3L aquaria with bio ball filters (CBB1-S; Pentair, Apopka, FL) to simulate natural stream substrate. Exogenous feeding fish were removed from the simulated substrate and held in 3L aquaria, while juvenile fish were held in the larger 18-gallon tanks. Each treatment group was raised in three replicate tanks, and by the start of behavior experiments each treatment had one to three replicates depending on mortality during the memory formation period. Odorants were mixed and replaced daily in 3L of hatchery water to ensure desired

concentrations were met. A peristaltic pump was used to pump the odor mixture into a head tank supplying water to tanks receiving the experimental odorants. Rhodamine was pumped into the head tank and measured using a hand-held DataBank datalogger and Cyclops-7 Optical Rhodamine Dye Tracer (Turner Designs, Sunnyvale, CA) to validate even mixing of odorants in the head tank and even distribution of odorants to all tanks. Time of odorant mixture replacement and the volume of odorant mixture remaining each day was used to track daily odorant concentrations across all tanks. Daily odorant concentrations for PEA ranged from 0 M on days where the pump failed (this occurred on three different days) to 1.04×10^{-7} M and 0 M to 9.90×10^{-11} M for morpholine, with an average concentration of $9.71 \times 10^{-8} \pm 2.22 \times 10^{-9}$ M (mean ± SE) for PEA and $9.24 \times 10^{-11} \pm 2.11 \times 10^{-12}$ M (mean ± SE) for morpholine over the duration of the experiment.

Behavior experiments

Juvenile lake sturgeon swimming and activity behaviors were observed in response to PEA and morpholine exposure as a test of olfactory memory of the artificial natal odorants. Twenty individuals were observed from each treatment group, with an equal number of individuals observed for each replicate tank. Trials took place in a cylindrical tank with a 1975.8 cm^2 base filled with 3 L of groundwater from the facility (Figure A1.1). Four identical arenas were used to measure behaviors, allowing for multiple trials to be run concurrently. For each trial, one individual fish was removed from its housing tank and a digital photo was taken for body length measurements. The fish was then acclimated to the enclosure for seven and a half minutes, and videos were recorded for five minutes after acclimation to measure pre-odor behaviors. Odorant solutions were created to reach the desired concentrations of 1.04×10^{-7} M for PEA and 9.90×10^{-11} M for morpholine in the behavioral arena. A volume of 100ml of

odorants stock solution was added using two 50ml syringes. Dye tests were used during method development to ensure odorants mixed evenly in the arena. One minute after the initial addition of odorants, another five-minute video was recorded to measure behaviors post-odor application. Fish were then removed from the enclosures, and enclosures were thoroughly rinsed with groundwater before the next trial began. To prevent outside stimuli from affecting fish behaviors, fish were observed in the evening outside of working hatchery hours, under red lighting. Length was also measured for each fish with the ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.; http:// rsbweb.nih.gov/ij/) to ensure differences in behavior were not solely resulting from physical differences between individuals. Videos were analyzed using Loligo v.4.0 tracking software (Loligo Systems, Viborg, Denmark; https://www.loligosystems.com/software), which recorded average velocity (cm/s), average acceleration (cm/s^2), average deacceleration (cm/s^2), time active (s), time active (%), time inactive (s), time inactive (%), and total distance traveled (cm).

Fish never exposed to the experimental odorants and fish exposed during all four stages were observed first. Individuals from these treatment groups were first observed after the addition of a positive stimulus (bloodworm odorants) and a control stimulus (groundwater from the facility) to ensure our behavioral assay could identify behavioral differences in response to stimuli. After method validation, individuals from these treatment groups were then observed to measure behavioral responses to the experimental odorants, PEA and morpholine. The order for observations of all other treatment groups was randomized.

Statistical analyses

All analyses were conducted using *R* (v4.1.2; R Core Team, 2021). To identify relationships between behavioral metrics and to select an informative metric to use in our

analysis, we calculated correlations between response variables using the *corrplot* package (v0.92; Wei and Simko, 2021). Using the absolute value of Pearson correlation coefficients, we found strong pairwise correlations ($|r| \geq 0.89$) between average velocity, average acceleration, average deacceleration, and total distance traveled variables (Figure 1.1). We also found strong correlations ($|r| \geq 0.93$) between time active and time inactive measures. There was a moderate correlation ($|r| > 0.62$) between distance traveled and all time active and inactive measures. Pairwise correlations between all potential response variables were non-zero ($p < 0.001$). Due to its correlation with the other response variables, total distance traveled was selected as the single representative behavior to be used in our statistical analyses of odorant response.

	Avg. acceleration	Avg. deacceleration	Time active (%)	Time active (s)	Time inactive (s)	Time inactive (%)	Distance Traveled
Avg. velocity	**0.98**	**-0.98**	**0.36**	**0.35**	**-0.34**	**-0.36**	**0.89**
Avg. acceleration		**-1.00**	**0.27**	**0.26**	**-0.27**	**-0.27**	**0.84**
Avg. deacceleration			**-0.27**	**-0.26**	**0.27**	**0.27**	**-0.84**
Time active (%)				**0.99**	**-0.97**	**-1.00**	**0.66**
Time active (s)					**-0.93**	**-0.99**	**0.66**
Time inactive (s)						**0.97**	**-0.63**
Time inactive (%)							**-0.66**

Figure 1.1. Matrix showing pairwise correlations between all measured post-odor behavioral responses of juvenile lake sturgeon from the Loligo tracking software. All correlations were non-zero ($p<0.001$).

Prior to statistical analysis, we checked for visual and statistical outliers in both pre-odor and post-odor distance traveled. Outliers were suspected in some cases based on lighting variation and blemishes on the tank background that influenced fish tracking in the Loligo v.4.0 tracking software. Four trials were removed from the analysis because of tracking related issues or incomplete video recordings. Visual outliers were identified when an individual fish showed behavioral responses that were behaviorally questionable during both the pre-odor and post-odor periods. Statistical outliers were identified using a Grubb's Test with the *outliers* package (v0.14; Komsta, 2011) and observations were considered for removal when both pre-odor and post-odor distance traveled measures were significant outliers. Two additional trials were only removed based on both visual and statistical criteria.

We modeled post-odor distance traveled under a normal distribution using robust linear regression as a function of a variety of predictor variables measured throughout our experiments. Normality of residuals and homoscedasticity were assessed following model selection for a traditional linear regression and the model did not meet the assumptions; specifically, we observed multiple highly influential (high leverage) observations in our model based on the residual quantile-quantile and residuals vs. leverage plots (Chatterjee and Hadi, 1986) (Figure A1.2). Based on these findings, we performed robust linear regression models using an M estimator, which downweighs highly influential observations without removing observations from the analysis (Filzmoser and Nordhausen, 2021). Models were compared to select fixed effects to include in full model predictions and inference. To account for individual variation in swimming behaviors and activity, pre-odor distance traveled was included as a predictor variable in all but the null model. The fixed effects included individual length, pre-odor distance traveled, treatment group, and their pairwise interactive effects. Models were compared using Akaike

Information Criterion – small sample size correction (AICc) with the *AICcmodavg* package (v2.3-1; Mazerolle, 2020). All models within two AICc of the top model were considered (Tredennick et al., 2021).

Our experimental design incorporated two nuisance grouping factors, the tank in which fish were raised before the initiation of behavior experiments and the arena used for observations, which we incorporated into model interpretation to account for non-independence of individuals based on these factors. Robust linear mixed models were run based on results from the fixed effects model selection using the *robustlmm* package (Koller, 2016). Robust linear mixed models included arena as a random intercept, tank as a random intercept, or both arena and tank as crossed random effects. Tank and arena were low-level random effects, with tank groupings ranging from one to three for each treatment and only four unique arenas used for behavior experiments. Given we were not interested in making inferences on the random effects, we included them for model interpretation (Gomes, 2022). Figures were produced for the focal model using the *ggplot2* (Wickham, 2016) and *cowplot* (v1.1.1; Wilke, 2020) packages. Predictions based on robust linear models were made using the *predict.rlm* function from the *MASS* package (Venables and Ripley, 2002).

RESULTS

Lake sturgeon reared in PEA and morpholine during early life stages traveled a greater distance after exposure to the odorants in our behavioral experiments compared to naïve individuals. AICc values indicated the best-fit model included pre-odor distance traveled, treatment, and their interaction (Table 1.2). The intercept estimates of our top models indicate post-odor behavior for individuals with low pre-odor behavior measures differed between treatment groups (Table 1.3). Median pre-odor and post-odor distance traveled also differed across treatment groups (Figure 1.2) and there was a positive correlation between pre-odor and post-odor total distance traveled across all treatments, though this relationship varied by treatment (Table 1.3). All treatment groups, other than fish exposed during the juvenile stage only, had a larger increase in post-odor distance traveled with a unit increase in pre-odor distance traveled (slope) when compared with fish never exposed to PEA or morpholine prior to experiments (Figure 1.3A). With a mean pre-odor distance of 2634.95 ±163.040 cm (mean ± SE), all treatment groups had higher predicted post-odor distance traveled when compared to the control group (Figure 1.3B). Fish exposed during the exogenous feeding stage only had the highest predicted post-odor distance traveled response at the mean pre-odor distance traveled (55.99 % larger than the control, followed by fish exposed during the free-embryo stage only (42.29 % larger than control). Predicted post-odor distance traveled responses at the mean pre-odor distance and slope estimates were not larger for fish exposed during consecutive stages when compared to fish exposed during the free-embryo or exogenous feeding stages only (Figure 1.3).

Table 1.2. Comparison of AICc differences of robust linear regression models for post-odor distance traveled responses associated with different fixed-effects. AICc differences are calculated in reference to the model with the lowest AICc. Fixed effects included treatment, pre-odor distance, total length of the individual, and pairwise interactions between the independent variables.

Model	ΔAICc
Treatment + Pre-odor distance + Treatment*Pre-odor distance	0
Treatment + Pre-odor distance + Length + Treatment * Pre-odor distance	2.71
Pre-odor distance	3.53
Length + Pre-odor distance + Length * Pre-odor distance	5.42
Length + Pre-odor distance	5.48
Treatment + Pre-odor distance	13.34
Treatment + Pre-odor distance + Length	15.57
Treatment + Pre-odor distance + Length + Length * Pre-odor distance	16.94
Treatment + Pre-odor distance + Length + Treatment * Pre-odor distance + Length * Pre-odor distance + Treatment * Length	18.26
Treatment + Pre-odor distance + Length + Treatment*Length	23.4
Intercept only	184.45

Table 1.3. Parameter estimates and standard errors (SE) for the robust linear regression model of post-odor distance traveled based on treatment group (e.g., juvenile [juv.]), pre-odor distance traveled, and the interaction between treatment and pre-odor distance traveled. Estimates on the left are from the fixed-effects only models and estimates on the right are from a robust linear mixed model with the arena used for observations as a random intercept.

Parameter	Estimate	SE
Fixed-only		
Intercept	521.229	283.05
Egg	-254.647	424.32
Hatch	-720.232	427.00
Larvae	-802.955	418.29
Juv.	423.184	402.24
Egg-Hatch	-360.368	404.10
Larvae-Juv.	276.980	393.30
Egg-Hatch-Larvae	-311.486	394.62
Hatch-Larvae-Juv.	480.305	393.42
Larvae-Juv. * Pre-odor dist.	0.106	0.12
Hatch-Larvae-Juv. * Pre-odor dist.	0.118	0.12
Egg-Hatch-Larvae-Juv. * Pre-odor dist.	0.180	0.14
Egg-Hatch-Larvae-Juv.	-108.347	430.44
Pre-odor dist.	0.440	0.10
Egg * Pre-odor dist.	0.223	0.16
Hatch * Pre-odor dist.	0.543	0.14
Larvae * Pre-odor dist.	0.662	0.14
Juv. * Pre-odor dist.	-0.045	0.12

Table 1.3 (Cont'd)

Egg-Hatch * Pre-odor dist.	0.366	0.13
Egg-Hatch-Larvae * Pre-odor dist.	0.372	0.13
Fixed + Arena as a random intercept		
Intercept	858.464	329.28
Egg	-417.863	435.75
Hatch	-877.482	438.37
Larvae	-1080.007	433.89
Juv.	281.279	418.70
Egg-Hatch	-569.650	417.14
Larvae-Juv.	144.816	403.88
Egg-Hatch-Larvae	-498.138	405.07
Hatch-Larvae-Juv.	312.664	403.80
Egg-Hatch-Larvae-Juv.	-423.665	441.74
Pre-odor dist.	0.246	0.10
Egg * Pre-odor dist.	0.339	0.16
Hatch * Pre-odor dist.	0.683	0.15
Larvae * Pre-odor dist.	0.807	0.14
Juv. * Pre-odor dist.	0.102	0.13
Egg-Hatch * Pre-odor dist.	0.518	0.13
Larvae-Juv. * Pre-odor dist.	0.254	0.12
Egg-Hatch-Larvae * Pre-odor dist.	0.515	0.14
Hatch-Larvae-Juv. * Pre-odor dist.	0.264	0.13
Egg-Hatch-Larvae-Juv. * Pre-odor dist.	0.388	0.14

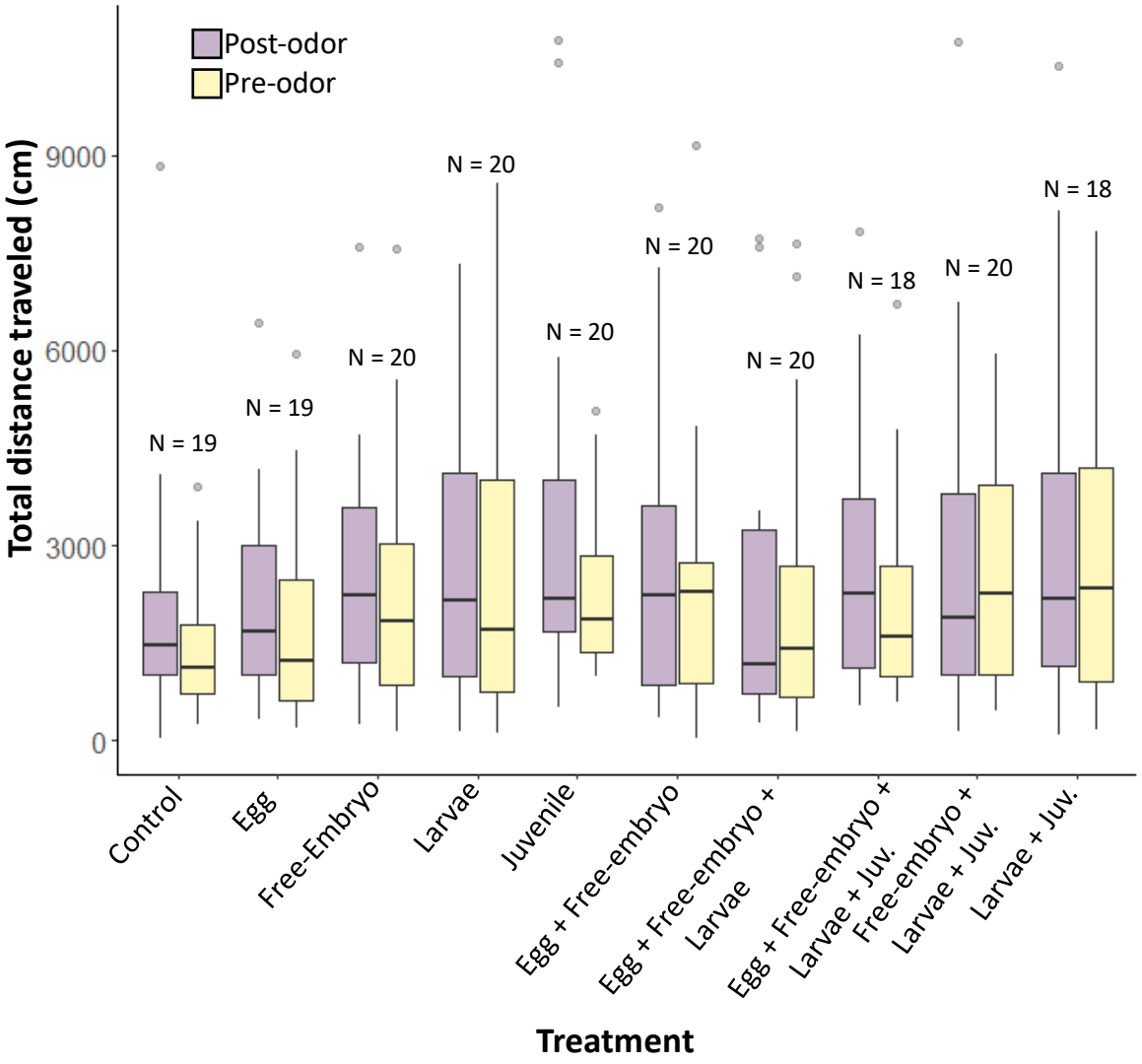

Figure 1.2. Boxplot of distance traveled (mean + SE) behaviors before (yellow) and after (purple) odor application for each treatment group (see Table 1.1 for diagram and information on exposure duration). Black horizontal bars represent the median and points represent outliers. Whiskers represent variability outside of upper and lower quartiles. Numbers above the boxplots indicate sample size for each treatment group.

