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PRIMARY RESEARCH PAPER

Development of non-lethal monitoring of stable isotopes in asp (*Leuciscus aspius*): a comparison of muscle, fin and scale tissues

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Abstract We explored whether fin clips and scales can be used as potential non-lethal alternatives to muscle tissue for examining the isotopic composition of asp *Leuciscus aspius*, a locally threatened freshwater species. Dorsal fin clips, scales and muscle plugs were collected from two asp populations and subsequently analysed for nitrogen and carbon stable isotopes. Both fins and scales were consistently depleted in ^{15}N and enriched in ^{13}C relative to muscle. A linear regression found that the isotope values in asp fins and scales were significantly related to those in the muscle tissue. These results indicate that fins and scales have the potential to be a substitute for muscle in stable isotope studies of asp, thus providing a non-destructive sampling method for this species. Nevertheless, to determine reliable conversion factors between tissues, a subset of individuals covering a sufficiently wide range of body sizes may need to be sacrificed for any given population.

Keywords Fin clips · Fish scales · Fractionation · Non-lethal sampling · Stable isotopes · Threatened species

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Introduction

Naturally occurring stable isotopes, especially those of carbon and nitrogen, are now routinely used in studies of aquatic food webs. The most common applications of stable carbon and nitrogen isotope measurements are to determine trophic interactions among organisms (Clarke et al., 2005; McIntyre et al., 2006; Hayden et al., 2014), to quantify energy flow through ecological communities (Vander Zanden et al., 1999; Finlay et al., 2002; Karlsson & Byström, 2005), to trace nutrient pollution (Schlacher et al., 2005; Anderson & Cabana, 2006; Xu & Zhang, 2012) and to predict contaminant bioaccumulation (Cabana & Rasmussen, 1994; Kidd et al., 2001). For fish, dorsal white muscle has been the tissue traditionally used in stable isotope analyses (Pinnegar & Polunin, 1999). However, sampling of muscle tissue is usually destructive and requires killing the fish. Lethal research sampling causes ethical issues and should be avoided, especially in cases of endangered and locally rare species or populations of exceptional value. Hence, to eliminate

unnecessary mortality of sampled animals, the use of non-lethal methods in stable isotope studies of fish has recently received much attention (Kelly et al., 2006; Church et al., 2009; Willis et al., 2013). Particularly, fin clips and scales are the tissues that can be relatively easily obtained by non-lethal sampling, and several studies have shown that isotopic signals of fins and scales were correlated with those of muscle (e.g., Sanderson et al., 2009; Hanisch et al., 2010; Jardine et al., 2011; Fincel et al., 2012; Tronquart et al., 2012). It has also been demonstrated that isotopic offsets between tissues may differ, not only among species but also even among populations and life-history stages of the same species (Kelly et al., 2006; Sinnatamby et al., 2008; Blanco et al., 2009; Graham et al., 2013). However, despite the increasing interest in using non-destructive methods, information on the relationship between isotope signatures of muscle and those of non-lethally sampled tissues is still lacking for many species and populations of concern. To help fill this gap, the present study explored the use of fin clips and scales as a tool for examining isotopic composition of asp *Leuciscus aspius* (Linnaeus, 1758), a rarely studied species from the family Cyprinidae.

Asp is a specialist piscivore, inhabiting large–medium-sized lowland rivers and large lakes in Eurasia (Kottelat & Freyhof, 2007). The IUCN Red List conservation status of this species is Least Concern (Freyhof & Kottelat, 2008). Nevertheless, asp is included among species protected under the EU Habitats Directive (Natura 2000 network), as it is locally threatened, particularly by alteration of river morphology. In the Czech Republic, asp is not only relatively abundant in many rivers and reservoirs, partially due to artificial enhancement through stocking, but also because it thrives well under the prevalent eutrophic conditions (Vašek et al., 2013). Asp is valued both as a game fish and as a species used to induce top-down control on lower trophic levels (Donabaum et al., 1999; Vašek et al., 2013).

