Effects of size and diet on stable hydrogen isotope values (δD) in fish: implications for tracing origins of individuals and their food sources

David X. Soto, Leonard I. Wassenaar, Keith A. Hobson, and Jordi Catalan

Abstract: Recent studies suggest that stable hydrogen isotope ratios (δD) of fish can be used to track their watershed origin and the relative contributions of source material. Both applications assume that there is no metabolic or trophic effect on tissue δD. We studied the local variation of δD values in four fish species of contrasting size and feeding habits in an isotopically homogenous reservoir (Flix, Spain). Other isotopic values ($\delta^{15}N$, $\delta^{13}C$) and trace metal content were measured as indicators of trophic and bioaccumulation patterns. In addition, isotopic values (δD, $\delta^{15}N$, $\delta^{13}C$) of other food web components were measured for comparison. Muscle δD values differed greatly among fish species: European catfish, *Silurus glanis* (–131‰); common carp, *Cyprinus carpio* (–141‰); rudd, *Scardinius erythrophthalmus* (–158‰); and roach, *Rutilus rutilus* (–163‰). The influence of fish size and trophic position affected the fish δD values. Possible mechanisms that drive δD variation among fish might be a metabolic effect and (or) the compounding effect of ambient water δD on exchangeable H at each trophic stage. Our findings showed that size and feeding habits are factors that should be controlled when tracing origins of fish or their dependence on nutrient inputs using δD measurements.

Résumé : Des études récentes indiquent que les rapports d'isotopes stables d'hydrogène (δD) des poissons peuvent servir à retracer leur bassin versant d'origine et les contributions relatives des matériaux sources. Ces deux utilisations présupposent qu'il n'y a pas d'effet métabolique ou trophique sur le δD des tissus. Nous avons étudié la variation locale des valeurs de δD chez quatre espèces de poissons de tailles et d'habitudes alimentaires très différentes dans un réservoir homogène par ses isotopes (Flix, Espagne). Nous avons déterminé les autres valeurs isotopiques ($\delta^{15}N$, $\delta^{13}C$) et les concentrations des métaux en trace comme indicateurs des patrons trophiques et des patrons de bioconcentration. Nous avons, de plus, mesuré les valeurs isotopiques (δD, $\delta^{15}N$, $\delta^{13}C$) des autres composantes du réseau alimentaire pour établir des comparaisons. Les valeurs de δD du muscle varient considérablement d'une espèce de poisson à une autre : le silure glane, *Silurus glanis* (–131 ‰), la carpe commune, *Cyprinus carpio* (–141 ‰), le rotengle, *Scardinius erythrophthalmus* (–158 ‰) et le gardon, *Rutilus rutilus* (–163 ‰). La taille du poisson et sa position trophique affectent les valeurs de δD du poisson. Les mécanismes explicatifs possibles de la variation de δD chez les poissons pourraient inclure un effet métabolique et (ou) l'effet cumulatif du δD de l'eau ambiante sur l'H échangeable à chaque stade trophique. Nos résultats démontrent qu'il faut tenir compte de la taille et des habitudes alimentaires pour retracer à l'aide de mesures de δD les origines des poissons ou leur dépendance des apports de nutriments.

[Traduit par la Rédaction]

Introduction

Stable isotopes are currently broadly applied in ecological studies. Measurements of stable isotopes of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and sulfur ($\delta^{34}S$) are used as indicators of trophic level and dietary sources in aquatic food webs (Peterson and Fry 1987; Hesslein et al. 1991; France 1995). Stable isotopes of hydrogen (δD) have been used to track animal migration (Hobson and Wassenaar 2008) and movements of humans (O'Brien and Wooller 2007; Ehleringer et al. 2008) among isotopically distinct regions. It has been suggested that δD values of fish and invertebrate tissues reflect those of ambient water and can therefore be used to infer the watershed origin of fish as they should reflect the main host stream or lake in which the tissue developed (Whitledge et al. 2006, 2007; Myers et al. 2011). The use of δD for distinguishing among allochthonous and autochthonous energy inputs to aquatic ecosystems has also been proposed (Doucett et al. 2007). Both applications assume negligible trophic effects on hydrogen isotopes. However, patterns of δD related to trophic levels are suggested by Birchall et al. (2005) and Reynard and Hedges (2008). A few controlled-diet experi-