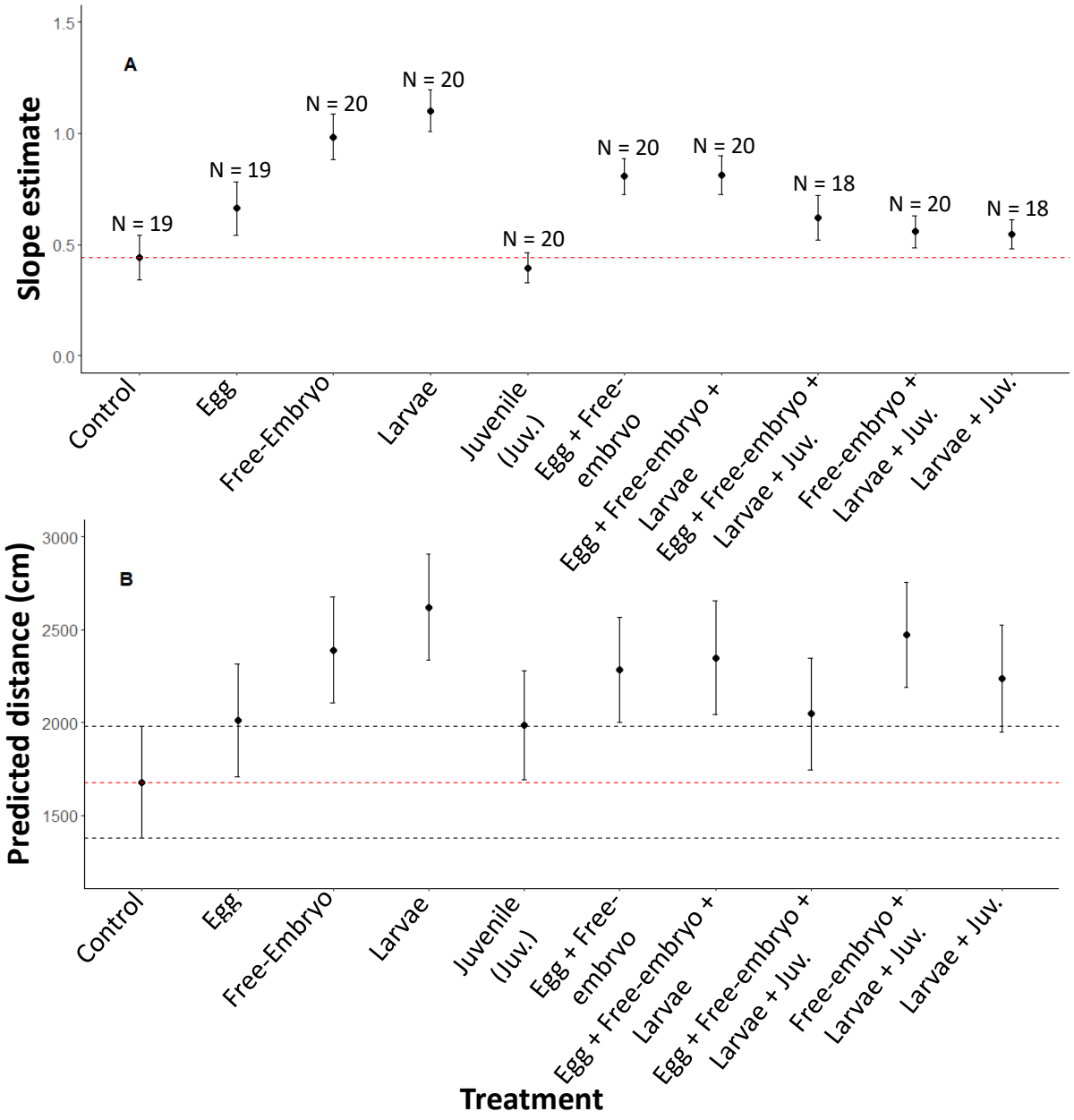

Figure 1.3. Slope estimates for the relationship between pre-odor distance traveled (A) and predicted post-odor distance traveled at the mean of 2634.95 cm after 1000 simulations (B) based on the robust linear model relating post-odor distance to treatment, pre-odor distance, and the interaction between treatment and pre-odor distance. Error bars represent one standard error of the slope estimates (A) and 95% confidence intervals of predicted responses (B). Red dashed horizontal lines were included for comparison between the control and other treatment groups and represent the estimated slope value and predicted post-odor distance for the control. Black dashed horizontal lines represent the value of the lower and upper 95% confidence intervals of the post-odor distance prediction for the control group. Numbers above the estimates represent sample sizes for each treatment.

Results were considered for both the fixed effects and mixed models as there is not an accurate method for robust linear mixed model comparisons (Koller, 2016). Both mixed effects models include tank as a random intercept, which yielded an estimate term of 0 for the tank factor. To prevent overfitting, we focused on the fixed effects model and the model including the random intercept of arena only (Table 1.3). The fixed effects model was used for predictions and inference as it supported confidence interval estimation. Pre-odor behaviors differed for individuals of each treatment group, which we accounted for by including pre-odor distance traveled as a covariate in our models (Figure 1.2).

DISCUSSION

Findings support our hypothesis that lake sturgeon form olfactory memory during early ontogeny and demonstrate that exposure to odorants during this period influences behavioral responses to these odorants by juveniles. Fish exposed to PEA and morpholine during the free-embryo or the exogenous feeding stages (or both) had the strongest behavioral responses to the experimental odorants when accounting for pre-odor behaviors. Behavioral responses to PEA and morpholine by fish previously exposed to the odorants differed from the control fish that had not previously been exposed to PEA and morpholine, indicating behavioral responses to the odorants represented olfactory memory rather than responses to a novel stimulus. Post-odor distance traveled responses depended on treatment and pre-odor distance traveled measures. Pre-odor behaviors varied across all treatments, but at the mean pre-odor response we predicted fish exposed during the exogenous feeding stage or free-embryo stages only would have the highest post-odor response. Slope estimates from our analyses indicated that fish exposed during the exogenous feeding stages or free-embryo stages only would have the largest increase in post-odor distance traveled with an increase in pre-odor activity. Results align with estimates for an olfactory imprinting period based on changes in organ development and forebrain growth in lake sturgeon (Dang and Zhang, 2014). There did not seem to be an additive effect of exposure during additional stages as groups exposed during single stages had larger predicted behavioral responses than those exposed during consecutive stages, which contrasts with findings that the duration of exposure may be critical to olfactory imprinting during the smolt stage in sockeye salmon (*Oncorhynchus nerka*) (Havey et al., 2017). Findings are consistent with other studies of olfactory imprinting that indicate olfactory imprinting occurs during specific development stages, rather than the length of exposure (Gerlach et al., 2019).

Our experiments provide behavioral evidence for multiple olfactory imprinting periods in lake sturgeon. At the free-embryo stage, lake sturgeon remain near the spawning site burrowed in the substrate and are exposed to natal stream odors at this location (Kempinger, 1988). Natal stream odors could differ for lake sturgeon during the exogenous feeding larvae stage, which is a period when lake sturgeon leave the substrate and drift downstream (Auer and Baker, 2002). In Pacific and Atlantic salmon, olfactory imprinting is known to occur during parr-smolt transformation which is an important period of behavioral, endocrine, and physiological changes (Hasler and Scholz, 1983; Morin et al., 1989). Recent studies have also provided evidence for olfactory imprinting at earlier stages, which supports hypotheses that olfactory imprinting may occur sequentially at multiple developmental stages and guides natal homing to not only a specific river but a specific natal site (Armstrong et al., 2021; Bett and Hinch, 2016; Dittman et al., 2015). Future experiments may be able to provide a more precise estimate of a period when olfactory memory is formed by separating the four exposure stages into smaller groups under specific developmental criteria. It's possible that olfactory imprinting in lake sturgeon occurs during the transition from the free-embryo to the exogenous feeding larvae stages. Exposure periods in our experiments may not have begun at the exact start of a developmental stage as we were not able to perfectly track and immediately administer odorants as soon as individuals hatched or began feeding exogenously. We used calculations of development rates (Eckes et al., 2015) and physiological changes to determine when most individuals hatched or transitioned to exogenous feeding. We also used full siblings to decrease inter-family variation in our experiments. There is considerable evidence for additive genetic variation in lake sturgeon phenotypic traits and physiology that warrant further consideration in future studies (Dammerman et al., 2015; 2020; Wassink et al., 2019; 2020).

Straying from natal streams may have historically reduced the probability of local extirpation and increased genetic diversity across the Great Lakes. However, interbreeding between members of genetically distinct populations may lead to outbreeding depression, which is the reduction in fitness because of the breakdown of coadapted genotypes that are adapted to species and (largely natal) environments (Edmands, 2007). This is a concern for the conservation and management of lake sturgeon (DeHaan et al., 2006; Homola et al., 2012; Welsh et al., 2010) and streamside rearing facilities have been implemented under the assumption that fish exposed to natal stream odors during early developmental periods will be more likely to return to natal rivers as adults to reproduce (Hayes and Caroffino, 2012; Holtgren et al., 2007). Our experiments provide evidence for olfactory imprinting during early ontogeny in lake sturgeon and demonstrate a methodology for exploring olfactory memory formation in juvenile lake sturgeon. These methods differ from traditional odorant preference and choice experiments used to study olfactory imprinting in adult salmon (Bett and Hinch, 2016) but may better represent the behaviors of juvenile lake sturgeon during a period when river choice or preference is not present. Future studies into olfactory memory in lake sturgeon could focus on behaviors of older individuals or expand the lag time between the memory formation period and behavior experiments to explore memory retention over a longer period. A better understanding of the time of olfactory imprinting in lake sturgeon may inform lake sturgeon rearing methodologies, particularly whether fish collected from larval drift in one river can develop olfactory memory for odorants to another river. Most SRFs utilize lake sturgeon collected during larval drift to mimic the genetic make-up of naturally produced cohorts (Holtgren et al., 2007). Our results suggest these fish may have already developed olfactory memory of natal stream odorants, which could affect the success of olfactory imprinting guided site fidelity to other target streams.

Further exploration is warranted to compare olfactory memory of lake sturgeon fertilized in an SRF to lake sturgeon acquired in larval drift, particularly in facilities that rely on lake sturgeon from other rivers.

APPENDIX

Figure A1.1. Example of a behavioral arena used for measuring responses of juvenile lake sturgeon to experimental odorants.

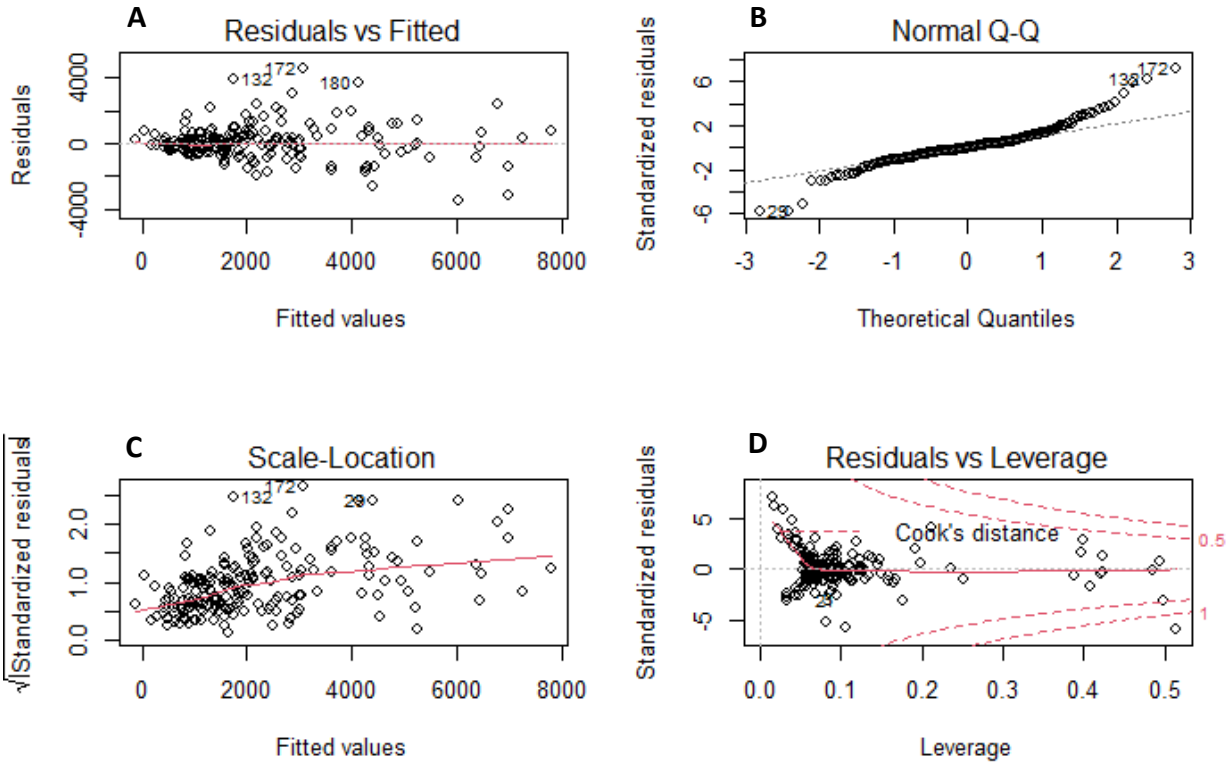

Figure A1.2. Residual plots used for checking assumptions of normality and homoscedasticity after using robust linear regression methods to reduce effects of high-leverage observations for the model relating post-odor distance to treatment, pre-odor distance, and the interaction of treatment and pre-odor distance. Plots represent the relationship between residuals and predicted responses for each observation (A), normal quantile-quantile plot (B), the relationship between the square-root of standardized residuals and predicted responses (C), and the relationship between standardized residuals and leverage of individual observations (D).