The aim of this study was to examine whether fins and scales can be used as non-lethal alternatives to muscle tissue in carbon and nitrogen stable isotope analyses of asp. The specific objectives were to (1) explore how fins and scales compare with muscle tissue in their isotope signatures, (2) develop appropriate correction factors allowing reciprocal use of different tissues in future stable isotope studies, (3) explore whether differences in isotope signatures between tissues vary with asp length, and (4) determine whether the isotopic composition differs between asp males and females.

Materials and methods

Study area

Asp were collected from Želivka Reservoir (dam coordinates: 49°43′31″N, 15°05′21″E) and Římov Reservoir (dam coordinates: 48°51′00″N, 14°29′28″E), in the Czech Republic. Both reservoirs are of canyon-type character, having elongated morphology and pronounced internal gradients of depth and productivity (Vašek et al., 2016). Želivka Reservoir has a surface area of 1430 ha, length of 31 km and maximum depth of 54 m. Římov Reservoir has a surface area of 210 ha, length of 10 km and maximum depth of 43 m. Both reservoirs contain fish communities dominated by cyprinids [bream *Abramis brama* (Linnaeus, 1758), roach *Rutilus rutilus* (Linnaeus, 1758), and bleak *Alburnus alburnus* (Linnaeus, 1758)] accompanied by perch *Perca fluviatilis* Linnaeus, 1758 and ruffe *Gymnocephalus cernua* (Linnaeus, 1758) (Vašek et al., 2016). Želivka Reservoir hosts a viable, wild population of asp (Vejřík et al., 2014) and is therefore protected as a Natura 2000 site (site code CZ0214016). Each year, a portion of this wild population is utilised as brood stock in a local hatchery, which produces pond-reared, 1-year-old asp used for enhancing the populations in other reservoirs. Římov Reservoir has also a naturally reproducing asp population (Blabolil et al., 2016) which is, however, more or less regularly supported by stocking pond-reared fingerlings originating from the wild Želivka's brood stock.

Sample collection and preparation

Mature asp [aged ≥5+, standard length (SL) range 410–650 mm] of the Želivka Reservoir were collected in April 2013, shortly after they entered their spawning ground at the major tributary. Twenty one individuals (19 males and 2 females), captured with a boat mounted electro-fishing unit, were sacrificed to obtain samples for stable isotope analysis. A total of 27 mostly juvenile and subadult asp (aged between 1+ and 4+, SL range 158–370 mm) were collected in August 2014 at different sites of the Římov Reservoir

using gillnets and shore seine nets. All these asp were euthanised, measured and weighed. Otoliths were dissected and used to age the fish. White dorsal muscle tissue, a dorsal fin clip (a tip of the dorsal fin) and 5–10 scales from above the lateral line were collected from each asp. All tissues were kept frozen (−20°C).

Prior to the stable isotope analysis, the muscle and fin samples were oven dried (60°C for 48 h) and pulverised in a mixer mill (Retsch MM 200). Whole fin clips were homogenised, which means that these samples contained all fin tissue elements, including bony rays. Scale samples were submersed in distilled water and gently cleaned from epidermal skin, mucus and adhered material with a scalpel under a binocular microscope. To correct for the potential influence of inorganic carbonates, the scale samples were acidified for 2 min in 1.2 M hydrochloric acid, rinsed five times in distilled water and oven dried (Perga & Gerdeaux, 2003). Only the outermost area of the scale, representing growth of fish during the last 1–2 years, was cut off and analysed. Two–five scales from each individual usually provided a sufficient amount of sample material for stable isotope analysis ($\sim$1 mg).

To maximally protect the wild asp population in the Želivka Reservoir, males were primarily sacrificed to obtain muscle samples for stable isotope analysis. As a consequence of this, only fin samples were used to compare the isotope composition between males and females. For this purpose, dorsal fin clips were taken from another 13 females (SL range 510–650 mm) captured on the spawning ground at the major tributary of the Želivka Reservoir in April 2013. After measuring, weighing and fin clipping, these females were released alive. The fin samples were stored and prepared in the same way as described above.

