Experimental Determination of the Limits of Using Stable Isotopes to Distinguish Steelhead and Rainbow Trout Offspring

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Experimental Determination of the Limits of Using Stable Isotopes to Distinguish Steelhead and Rainbow Trout Offspring

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Abstract
Salmonid ecology and conservation projects often necessitate distinguishing the offspring from anadromous or nonanadromous mothers, but the forms are often physically and genetically similar. We reared *Oncorhynchus mykiss* fry produced by anadromous mothers on one of two diets: one rich in and the other depleted in marine nutrients. We then sampled and analyzed fin and muscle tissues from fish fed both diets for nitrogen stable isotopes over time. Tissue nitrogen values from fish on the marine-nutrient-depleted diet indicated the size and time cutoffs at which offspring of anadromous *O. mykiss* no longer reflected a marine origin (muscle tissue cutoff: 0.81 g, 50.8 mm, and 29.4 d postfeeding; fin tissue cutoff: 0.65 g, 45.9 mm, and 35.3 d). Salmonids smaller than 50 mm cannot provide a sufficiently large fin sample for isotopic analysis without significant risk of mortality. Therefore, our experiment did not reveal a period when fish were large enough to provide a nonlethal fin clip but still maintain their maternal nitrogen isotope signature. However, fish growing slower than those we examined would likely be distinguishable for a longer period of time than our data indicated.

Salmonids exhibit a wide diversity of migratory patterns along a continuum from obligate anadromy to freshwater residency (Rounsefell 1958; Quinn and Myers 2004; Pavlov and Savvaitova 2008). For example, Pink Salmon *Oncorhynchus gorbuscha* and Chum Salmon *O. keta* have no observed nonanadromous populations in their native range, whereas Gila Trout *O. gilae*, Apache Trout *O. apache*, Mexican Golden Trout *O. chrysogaster*, and some subspecies of Cutthroat Trout *O. clarkii* and Rainbow Trout *O. mykiss* are invariably nonanadromous (Behnke 2002). However, many salmonid species have both anadromous and nonanadromous individuals, either in discrete populations displaying one of these migration patterns or as individuals within populations (Jonsson and Jonsson 1993; Dodson et al. 2013; Slout et al. 2014). Species that exhibit alternative migration patterns are found in all the main genera of salmonids (*Salvelinus, Salmo*, and *Oncorhynchus*), but the past decade has seen especially close examination of the differentiation between forms and the processes controlling anadromy and residency in *O. mykiss*, known as Rainbow Trout when nonanadromous and steelhead when anadromous (reviewed by Kendall et al. 2015). During the juvenile phase (i.e., prior to seaward migration by steelhead), the two forms cannot be distinguished. They are physically identical and they may not show genetic differences (e.g., Heath et al. 2008), which is consistent with observations that the two forms interbreed (McMillan et al. 2007) and that each

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form can produce the other (e.g., Courter et al. 2013). As a result, it has been difficult to answer important questions about the two forms within rivers, though it has long been recognized that many important conservation questions hinge on being able to distinguish between them (Neave 1944; e.g., determining the presence and abundance of both life history types in mixed populations: Zimmerman et al. 2009; determining the extent of steelhead recolonization and movement in mixed populations: Winans et al. 2010).