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CHAPTER 2:

Chemical and behavioral studies on the potential role of amino acids in olfactory imprinting to natal stream odors by lake sturgeon (*Acipenser fulvescens*)

ABSTRACT

Natal homing during spawning migrations is an important mechanism supporting localized adaptions to spawning sites in many fish species and straying from these sites can reduce the overall fitness of interbreeding populations. Olfactory imprinting is one mechanism guiding natal homing and has been extensively studied in Pacific salmon. The odorants guiding this process are unknown and will likely be river specific and stable over time. Several studies have suggested the ability of salmon to olfactorily imprint on amino acids. Lake sturgeon is another species believed to olfactorily imprint and roughly 20 years separate the period when imprinting is expected to occur and the age at which adults first spawn. We investigated the variability of amino acid profiles across tributaries of the Great Lakes, specifically looking at differences in profiles between rivers, across years, and between sample periods within a year. We also tested the ability of lake sturgeon to develop olfactory memory for amino acids profiles replicating the profiles of different rivers. This study provides evidence of spatial (river to river) and temporal variability (within and between years) of the amino acid profiles of twenty-three tributaries of the Great Lakes. We found that previous exposure to the amino acid profile of the Oconto River affected behavioral responses to amino acids and river water by juvenile lake sturgeon but did not observe evidence that these responses were dependent on a specific amino acid profile. These findings do not support the role of amino acid profiles in lake sturgeon olfactory imprinting. Further studies are needed to identify which environmental factors contribute to the variability in amino acid profiles across the great lakes and whether this variability has a significant impact on olfactory imprinting and natal homing in lake sturgeon.

INTRODUCTION

Natal homing mediated by olfactory imprinting to stream-specific odors during early life stages is an important reproductive strategy that has been well document in Pacific salmon (Dittman and Quinn, 1996), and hypothesized to occur in many teleost fish (Bett and Hinch, 2016; Cathcart, 2021). Populations of Pacific salmon exhibit adaptive genetic variation (Ricker, 1972), driven, in part, by homing to natal rivers, tributaries, and even specific stream stretches (Quinn, 2005; Quinn et al., 2006). Behavioral and migratory experiments have shown the ability for developing salmon to imprint on artificial odorants in natal water during early development, such as phenethyl alcohol (PEA) (Dittman et al., 1996; Nevitt et al., 1994; Scholz et al., 1976), morpholine (Scholz et al., 1976), and a mixture of amino acids and PEA (Havey et al., 2017).

The chemical signature in natal stream water learned by salmon may be composed, in part, of amino acids (Shoji et al., 2000; 2003; Yamamoto et al., 2013; Chen et al., 2017). Amino acids are potent odorants that provide information to aquatic organisms (Hara, 1992; Thomas, 1997), and derive from biofilms, dissolved organic materials, and the sediment from terrestrial habitat of aquatic ecosystems (Ishizawa et al., 2010; Thomas and Eaton, 1996; 1997). Stream-specific chemical profiles consisting of amino acids and other molecules may arise from unique biotic (*i.e.,* plant or microbial communities) and abiotic (watershed geology) characteristics of a stream, though notably little is known about what contributes to a stream's distinct odor (Ueda, 2011). Natal homing mediated by learned stream odors relies on temporally stable and geographically distinct odorants that distinguish different streams (Bett and Hinch, 2016). Such odors, present at the time of imprinting, typically expose individuals during early life stages, and match the odorants present at the time of return during stream reproductive migrations as adults (Dittman and Quinn, 1996). The few studies investigating the role of amino acids in olfactory

imprinting indicate that amino acid profiles (stream-specific profiles consisting of dissolved and particulate amino acids) are stable across years; however, these studies have not compared how amino acid profiles may vary between potential spawning streams (Chen et al., 2017; Shoji et al., 2000; Yamamoto et al., 2013; Yamamoto and Ueda, 2009). To further evaluate the role of amino acids in stream discrimination via olfactory cues, more information is needed on the spatial and temporal variability in amino acid profiles.

Although much of our understanding of olfactory imprinting comes from Pacific salmon, evidence from other taxa (*i.e.* Atlantic salmon [*Salmo salar*], alewife [*Alosa pseudoharengus*]) indicates it may be commonly used by homing fishes (Sutterlin and Gray, 1973; Thunberg, 1971; Horrall, 1981; Bett and Hinch, 2016). Lake sturgeon (*Acipenser fulvescens*) are characterized by genetically distinct populations, likely attributed to reproductive isolation resulting from homing to natal streams to spawn (McQuown et al., 2003; DeHaan et al., 2006; Welsh et al., 2008; Homola et al., 2012; Donofrio et al., 2017). Current stocking programs in the Great Lakes use streamside rearing facilities (SRFs) to expose fish to natal stream odorants during early life stages based on the assumption that stream-specific odorants will guide future homing in adults during spawning migrations (Holtgren et al., 2007). Assumptions underlying widely used management prescriptions are fundamentally based on a poor understanding of the mechanisms underlying olfactory imprinting by lake sturgeon. As in Pacific salmon and other migratory species, one major gap in our understanding of imprinting by lake sturgeon is the identities of potential molecules that guide homing. The objective of this study is to characterize the role amino acids may play in lake sturgeon olfactory imprinting by 1) quantifying the spatial and temporal variation of amino acids in Great Lakes tributaries; and 2) examining the role of amino acids in behavioral discrimination between natal and non-natal river waters.

We hypothesize that amino acids guide natal homing to streams in lake sturgeon. To test our hypothesis, we tested the prediction that differences in amino acid profiles are larger among streams than between sample periods, both within a year and between years. Additionally, we tested the prediction that juvenile lake sturgeon discriminate between streams using amino acid profiles. Here we describe evidence of temporal and spatial variability of amino acid profiles of Great Lakes tributaries and document the first attempt at observing the development of olfactory memory to amino acids in juvenile lake sturgeon.

METHODS

Chemical analyses of amino acids in stream water

Water sampling

In each of the three years from 2019 to 2021, we collected water samples to characterize amino acid profiles of Great Lakes tributaries and tested whether profiles varied among rivers and between years. Water samples (1 L in 2019, 500 ml in 2020 and 2021) were collected using wide-mouth Nalgene bottles from rivers with existing or extirpated lake sturgeon spawning populations (Holey et al., 2000) or based on their proximity to sturgeon spawning rivers. Nineteen tributaries of Lake Michigan, one tributary of Lake Huron, and three tributaries of Lake Superior were sampled (Figure 2.1). Samples were collected from river access points closest to the known Lake Sturgeon spawning locations, typically below the downstream most dam or impoundment. Water samples were held on ice, and within ten hours stored at -20°C until processing. Samples were collected during two sample periods each year. In 2019, a single water sample was collected from each tributary during two sample periods (April 20 to May 19 and June 3 to June 16). These sample periods represented the start and end of the lake sturgeon spawning season in the Great Lakes. In 2020, triplicate water samples were collected from each tributary during two sample periods (June 4 to June 9 and October 9 to October 10). Field season delays in 2020 during the beginning of the Covid pandemic meant our first sample period did not occur until the end of lake sturgeon spawning seasons in the Great Lakes and the second sample was collected in the fall to ensure we sampled two time points within a year. In 2021, triplicate water samples were collected from a subset of the selected tributaries during three sample periods (April 11 to April 16, June 24 to June 27, and July 20 to July 26) to accompany our behavior experiments.

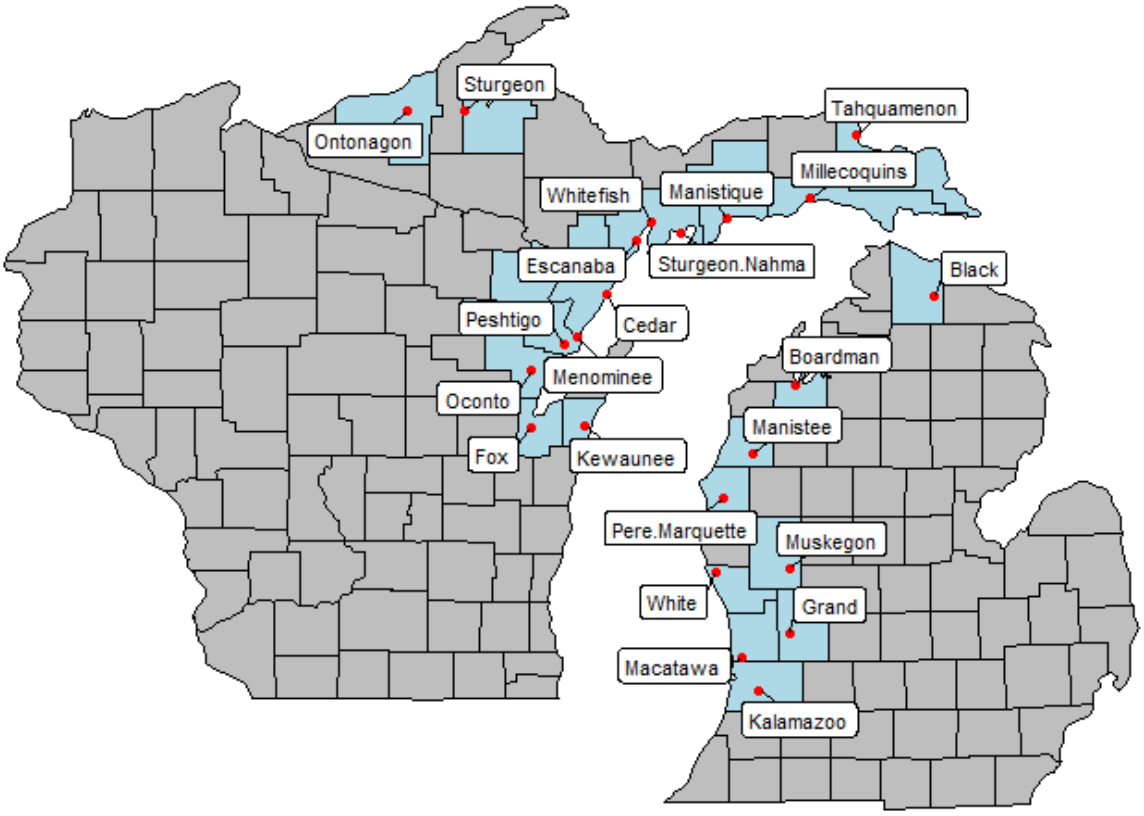

Figure 2.1. Map of water sampling locations for Great Lakes tributaries. The red dots represent the coordinates of the sample site for the labeled tributary.

Water sample extractions

Amino acids from each river water sample were extracted based on the method developed by Tang et al. (2014). Briefly, a 200 ml portion of the water sample was filtered through Whatman GF/C glass fiber filters. The pH of the filtered samples was adjusted to 2.8 using acetic acid. For solid phase extraction (SPE), Oasis MCX cartridges (6cc barrel size, 500 mg sorbent, 60 μm particle size) were conditioned with 5 ml methanol followed by 5 ml of 0.1M acetic acid. The pH adjusted water samples were then loaded onto the SPE cartridges. After loading, the cartridges were washed with 5 ml of 0.1M acetic acid after which they were dried under vacuum for 30 min. Finally, 5 ml of methanol containing 5% ammonium hydroxide was used for the elution of amino acids. The eluent was then evaporated to dryness under vacuum

and was reconstituted in 100 µl of 50% methanol in water with 10mM PFHA for LC-MS/MS analysis. All chemicals were purchased from Millipore Sigma (St. Louis, MO).

LC-MS/MS Analyses

Amino acids were analyzed using a previously described LC-MS/MS method (Gu et al., 2007) with slight modifications. A Waters Acquity UPLC system connected to a Waters Xevo TQS-micro Triple Quadrupole mass spectrometer was used for the quantification. Chromatographic separation was achieved using an Acquity UPLC HSS T3 (2.1 x 100 mm, 1.7 µm particle size) column attached with a 0.2 µm pre-column filter. A 10mM solution of perfluoroheptanoic acid (PFHA) in water was used as solvent A and acetonitrile was used as solvent B. The gradient was maintained as follows, 0-1 min: 0% B; 8 min: 65% B; 8.01-9 min: 90% B; 9.01-13 min: 0% B. The column temperature was maintained at 40 º C and the flow rate at 0.3 ml/min. The injection volume was 5 µl. All amino acids were analyzed by electrospray ionization in the positive mode. The ESI-MS/MS parameters were set as follows, capillary voltage: 1 kV; desolvation temperature: 350 °C; desolvation gas flow: 800 L/h; and cone gas flow: 40 L/h. The multiple reaction monitoring (MRM) and other parameters are provided as appendices (Table A2.1). Data acquisition was performed in three time resolved windows (0-4.5 min, 4.5-6.5 min, and 6.5-13 min) to allow sufficient dwell time for each amino acid (Major et al., 2020). MassLynx 4.2 software was used for data acquisition and data were processed using TargetLynx XS. Amino acid concentrations below the limit of quantification (LOQ) were converted to half the LOQ for the given amino acid.

Statistical analyses of the spatial and temporal variability in the composition of amino acid profiles among rivers

To investigate inter-annual and site-specific variation in river amino acid profiles, we compared the proportional abundance of amino acids for Great Lakes tributaries sampled in 2019 and 2020 during the June sampling period. Amino acid profiles were created for each water sample by calculating the proportional abundance of each measured amino acid in the sample. We used the proportional abundance of each amino acids to create amino acid profiles as we were interested in how the relative ratios of components in the amino acid mixtures differed between samples, rather than the concentrations of individual amino acids as fish show olfactory responses to a mixture of amino acids that differ from responses to any one amino acid from the mixture (Caprio et al., 1989; Zielinski and Hara, 2006). To compare the spatial and annual dissimilarity of amino acid profiles, we compared Bray-Curtis dissimilarity matrices between rivers in 2019, between rivers in 2020, and between years for the same river. The Bray-Curtis dissimilarity measure was used to synthesize differences in the proportional abundance of all amino acids between water samples into a single measure of inter-sample amino acid profile dissimilarity (Ricotta and Podani, 2017). We used a mantel test implemented using the *vegan* package to estimate the correlations between pairwise inter-sample Bray-Curtis dissimilarity of amino acid profiles between rivers in 2019 and 2020 and test whether the dissimilarity of amino acid profiles between rivers was consistent across years (Legendre and Legendre, 2012). Prior to running analyses using Bray-Curtis dissimilarity, we confirmed that dissimilarity between samples was consistent when comparing amino acid profiles based on the proportional abundance of each amino acid and the molarity of each amino acid. We used a mantel test to estimate the correlation between pairwise Bray-Curtis dissimilarities of samples based on

proportional abundance of each amino acid and based on molarity of each amino acid. We found a significant correlation between the pairwise Bray-Curtis dissimilarities of samples based on the proportional abundance of amino acids and the molarity of amino acids (mantel $r = 0.671$, $p < 0.001$), suggesting dissimilarity estimates were consistent for both measures of amino acid profiles. All analyses of Bray-Curtis dissimilarity between samples used amino acid profiles representing the proportional abundance of each amino acid in the sample.

We used a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017) using the Adonis method of the *vegan* package (v2.5-7; Oksanen et al., 2020) to assess whether there were differences in Bray-Curtis dissimilarity of amino acid profiles between rivers, sample year, and their interaction: Dissimilarity ~ Year + River + Year*River. The PERMANOVA identifies differences in the composition of Bray-Curtis dissimilarity between groups (river, sample year, and sample year and river) and assumes the variance of Bray-Curtis dissimilarities are equal between groups. We used a beta dispersion test from the *vegan* package, to test for homogeneity of dispersion among rivers and years to ensure assumptions were met for the PERMANOVA and that results were based on differences in the centroid of Bray-Curtis dissimilarities between groups rather than differences in the heterogeneity of Bray-Curtis dissimilarities among groups (Oksanen, 2009). We used the amino acid profiles of water samples collected in 2020 during the June and October sample periods to compare the spatial and temporal variability of amino acid profiles for Great Lakes tributaries within a year. Specifically, we used a PERMANOVA to assess whether there were differences in Bray-Curtis dissimilarity of amino acid profiles between water samples based on rivers, sample period, and their interaction: Dissimilarity ~ Sample Period + River + Sample Period*River.

All analyses were conducted using *R* (v4.1.2; R Core Team, 2021). We developed a heatmap using the *stats* package (R Core Team, 2021) to visualize the relationship between water samples from each river and sample period or year and the contribution of each amino acid to the Bray-Curtis dissimilarity of amino acid profiles. Amino acids were also organized based on their side-chain classes into non-polar neutral, polar neutral, acidic polar, and basic polar to explore whether specific amino acid classes, to which olfactory responses may vary in fish (Hara, 1994), explained the spatial and temporal variability of amino acid profiles in Great Lakes tributaries.