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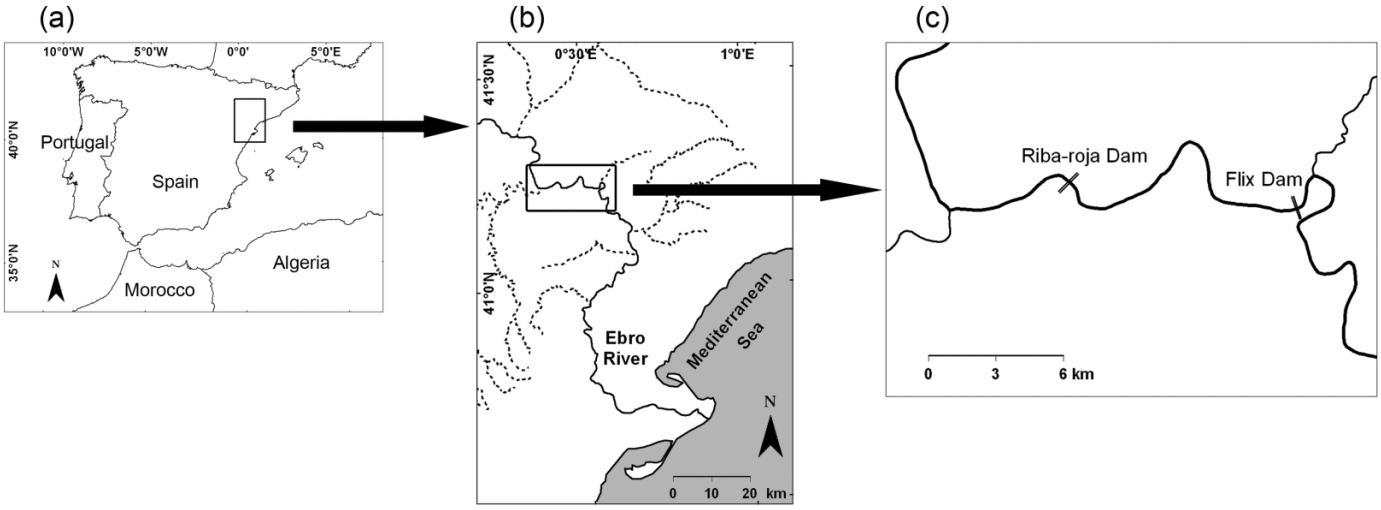
Fig. 1. (*a*) Location of the study area within the Iberian Peninsula, (*b*) location of the Flix reservoir within the lower Ebro River basin, and (*c*) a detailed map of the marked area of (*b*).



ments on terrestrial insects and aquatic organisms suggested that δD trophic enrichment does not occur (Hobson et al. 1999*b*; Solomon et al. 2009). These experimental studies suggest that tissue δD values are influenced by both food and environmental water. Further studies are required to fully understand the mechanisms and controlling factors that determine variation in δD values as these will determine to a large degree how effectively this isotope can be used to trace origins of fish and the nutrients that they use.

Part of the current uncertainty about δD measurements may be due to inconsistency among laboratory methods used for δD measurements. Some authors remove lipids prior to δD analysis (e.g., Jardine et al. 2009; this study), but others do not (e.g., Doucett et al. 2007). Lipids are greatly depleted in deuterium and, depending on lipid content, can significantly impact whole-tissue δD measurements. The deuterium content of lipids is much lower than that of proteinaceous tissues in general (60‰ lower than muscle tissue; Hobson et al. 1999*a*). Differences between non-lipid- and lipid-extracted values in fish muscle can therefore range up to 57‰ (mean, 10.5‰; $n = 84$; L.I. Wassenaar and K.A. Hobson, unpublished freshwater fish data from Lake Winnipeg, Canada). Large δD differences between lipids and water are observed in different types of organisms (Sternberg 1988; Zhang et al. 2009). Moreover, δD measurements need to be conducted using appropriate reference materials that match closely with sample matrix and the fraction of exchangeable hydrogen of the samples of interest (Chesson et al. 2009).

In the current study, we assessed the δD variation in lipid-free muscle within a single fish community in which the hydrogen isotopic composition of ambient water was relatively constant. Our aim was to evaluate to what extent local inter- and intra-specific variability could undermine the application of fish δD as a tracer of watershed origin or their food sources and, eventually, which factors should be taken into consideration to avoid misinterpretations. We investigated δD patterns in coexisting fish populations of four species of contrasting size and feeding habits: European catfish, *Silurus glanis*; common carp, *Cyprinus carpio*; roach, *Rutilus rutilus*; and rudd, *Scardinius erythrophthalmus*. To avoid the potential confounding effect of seasonal and spatial hydrogen isotopic variation in ambient river water and species movement, we performed the study in a reservoir of the Ebro River in which (*i*) the influence of environmental water δD on these fish tissues was the same for all species, (*ii*) the isotopic composition of the river water was known to be homogenous due to storage in upstream large reservoirs, and (*iii*) fish migrations were constrained by up- and down-stream dams. Thus, any differences in tissue δD values should be due to local processes related to fish diet and (or) physiology.

To complement our approach, we measured other tracers: stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) in all fish and food sources as trophic indicators. We also determined concentrations of Hg and As in fish as additional indicators of bioaccumulation and exchange related to diet and physiological constraints. Hg and As tend to show opposite behavior as bioaccumulation tracers with increasing trophic level (Cabana and Rasmussen 1994; Kuroiwa et al. 1994; Suhendrayatna et al. 2002). Relationships between Hg concentrations and size can be attributed to fish growth efficiency within a population (Trudel and Rasmussen 2006).

Our approach allowed us to identify the factors that influence δD values in aquatic consumers by controlling for potential movements and to assess the applicability of the δD approach to tracing watershed origins or food sources.

Materials and methods

Study area

The Ebro River is located in the northeastern Iberian Peninsula (southwest Europe). Our study was carried out in the Flix reservoir, located within the lower Ebro River basin (Fig. 1). The Flix reservoir is relatively small (11×10^6 m^3), has a water residence time of 0.3 days (Navarro et al. 2006), and is preceded by the Mequinenza and Riba-roja reservoirs, which have large storage capacities of 1534×10^6 and 207×10^6 m^3, respectively. The maximum depth of the Flix reservoir is <10 m; therefore, the photic zone generally extends to the bottom, resulting in high macrophyte and benthic algal production. The Riba-roja reservoir is located 13 km upstream of the Flix dam. As no fish ladders are present at either dam, fish populations in the Flix reservoir are effectively

isolated from populations above and below the reservoir, thereby preventing sampling of migrant individuals.