Stable isotope analysis

Stable isotope ratios of carbon and nitrogen and the elemental composition of all samples were determined at the Iso-Analytical Limited (Crewe, Cheshire, UK) using a Europa Scientific 20–20 isotope ratio mass spectrometer coupled with an elemental analyser. Vienna Pee Dee Belemnite and atmospheric N_2 were used as the international standards for carbon and nitrogen, respectively. The working standard for our samples was NBS-1577B (powdered bovine liver, $\delta^{13}C_{V\text{-}PDB} = -21.60‰$, $\delta^{15}N_{Air} = 7.65‰$). NBS-1557B was calibrated in-house as a secondary reference material and is directly traceable to IAEA-CH-6 (sucrose, $\delta^{13}C_{V\text{-}PDB} = -10.43‰$) and IAEA-N-1 (ammonium sulphate, $\delta^{15}N_{Air} = 0.40‰$). Isotope ratios of the tissue samples were expressed in conventional delta notation ($\delta^{15}N$, $\delta^{13}C$) as parts per thousand (‰) differences from the international standard. The analytical error, estimated from replicated runs of the reference material, was less than 0.1 and 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. Data were not corrected for lipids since elemental carbon–nitrogen (C:N) ratios of all tissues were <4, indicating a low lipid content (Hoffman et al., 2015).

Data analysis

Paired *t*-tests were run to determine whether fin and scale isotope values differ from those of muscle. Mean C:N ratios were compared among the three tissue types using a one-way repeated measures analysis of variance (ANOVA) with multiple comparisons (Tukey test). The isotopic offsets between tissues, defined as the differences between fin and muscle or scale and muscle isotope values, were compared between Želivka and Římov asp with an independent two-sample *t*-test. Relationships between isotope values of fins or scales and those of muscle tissue were examined by linear regression analysis. Linear regressions were also applied to test if the isotopic offsets between tissues were influenced by asp body size. Male and female Želivka asp were compared for body size and mass using an independent two-sample *t*-test. Analysis of covariance (ANCOVA), with sex as the fixed factor and fish SL as the covariate, was performed to examine whether fin clips of male and female Želivka asp differ in nitrogen and carbon stable isotope values and C:N ratios. All statistical tests were carried out with STATISTICA 9.1 (StatSoft, Inc., Tulsa, OK, USA, 2010).

Results

The $\delta^{15}N$ and $\delta^{13}C$ isotope values in fins and scales of asp were significantly different from those in their muscle tissue (Table 1). Both fins and scales were depleted in ^{15}N and enriched in ^{13}C relative to the muscle, but the magnitude of these differences was generally greater in scales compared with fins (Table 1). The Želivka and Římov asp did not differ

Table 1 Mean ± SD values of $\delta^{15}N$, $\delta^{13}C$ and the C:N ratios measured in muscle, fins and scales of Želivka and Římov asp

Population	Tissues	$\delta^{15}N$ (‰)	$\delta^{15}N$ offset (‰)	$\delta^{13}C$ (‰)	$\delta^{13}C$ offset (‰)	C:N
Želivka asp ($n = 21$)	Muscle	16.88 ± 0.45		−24.06 ± 0.58		3.39 ± 0.22^{A}
	Fin	15.97 ± 0.45	−0.91 ± 0.38***	−22.64 ± 0.63	+1.42 ± 0.39***	3.66 ± 0.16^{B}
	Scale	15.07 ± 0.31	−1.82 ± 0.41***	−20.94 ± 0.72	+3.12 ± 0.62***	2.82 ± 0.04^{C}
Římov asp ($n = 27$)	Muscle	14.06 ± 0.53		−21.45 ± 0.59		3.22 ± 0.05^{A}
	Fin	13.03 ± 0.59	−1.04 ± 0.19***	−20.08 ± 0.57	+1.37 ± 0.41***	3.74 ± 0.10^{B}
	Scale	12.58 ± 0.60	−1.48 ± 0.27***	−18.20 ± 0.49	+3.26 ± 0.37***	2.81 ± 0.05^{C}