Behavioral responses to stream water and synthesized amino acids

Overview

Two sets of behavioral experiments were used to evaluate the role of amino acids in olfactory imprinting to stream odors. The first set of behavioral experiments tested whether lake sturgeon respond to natural stream water after being exposed to synthesized mixtures of amino acids recreated based on the amino acid profiles determined in the natural stream water. The second set of behavioral experiments tested whether lake sturgeon discriminate between streams based upon their odor and whether amino acids enable this discrimination. In Experiment 1, lake sturgeon were hatched and reared at the Black Lake Sturgeon Rearing Facility near Onaway, Michigan, USA in 50 micron filtered Black River water modified with mixtures of amino acids that recreated the proportional amino acid profiles in the Oconto River and Cedar River. These rivers were selected based on the relative pairwise dissimilarity in amino acids between the Cedar, Oconto, and Black Rivers, when compared with other rivers sampled in 2019 (see Chapter 1). Water mixtures were subsequently used to evaluate behavioral responses to exposure of the synthesized amino acid mixtures relative to natural stream water. In Experiment 2, lake

sturgeon were reared at the Ontonagon River Streamside Rearing Facility in Bergland, Michigan, USA in Ontonagon River water and later tested for behavioral responses to natural river water from the Ontonagon River and nearby rivers and to mixtures of amino acids recreated based on the amino acid profiles in the Ontonagon River and nearby rivers. The Sturgeon and Tahquamenon Rivers were selected for these experiments as they were the two other tributaries of Lake Superior where we collected water samples from 2019 to 2021. The Ontonagon, Sturgeon, and Tahquamenon rivers currently or have previously supported lake sturgeon populations (Holey et al., 2000) and represent potential spawning locations for migrating lake sturgeon in Lake Superior.

We observed the behavior of juvenile lake sturgeon (> 70 days post-fertilization) in both experiments following methods previously used to document olfactory memory behaviors in lake sturgeon (Chapter 1). All experimental animals were used and experiments were conducted under approved Michigan State University Animal Use and Care Committee protocols (AUF PROTO202000023/AMEND202100062).

Experiment 1: Responses of lake sturgeon reared in water activated with artificial amino acid mixtures

Behavioral experiments at the Black Lake Sturgeon Rearing Facility were conducted from July 16 to July 21, 2021. Lake sturgeon used in the first set of experiments were reared from egg fertilization through behavior experimentation at the Black River Sturgeon Rearing Facility (BLSRF) in Onaway, MI, USA which operates as a flow-through streamside rearing facility using water supplied directly from the Upper Black River at ambient temperature. Eggs and sperm from a single male and female were sampled from lake sturgeon in the Upper Black River on May 4, 2021. Eggs were fertilized within the day following standardized hatchery

procedures (Bauman et al., 2015; Crossman et al., 2011). Use of full siblings was expected to reduce variation due to additive genetic (family) effects (Dammerman et al., 2015; 2020).

Fish were raised under three different odorant conditions at the BLSRF. Control fish (fish not exposed to amino acid odorants) were raised in 50 micron filtered Black River hatchery water. Cedar River fish were exposed to an artificial odor profile based on average concentrations of fifteen amino acids from Cedar River water samples in 2019. Oconto River fish were exposed to an artificial odor profile based on average concentrations of fifteen amino acids from Oconto River water samples in 2019 (Table 2.1). We applied solutions of each amino acid into tanks to reach concentrations at 10x of what we quantified in 2019 to offset background amino acid profiles of the hatchery water and ensure the proportional abundance of each amino acid was consistent. Amino acid solutions were created by dissolving solid amino acids in either water, hydrochloric acid (HCl), or ammonia hydroxide (NH4OH), depending on recommendations from Millipore Sigma (St. Louis, MO) (Table 2.1). Rhodamine was pumped into the head tank and measured using a hand-held DataBank datalogger and Cyclops-7 Optical Rhodamine Dye Tracer (Turner Designs, Sunnyvale, CA) to validate even mixing of odorants in the head tank and even distribution of odorants to all tanks.

Table 2.1. Amino acid profiles from 2019 used to develop artificial river water for behavior experiments compared to amino acid profiles measured in 2021. Artificial concentrations (Artificial - con.) represent the molarity of each amino acid for each river (based on the average of April and June water samples in 2019). The proportion of each amino acid (Artificial - Proportions) represents the amino acid profiles for 2019 rivers. 2021 amino acid profiles (Actual - Prop) were created by averaging the concentrations in 2021 samples for each river and then calculating the proportional abundance of each amino acid. "% change" from 2019 to 2021 was calculated using the proportional abundance of each amino acid.

River	Sample	N	Valine[W]	Leucine[H]	Isoleucine[A]
Cedar	Artificial - Con. (M)	2	2.72E-08	5.26E-08	4.58E-08
	Artificial - Prop.		0.112	0.217	0.189
	Actual - Prop.	8	0.169	0.149	0.155

Table 2.1 (Cont'd)

	SE		0.024	0.019	0.023
	% Change		51.252	-31.311	-17.980
Oconto	Artificial - Con. (M)	2	5.85E-08	2.02E-08	1.74E-08
	Artificial - Prop.		0.146	0.050	0.043
	Actual - Prop.	9	0.264	0.081	0.093
	SE		0.026	0.012	0.012
	% Change		80.685	60.621	113.802
Ontonagon	Artificial - Con. (M)	2	7.83E-08	2.98E-08	2.87E-08
	Artificial - Prop.		0.136	0.052	0.050
	Actual - Prop.	9	0.050	0.038	0.028
	SE		0.011	0.006	0.004
	% Change		-63.614	-26.601	-43.036
Sturgeon	Artificial - Con. (M)	2	4.75E-08	2.69E-08	2.29E-08
	Artificial - Prop.		0.062	0.035	0.030
	Actual - Prop.	9	0.041	0.036	0.026
	SE		0.004	0.005	0.003
	% Change		-34.414	3.712	-11.280
Tahquamenon	Artificial - Con. (M)	2	7.56E-07	5.42E-07	1.87E-07
	Artificial - Prop.		0.191	0.137	0.047
	Actual - Prop.	9	0.066	0.047	0.034
	SE		0.017	0.008	0.007
	% Change		-65.197	-65.305	-28.630
River	**Sample**	**N**	**Valine[W]**	**Leucine[H]**	**Isoleucine[A]**
Cedar	Artificial - Con. (M)	2	6.10E-09	6.00E-10	9.00E-10
	Artificial - Prop.		0.025	0.002	0.004
	Actual - Prop.	8	0.057	0.015	0.003
	SE		0.004	0.004	0.000
	% Change		125.725	508.930	-24.491
Oconto	Artificial - Con. (M)	2	3.95E-08	1.80E-09	9.50E-10
	Artificial - Prop.		0.099	0.004	0.002
	Actual - Prop.	9	0.080	0.005	0.002
	SE		0.008	0.002	0.000
	% Change		-18.820	19.806	-12.004
Ontonagon	Artificial - Con. (M)	2	7.61E-08	2.23E-09	1.80E-10
	Artificial - Prop.		0.133	0.004	0.000
	Actual - Prop.	9	0.076	0.006	0.004
	SE		0.004	0.001	0.001

Table 2.1 (Cont'd)

	% Change		-42.524	59.376	1220.410
Sturgeon	Artificial - Con. (M)	2	3.05E-08	6.45E-09	1.40E-09
	Artificial - Prop.		0.040	0.008	0.002
	Actual - Prop.	9	0.076	0.006	0.005
	SE		0.005	0.001	0.001
	% Change		90.464	-25.516	172.119
Tahquamenon	Artificial - Con. (M)	2	2.24E-07	1.40E-08	9.60E-09
	Artificial - Prop.		0.056	0.004	0.002
	Actual - Prop.	9	0.078	0.006	0.003
	SE		0.005	0.001	0.001
	% Change		39.105	63.458	28.419
River	**Sample**	**N**	**Valine[W]**	**Leucine[H]**	**Isoleucine[A]**
Cedar	Artificial - Con. (M)	2	2.47E-08	2.92E-08	6.00E-09
	Artificial - Prop.		0.102	0.120	0.025
	Actual - Prop.	8	0.025	0.069	0.006
	SE		0.009	0.015	0.002
	% Change		-75.076	-42.879	-75.273
Oconto	Artificial - Con. (M)	2	5.72E-08	2.53E-08	2.85E-09
	Artificial - Prop.		0.143	0.063	0.007
	Actual - Prop.	9	0.026	0.056	0.007
	SE		0.003	0.008	0.001
	% Change		-81.944	-11.671	-7.097
Ontonagon	Artificial - Con. (M)	2	1.05E-07	4.25E-08	2.18E-09
	Artificial - Prop.		0.183	0.074	0.004
	Actual - Prop.	9	0.107	0.022	0.017
	SE		0.018	0.004	0.002
	% Change		-41.477	-70.904	334.586
Sturgeon	Artificial - Con. (M)	2	2.54E-07	3.96E-08	1.64E-08
	Artificial - Prop.		0.331	0.052	0.021
	Actual - Prop.	9	0.088	0.019	0.021
	SE		0.012	0.002	0.003
	% Change		-73.454	-63.275	-3.507
Tahquamenon	Artificial - Con. (M)	2	1.82E-07	5.34E-07	1.18E-08
	Artificial - Prop.		0.046	0.135	0.003
	Actual - Prop.	9	0.081	0.019	0.018
	SE		0.016	0.002	0.002
	% Change		77.098	-85.856	504.087

Table 2.1 (Cont'd)

River	Sample	N	Valine[W]	Leucine[H]	Isoleucine[A]
Cedar	Artificial - Con. (M)	2	5.35E-10	1.62E-08	2.62E-09
	Artificial - Prop.		0.002	0.067	0.011
	Actual - Prop.	8	0.059	0.035	0.015
	SE		0.008	0.004	0.005
	% Change		2583.816	-47.628	42.598
Oconto	Artificial - Con. (M)	2	3.20E-10	5.99E-08	1.96E-08
	Artificial - Prop.		0.001	0.150	0.049
	Actual - Prop.	9	0.068	0.061	0.016
	SE		0.009	0.012	0.003
	% Change		8453.763	-59.109	-67.243
Ontonagon	Artificial - Con. (M)	2	9.05E-10	4.98E-08	6.57E-08
	Artificial - Prop.		0.002	0.087	0.114
	Actual - Prop.	9	0.166	0.023	0.036
	SE		0.031	0.006	0.003
	% Change		10425.849	-73.264	-68.388
Sturgeon	Artificial - Con. (M)	2	4.05E-10	4.21E-08	6.65E-08
	Artificial - Prop.		0.001	0.055	0.087
	Actual - Prop.	9	0.201	0.015	0.038
	SE		0.028	0.001	0.003
	% Change		37959.128	-73.547	-56.011
Tahquamenon	Artificial - Con. (M)	2	8.30E-10	4.70E-09	1.13E-06
	Artificial - Prop.		0.000	0.001	0.285
	Actual - Prop.	9	0.160	0.024	0.037
	SE		0.033	0.007	0.003
	% Change		76579.033	1943.102	-86.920
River	**Sample**	**N**	**Valine[W]**	**Leucine[H]**	**Isoleucine[A]**
Cedar	Artificial - Con. (M)	2	1.67E-08	9.75E-10	3.00E-10
	Artificial - Prop.		0.069	0.004	0.001
	Actual - Prop.	8	0.051	0.072	0.002
	SE		0.009	0.020	0.001
	% Change		-25.318	1687.163	28.897
Oconto	Artificial - Con. (M)	2	5.64E-08	1.78E-08	2.05E-09
	Artificial - Prop.		0.141	0.044	0.005
	Actual - Prop.	9	0.069	0.100	0.002
	SE		0.007	0.008	0.000

Table 2.1 (Cont'd)

	% Change		-50.825	123.848	-63.612
Ontonagon	Artificial - Con. (M)	2	4.11E-08	3.54E-08	2.80E-10
	Artificial - Prop.		0.072	0.062	0.000
	Actual - Prop.	9	0.134	0.153	0.003
	SE		0.014	0.026	0.001
	% Change		86.365	148.639	487.384
Sturgeon	Artificial - Con. (M)	2	8.74E-08	1.07E-07	5.30E-10
	Artificial - Prop.		0.114	0.139	0.001
	Actual - Prop.	9	0.136	0.150	0.005
	SE		0.012	0.023	0.001
	% Change		18.778	7.644	661.800
Tahquamenon	Artificial - Con. (M)	2	7.71E-08	1.28E-07	1.12E-08
	Artificial - Prop.		0.019	0.032	0.003
	Actual - Prop.	9	0.102	0.188	0.006
	SE		0.013	0.030	0.001
	% Change		423.666	484.473	95.140
River	**Sample**	**N**	**Valine[W]**	**Leucine[H]**	**Isoleucine[A]**
Cedar	Artificial - Con. (M)	2	4.70E-09	3.9E-09	3.9E-09
	Artificial - Prop.		0.019	0.016	0.016
	Actual - Prop.	8	0.063	0.007	0.040
	SE		0.007	0.003	0.016
	% Change		225.293	-57.241	147.031
Oconto	Artificial - Con. (M)	2	1.70E-09	9.45E-09	9.40E-09
	Artificial - Prop.		0.004	0.024	0.024
	Actual - Prop.	9	0.032	0.006	0.021
	SE		0.003	0.001	0.008
	% Change		655.318	-75.476	-8.668
Ontonagon	Artificial - Con. (M)	2	1.43E-09	3.90E-09	1.01E-08
	Artificial - Prop.		0.002	0.007	0.018
	Actual - Prop.	9	0.024	0.014	0.072
	SE		0.004	0.003	0.014
	% Change		869.382	105.042	306.681
Sturgeon	Artificial - Con. (M)	2	1.13E-08	2.25E-09	3.90E-09
	Artificial - Prop.		0.015	0.003	0.005
	Actual - Prop.	9	0.023	0.019	0.067
	SE		0.004	0.003	0.016

Table 2.1 (Cont'd)

	% Change		54.078	542.323	1218.174
Tahquamenon	Artificial - Con. (M)	2	7.52E-08	8.25E-09	7.40E-08
	Artificial - Prop.		0.019	0.002	0.019
	Actual - Prop.	9	0.026	0.019	0.064
	SE		0.003	0.003	0.017
	% Change		37.830	819.474	243.803
River	**Sample**	**N**	**Valine^{W}**	**Leucine^{H}**	**Isoleucine^{A}**
Cedar	Artificial - Con. (M)	2			
	Artificial - Prop.				
	Actual - Prop.	8	0.005	0.005	
	SE		0.001	0.001	
	% Change				
Oconto	Artificial - Con. (M)	2			
	Artificial - Prop.				
	Actual - Prop.	9	0.006	0.005	
	SE		0.001	0.001	
	% Change				
Ontonagon	Artificial - Con. (M)	2			
	Artificial - Prop.				
	Actual - Prop.	9	0.014	0.013	
	SE		0.002	0.003	
	% Change				
Sturgeon	Artificial - Con. (M)	2			
	Artificial - Prop.				
	Actual - Prop.	9	0.016	0.012	
	SE		0.002	0.002	
	% Change				
Tahquamenon	Artificial - Con. (M)	2			
	Artificial - Prop.				
	Actual - Prop.	9	0.013	0.009	
	SE		0.002	0.002	
	% Change				

Note: Superscripts denote the solvent used for mixing solutions for each amino acid. "W" represents amino acids dissolved in water, "H" represents amino acids dissolved in hydrochloric acid, and "A" represents amino acids dissolved in ammonia hydroxide. Asterisks indicate amino acids not included in artificial river water solutions.