Available data indicate that the Ebro River has low spatial variability in water δD. During the summer of 2008, water δD values in the lower basin were –64.6‰ ± 1.2‰ (n = 4), and during the period 2002–2003, δD values throughout the Ebro River watershed were –56‰ ± 6.1‰ (n = 14) (Confederación Hidrográfica del Ebro – Centro de Estudios y Experimentación de Obras Públicas 2003). This low spatial and seasonal isotopic variation results from storage and mixing in upstream large reservoirs and is much lower than the variation observed in the reservoir fish populations in our study (see below). Generally, seasonal variation can be larger in streams where the main source of water is precipitation (e.g., reaches of first order) than in those where it is groundwater. Nielson and Bowen (2010) report a δD variability range of 15‰ from spatially and seasonal distributed values in Great Salt Lake. Data on δD water in Weser River, Germany, show a standard deviation (SD) of <3‰ and ranges of <15‰ in most locations during a 5-year sampling (Koeniger et al. 2009). Therefore, we concluded that the δD variability in Ebro River is also low.

Sampling and sample treatment

Fish sampling was carried out in July 2006, but some (n = 11) catfish were captured in February 2006. Fish were captured in the littoral zone by daylight boat electrofishing (Carol et al. 2006) and were kept alive in oxygenated tanks until processing. Each individual was measured and weighed. Muscle tissue of 20 catfish (*Silurus glanis*), 30 carp (*Cyprinus carpio*), 20 rudd (*Scardinus erythrophthalmus*), and six roach (*Rutilus rutilus*) individuals was dissected and analyzed. Potential food sources of those fish species were also collected. Three sources of autochthonous organic matter were sampled: epilithic biofilms (collected using a soft brush), submerged macrophytes (hand collected), and seston (reservoir water sampled using Niskin bottles at 2–3 m depth and filtered through glass fiber filters). Zooplankton was sampled by means of horizontal transects at a 1 m depth using a plankton net with 200 μm mesh; small macroinvertebrates (i.e., Odonata, Gasteropoda, Coleoptera, etc.) were sampled by disturbing the littoral zone over common reed (*Phragmites australis*) and submerged macrophytes; zebra mussels (*Dreissena polymorpha*) were removed from stones or rizhomes of common reed; crayfish (*Procamburus clarkii*) were sampled using an elastic net overnight; and small fish (<70 mm) of two different species (*Scardinius erythrophthalmus*, n = 12; *Gambusia holbrooki*, n = 20) were sampled by electrofishing and processed whole, in contrast to large fish, which were muscle-subsampled. All samples were frozen at –20 °C, freeze-dried, and then homogenized.

Stable isotope analyses

For $\delta^{15}N$ and $\delta^{13}C$ analyses, samples were weighed into tin capsules and analysed on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope-ratio mass spectrometer using standard techniques. Samples for δD measurements were lipid-extracted by soaking and multiple rinses in a solution of 2:1 (*v*/*v*) chloroform–methanol and weighed into silver capsules. We used the comparative equilibration method to determine the nonexchangeable hydrogen isotopic composition (Wassenaar and Hobson 2000, 2003) by using three laboratory fish muscle standards (Florida garr (FLG), lake trout (LAT), and Atlantic salmon (ATS)) that were previously calibrated offline for their nonexchangeable δD values (fraction of H exchangeable for fish muscle is 18.5% ± 2.1%; L.I. Wassenaar, Environment Canada, unpublished data). Measurements of δD of samples and standards were performed on H_2 derived from 1350 °C flash pyrolysis and continuous-flow isotope-ratio mass spectrometry (CF-IRMS). Pure H_2 was used as the sample analysis gas and the isotopic reference gas. A Hekatech high-temperature oxygen (HTO) analyzer was used to automatically pyrolyse muscle samples to a single pulse of H_2 gas (and N_2 and CO gas). The resolved H_2 sample pulse was then introduced to the isotope-ratio mass spectrometer (Micromass Isoprime (Manchester, England) with electrostatic analyzer) via an open-split capillary (Wassenaar and Hobson 2006). Water isotope samples were analyzed using an OA-ICOS water isotope analyzer (Los Gatos model 908-0008) coupled to a CTC LC-PAL liquid autosampler for D/H ratios measurements of H_2O (Lis et al. 2008).

All isotopic results are expressed in the typical delta notation, in units of per mil (‰). Nitrogen and carbon isotopic analyses were reported relative to Air and PDB standards, respectively. Hydrogen isotopic analyses were performed at Environment Canada in Saskatoon (Saskatchewan, Canada) and normalized to Vienna Standard Mean Ocean Water (VSMOW). Repeated analyses of isotope laboratory standards yielded an analytical precision better than ±0.2‰ for nitrogen, ±0.3‰ for carbon, and ±2.0‰ and ±0.3‰ (Lis et al. 2008) for hydrogen in tissue and water samples, respectively.