Mean ± SD offsets are the differences in $\delta^{15}N$ and $\delta^{13}C$ between fin and muscle or scale and muscle. Asterisks indicate a significant difference from the value for muscle ($P < 0.001$, paired t-test). Distinct letters (A–C) indicate a significant difference among tissues ($P < 0.001$, ANOVA, followed by Tukey test)

in the $\delta^{15}N$ offset between fin and muscle (t-test: $t_{46} = 1.49$, $P = 0.11$) but they significantly differed in the $\delta^{15}N$ offset between scale and muscle (t-test: $t_{46} = -3.41$, $P < 0.002$). The two populations did not differ in the $\delta^{13}C$ offset between fin and muscle (t-test: $t_{46} = 0.45$, $P = 0.65$), nor scale and muscle (t-test: $t_{46} = -0.93$, $P = 0.36$). The C:N ratios of both Želivka and Římov asp significantly differed among the types of tissue, being highest in fins, intermediate in muscle and lowest in scales (Table 1).

Linear regression demonstrated that muscle $\delta^{15}N$ and $\delta^{13}C$ values were significantly related to those measured from fins and scales (Fig. 1). Fins and scales of Římov asp were a strong predictor of muscle $\delta^{15}N$ ($R^2 = 0.81$–0.90; Table 2) but a moderate predictor of muscle $\delta^{13}C$ ($R^2 = 0.57$–0.61; Table 2). In Želivka asp, fins were moderate predictors of muscle $\delta^{15}N$ and $\delta^{13}C$ values ($R^2 = 0.42$–0.64; Table 2), whereas scales were comparatively poorer predictors of muscle $\delta^{15}N$ and $\delta^{13}C$ values ($R^2 = 0.22$–0.31; Table 2).

No significant trend in isotopic offsets between fin and muscle or scale and muscle with fish body length was usually evident in Želivka asp (linear regressions: $P > 0.05$; Fig. 2). The only exception was a positive relationship between asp body length and the $\delta^{13}C$ scale–muscle offset ($R^2 = 0.30$, $P < 0.05$; Fig. 2). In Římov asp, the $\delta^{15}N$ offset between fin and muscle was positively related to body length ($R^2 = 0.19$, $P < 0.05$; Fig. 2) but the $\delta^{15}N$ offset between scale and muscle was unrelated to body length ($R^2 = 0.05$, $P = 0.28$; Fig. 2). Further, in Římov asp, the $\delta^{13}C$ offsets between fin and muscle and between scale and muscle were both positively affected by body length ($R^2 = 0.45$ and 0.57, respectively, both $P < 0.001$; Fig. 2).

The males from the Želivka Reservoir reached a SL of 505 ± 52 mm and mass of 1.9 ± 0.6 kg (mean ± SD). The females from the same reservoir were significantly larger (t-test: $t_{32} < -3.96$, $P < 0.001$), and their mean SL and mass were 570 ± 42 mm and 2.8 ± 0.7 kg, respectively. No significant difference between sexes was found in terms of fin $\delta^{15}N$, with mean (±SD) values of 16.0 ± 0.5‰ for males and 15.9 ± 0.4‰ for females (ANCOVA: $F_{1,31} = 1.75$, $P = 0.20$). However, the fin $\delta^{13}C$ did differ significantly between sexes, with the mean values being −22.7 ± 0.6‰ for males and −22.0 ± 0.6‰ for females (ANCOVA: $F_{1,31} = 4.47$, $P < 0.05$). No difference between sexes was found in the fin C:N ratio (ANCOVA: $F_{1,31} = 0.48$, $P = 0.49$), with the mean values being 3.7 ± 0.2 and 3.6 ± 0.2 for males and females, respectively.