Fish from each treatment group were raised during all life stages in each of three replicate tanks from fertilization through the start of experiments. Fertilized eggs were placed in McDonald jars (Pentair, Apopka, FL), at hatch, fished were held in 3L aquaria with bio ball filter media (CBB1-S; Pentair, Apopka, FL) added as simulated gravel substrate, exogenous feeding fish were removed from the simulated gravel and held in 3L aquaria, and juvenile fish were held in larger 18-gallon tanks. Exposure to artificial river water began after eggs hatched, at twelve days post-fertilization. This exposure period aligns with previously identified olfactory memory formation stages in lake sturgeon which occur after hatch during the free-embryo and exogenous feeding stages (Chapter 1). Amino acid solutions were mixed and replaced daily. A peristaltic pump was used to pump the odor mixture into a head tank supplying water to tanks receiving the experimental odorants. The mean proportion of the desired concentrations of amino acids dosed was 0.902 ± 0.03 (mean ± SE) for artificial Cedar River water and 0.949 ± 0.04 (mean ± SE) for artificial Oconto River water.

Juvenile lake sturgeon swimming and activity behaviors were observed in response to natural and artificial river waters as a test of olfactory memory and river discrimination based on amino acid profiles. Twenty individuals were observed from each treatment group, with an equal number of individuals observed for each replicate tank. Trials took place in a cylindrical tank with a 1975.8 cm^2 base filled with 3 L of groundwater from the BLSRF (Chapter 1). Four identical arenas were used to measure behaviors, allowing for multiple trials to be run concurrently. For each trial, one fish was removed from its housing tank and a digital photo was taken for body length measurements. The fish was then acclimated to the enclosure for five minutes. Videos were recorded for five minutes after the seven-and-a-half-minute acclimation period to measure pre-odor behaviors. A volume of 100 ml of odors was added using two 50ml

syringes after the five-minute pre-odor observation period, twelve and a half minutes after the fish was introduced to the tank. Odors included groundwater from the BLSRF (control), artificial Cedar River water, artificial Oconto River water, natural Cedar River water, and natural Oconto River water. Cedar and Oconto River waters were collected the week of experiments. The natural river water was added directly to the arena, with a dilution of 1 ml of river water to 30 ml of water in the arena. Artificial river water consisted of an odor solution that was added to the arena to reach concentrations matching artificial amino acid profiles based on 2019 water samples from each river (Table 2.1). Dye tests were used during method development to ensure odorants mixed evenly in the arena. One minute after the initial addition of odorants, another five-minute video was recorded to measure behaviors post-odor application. Fish were then removed from the enclosures, and enclosures were thoroughly rinsed with groundwater from the BLSRF before the next trial began. To prevent outside stimuli from affecting fish behaviors, fish were observed in the evening outside of working hatchery hours, under red lighting. Length was also measured for each fish with the ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A., http:// rsbweb.nih.gov/ij/) to ensure differences in behavior were not solely resulting from physical differences between individuals. Videos were analyzed using Loligo v.4.0 tracking software (Loligo Systems, Viborg, Denmark; https://www.loligosystems.com/software), which recorded average velocity (cm/s), average acceleration (cm/s^2), average deacceleration (cm/s^2), time active (s), time active (%), time inactive (s), time inactive (%), and total distance traveled (cm)

To identify relationships between behavioral (movement) metrics and to select an informative metric to use in our analysis, we calculated correlations between response variables using the *corrplot* package (v0.92; Wei and Simko, 2021). Using the absolute value of Pearson

correlation coefficients, we found strong pairwise correlations between average velocity, average acceleration, average deacceleration, and total distance traveled variables for the BLSRF experiments ($|r| \geq 0.80$) (Figure 2.2). We also found strong correlations between time active and time inactive measures (BLSRF: $|r| \geq 0.94$). There was a moderate correlation between distance traveled and all time active and inactive measures BLSRF: $|r| \geq 0.59$). We used a correlation test from the *psych* package (v2.2.5; Revelle, 2022) to identify significant non-zero correlations between behavior metrics. All pairwise correlations between potential response variables were non-zero for the BLSRF experiments ($p<0.05$) (Figure 2.2). Due to its comparatively lower correlation with the other response variables, total distance traveled was selected as the single representative behavior to be used in statistical analyses.

	Avg acceleration	Avg deacceleration	Time active(%)	Time active (s)	Time inactive (s)	Time inactive (%)	Distance Traveled
Avg velocity	**0.97**	**-0.97**	**0.31**	**0.29**	**-0.32**	**-0.31**	**0.90**
Avg acceleration		**-1.00**	**0.15**	**0.13**	**-0.18**	**-0.15**	**0.80**
Avg deacceleration			**-0.15**	**-0.13**	**0.18**	**0.15**	**-0.80**
Time active(%)				**0.99**	**-0.98**	**-1.00**	**0.61**
Time active (s)					**-0.94**	**-0.99**	**0.61**
Time inactive (s)						**0.98**	**-0.59**
Time inactive (%)							**-0.61**

Figure 2.2. Matrix showing pairwise correlations between all measured post-odor behavioral responses of juvenile lake sturgeon from the Loligo tracking software for the artificial amino acid profile experiments at the Black River. All correlations were non-zero ($p<0.05$).

Prior to statistical analysis, we checked for visual and statistical outliers in both pre-odor and post-odor distance traveled. Outliers were suspected in some cases based on lighting variation and blemishes on the tank background that influenced fish tracking in the Loligo software. Seven trials were removed from the analysis of BLSRF experiments because of tracking related issues or incomplete video recordings. Visual outliers were identified when an individual fish showed behavioral responses (such as average velocity and acceleration) that were larger than other observations and were improbable for a juvenile lake sturgeon (Downie

and Kieffer, 2017) during both the pre-odor and post-odor periods. Statistical outliers were identified using a Grubb's Test with the *outliers* package (v0.14; Komsta, 2011) and observations were considered for removal when both pre-odor and post-odor distance traveled measures were significant outliers. Five additional trials were removed based on both visual and statistical criteria from the BLSRF experiments.

We modeled post-odor distance traveled under a normal distribution through a robust linear regression as a function of a variety of predictor variables measured throughout our experiments. Normality of residuals and homoscedasticity were assessed following model selection for a traditional linear regression and the models did not meet the assumptions; specifically, we observed highly influential (high leverage) observations in our model based on the residual quantile-quantile and residuals vs. leverage plots (Chatterjee and Hadi, 1986) (Figure A2.1; A2.2). Based on these findings, we performed robust linear regression models using an M estimator, which downweighs highly influential observations without removing any observations from the analysis (Filzmoser and Nordhausen, 2021). Models were compared to select which fixed effects to include in full model predictions and inference. To account for individual variation in swimming behaviors and activity, pre-odor distance traveled was included as a predictor variable in all but the null model. For the BLSRF experiments, fixed effects included individual body length, pre-odor distance traveled, treatment group or the water fish were raised in (Black River water, artificial Cedar River water, or artificial Oconto River water), odor applied to the arena during behavior experiments (groundwater, an artificial river water, or a natural river water) and their pairwise interactive effects. Models were compared using Akaike Information Criterion – small sample size correction (AICc) with the *AICcmodavg* package

(v2.3-1; Mazerolle, 2020). All models within two AICc of the top model were considered (Tredennick et al., 2021).

Our experimental design at the Black Lake SRF incorporated two nuisance grouping factors, the tank in which fish were raised prior to experiments and the arena used for observations, which we incorporated into model interpretation to account for non-independence of individuals based on these factors. Our experimental design at the Ontonagon SRF incorporated one nuisance grouping factor, the behavioral arena used for each individual fish which subsequently accounts for the effect of family as fish from each of the four families were used in only one arena each. Robust linear mixed models were run based on results from the fixed effects model selection using the *robustlmm* package (Koller, 2016). Robust linear mixed models included arena as a random intercept, tank as a random intercept, or both arena and tank as crossed random effects for the BLSRF experiments. Tank and arena were low-level random effects, with tank groupings ranging from one to three for each treatment and only four unique arenas used for behavior experiments. Given we were not interested in making inferences on the random effects, we included them for model interpretation (Gomes, 2022). Figures were produced for the focal model using the *ggplot2* (Wickham, 2016) and *cowplot* (v1.1.1 Wilke, 2020) packages. Predictions based on robust linear models were made using the *predict.rlm* function from the *MASS* package (Venables and Ripley, 2002).

Experiment 2: Behavior of lake sturgeon reared in natural stream water

Fish for the second set of experiments were raised at the Ontonagon SRF on the Ontonagon River in Bergland, Michigan, USA operated by the U.S. Fish and Wildlife Service. Individuals from four different families reared from egg stage within the facility were used for behavioral observations. We conducted behavior experiments at the Ontonagon SRF from July

24 to July 26, 2021. These experiments followed the same protocols as those at the Black Lake SRF, but due to limited space at the Ontonagon streamside rearing facility, behavioral observations were observed outside of the facility in a tent during the afternoon and evening. We recorded behavioral responses for fish raised in the Ontonagon SRF to seven different odors including, ground water (n=12) from the Black Lake SRF, natural Ontonagon River water (n = 13), natural Sturgeon River water (n= 11), natural Tahquamenon River water (n= 11), and artificial river waters for the Ontonagon (n = 14), artificial Sturgeon (n= 13), and artificial Tahquamenon rivers waters (n= 14). Natural river water was added directly to the arena, with a dilution of 1 ml of river water to 30 ml of water in the arena. Artificial river water consisted of an odorant solution that was added to the background groundwater of arena to reach concentrations matching artificial amino acid profiles based on 2019 water samples from each river (Table 2.1).

Data for Experiment 2 were analyzed using the same methods as in Experiment 1. As in Experiment 1, total distance traveled was selected as the single representative behavior to be used in our statistical analyses. We found strong pairwise correlations between average velocity, average acceleration, average deacceleration, and total distance traveled variables for the Ontonagon (ONT) SRF experiments ($|r| \geq 0.89$) (Figure 2.3), strong correlations between time active and time inactive measures (ONT: $|r| = 1$), and a moderate correlation between distance traveled and all time active and inactive measures (ONT: $|r| \geq 0.46$). Pairwise correlations between acceleration/deacceleration measures and activity measures were not significant ($p>0.05$) for the ONT experiments. All other pairwise correlations between potential response variables were non-zero ($p<0.05$) (Figure 2.3). No trials were removed from the analysis of ONT experiments due to tracking issues. Three trials were removed as outliers based on both

visual and statistical criteria from the ONT experiments. One additional statistical outlier was removed from the ONT experiments based on extreme pre-odor velocity, acceleration, and distance traveled measures that were larger than other observations and were improbable for a juvenile lake sturgeon (Downie and Kieffer, 2017). For the ONT experiments, fixed effects included individual length, pre-odor distance traveled, odor applied to the arena, and their pairwise interactive effects. Only arena was included as a random intercept for robust linear models in the ONT experiments.

	Avg acceleration	Avg deacceleration	Time active(%)	Time active (s)	Time inactive (s)	Time inactive (%)	Distance Traveled
Avg velocity	**0.96**	**-0.96**	**0.27**	**0.27**	**-0.27**	**-0.27**	**0.97**
Avg acceleration		**-1.00**	**0.04***	**0.04***	**-0.04***	**-0.04***	**0.89**
Avg deacceleration			**-0.05***	**-0.05***	**0.06***	**0.05***	**-0.90**
Time active(%)				**1.00**	**-1.00**	**-1.00**	**0.46**
Time active (s)					**-1.00**	**-1.00**	**0.46**
Time inactive (s)						**1.00**	**-0.46**
Time inactive (%)							**-0.46**

Figure 2.3. Matrix showing pairwise correlations between all measured post-odor behavioral responses of juvenile lake sturgeon from the Loligo tracking software for the Ontonagon Streamside Rearing Facility experiments at the Black River. Asterisks represent correlations that were not significant (p>0.05).

RESULTS

Spatial and temporal variability of amino acid profiles of Great Lakes tributaries

Analyses of river water samples yielded nanomolar concentrations for eighteen amino acids from samples collected in 2019 and twenty amino acids in samples processed in 2020 and 2021. We found from our analyses of the Bray-Curtis dissimilarity in amino acid profiles of Great Lakes tributaries in June of 2019 and 2020 that both river and year explained the variability in dissimilarity between samples (Table 2.2). We found that the river sampled accounted for the most variability in Bray-Curtis dissimilarities between amino acid profiles of two water samples (pseudo $F = 2.322$, $p < 0.05$) (31.1%), the sample year accounted for 21.6% (pseudo $F = 34.474$, $p < 0.05$), and 21.7% was explained by the interaction between river and year (pseudo $F = 1.625$, $p < 0.05$). Beta dispersion tests indicate the assumptions of the PERMANOVA analyses were met as there were no significant differences in the dispersion between samples of different rivers, samples of different years, or samples of different rivers and years (Table 2.2).

Table 2.2. Results for the PERMANOVA comparing the effects of year, river, and their interaction on the Bray-Curtis dissimilarity of amino acid profiles between samples collected from Great Lakes tributaries in June of 2019 and 2020. The beta dispersion test shows homogeneity of the dispersion of Bray-Curtis dissimilarity between rivers and years.

PERMANOVA: Dissimilarity ~ Year + River + Year*River						
Factors	*Df*	*Sum Sq.*	*Mean Sq.*	*F value*	R^2	*p value*
Year	1	1.673	1.673	35.474	0.216	0.001
River	22	2.409	0.109	2.322	0.311	0.001
Year*River	22	1.685	0.077	1.625	0.218	0.005
Residuals	42	1.981	0.047	0.256		
Beta dispersion test of homogeneity between factor levels						
Factors	*Df*	*Sum Sq.*	*Mean Sq.*	*F value*	*p value*	
Year	1	0.015	0.015	1.522	0.221	
Residuals	86	0.875	0.010			
River	22	0.132	0.006	0.271	0.999	
Residuals	65	1.439	0.022			

Comparisons of the Bray-Curtis dissimilarity matrices between rivers in 2019, rivers in 2020, and between the same river across years also indicate that the Bray-Curtis dissimilarity in amino acid profiles between rivers within a year is greater than the dissimilarity in amino acid profiles for a river across years (Table 2.3). The average of the pairwise Bray-Curtis dissimilarities between rivers in 2019 was 0.384 ± 0.009 (mean ± SE), compared to 0.335 ± 0.003 (mean ± SE) for the pairwise Bray-Curtis dissimilarities between rivers in 2020, and 0.377 ± 0.013 (mean ± SE) for the Bray-Curtis dissimilarities between same rivers in 2019 and 2020. In addition, a mantel test indicated the Bray-Curtis dissimilarity between river amino acid profiles was not consistent across years, as we found no significant correlation between the level of dissimilarities for rivers in 2019 and the level of dissimilarities for rivers in 2020 (mantel r = 0.116, p = 0.096).

Table 2.3. Table of the average Bray-Curtis dissimilarities of amino acid profiles between rivers and years from water samples collected in June of 2019 and 2020. The outlined cells represent the average Bray-Curtis dissimilarity between the same river in 2019 and 2020. The cells above the outlined cells represent the Bray-Curtis dissimilarities between rivers in June 2019, based on single water samples. The cells below the outlined cells represent the average Bray-Curtis dissimilarities between rivers in June 2020 among all replicate water samples.