Trace metal analyses

Each fish muscle sample (~70 mg) was digested in 2 mL concentrated HNO_3 (65% Merck Suprapur) and 1 mL H_2O_2 (30% Merck Suprapur) in closed Teflon vessels for two periods of 16 h at 95 °C. After digestion, samples were diluted with deionized water (30 mL) for trace element analysis. A triplicate of blanks was run with each batch of samples.

Analyses of Hg and As were determined by inductively coupled plasma mass-spectrometry (ICP-MS). Determination of metals was performed with a Perkin-Elmer Elan 6000 ICP-MS. Certified ICP standards were used in the calibration of standard curves. Analytical accuracy was checked by a standard reference material (DORM-2, National Research Council of Canada). Accuracy (n = 18) was ±10% for Hg and As. The trace metal concentrations were expressed in dry mass.

Numerical analyses

Trophic position of food web components were estimated by the equation below (Post 2002). We adjusted the estimation to the isotopic baseline of primary consumers using zebra mussels as the pelagic food web reference and snails as the littoral zone reference. We assumed no trophic fractionation in carbon isotopes.

$$\text{TP} = 2 + \frac{\delta^{15}N_{\text{consumer}} - [\delta^{15}N_{\text{snail}}\,\alpha + \delta^{15}N_{\text{mussel}}\,(1-\alpha)]}{\Delta\delta^{15}N} \quad (1)$$

where TP is trophic position, $\delta^{15}N_{\text{consumer}}$ is the $\delta^{15}N$ value of the consumer, $\delta^{15}N_{\text{snail}}$ and $\delta^{15}N_{\text{mussel}}$ are the mean $\delta^{15}N$ va-

lues of snails and zebra mussels, respectively, $\Delta\delta^{15}N$ is the mean trophic discrimination factor for $\delta^{15}N$ (4.8‰ for rudd, the herbivorous fish species (Mill et al. 2007), and 3.4‰ for the rest of aquatic consumers (Post 2002)), and α is the proportion of nutrients in the consumer derived from the base of littoral food web that is estimated according to

$$(2) \qquad \alpha = (\delta^{13}C_{consumer} - \delta^{13}C_{mussel})/(\delta^{13}C_{snail} - \delta^{13}C_{mussel})$$

where $\delta^{13}C_{consumer}$ is the $\delta^{13}C$ value of the consumer and $\delta^{13}C_{mussel}$ and $\delta^{13}C_{snail}$ are the mean $\delta^{13}C$ values of snails and zebra mussels, respectively. For TP calculations, the values of $\delta^{13}C_{consumer}$ were normalized for the effects of lipids on carbon isotopes using eq. 3 of table 1 of Post et al. (2007) for aquatic animals.

Fish length and the trace metal concentrations were $\log_{10}$-transformed in all statistical analyses. Relationships between δD and the other measured variables were examined by Pearson's correlation coefficients. General linear models (GLM) were used to determine which factors influenced fish δD values. We modeled size and trophic effects on δD values and Hg and As concentrations for fish to determine the factors driving that δD variability. We selected the models using the Akaike information criterion (AIC), and we usually removed nonsignificant variables ($p > 0.05$). We studied the effect of fish length and trophic information (α, TP, and their interaction). Then, because colinearity between length and trophic effects occurs, the residuals of the model between length and trophic information were used to show the effect of size excluding trophic effects.

Results

Patterns of δD, $\delta^{15}N$, and $\delta^{13}C$ values throughout the food web, fish, and their potential food sources differed markedly (Fig. 2). The range of δD values for all fish muscle spanned 49‰ (–169‰ to –118‰). A comparison of the four fish species revealed a decreasing order for δD values as follows: catfish (–131‰ ± 8‰, mean ± SD), carp (–141‰ ± 5‰), rudd (–158‰ ± 8‰), and roach (–163‰ ± 5‰). The δD values differed among species, except between rudd and roach (analysis of variance (ANOVA), $F = 70.28$; Bonferroni's test, $p < 0.01$). A large range of δD values (ca. –200‰ to –100‰) was found for the main components of the food web. Among the potential food items for fish, macrophytes and zebra mussel had high δD values, and zooplankton and biofilm had low δD values (Fig. 2). Although $\delta^{15}N$ and $\delta^{13}C$ values showed a large overlap among the four fish species, some differences were significant. Carp had lower $\delta^{15}N$ and $\delta^{13}C$ values than catfish and rudd, and roach had lower $\delta^{13}C$ values than catfish (ANOVA, Bonferroni's test, $p < 0.05$). Our $\delta^{15}N$ and $\delta^{13}C$ measurements were not useful in separating isotopically among species, and we were unable to model fish diet using conventional C and N stable isotope mixing models (e.g., Phillips and Gregg 2003).

There was a high correlation between the δD values of fish and fish length (Table 1; Fig. 3*a*) and Hg concentrations (Table 1; Fig. 3*d*). In addition, trophic indicators (α and TP) were correlated to Hg concentrations positively and to As concentrations negatively (Table 1). Of note was the high correlation between fish length and Hg concentrations.

We statistically modeled the variability in fish δD values to assess the influences of size and trophic effects. To avoid negative values, instead of α, the variable ($\alpha + 1$) was used in the models as a trophic tracer indicating the littoral origin of food sources (see Fig. 3*c*). Firstly, we found a high variation of fish length explained by trophic indicators (ca. 20%; model 1 in Table 2). Therefore, this collinearity was removed using the residuals of this relationship.