Stable isotope analysis is a useful tool for classifying organisms based on their trophic level and the environment in which they feed (Fry 2006). Organisms feeding in marine and freshwater environments are characterized by different ratios of heavy-to-light nitrogen ($\delta^{15}N$), among other elements (France 1995; Doucett et al. 1999). As aquatic consumers capture food in their environment and incorporate prey nutrients into their own tissues, $\delta^{15}N$ is mostly conserved, though altered by trophic fractionation (3.4%; Vander Zanden and Rasmussen 1999; Post 2002). Therefore, we can infer where an individual fish has been feeding based on the nitrogen signature of its body tissues after accounting for trophic position. Further, when female salmon undergo oogenesis, the marine nitrogen signature is expressed in their eggs (Doucett et al. 1999). Stable isotope analysis can therefore be used to determine the maternal origin (anadromous or nonanadromous) of juveniles in a stream (Ciancio et al. 2008; Jardine et al. 2008; Briers et al. 2013). Offspring of anadromous mothers will be “enriched,” or will have a larger heavy-to-light nitrogen ratio ($\delta^{15}N$) than freshwater-origin conspecifics (Jardine et al. 2008). The maternal signal becomes diluted as fish grow (Doucett et al. 1999) or tissues turn over (Heady and Moore 2013; Vander Zanden et al. 2015), so stable isotope analysis has limitations for determining a fish’s maternal origin as time passes and juveniles feed and grow in freshwater.

Samples for isotope analysis can be extracted from a number of different fish tissues, including muscle, fin, mucus, and liver (Church et al. 2009; Heady and Moore 2013). In small fish, muscle tissue sampling can be lethal, which is problematic when working with threatened or endangered populations. Sanderson et al. (2009) reported that the isotopic signatures of fin and muscle tissues were highly correlated, suggesting that fin tissue can be a viable, nonlethal alternative to other tissue sampling methods (see also McIntyre and Flecker 2006; Hanisch et al. 2010; Curry et al. 2014). Fins regenerate rapidly and a caudal fin sample is not lethal for fish $> 50$ mm FL (Sanderson et al. 2009). A conflict therefore arises between two considerations when juvenile salmonids are sampled in a mixed population to determine which individuals were produced by anadromous mothers and which by nonanadromous mothers. Thus, there is a need to sample juveniles when they are young and small enough to still have a stable isotope signature indicative of maternal origin, yet large enough to survive the sampling process.

In this paper we report an experiment designed to determine the decay function (in body length, weight, and time after exogenous feeding) of the nitrogen isotope signature in anadromous *O. mykiss* fry fed a marine-nutrient-depleted diet. Fry from anadromous mothers were fed the depleted diet and compared with fry fed a nitrogen-enriched marine-origin diet, and with $\delta^{15}N$ values of nonanadromous trout from other studies. Our first goal was to identify the point at which offspring from anadromous mothers could no longer be identified as marine origin via nitrogen isotope analysis. The second goal was to determine whether *O. mykiss* small enough to be distinguished as offspring of anadromous or nonanadromous mothers were large enough to provide an adequate caudal fin sample and survive.

**METHODS**

*Fish rearing.*—On February 4, 2013, 500 eyed *O. mykiss* embryos from a population of steelhead were acquired from the Tokul Creek Hatchery, Fall City, Washington. These fish were incubated at the University of Washington hatchery in Heath trays (MariSource, Fife, Washington) at 11°C. Embryos began hatching during the week of February 11, and on March 4 (at mean FL $\approx 27$ mm) fry were moved from the incubation tray to two rearing troughs ($61 \times 30.5 \times 243.8$ cm; working depth, 20.3 cm; volume, $\sim 303$ L; MariSource) at ambient Lake Washington surface water temperature (range, 7–13°C). The fry were equally divided into control and treatment feed groups, each in a separate trough. On April 16 (at mean FL $\approx 48$ mm), fish were transferred from troughs to large tanks ($78.7 \times 78.7 \times 66$-cm semisquare tank (SSFB-3226); Poly-Tank, Litchfield, Minnesota), again separated by treatment. Daily rations were calculated using a maximum growth feeding model, administered over five feeding sessions per day (Wester 2001). The feeding model calculated a per-capita food ration, and was adjusted midexperiment to account for differential fish mortality in the two treatments (control mortality rate: 0.47 fish/d; treatment mortality rate: 0.98 fish/d). The experiment ended on April 28, 2013.