	Black	Boardman	Cedar
Black	0.312	0.338	0.149
Boardman	0.257	0.389	0.296
Cedar	0.342	0.326	0.319
Escanaba	0.544	0.506	0.341
Fox	0.355	0.339	0.256
Grand	0.291	0.313	0.313
Kalamazoo	0.386	0.390	0.324
Kewaunee	0.211	0.251	0.308
Macatawa	0.534	0.496	0.318
Manistee	0.263	0.277	0.340
Manistique	0.321	0.316	0.376
Menominee	0.393	0.389	0.375
Millecoquins	0.248	0.256	0.276
Muskegon	0.330	0.308	0.302
Oconto	0.312	0.306	0.248

Table 2.3 (Cont'd)

Ontonagon	0.310	0.312	0.358
Pere.Marquette	0.200	0.232	0.372
Peshtigo	0.272	0.274	0.291
Sturgeon	0.233	0.280	0.385
Sturgeon.Nahma	0.260	0.284	0.372
Tahquamenon	0.292	0.329	0.480
White	0.236	0.292	0.308
Whitefish	0.226	0.262	0.329
	Escanaba	Fox	Grand
Black	0.200	0.520	0.470
Boardman	0.233	0.567	0.397
Cedar	0.195	0.482	0.349
Escanaba	0.486	0.511	0.510
Fox	0.362	0.441	0.523
Grand	0.488	0.317	0.445
Kalamazoo	0.408	0.315	0.363
Kewaunee	0.501	0.325	0.268
Macatawa	0.266	0.327	0.457
Manistee	0.500	0.327	0.314
Manistique	0.500	0.373	0.370
Menominee	0.387	0.365	0.416
Millecoquins	0.431	0.274	0.273
Muskegon	0.433	0.319	0.358
Oconto	0.349	0.254	0.308
Ontonagon	0.489	0.345	0.370
Pere.Marquette	0.606	0.385	0.282
Peshtigo	0.422	0.309	0.333
Sturgeon	0.557	0.367	0.311
Sturgeon.Nahma	0.525	0.351	0.325
Tahquamenon	0.669	0.487	0.402
White	0.483	0.319	0.290
Whitefish	0.503	0.336	0.287
	Kalamazoo	Kewaunee	Macatawa
Black	0.382	0.274	0.503
Boardman	0.388	0.308	0.384
Cedar	0.328	0.199	0.391
Escanaba	0.451	0.339	0.493
Fox	0.514	0.509	0.508
Grand	0.128	0.223	0.163
Kalamazoo	0.426	0.146	0.215

Table 2.3 (Cont'd)

Kewaunee	0.356	0.329	0.327
Macatawa	0.345	0.492	0.246
Manistee	0.379	0.234	0.493
Manistique	0.451	0.324	0.525
Menominee	0.441	0.392	0.422
Millecoquins	0.349	0.233	0.433
Muskegon	0.376	0.314	0.428
Oconto	0.307	0.280	0.333
Ontonagon	0.410	0.293	0.494
Pere.Marquette	0.418	0.189	0.594
Peshtigo	0.381	0.266	0.434
Sturgeon	0.420	0.242	0.563
Sturgeon.Nahma	0.411	0.269	0.537
Tahquamenon	0.507	0.317	0.681
White	0.358	0.242	0.467
Whitefish	0.365	0.218	0.503
	Manistee	Manistique	Menominee
Black	0.149	0.397	0.220
Boardman	0.246	0.155	0.284
Cedar	0.117	0.344	0.197
Escanaba	0.233	0.291	0.283
Fox	0.511	0.526	0.517
Grand	0.392	0.396	0.326
Kalamazoo	0.335	0.398	0.240
Kewaunee	0.212	0.333	0.160
Macatawa	0.432	0.352	0.356
Manistee	0.367	0.298	0.177
Manistique	0.291	0.399	0.332
Menominee	0.369	0.321	0.448
Millecoquins	0.239	0.259	0.312
Muskegon	0.324	0.351	0.368
Oconto	0.310	0.364	0.354
Ontonagon	0.230	0.274	0.345
Pere.Marquette	0.236	0.307	0.416
Peshtigo	0.257	0.286	0.318
Sturgeon	0.210	0.265	0.361
Sturgeon.Nahma	0.229	0.234	0.326
Tahquamenon	0.347	0.372	0.484
White	0.283	0.331	0.382
Whitefish	0.267	0.313	0.380

Table 2.3 (Cont'd)

	Millecoquins	Muskegon	Oconto
Black	0.499	0.536	0.223
Boardman	0.387	0.562	0.393
Cedar	0.381	0.478	0.208
Escanaba	0.528	0.547	0.264
Fox	0.530	0.100	0.480
Grand	0.120	0.543	0.450
Kalamazoo	0.182	0.541	0.397
Kewaunee	0.293	0.530	0.276
Macatawa	0.105	0.528	0.480
Manistee	0.422	0.485	0.255
Manistique	0.377	0.543	0.426
Menominee	0.358	0.502	0.241
Millecoquins	0.329	0.556	0.466
Muskegon	0.283	0.479	0.553
Oconto	0.225	0.287	0.419
Ontonagon	0.261	0.328	0.341
Pere.Marquette	0.264	0.341	0.366
Peshtigo	0.236	0.288	0.295
Sturgeon	0.240	0.339	0.338
Sturgeon.Nahma	0.218	0.337	0.311
Tahquamenon	0.363	0.415	0.432
White	0.257	0.333	0.283
Whitefish	0.222	0.308	0.245

	Ontonagon	Pere.Marquette	Peshtigo
Black	0.390	0.195	0.295
Boardman	0.278	0.382	0.283
Cedar	0.399	0.209	0.239
Escanaba	0.242	0.249	0.372
Fox	0.522	0.521	0.525
Grand	0.595	0.510	0.199
Kalamazoo	0.571	0.415	0.230
Kewaunee	0.461	0.304	0.205
Macatawa	0.568	0.501	0.255
Manistee	0.355	0.205	0.210
Manistique	0.302	0.431	0.334
Menominee	0.398	0.206	0.210
Millecoquins	0.595	0.540	0.230
Muskegon	0.563	0.525	0.493
Oconto	0.361	0.107	0.392

Table 2.3 (Cont'd)

Ontonagon	0.350	0.368	0.476
Pere.Marquette	0.292	0.275	0.342
Peshtigo	0.245	0.275	0.379
Sturgeon	0.233	0.222	0.272
Sturgeon.Nahma	0.233	0.260	0.260
Tahquamenon	0.397	0.251	0.382
White	0.325	0.252	0.287
Whitefish	0.321	0.242	0.289
	Sturgeon	Sturgeon.Nahma	Tahquamenon
Black	0.375	0.211	0.475
Boardman	0.391	0.275	0.370
Cedar	0.379	0.142	0.407
Escanaba	0.215	0.286	0.450
Fox	0.643	0.501	0.561
Grand	0.674	0.340	0.438
Kalamazoo	0.613	0.361	0.459
Kewaunee	0.496	0.244	0.435
Macatawa	0.656	0.372	0.409
Manistee	0.392	0.183	0.376
Manistique	0.442	0.289	0.248
Menominee	0.452	0.214	0.407
Millecoquins	0.695	0.316	0.419
Muskegon	0.682	0.522	0.584
Oconto	0.385	0.251	0.484
Ontonagon	0.299	0.379	0.361
Pere.Marquette	0.380	0.254	0.478
Peshtigo	0.552	0.237	0.432
Sturgeon	0.290	0.479	0.547
Sturgeon.Nahma	0.173	0.335	0.351
Tahquamenon	0.275	0.290	0.457
White	0.268	0.269	0.351
Whitefish	0.238	0.240	0.281
	White	Whitefish	
Black	0.503	0.578	
Boardman	0.355	0.487	
Cedar	0.421	0.459	
Escanaba	0.537	0.605	
Fox	0.560	0.603	
Grand	0.186	0.142	
Kalamazoo	0.211	0.206	

Table 2.3 (Cont'd)

Kewaunee	0.342	0.323
Macatawa	0.154	0.190
Manistee	0.418	0.501
Manistique	0.356	0.487
Menominee	0.361	0.437
Millecoquins	0.127	0.158
Muskegon	0.570	0.611
Oconto	0.533	0.560
Ontonagon	0.602	0.691
Pere.Marquette	0.533	0.620
Peshtigo	0.230	0.302
Sturgeon	0.696	0.763
Sturgeon.Nahma	0.382	0.469
Tahquamenon	0.406	0.532
White	0.412	0.161
Whitefish	0.253	0.426

The relationship between year and river sampled can be visualized in the heatmap of amino acid profiles for water samples collected from Great Lakes tributaries in 2019 and 2020 (Figure 2.4), which suggests that specific amino acids of different classes (polar, non-polar, acidic, and basic) account for more of the variance in differences between years and specific rivers. The organization of samples in the heatmap based on the Bray-Curtis dissimilarity of amino acid profiles demonstrates differences in amino acid profiles of samples in 2019 and 2020. Non-polar amino acids were more abundant across all samples, though the contribution of specific amino acids to samples varied across years. Specifically, the non-polar neutral amino acids phenylalanine, leucine, and isoleucine as well as the polar neutral amino acid serine have higher relative abundances in 2019 samples, while the polar neutral amino acid glutamine and the polar basic amino acid histidine have higher relative abundances in 2020 samples. The heatmap indicates the abundance of specific amino acids differ between samples, though the

dissimilarity between specific rivers and the amino acids contributing to these differences differed between years (Figure 2.4).

Amino acid profiles for rivers sampled in 2020 also varied seasonally between June and October. A PERMANOVA of the samples collected in 2020 found that the Bray-Curtis dissimilarity differed by river, sample period, and their interaction (Table 2.4). Consistent with our comparison of the effects of river and year, we found that the river sampled explained the most variation in Bray-Curtis dissimilarities in 2020 water samples (38.95%) (Pseudo $F = 3.485$, $p < 0.05$), with 6.05 % explained by sample period (Pseudo $F = 11.910$, $p < 0.05$) and 14.35 % explained by the interaction between sample period and river (Pseudo $F = 1.569$, $p < 0.05$). Beta dispersion tests indicate the assumptions of the PERMANOVA analyses were met as there were no significant differences in the dispersion between samples of different rivers, samples of different sample periods, or samples of different rivers and sample periods (Table 2.4).

The relationship between sample period and rivers sampled can be visualized in the heatmap of amino acid profiles for water samples collected from Great Lakes tributaries in June and October 2020 (Figure 2.5). The organization of samples in the heatmap based on the Bray-Curtis dissimilarity of amino acid profiles does not show a clear separation of samples based on sample period across all samples, but rather we see small groupings of samples based on sample period that are more dissimilar to other samples from the same period than groupings of samples from the other sample period. There are no clear distinguishing amino acids contributing more to amino acid profiles of one sample period and overall, non-polar amino acids were more abundant than other amino acid classes in both sample periods. By and large, river to river comparisons and the specific amino acids contributing to differences between specific rivers were not consistent across sample periods.

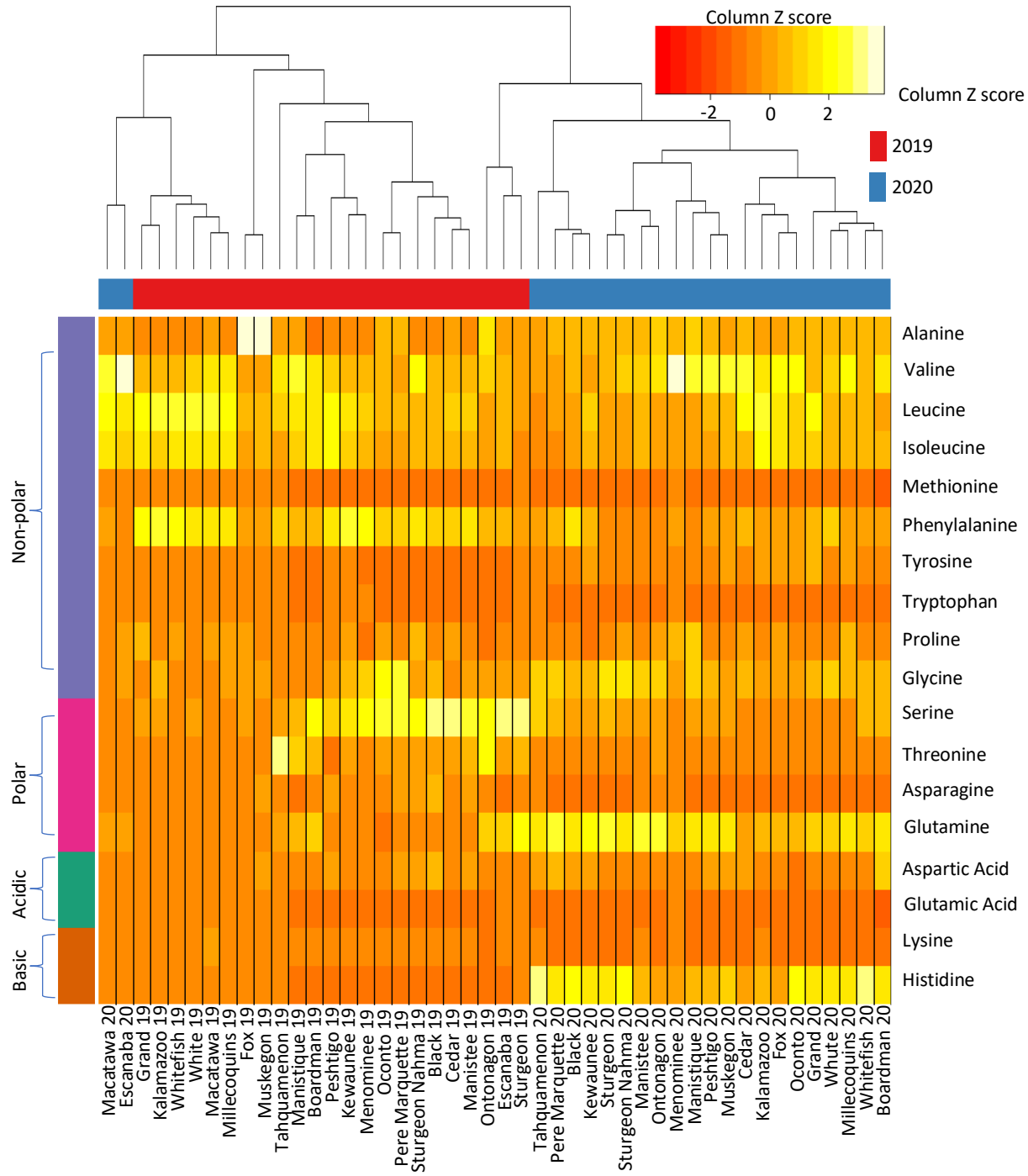

Figure 2.4. Heatmap of the amino acid profiles of the Great Lakes tributaries sampled in June of 2019 and 2020. Colors for each amino acid represent the proportional abundance of the amino acid in each sample, with red being the least abundant and white being the most abundant. Rows represent each of the eighteen amino acids included in the amino acid profiles for each sample in Bray-Curtis dissimilarity analysis. Samples at the bottom of the column are represented by the river name and year. The dendrogram (figure top) represents the Bray-Curtis dissimilarity between samples and the fewer nodes between two

Figure 2.4 (Cont'd)

samples, the more related they are based on their amino acid profiles. Amino acid types are color coded with the left color bar. Purple represents non-polar amino acids, pink represents polar amino acids, green represents acidic amino acids, and orange represents basic amino acids. Samples collected in 2019 are represented by the red sections of the top color bar and samples collected in 2020 are represented by the blue sections.

Table 2.4. Results for the PERMANOVA comparing the effects of sample period, river, and their interaction on the Bray-Curtis dissimilarity of amino acid profiles between samples collected from Great Lakes tributaries in 2020. The beta dispersion test shows homogeneity of the dispersion of Bray-Curtis dissimilarity between sample periods and rivers.