Fig. 2. Box plots of the values of (*a*) δD, (*b*) $\delta^{15}N$, and (*c*) $\delta^{13}C$ for each fish species and their potential food sources from the Flix reservoir. The box plots depict the minimum and maximum values (whiskers), the lower and upper quartiles (25th and 75th percentiles), and the median (central line inside the box). Values more than 1.5 IQRs (interquartile range) are labelled as outliers (solid circles with a line). Dotted lines represent the location of each *x*-axis label to help the visualization of the boxes. Solid triangles depict the values of (*a*), (*b*), and (*c*) for macrophyte, biofilm, and seston samples. Macroinv., macroinvertebrates; Z. mussel, zebra mussel; Zoopl., zooplankton; Macroph., macrophytes.

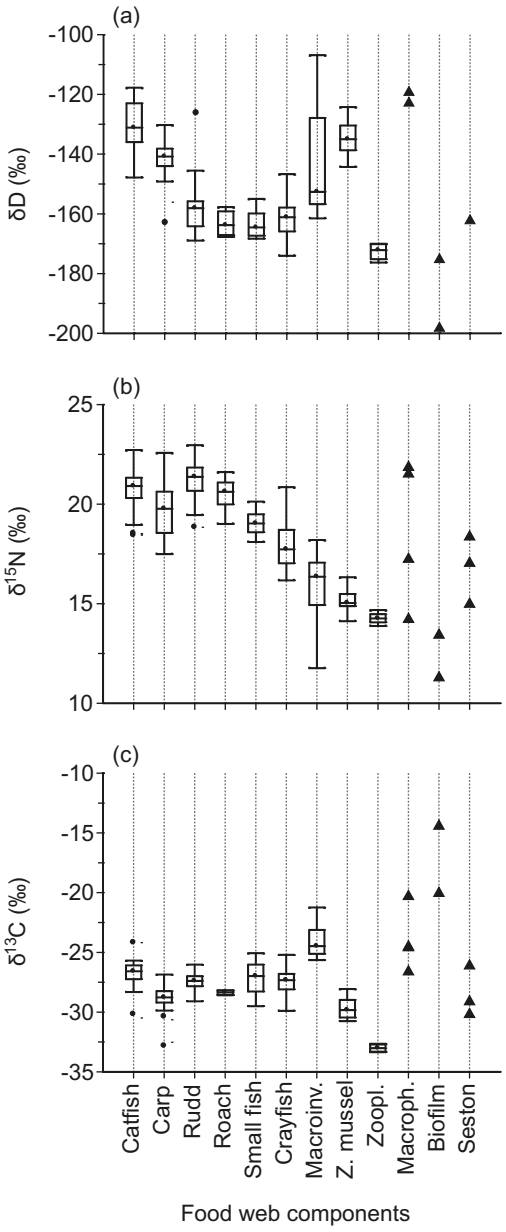
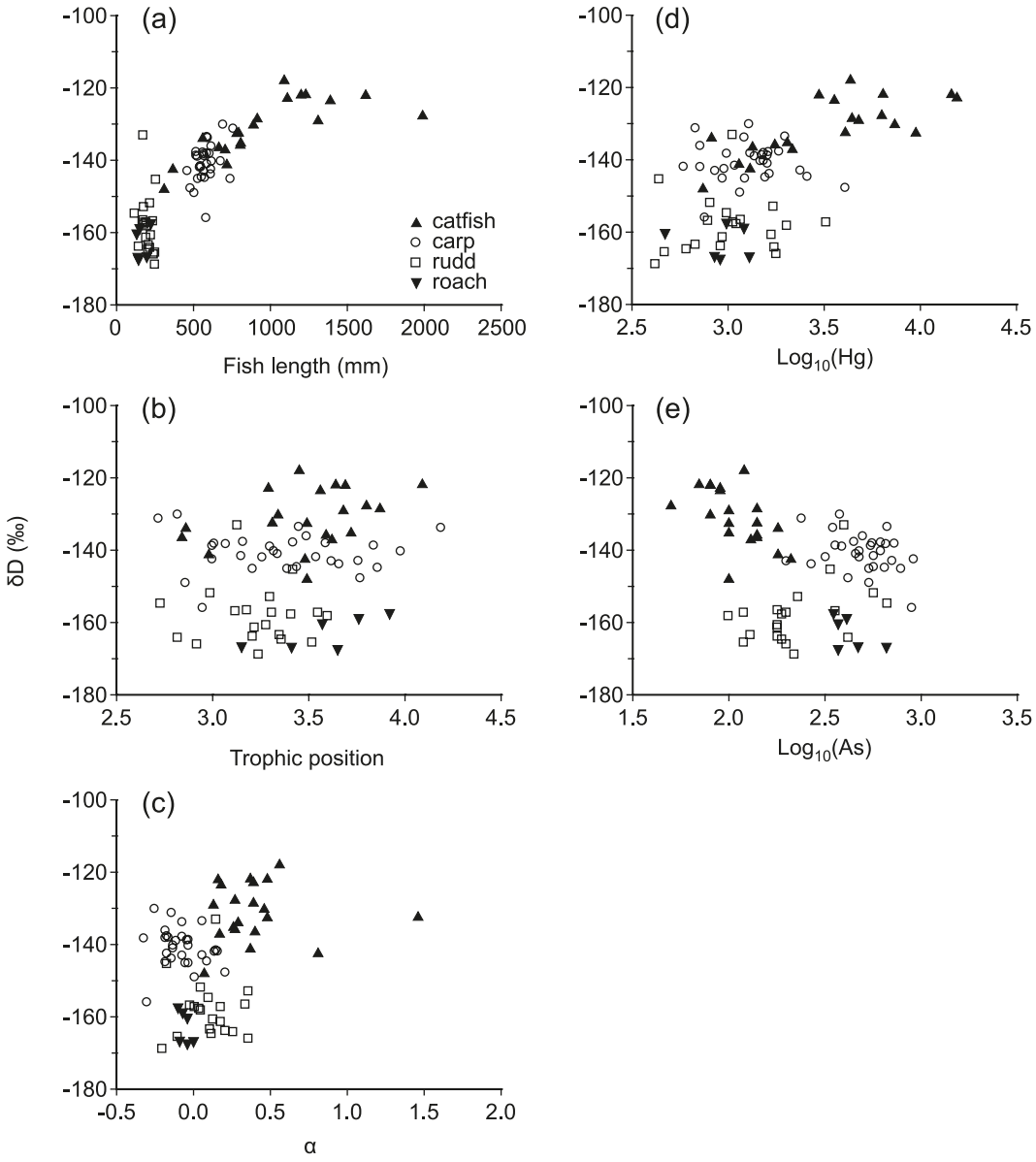