Discussion

Asp fins and scales were found to be lower in ^{15}N and richer in ^{13}C compared with muscle tissue. Previous studies done on other fish species have also observed that fins and scales were usually depleted in ^{15}N and enriched in ^{13}C relative to muscle (Kelly et al., 2006; Tronquart et al., 2012; Willis et al., 2013; Cano-Rocabayera et al., 2015). In both asp populations, the mean isotopic differences between scales and muscle were considerably larger than the mean isotopic differences between fins and muscle. Unlike fins, asp scales were acid treated to remove inorganic carbonates, which might potentially affect their isotopic composition. However, since Syväranta et al. (2008) and Ventura & Jeppesen (2010) reported that

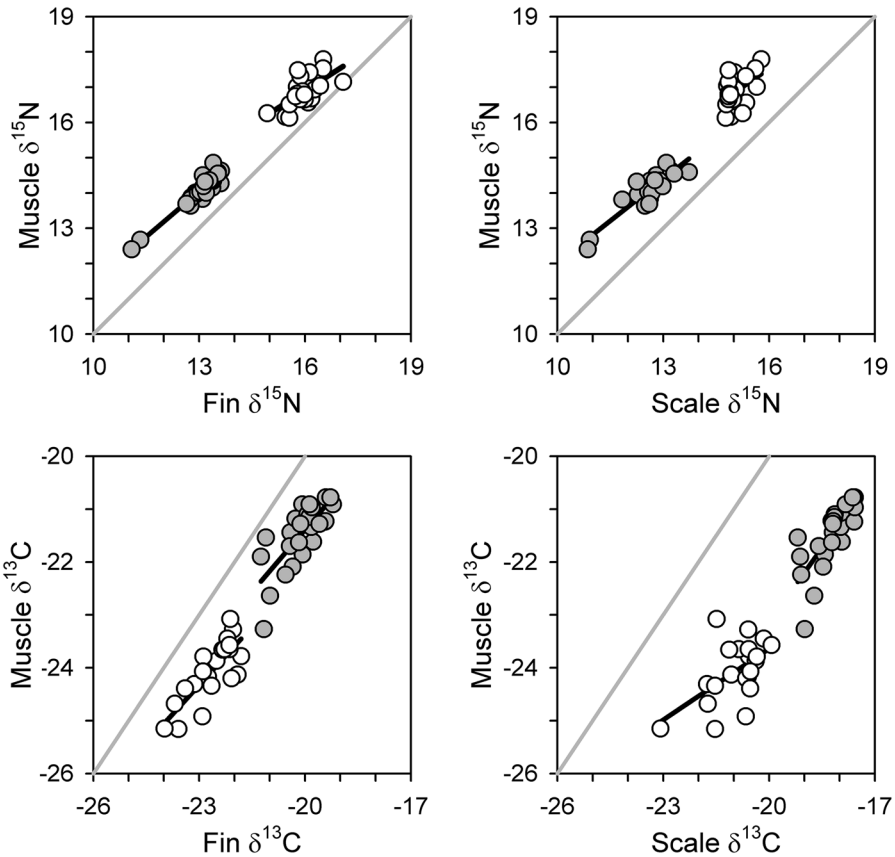

Fig. 1 Linear regressions of $\delta^{15}N$ and $\delta^{13}C$ values in muscle on those of fins and scales for Želivka (*open circles*) and Římov (*shaded circles*) asp. The *black lines* indicate the regression lines where significant relationships were found ($P < 0.05$). The *grey lines* represent the 1:1 reference line at which both tissue types have identical values

Table 2 Linear regression equations for Želivka and Římov asp to convert $\delta^{15}N$ and $\delta^{13}C$ values in fins and scales to those of muscle

Population	Equations	95% CI of slope	R^2	P
Želivka asp ($n = 21$)	Muscle $\delta^{15}N = 0.6443 \times$ fin $\delta^{15}N + 6.5913$	0.30–0.99	0.42	<0.002
	Muscle $\delta^{15}N = 0.6906 \times$ scale $\delta^{15}N + 6.4776$	0.11–1.27	0.22	<0.05
	Muscle $\delta^{13}C = 0.7346 \times$ fin $\delta^{13}C - 7.4302$	0.49–0.98	0.64	<0.001
	Muscle $\delta^{13}C = 0.4486 \times$ scale $\delta^{13}C - 14.6683$	0.15–0.75	0.31	<0.01
Římov asp ($n = 27$)	Muscle $\delta^{15}N = 0.8545 \times$ fin $\delta^{15}N + 2.9302$	0.74–0.97	0.90	<0.001
	Muscle $\delta^{15}N = 0.7889 \times$ scale $\delta^{15}N + 4.1383$	0.64–0.94	0.81	<0.001
	Muscle $\delta^{13}C = 0.7783 \times$ fin $\delta^{13}C - 5.8222$	0.51–1.04	0.57	<0.001
	Muscle $\delta^{13}C = 0.9247 \times$ scale $\delta^{13}C - 4.6264$	0.63–1.22	0.61	<0.001