PERMANOVA: Dissimilarity ~ Sample Period + River + Sample Period*River						
Factors	*Df*	*Sum Sq.*	*Mean Sq.*	*F value*	R^2	*p value*
Sample period	1	0.4469	0.44692	11.9096	0.06051	0.001
River	22	2.8769	0.13077	3.4847	0.3895	0.011
Sample period*River	18	1.06	0.05889	1.5693	0.14352	0.015
Residuals	80	3.0021	0.03753	0.40646		
Beta dispersion test of homogeneity between factor levels						
Factors	*Df*	*Sum Sq.*	*Mean Sq.*	*F value*	*p value*	
Sample period	1	0.015	0.015	1.522	0.221	
Residuals	86	0.875	0.010			
River	22	0.132	0.006	0.271	0.999	
Residuals	65	1.439	0.022			

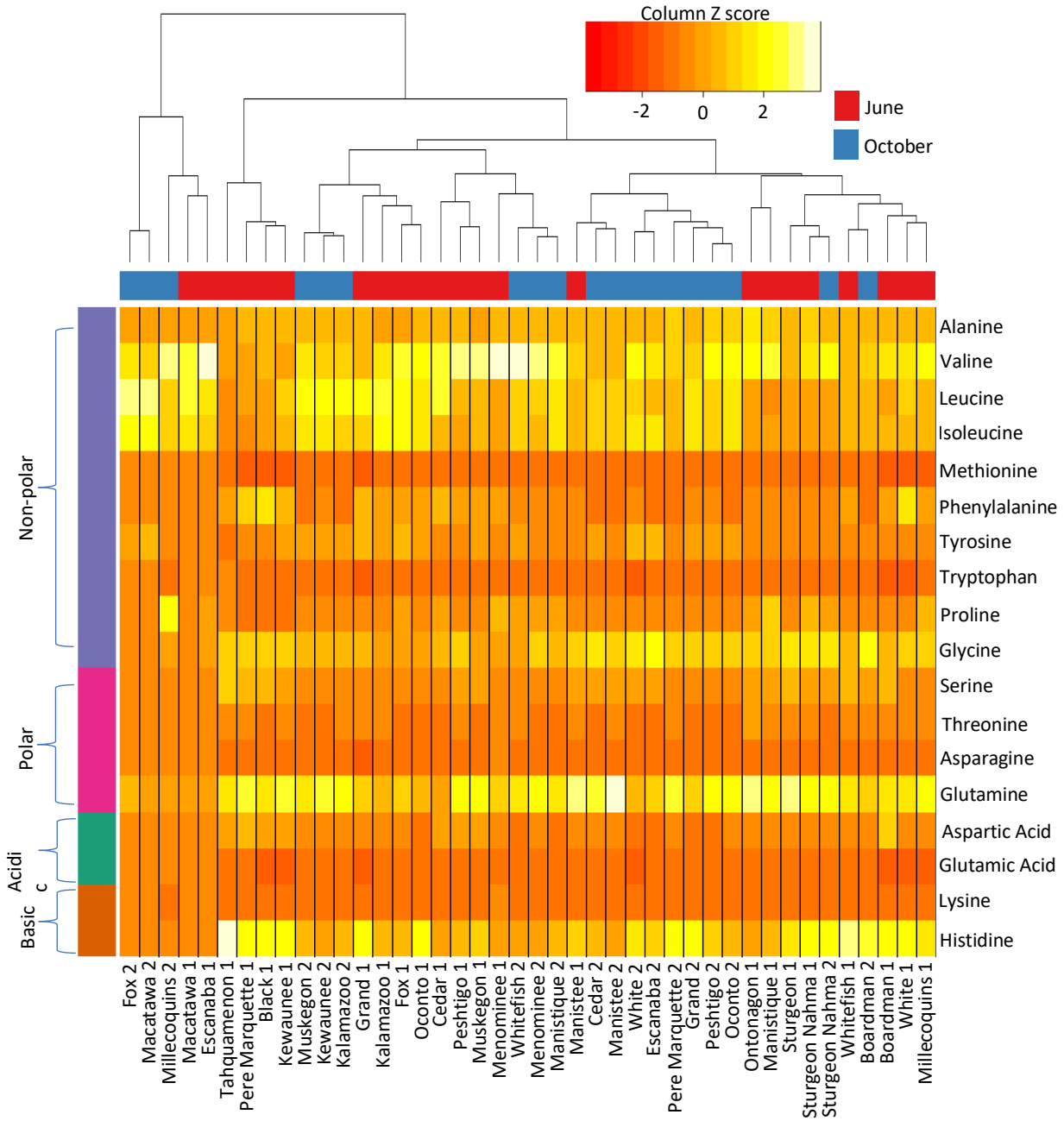

Figure 2.5. Heatmap of the amino acid profiles of the Great Lakes tributaries sampled in June and October of 2020. Colors for each amino acid represent the proportional abundance of the amino acid in each sample, with red being the least abundant and white being the most abundant. Rows represent each of the eighteen amino acids included in the amino acid profiles for each sample in Bray-Curtis dissimilarity analysis. Samples at the bottom of the column are represented by the river name and sample period (1- June, 2-October). The dendrogram (figure top) represents the Bray-Curtis dissimilarity between samples and the fewer nodes between two samples, the more related they are based on their amino acid profiles. Amino acid types are color coded with the left color bar. Purple represents non-polar amino acids, pink represents polar amino acids, green represents

Figure 2.5 (Cont'd)

acidic amino acids, and orange represents basic amino acids. Samples collected in June are represented by the red sections of the top color bar and samples collected in October are represented by the blue sections.

Behavioral responses to natural stream water and synthesized amino acid mixtures

Experiment 1: Responses of lake sturgeon reared in water activated with artificial amino acid mixtures

Model comparison for fixed effects yielded two potential models, the model including the effect of pre-odor distance traveled only and the model including the effects of pre-odor distance traveled and treatment (Table 2.5). Here we report results for the model including the effects of treatment and pre-odor distance traveled. We considered results for fixed effects-only and mixed models and found mixed effects models including tank as a random intercept, yielded an estimate term of 0 for tank. To prevent overfitting, we focused on the fixed effects model and the model including the random intercept of arena (Table 2.6).

Table 2.5. Model selection for the Black Lake Sturgeon Rearing Facility Experiments. Comparison of AICc differences of robust linear regression models for post-odor distance traveled responses based on different fixed effects. AICc differences are calculated in reference to the model with the lowest AICc. Fixed effects included treatment, pre-odor distance, odor added during behavior experiments, total length of the individual, and pairwise interactions between the independent variables.

Model	ΔAICc
Pre-odor distance	0.00
Treatment + Pre-odor distance	1.30
Treatment + Pre-odor distance + Treatment*Pre-odor distance	2.27
Odor + Pre-odor distance	5.66

Table 2.5 (Cont'd)

Odor + Treatment + Pre-odor distance	6.96
Treatment + Pre-odor distance + Odor + Treatment*Pre-odor distance	7.68
Odor + Treatment + Length + Pre-odor distance	7.76
Odor + Pre-odor distance + Odor*Pre-odor distance	12.32
Treatment + Pre-odor distance + Odor + Odor*Pre-odor distance	13.10
Odor + Treatment + Pre-odor distance + Odor*Treatment	17.84
Odor + Treatment + Pre-odor distance + Odor*Treatment + Odor*Pre-odor distance + Treatment*Pre-odor distance	22.58
Odor + Treatment + Length + Pre-odor distance + Odor*Treatment + Odor*Pre-odor distance + Treatment*Pre-odor distance	23.82

Table 2.6. Robust linear regression results for the Black Lake Sturgeon Rearing Facility behavior experiments. Parameter estimates and standard errors for the robust linear regression model of post-odor distance traveled based on treatment group and pre-odor distance traveled. Estimates on the left are from the fixed-effects only models and estimates on the right are from a robust linear mixed model with the arena used for observations as a random intercept. Predictions for post-odor distance traveled at the mean pre-odor distance (1583.59 cm) are based on the fixed-effects only model.

Fixed-only			**Fixed + Arena as a random effect**		
Parameter	*Estimate*	*SE*	*Parameter*	*Estimate*	*SE*
Intercept	161.056	64.54	Intercept	163.374	71.49
Trt. Control	-75.743	61.84	Trt. Control	-78.998	63.47
Trt. Oconto	59.872	61.67	Trt. Oconto	58.187	63.24
Pre-odor distance	0.965	0.03	Pre-odor distance	0.965	0.03

Table 2.6 (Cont'd)

Predicted post-odor distance at mean pre-odor distance for each treatment			
Treatment	*Estimate*	*Lower 95% CI*	*Upper 95% CI*
Trt. Cedar	1689.786	1607.92	1771.65
Trt. Control	1614.043	1532.22	1695.86
Trt. Oconto	1749.657	1667.99	1831.33

Intercept estimates for the model relating post-odor distance traveled to pre-odor distance traveled and treatment indicate that behavioral responses to artificial and natural river waters differed for lake sturgeon raised in artificial Cedar River water, artificial Oconto River water, and Black River hatchery water (Table 2.6). Although odor was not included in the top model, we found that previous exposure to artificial Oconto River water during early development led to increased behavioral responses by juvenile fish in our behavior experiments. At the mean pre-odor distance traveled of 1583.59 cm, our model predicted that fish raised in artificial Oconto River water will have the largest post-odor distance traveled behavioral response when not accounting for odor exposure, followed by fish previously exposed to artificial Cedar River water (Table 2.6).

To further explore the effect of odor on behavioral responses of individuals across treatment groups, we also analyzed a robust linear regression model relating post-odor distance traveled to pre-odor distance traveled, the exposure treatment group of fish during development, the odor applied during the behavior experiment, and all pairwise interactions of the three selected predictors. To compare the effect of pre-odor distance traveled on post-odor distance traveled for each treatment group under each odor condition, we visualized slope estimates from the robust linear regression (Figure 2.6). Although this model was not selected through model comparison, it demonstrates the variability in responses to odorants for all individuals, regardless

of treatment group. Specifically, we found that fish from all treatment groups had highest slope estimates when the artificial Oconto River water was added to the behavioral experiments.

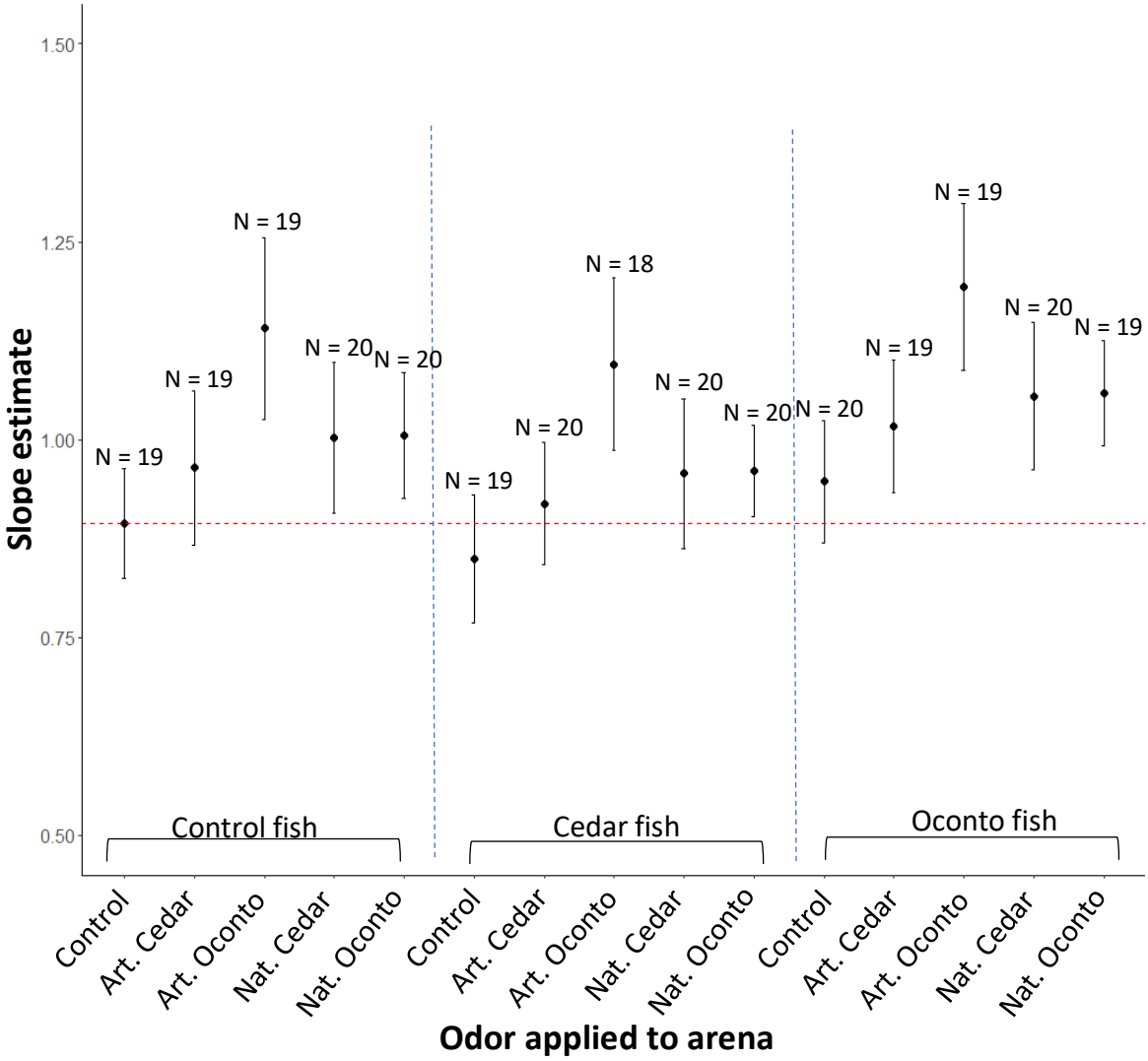

Figure 2.6. Slope estimates for the relationship between pre-odor distance traveled based on the robust linear model relating post-odor distance to treatment, odor applied to the arena, pre-odor distance, and all pairwise interactions. Error bars represent standard error of the slope estimates. Dashed horizontal red lines were included for comparison between the control and other treatment groups and represent the estimated slope value. and predicted post-odor distance for the control. Blue dashed horizontal lines separate treatment groups.

Experiment 2: Behavior of lake sturgeon reared in natural stream water

Exposure to odor during behavioral assays did not predict post-odor distance traveled in Experiment 2. Model selection for these experiments yielded one top model, which included the effect of pre-odor distance traveled only (Table 2.7). We considered results for the fixed effects model as well as a mixed model including behavioral arena as a random intercept (Table 2.8). Based on the fixed effects model, we estimated a positive relationship between pre-odor and post-odor distance traveled, with a 0.676 ± 0.08 (slope + SE) increase in post-odor distance traveled with a unit increase in pre-odor distance. The odor fish were exposed to during the behavior experiments was not included in our top model, so we cannot report how the artificial and natural river waters affected the behavior of Ontonagon SRF fish.

Table 2.7. Model selection for the Ontonagon Streamside Rearing Facility Experiments. Comparison of AICc differences of robust linear regression models for post-odor distance traveled responses based on different fixed-effects. AICc differences are calculated in reference to the model with the lowest AICc. Fixed effects included pre-odor distance, odor added during behavior experiments, total length of the individual, and pairwise interactions between the independent variables.

Model	ΔAICc
Pre-odor distance	0
Length + Pre-odor distance	2.2
Length + Pre-odor distance + Length*Pre-odor distance	4.38
Odor + Pre-odor distance	11.51
Odor + Pre-odor distance + Length	14.16
Odor + Pre-odor distance + Length + Length*Pre-odor distance	16.92
Odor + Pre-odor distance + Odor*Pre-odor distance	17.63
Odor + Pre-odor distance + Length + Odor*Pre-odor distance	20.17

Table 2.7 (Cont'd)

Odor + Pre-odor distance + Length + Odor*Length	29.24
Intercept only	35.23
Odor + Pre-odor distance + Length + Odor*Pre-odor distance + Length*Pre-odor distance + Odor*Length	45.6

Table 2.8. Robust linear regression results for the Ontonagon Streamside Rearing Facility behavior experiments. Parameter estimates and standard errors for the robust linear regression model of post-odor distance traveled based on pre-odor distance traveled. Estimates on the left are from the fixed-effects only models and estimates on the right are from a robust linear mixed model with the arena used for observations as a random intercept.