Table 1. Correlation matrix of δD values and their potential predictors (length, α, TP, Hg, and As) for the fish from the Flix reservoir ($N = 76$).

	δD	Log_{10}(length)	α	TP	Log_{10}(Hg)
Log_{10}(length)	0.88				
α	0.27	0.19			
TP	0.20	0.21	0.04		
Log_{10}(Hg)	0.60	0.58	0.53	0.31	
Log_{10}(As)	–0.26	–0.26	–0.57	–0.28	–0.49

Note: TP, trophic position; α, the proportion of nutrients in the consumer derived from the base of littoral food web.

Fig. 3. Relationship between δD values of fish from the Flix reservoir and potential predictor variables: (*a*) fish length, (*b*) trophic position (TP), (*c*) α, (*d*) $\log_{10}$(Hg), and (*e*) $\log_{10}$(As).



An influence of both trophic position and size effects on fish δD values occurred (models 2–4 in Table 2). Trace metal concentrations (Hg and As) were primarily related to trophic effects, and some influence of size on [Hg] was found (models 5–10 in Table 2). Fish δD values and [Hg] were influenced positively by fish size and the interaction between trophic position (TP) and the variable ($\alpha + 1$) and were negatively influenced by the parameters TP and ($\alpha + 1$). In contrast, [As] was only influenced by the interaction between TP and ($\alpha + 1$).

Table 2. Modeling of size and trophic position effects on δD values and Hg and As concentrations for fish to determine the factors driving the δD variability.

	Effect	Linear model	R^2/adjusted R^2	AIC
Collinearity	TP on length	1. $\text{Log}_{10}(\text{length}) \sim -\text{TP} - (\alpha + 1) + \text{TP}:(\alpha + 1)$	0.20/0.17	
δD	Length	2. $\delta\text{D} \sim +\log_{10}(\text{length})$	0.77/0.77	501.98
	TP	3. $\delta\text{D} \sim -\text{TP} - (\alpha + 1) + \text{TP}:(\alpha + 1)$	0.22/0.19	599.08
	Size + TP	4. $\delta\text{D} \sim +\text{resid(model 1)} - \text{TP} - (\alpha + 1) + \text{TP}:(\alpha + 1)$	0.79/0.77	503.23
Log_{10}(Hg)	Length	5. $\text{Log}_{10}(\text{Hg}) \sim +\log_{10}(\text{length})$	0.33/0.32	23.27
	TP	6. $\text{Log}_{10}(\text{Hg}) \sim -(\alpha + 1) - \text{TP} + \text{TP}:(\alpha + 1)$	0.43/0.41	14.91
	Size + TP	7. $\text{Log}_{10}(\text{Hg}) \sim +\text{resid(model 1)} - \text{TP} - (\alpha + 1) + \text{TP}:(\alpha + 1)$	0.56/0.54	–2.94
Log_{10}(As)	Length	8. $\text{Log}_{10}(\text{As}) \sim -\log_{10}(\text{length})$	0.07/0.05	43.46
	TP	9. $\text{Log}_{10}(\text{As}) \sim -\text{TP}:(\alpha + 1)$	0.39/0.38	10.86
	Size + TP	10. $\text{Log}_{10}(\text{As}) \sim -\text{resid(model 1)}^a - \text{TP}:(\alpha + 1)$	0.40/0.38	12.38

Note: The studied predictor variables are $\log_{10}$(length), trophic position (TP), $(\alpha + 1)$, and the interaction between trophic position and % littoral origin (TP:$(\alpha + 1)$). Before each predictor variable, the symbol of that influence (+ or –) is depicted. In each case, best models were selected using the AIC values as a measure of the relative goodness of fit of the statistical model (lower AIC values) and removing nonsignificant variables ($p > 0.05$). The coefficients of determination (R^2) and the adjusted value for the number of explanatory terms (adjusted R^2) in each model are shown as the proportion of variability that is accounted for by the statistical model. Because of the collinearity between size and trophic effects, the residuals (resid) of their relationship (model 1) were used to show the effect of size removing the explained variation by trophic effects in next models.