Control fish were fed commercial fish feed (Bio-Oregon, Longview, Washington; BioVita Starter size #0, #1) containing marine-origin nutrients (Table 1). Treatment fish were fed an experimental marine-nutrient-depleted diet formulated to reflect a freshwater nitrogen signature (Table 2). The diets were similar in proximate composition and energy content (Table 1). The treatment feed was produced using the micro-extrusion maruzenization process (Barrows and Lelli 2006). Dry ingredients were finely ground to a particle size of $< 200 \mu$m using an air-swept pulverizer (Model 18H; Jacobson, Minneapolis, Minnesota) and a ball mill (Twin deck; U.S. Stoneware, East Palestine, Ohio), and then blended to form a homogenous mixture. After addition of the liquid ingredient and water, the resulting dough was cold-extruded to form 0.5-mm-diameter strands using a side-discharge extruder.
(LCI, Raleigh, North Carolina). The strands were then placed on a rotating disk to produce pellets approximately 0.5 mm long. The pellets were dried with forced air at room temperature (25°C) for 24 h to <10% moisture and then screened to remove fines. Feeds were kept in sealed plastic containers at room temperature and were refrigerated after opening.

Samples of ground feeds were analyzed for proximate composition using standard methods (AOAC 1990). Samples were dried in a convection oven at 105°C for 12 h to determine moisture level and were analyzed for crude protein (total nitrogen \times 6.25) using a LECO FP-428 nitrogen analyzer (LECO Instruments, St. Joseph, Michigan). Crude lipid was analyzed using an ANKOM XT15 extraction apparatus (ANKOM Technology, Macedon, New York), which used petroleum ether as the extracting solvent, and ashed by incineration at 600°C for 4 h in a muffle furnace. Energy content of samples was determined using a Parr Adiabatic Calorimeter (Parr Instruments, Moline, Illinois). Feed samples were analyzed for amino acid content by the University of Missouri Analytical Laboratory and for mineral content by the University of Idaho Analytical Science Laboratory. All essential amino acids and minerals exceeded the dietary requirements of Rainbow Trout (NRC 2011).

**Tissue sampling.**—Every 6 d, nine fish from each treatment were selected at random and euthanized with an overdose of tricaine methanesulfonate (MS-222) buffered to neutral pH with sodium bicarbonate. Fish were weighed and measured (FL), and caudal fin and dorsal muscle tissue samples were taken from each individual. Due to the small fish size, caudal fin clips were combined into composite samples, each containing fin clips from three fish (Figure 1). Samples were weighed and frozen for subsequent processing and analysis. Fin and muscle tissue samples were freeze-dried for 24 h and ground into a homogeneous powder. Aliquots of dried sample (0.4–0.6 mg) were weighed, sealed in tin capsules (5 \times 9 mm; Costech Analytical Technologies, Valencia, California), and sent to the University of Washington IsoLab for \( ^{15} \text{N} \) isotope analysis using mass spectrometry (Costech ECS 4010 Elemental Analyzer [Costech Analytical Technologies], ThermoFinnigan Conflo III Interface [Thermo Electron Corporation, Waltham, Massachusetts], ThermoFinnigan MAT 253 Isotope Ratio Mass Spectrometer [Thermo Fisher Scientific, Waltham, Massachusetts]). Nitrogen isotope ratios were reported relative to atmospheric nitrogen (Air-N\(_2\)).

**Analysis.**—Because the fish were offspring of anadromous mothers, we expected that the fish would initially show \( ^{15} \text{N} \) values consistent with their marine origin, but the values in the control and treatment groups would diverge as time passed and the fish grew. We were particularly interested in the rate of \( ^{15} \text{N} \) decrease for the treatment group as a function of time and fish size, so we used nonlinear least squares to fit an exponential decay function to the \( ^{15} \text{N} \) data for muscle and fin tissue versus weight, length, and time since feeding began (nls2 package in R: Grothendieck 2013; R Development Core Team 2014). Data were fit to the function:

\[
y = a + be^{-cx}.
\]

where \( y \) is \( ^{15} \text{N} \) (‰), \( a \) is the horizontal asymptote, \( b \) is the difference between the \( ^{15} \text{N} \) value of the function at \( x = 0 \) and the horizontal asymptote, \( c \) is the rate of isotopic change, and \( x \) is the weight (g), length (mm), or time (d) since exogenous feeding began (Hobson and Clark 1992).