Fixed-only			**Fixed + Arena as a random effect**		
Parameter	*Estimate*	*SE*	*Parameter*	*Estimate*	*SE*
Intercept	1147.483	271.00	Intercept	1129.000	269.70
Pre-odor distance	0.676	0.08	Pre-odor distance	0.680	0.08

DISCUSSION

Spatial and temporal variability of amino acid profiles of Great Lakes tributaries

Analyses of the amino acid profiles of twenty-three Great Lakes tributaries support our prediction that amino acid profiles differed between current and historical lake sturgeon spawning streams and that the variation in amino acid profiles among streams was greater than the variation of amino acid profiles between and within years. However, differences in amino acid profiles between rivers were not consistent over years or sample periods which supports temporal instability of river specific amino acid profiles. Specifically, our results demonstrate that the Bray-Curtis dissimilarity in amino acid profiles between rivers was dependent on year and sample period. We found no significant correlation between the pairwise Bray-Curtis dissimilarities of rivers sampled in June of 2019 and 2020. Our findings were consistent with studies of amino acid profiles in the Mississippi and Pearl Rivers, which found that the composition and abundance of amino acids varied across time (Duan & Bianchi, 2007). Yamamoto et al. (2013) found that stream specific amino acid profiles varied between single years but showed that amino acid profiles across the multi-year spawning interval for chum salmon (*Oncorhynchus keta*). Our study documented differences in the composition of amino acid profiles between only two years, for select Great Lakes tributaries that are relevant to lake sturgeon. It is possible that amino acid profiles for the rivers we sampled are consistent over a longer period and the variability in amino acid profiles we observed may not translate to rivers occupied by other olfactory imprinting species.

Future studies could benefit from increasing the frequency and replication of sample collection at a given site and by studying the composition of amino acids in Great Lakes tributaries over multiple years. Additionally, it is unclear whether the annual variation in amino

acid profiles observed would impact olfactory imprinting guided natal homing or whether lake sturgeon could discriminate this level of variation. The specificity of natal odorant profiles needed to accurately guide natal stream homing in fish is unknown. Studies focused on the olfactory or behavioral differentiation of increasingly different or variable amino acid profiles could identify an acceptable range of amino acid profiles for supporting olfactory imprinting. Heatmap visualizations of the dissimilarity in amino acid profiles of our samples indicated specific amino acids of different charge classes that differentiated rivers and years (Figure 2.4), however differences between rivers sampled during different sample periods in 2020 were less clear (Figure 2.5). Differences in the proportional abundance of specific amino acids between specific rivers and/or time may indicate amino acids used by lake sturgeon to differentiate between rivers. Amino acids of different classes may induce different olfactory responses in fish (Hara, 1994), but it is unclear whether these differences could guide natal site differentiation and should be explored in future studies on the role of amino acids in olfactory imprinting.

Behavioral responses to natural stream water and synthesized amino acid mixtures

Results from the analysis of behavioral responses to natural and artificial river waters by fish raised in artificial Cedar and Oconto River waters did not support our prediction that lake sturgeon develop olfactory memory to amino acid profiles during early development or that they can discriminate between artificial river waters. We observed differences in the post-odor distance traveled measure between fish raised in artificial Oconto, artificial Cedar, and Black River hatchery water, but the effect of odor application in the experiments was not included based on AICc model selection. Specifically, we found that when not accounting for the odor added to the behavioral arena, fish previously exposed to artificial Oconto River water had larger predicted post-odor distance traveled measures compared to control fish or fish raised in artificial

Cedar River water. This suggests previous exposure to amino acids during early life development may affect fish behavior, however, it is unclear whether increased behavior resulted from olfactory memory formation. Further exploration of a robust linear model including the effects of treatment group, odor applied to the behavioral arena, pre-odor distance traveled, and all interactions indicated that behavioral responses by fish from all treatment groups varied and found that behavioral responses were elevated to artificial Oconto River water in all treatment groups. While these findings do not support olfactory memory formation to amino acid profiles during early development in lake sturgeon, they provide evidence for the behavioral effects of exposure to amino acids on juvenile lake sturgeon. We also found no evidence to support our prediction that lake sturgeon discriminates between river waters (natural or artificial) in lake sturgeon raised at the Ontonagon Streamside Rearing Facility or whether fish can discriminate between rivers based solely on amino acid profiles. Model selection for the analysis of behaviors by Ontonagon SRF raised individuals found the top model to include the effect of pre-odor distance traveled only. Overall, we saw a correlation between pre- and post-odor activity, which was consistent with the Cedar and Oconto artificial river water experiments, but we are unable to say whether the activity was dependent on the odor added during the behavior experiments.

Olfactory imprinting to artificial amino acid profiles during parr-smolt transformation has been observed in salmon (Shoji et al., 2000; 2003; Yamamoto et al., 2013; Chen et al., 2017), though these studies focused on behavioral responses to amino acids in adult salmon that directly measured preference for and migration towards different odors. Our studies investigating olfactory memory of artificial amino acid profiles in lake sturgeon relied on behavioral observations of age-0 individuals, which required the use of alternative behavior measures such as distance traveled, to document responses to artificial and natural river water. Our application

of natural river water resulted in a dilution factor of one part river water to thirty parts groundwater and the dilution of amino acids and other odorants in the natural river water may have contributed to relatively low behavioral responses by fish in both experiment 1 and experiment 2. Chemical analysis done after behavioral experiments indicated that the proportional abundance of each of the fifteen amino acids used for creating artificial amino acid profiles differed between 2019, when samples were collected to determine amino acid profiles used to imprint lake sturgeon, and 2021, when behavioral responses to stream waters were tested (Table 2.1). Differences between the artificial amino acid profiles used in our experiments and the actual amino acid profiles for each river may have impacted fish behavior as the artificial river waters used did not perfectly replicate natural river water. We previously provided evidence for olfactory imprinting to artificial odorants during early ontogeny in lake sturgeon based on behavioral responses in juveniles (Chapter 1). The methods used in the BLSRF experiments were highly comparable to the methods used in Chapter 1 and results were not expected based on our previous evidence for olfactory imprinting in lake sturgeon. It is possible that behaviors related to olfactory memory of amino acid profiles differ from the experimental odorants used in Chapter 1 and that post-odor distance traveled was not an accurate indicator of olfactory memory to artificial amino acid profiles of a river. Behavior experiment methods at the Ontonagon SRF differed from those used in Chapter 1 and for observing behavioral responses by fish raised in artificial Cedar and Oconto River waters. Fish raised at the Ontonagon SRF were observed outside in fully lit conditions, which may have impacted overall activity and responses to the odorants.

Conclusions

Olfactory imprinting guided natal stream homing in lake sturgeon would require matching odor profiles over its relatively long-life span as over twenty years could separate the time of imprinting and the time of natal stream migration as adults to spawn (Bruch and Binkowski, 2002). Knowledge of the key odorants involved in olfactory memory formation could provide opportunities for alternative rearing methods, where odor profiles are recreated to match target stocking streams. Lake sturgeon stocking programs may benefit from a better understanding of olfactory memory in lake sturgeon and the key stream specific odors that guide natal stream migrations in adults, though the use of SRFs is a useful approach given the levels of uncertainty we have regarding olfactory imprinting in lake sturgeon. Lake sturgeon did not display behaviors suggesting olfactory memory was formed for amino acid profiles of select rivers in our experiments. We found evidence for spatial and temporal variability in stream specific amino acid profiles and further investigation is needed to assess whether amino acid profile variability impacts successful olfactory imprinting guided natal stream homing to stream specific amino acid profiles. Future studies would benefit from focusing on the variability of different classes of amino acids or on identifying the specific amino acids that differ between rivers and the causes of these differences. A recent study of bacterial communities of Lake Michigan tributaries found variation of bacterial community composition was associated with urban and agricultural land-use practices (Sanfilippo et al., 2021), which could signify the susceptibility of amino acids to land-use changes. Studies focused on the environmental basis for amino acid profiles may be able to explain the causes of amino acid profile variability and could highlight specific land-use factors contributing to changing natal stream odor profiles. Further

exploration into the effects of amino acid profile variability on olfactory imprinting related behaviors could elucidate the susceptibility of natal homing species to environmental changes.

APPENDIX

Table A2.1. LC-MS/MS multiple reaction monitoring and analysis parameters.

Compound	Parent ion	Product ion	Cone Voltage	Collision voltage	Retention time
Retention time window: 0 – 4.5 min					
Glycine	76	30	17	8	1.77
Alanine	90.1	44	17	8	3.12
Serine	106.1	60	19	10	1.53
Threonine	120.1	74	19	8	2.16
Cysteine	122	76	18	15	2.05
Asparagine	133.1	74	19	14	1.55
Aspartic acid	134.1	74	19	10	1.19
Glutamine	147.1	84	16	14	1.92
Glutamic acid	148.1	84	17	14	1.66
Retention time window: 4.5 – 6.5 min					
Proline	116	70	21	10	5.13
Valine	118.1	72	17	9	6.03
Methionine	150.1	104	19	9	5.71
Tyrosine	182.1	136.1	20	12	5.01
Retention time window: 6.5 – 13 min					
Leucine	132.1	86	19	9	7.03
Isoleucine	132.1	86	19	9	7.20
Lysine	147.1	84	19	14	8.07
Histidine	156.1	110	20	12	8.12
Phenylalanine	166.1	120	20	10	7.25
Arginine	175.1	70	24	18	8.27
Tryptophan	205.1	146	19	14	7.30

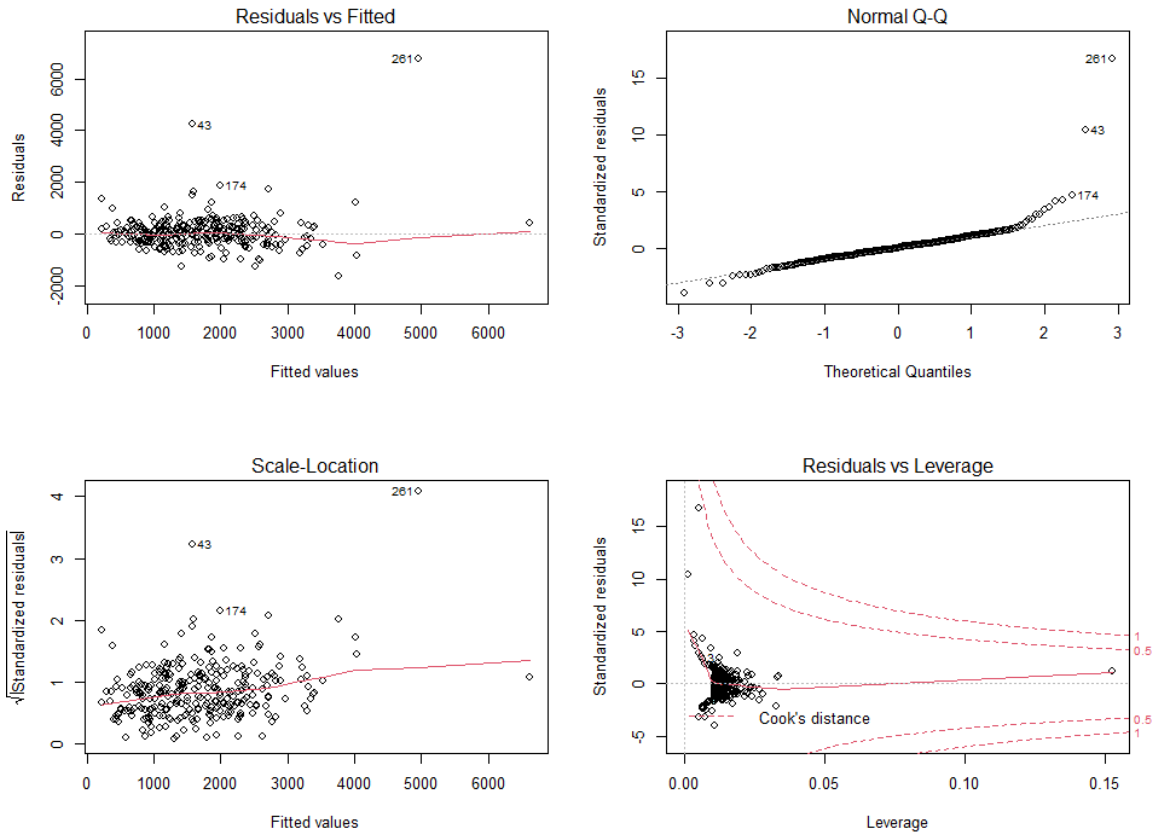

Figure A2.1. Residual plots used for checking assumptions of normality and homoscedasticity after using robust linear regression methods for the artificial amino acid profile experiments at the Black Lake Sturgeon Rearing Facility. Robust linear models were used to reduce effects of high-leverage observations for the model relating post-odor distance to treatment and pre-odor distance. Plots represent the relationship between residuals and predicted responses for each observation (A), normal quantile-quantile plot (B), the relationship between the square-root of standardized residuals and predicted responses (C), and the relationship between standardized residuals and leverage of individual observations (D).

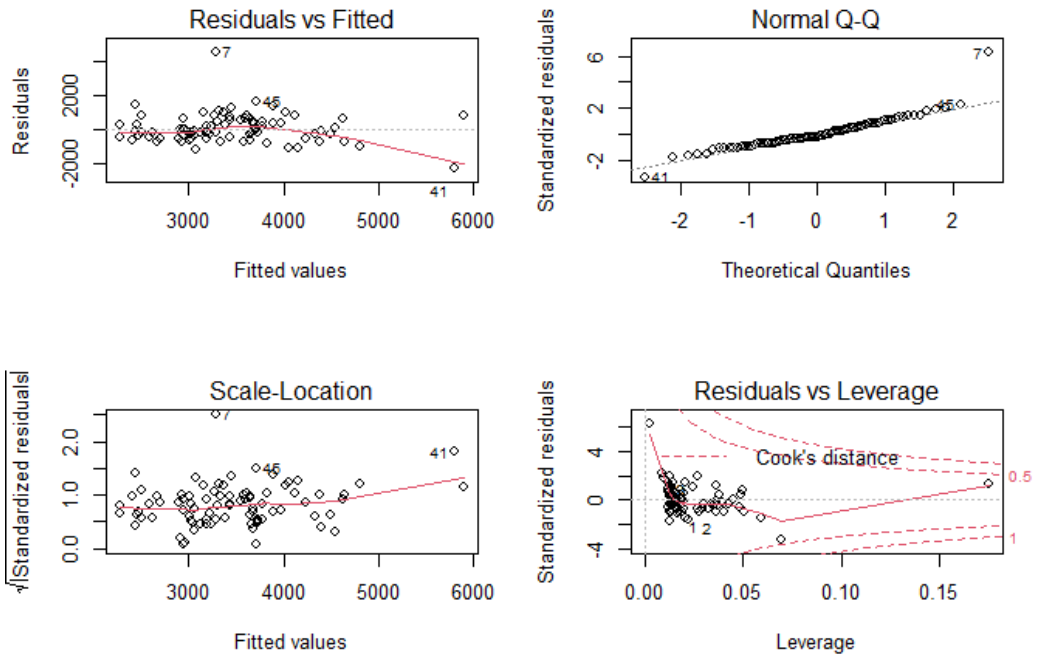

Figure A2.2. Residual plots used for checking assumptions of normality and homoscedasticity after using robust linear regression methods for the artificial amino acid profile experiments at the Ontonagon Streamside Rearing Facility. Robust linear models were used to reduce effects of high-leverage observations for the model relating post-odor distance to pre-odor distance. Plots represent the relationship between residuals and predicted responses for each observation (A), normal quantile-quantile plot (B), the relationship between the square-root of standardized residuals and predicted responses (C), and the relationship between standardized residuals and leverage of individual observations (D).

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