[a]Nonsignificant parameter in the best model.

Discussion

Our results showed that δD in lipid-extracted fish muscle tissue varied considerably among species and within populations in a single location, even when there was low isotopic variation in ambient water. This result warns against uncritically using δD for tracing watershed origins of organisms. For instance, using the regression model of Whitledge et al. (2006), linking fish tissue δD with water δD, the range of tissue values that we found would mistakenly correspond to a range in host water δD of nearly 100‰. Therefore, unless the factors that control tissue δD values, beyond ambient water, are taken into account, serious errors in assignment of fish to watershed origin may result. When tracing watershed origin of organisms, a size and trophic effect should be controlled, for example, by using fish of similar size and feeding habits. Moreover, studies using δD as a tracer of food sources or terrestrial inputs that do not take into account the influence of size and trophic position (TP), as well as the environmental water contribution to tissue H, should be avoided. A good example of the cautious use of δD as a tracer was provided by Cole et al. (2011), who assessed the terrestrial subsidies to pelagic consumers. They used zooplankton taxa from a similar TP that fed on some mixture of algal and terrestrial resources (treated as primary consumers) and a dietary water influence of 15%.

In our study, possible mechanisms that may have caused variation in fish tissue δD include (*i*) trophic deuterium discrimination in protein H, (*ii*) a metabolic effect related to size, and (*iii*) a compounding effect of isotope exchange with ambient water δD at each trophic level (Solomon et al. 2009). We considered each of these mechanisms in more detail below.

Trophic discrimination

Trophic discrimination in δD similar to δ^{15}N did not occur as δ^{15}N values were not related with δD values. Controlled experimental tests of δD isotopic discrimination under laboratory conditions have been made for monarch butterflies (*Danaus plexippus*; Hobson et al. 1999*b*) and aquatic consumers (Solomon et al. 2009). In both cases, δD showed negligible trophic enrichment. Trophic deuterium enrichment in carnivores is suggested by Birchall et al. (2005) and Reynard and Hedges (2008) based on bone collagen studies. They suggested that δD could be a trophic level indicator similar to δ^{15}N. However, in other cases, trophic level patterns are attributed to other mechanisms. Solomon et al. (2009) showed that consumers at higher trophic levels within distinct components of lake food webs such as zooplankton, zoobenthos, and fish also have higher δD values and provided an explanation based on a compounding effect of water δD at each trophic level (see below). Doucett et al. (2007) also found higher δD values in top-level predators (fish) than in lower trophic levels (macroinvertebrates) and attributed such difference to δD variation in diet items and heterogeneity among basal trophic levels.

Metabolic effect

Metabolic effects related to fish size may have contributed to the variance that we measured for fish muscle δD. An important influence of fish size on both δD values and [Hg] was observed, and [Hg] was also correlated with δD values. Tissue δD values could increase with time or age (which is allometrically related to size), similar to that observed for fish δ^{15}N (Overman and Parrish 2001). This mechanism could be due to the formation of new proteins by catabolic amino acids and the additive influence of H isotope exchange from environmental water into these amino acids. Therefore, the age of protein molecules is expected to play an important role. Catfish was the only species with a significant and positive correlation between δD and [Hg] or size, possibly because of their large size range. Whitledge et al. (2006) assessed the influence of fish length on standardized differences between mean ambient water δD and fish δD values and found no correlation. Therefore, further investigation is required to determine under which circumstances size could significantly influence differences in fish δD values.

Compounding effect

The compounding effect of ambient water δD with trophic level is a mechanism that may show an apparent trophic ef-

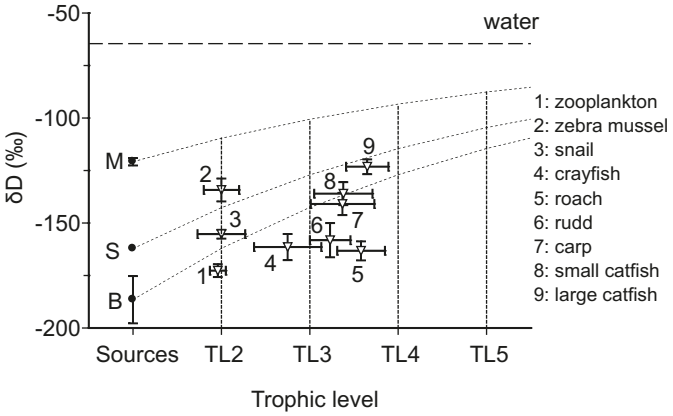
fect related to real dietary trophic position and food source. At each trophic level, there is an opportunity for the additive influence of H isotope exchange between consumer tissue and ambient water in addition to that which has already occurred in the diet (Solomon et al. 2009). Previous studies show that the contribution of ambient water H to consumer tissue in the form of exchangeable H varies widely among organisms and tissue type (Hobson et al. 1999*a*; Solomon et al. 2009; Wang et al. 2009). Hydrogen isotopes in fish body water are highly correlated with environmental water, and H transfer to free amino acids ultimately incorporates the ambient water isotopic influence into fish protein (Gasier et al. 2009). Thus, Solomon et al. (2009) suggest that trophic compounding, or accumulation of the effect of environmental water H along trophic levels, can also contribute to consumer tissue in aquatic food chains. They propose a model incorporating the effect of H isotopic exchange between water and ingested food at each trophic level. Their model predicts, assuming 20% exchangeable H in all tissues and prey items, that H in consumer fish tissues includes the 20% of H obtained from ambient water plus the 20% that their prey obtained from water, and so on. If this proposed model proves valid, fish δD should be correlated with the total contribution of environmental water to tissue H, which, in turn, depends on the trophic level of a consumer and the δD value of the ambient water and food source.