We used the horizontal asymptote value (\( a \)) as our experimental baseline, representing the isotope signature upon which our experimental population converged. The horizontal asymptote also represents the expected \( ^{15} \text{N} \) value of the offspring of nonanadromous \( O. \text{mykiss} \), if they were reared on our marine-nutrient-depleted treatment feed. We then used the
95% prediction interval for the exponential decay models to
determine at what cutoff point (length, weight, time) the off-
spring of anadromous *O. mykiss* could no longer be distin-
guished from the offspring of nonanadromous *O. mykiss* (i.e.,
when the lower 95% prediction interval intersected with the
experimental baseline). The experimental baseline 
\[ \delta^{15}N \] value
from our study may not represent that of a wild fish diet con-
sisting of a mix of insects and other natural prey. Accordingly,
we collected nitrogen isotope signature data for nonanadro-
mous *O. mykiss* and closely related Coastal Cutthroat Trout *O.
clarkii clarkii* from the literature and from collaborators in the
region to compare our laboratory results to those from field
studies.

The exponential decay function described above was fit to
the control data to allow visual comparison of control and
treatment decay curves. The extent of correlation between tis-
sue types within treatment groups was conducted using
Pearson’s product-moment correlation.

**RESULTS**

At the end of the experiment, control fish were 56.2 ±
1.1 mm (mean ± SE) in length and weighed 2.1 ± 0.1 g,
and treatment fish were 49.9 ± 2.6 mm and 1.5 ± 0.2 g.
Control and treatment groups diverged in \[ \delta^{15}N \] for both muscle
and fin tissue over the course of the experiment. The control
group, fed a commercial diet containing marine-origin
ingredients (8.97% ± 0.12%; diet mean ± SE), experienced
a reduction in \[ \delta^{15}N \] as they grew (i.e., compared with unfed
fry from the beginning of the study) but remained more
enriched than the treatment group fed the marine-nutrient-
depleted diet (4.45% ± 0.03%; diet mean ± SE; Figure 2).
Exponential decay models for treatment muscle tissue con-
verged on the experimental baseline at a fish weight of
0.81 g, length of 50.8 mm, or 29.4 d after the onset of
feeding. For fin tissue, this convergence occurred at 0.65 g,
45.9 mm, or 35.3 d (Table 3). The distribution of nitrogen
signatures for muscle and fin tissue overlapped and values
were strongly, positively correlated [control: \( r^2 = 0.75 \), fin
\[ \delta^{15}N = 1.05 \cdot (\text{muscle } \delta^{15}N) - 1.13 \); treatment: \( r^2 = 0.85 \),
fin \[ \delta^{15}N = 0.99 \cdot (\text{muscle } \delta^{15}N) - 0.38 \)], but fin tissues

tended to be less enriched than associated muscle tissues
(Figure 2). The experimental baseline \[ \delta^{15}N \] values for the
treatment tissues in our study ranged from 6.86% to 7.71%.
These values were towards the upper end of the range of sig-
natures found in regional wild nonanadromous populations of
*O. mykiss* and *O. clarkii* (Figure 3).

**DISCUSSION**

The first goal of this experiment was to establish size and
time cutoffs that would allow field researchers to identify fish
appropriate for specific sampling goals. Researchers attempt-
ing to distinguish between offspring of anadromous and nona-
nadromous *O. mykiss* should sample fish before the specified
cutoffs, i.e. before detectable levels of marine maternal nitro-
gen signature are lost. In contrast, investigators conducting
trophic studies (e.g., to compare food habits and prey selectiv-
ity) should sample fish after the specified cutoffs to avoid sam-
ping tissues influenced by latent maternal marine nitrogen
isotope values.