The compounding effect may be another possible mechanism contributing to the variation in fish δD that we observed along with the size effects that we described. We did not find a direct correlation between δD values and trophic position or α. However, the combination of TP and α (plus their interaction) had an influence on fish δD values. The relationship between δD values and trace metals (Hg, positively, and As, negatively) also indicated some trophic and size effects, as these trace metals can be biomagnified and biodiminished along the food chain (Cabana and Rasmussen 1994; Chen and Folt 2000).

To investigate the potential influence of the compounding effect, we modeled the theoretical δD values expected in linear food chains using our measured sources of primary production in the Flix reservoir (macrophytes, seston, and biofilm). We assumed that each consumer ate only from the previous lower trophic level and that the influence of environmental water to tissue H was constant (20%) for all consumers (Fig. 4). The theoretical values of δD for consumer tissues in each linear food chain (i.e., those based entirely on either biofilm, seston, or macrophytes) were calculated using eq. 3 of Solomon et al. (2009) and the water δD value of the reservoir. We placed our measured δD values of consumers using estimates of trophic position on this modeled depiction (Fig. 4). We separated large catfish (>100 cm), which feed mainly on crayfish and fish and so had the highest trophic position, from small catfish (<100 cm), which feed on crayfish, other invertebrates, and plant material (Carol et al. 2009).

The position of the two size classes of catfish (Fig. 4) corresponded well with their assumed diet. Among fish, we expected catfish to occupy the highest trophic position and rudd the lowest. However, our calculated trophic position of rudd was higher than expected. We suspect that this result was driven by consumption of macrophytes by rudd because

Fig. 4. Assessment of H compounding effect as a potential mechanism explaining δD variation: the δD values of tissue may vary depending on the trophic level of the consumer (TL) and the source of energy (Sources). Assuming a linear food chain in which each consumer eats only from the previous lower trophic level and that the compounding influence of environmental water is constant (20%), estimates of δD trophic trajectories starting at three dietary sources (macrophytes, M; seston, S; and biofilm, B) were calculated. Based on measured δD and trophic position estimates from $\delta^{15}N$ and $\delta^{13}C$, positions in the diagram of fish and other important dietary components are plotted. Sources and food web components are represented in the plot by solid circles and open inverted triangles, respectively. Reservoir water is represented by a broken horizontal line.



their diet is mainly based on detritus and plant material (García-Berthou and Moreno-Amich 2000). Macrophytes had relatively high $\delta^{15}N$ values in the reservoir and lower diet protein quality than other food items (i.e., fish), and our estimates of trophic position for rudd could therefore be equivocal (Fig. 4). Similarly, for carp and roach, we expected that they would occupy similar positions in Fig. 4 as they have similar feeding habits, but they had different δD values. Carp is a benthivorous species and likely fed on zebra mussel, macroinvertebrates, and biofilm, whereas roach likely fed more on zooplankton (García-Berthou 1999, 2001). Therefore, the different δD values between them were likely due to their size differences.

Our statistical model of the trophic influence on δD values was consistent with the theoretical δD modeling using trophic compounding (Fig. 4). The values of α were negatively related to δD values, and we found that the littoral primary consumers had more negative values than the more pelagic zebra mussel (Fig. 4). The interaction between trophic position and α was positively related to δD values, and the values of this interaction increased from the bottom left to the top right of Fig. 4. In contrast, trophic position had a negative influence in the model. Regarding the compounding effect isotope model shown (Fig. 4), the influence of water incorporation at each trophic step caused the overall trophic chain to move towards the isotopic composition of the ambient water, but the slope of the hydrogen isotopic enrichment at each trophic level diminished accordingly.

In conclusion, our data showed that variation of fish δD values, even within a single well-mixed reservoir, can be considerable, and such isotopic variation needs to be considered in studies using δD to infer watershed origins of fish and food sources. We concluded that the mechanisms that likely

drive this variation were related to size and compounding effects of ambient water δD. Therefore, fish size and their diet should be taken into account to compare populations from different locations when tracing watershed origins of fish using δD measurements. The use of δD measurements may eventually complement the more traditional use of $\delta^{13}C$ and $\delta^{15}N$ for evaluating trophic and food web structure (Jardine et al. 2009; Solomon et al. 2009; Finlay et al. 2010), but the fundamental mechanisms driving the local hydrogen isotopic variation in food webs and the uncertainties related to lipid content of each food web component remains poorly understood. More controlled experimental studies deciphering and quantifying the mechanisms and effects are needed before a more general application of δD measurements in ecological aquatic studies can be undertaken.

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