The isotopic signatures of the offspring of anadromous fish
fed a marine-nutrient-depleted diet converged rapidly on the
experimental baseline after the onset of feeding. The marine
signals in muscle and fin tissue were lost at similar, but not
identical, sizes and times. The natal marine nitrogen signature
of fin tissue was undetectable in fish ≥ 0.65 g and 45.9 mm
(Table 3). This was earlier than in muscle tissue, in which the
natal marine signature became undetectable at 0.81 g and 50.8 mm (Table 3). Therefore, muscle tissue sampled from a
0.70-g, 47-mm fish would indicate marine origin, but a fin sample from the same fish would not. Muscle tissue lost its marine signature after 29.4 d, but fin tissue indicated fish were of marine origin until 35.3 d (Table 3). Fin tissue turnover occurs more rapidly than in muscle tissue, so the accuracy of our time cutoffs may be affected by high variability in $\delta^{15}$N as a function of time, or by the composite analysis of our fin tissue samples (Heady and Moore 2013). Throughout the experiment, fin $\delta^{15}$N values were below or at the low end of the range of muscle $\delta^{15}$N values, which is consistent with other findings for salmonids (Sanderson et al. 2009). Due to the high variability in $\delta^{15}$N in muscle and fin tissues on any given day, and because we cannot know how long a wild fish has been feeding, we recommend using weight and/or length measurements as more dependable cutoffs for identifying potential fish to sample.

The second goal of this study was to determine whether fish smaller than the cutoff values could provide a nonlethal fin clip. According to Sanderson et al. (2009), a minimum of 42% of caudal fin tissue must be removed from small fish (<50 mm FL) to provide sufficient tissue for stable isotope analysis. This is likely a lethal injury and is not recommended.

### TABLE 3. Exponential decay equations and cutoff values for treatment models and tissue types in *O. mykiss*. $\delta^{15}$N represents the ratio of heavy to light nitrogen isotopes in a tissue. Exponential decay equations and cutoff values are as defined in the methods.

<table>
<thead>
<tr>
<th>Model</th>
<th>Model equation</th>
<th>Cutoff value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Muscle tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\delta^{15}$N versus weight</td>
<td>$y = 7.63 \times 10^{-2.96x} + 7.71$</td>
<td>0.81 g</td>
</tr>
<tr>
<td>$\delta^{15}$N versus length</td>
<td>$y = 47.36 \times 10^{-0.08x} + 7.02$</td>
<td>50.8 mm</td>
</tr>
<tr>
<td>$\delta^{15}$N versus time</td>
<td>$y = 5.29 \times 10^{-0.04x} + 7.43$</td>
<td>29.4 d</td>
</tr>
<tr>
<td><strong>Fin tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\delta^{15}$N versus weight</td>
<td>$y = 9.22 \times 10^{-3.80x} + 7.46$</td>
<td>0.65 g</td>
</tr>
<tr>
<td>$\delta^{15}$N versus length</td>
<td>$y = 93.81 \times 10^{-0.10x} + 6.90$</td>
<td>45.9 mm</td>
</tr>
<tr>
<td>$\delta^{15}$N versus time</td>
<td>$y = 5.97 \times 10^{-0.04x} + 6.86$</td>
<td>35.3 d</td>
</tr>
</tbody>
</table>
Fin $^{15}$N values converged on the experimental baseline at 45.9 mm. This was below the specified cutoff, indicating that there was no window for nonlethal sampling before maternal marine isotope signatures disappear from fin tissue. However, fish would be good candidates for trophic isotope studies once they reach nonlethal sampling size.

Many environmental factors affect the rate of convergence of $^{15}$N on the experimental baseline, and these factors will likely differ between our experiment and field conditions. The loss of maternal marine signature in the *O. mykiss* fry resulted from both metabolic tissue replacement and dilution from somatic growth, the latter process being more influential in small, rapidly growing fish (Hesslein et al. 1993; Doucett et al. 1999; Madigan et al. 2012). Our cutoff estimates for size (weight and length) are not expected to change between the laboratory and the field. Size cutoffs are mostly independent of growth rate, temperature, and fish metabolism because they are based on the amount of somatic tissue synthesized and the dilution of the maternal marine isotope signature, not the rate of synthesis or dilution. An exception to this could occur in fish with very low growth rates. The loss of maternal marine signature in these individuals will be mostly due to metabolic tissue turnover, not dilution from the addition of somatic tissue, so fish growing very slowly could equilibrate with a freshwater diet at a size smaller than our cutoff values. Variation in isotopic turnover rate and environmental factors such as temperature can influence the amount of time it takes for fry with anadromous mothers to reach our size cutoffs and adopt freshwater isotope signatures. Fish with higher metabolic rates (associated with warm water, as experienced in this study) also undergo more rapid tissue replacement and growth than conspecifics with lower metabolic rates (e.g., Zebrafish *Danio rerio*: Tarboush et al. 2006; Bluefin Tuna *Thunnus orientalis*: Madigan et al. 2012). Consequently, wild populations of *O. mykiss* in colder water and with lower rates of metabolism, growth, and tissue turnover than our experimental population would maintain their maternal nitrogen signature longer. Deviations in environmental conditions from those in our study will affect the accuracy of our estimated time cutoffs for both applications (i.e., distinguishing offspring of anadromous from nonanadromous mothers and also avoiding samples biased by maternal marine signals in trophic studies). Future studies should investigate how rates of metabolism, growth, and tissue...
turnover are affected by different feeding and temperature regimes.

The average isotopic value of food available to wild fish may be more or less enriched than our experimental diet, depending on the specific combination of insects and other prey items available in the stream, as well as environmental conditions. The baseline $\delta^{15}$N tissue values of fish on the experimental diet were higher than many values from regional wild *O. mykiss* and *O. clarkii* populations (Figure 3). The majority of isotopic turnover in young, rapidly growing fish is due to dilution of the maternal isotope signature by the addition of somatic tissue synthesized from the freshwater food source. Therefore, fish with the same growth rate should equilibrate at the same time and size regardless of their food source.

Similar studies of related salmonid species found results similar to ours. Jardine et al. (2008) used carbon and nitrogen isotopes to determine that Brook Trout *Salvelinus fontinalis* tissues maintained their maternal marine isotope signature for 1–2 months postemergence, or about 30 mm FL. Similarly, Curry (2005) found that the marine carbon signature of anadromous Brook Trout offspring was undetectable 20 d after emergence, at fork lengths < 30 mm. The equilibrium sizes reported in these studies are smaller than those we have established for *O. mykiss*. Fish size can influence the rate of isotopic turnover as smaller individuals generally turn over more rapidly than larger ones (McIntyre and Flecker 2006; Vander Zanden et al. 2015). It is possible that the Brook Trout were smaller at emergence than the *O. mykiss* in our study, resulting in more rapid turnover for the Brook Trout. Briers et al. (2013) found that it took Brown Trout *Salmo trutta* between 6 and 7 months to lose the maternal marine isotope signature. The time estimate may be longer than other predictions as a result of species-specific turnover rates (Briers et al. 2013). We recommend the use of our sampling cutoff values be restricted to *O. mykiss* because cutoffs for other species would be influenced by size- and species-specific turnover rates.

The cutoff values presented in this study will provide valuable sampling guidelines for researchers studying salmonid life history. Though nonlethal sampling is not possible in cut-off-sized fish, our guidelines can improve sampling efficiency by facilitating the identification of appropriately sized fish for different research questions. We hope that these guidelines will be used by researchers and managers studying conservation questions that require the ability to distinguish between offspring of migratory and nonmigratory fish, or those conducting trophic studies that need to know when maternal isotopic input is no longer detectable.

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