The 95% confidence intervals (CIs) of the regression slopes are also shown

untreated and acid treated cyprinid scales differed only slightly in isotope values, acid treatment is unlikely to be the primary cause of the differing isotope signatures of asp scales and fins. Instead, the differences suggest that isotopic fractionation might vary among the tissues. Such results are supported by the few studies of other fish species that simultaneously examined isotope signatures of muscle, fin and (untreated) scale tissues and revealed a closer similarity in isotopic composition between muscle and fins than between muscle and scales (Fincel et al., 2012; Cano-Rocabayera et al., 2015). These isotopic differences probably reflect the variability in biochemical components of the tissues (Vollaire et al., 2007), particularly the relative abundance of different amino acids (Pinnegar & Polunin 1999; Estrada et al., 2005).

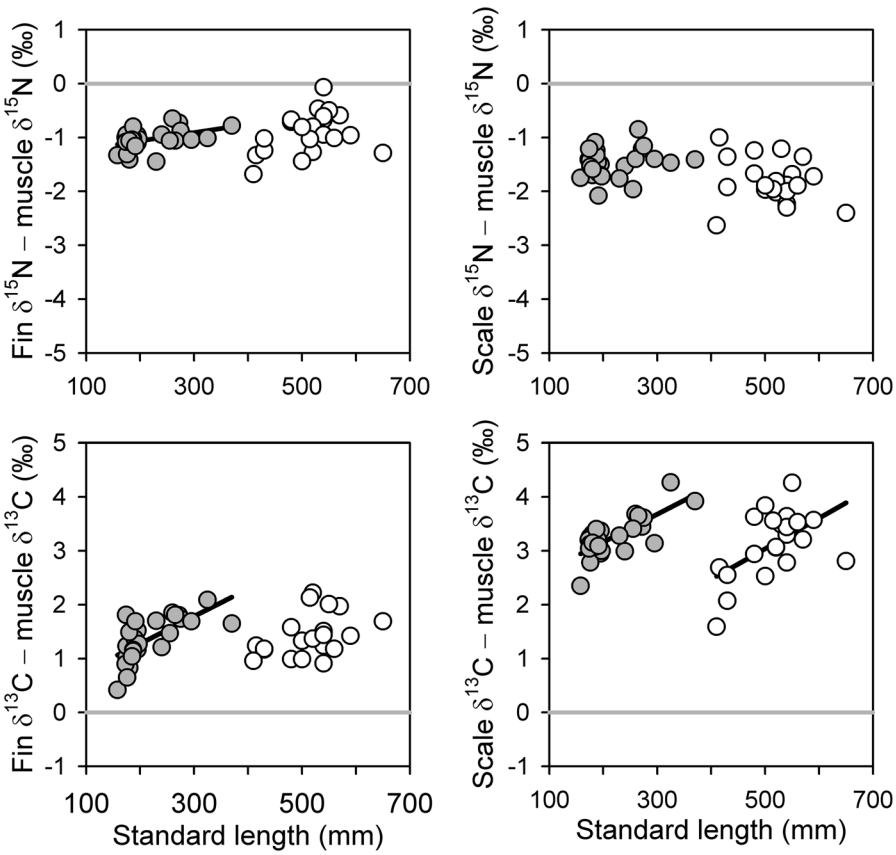

Fig. 2 Relationships between the $\delta^{15}N$ and $\delta^{13}C$ fin–muscle or scale–muscle offsets and body sizes of Želivka (*open circles*) and Římov (*shaded circles*) asp. The *black lines* indicate the regression lines where significant linear relationships were found ($P < 0.05$). The *grey lines* represent the zero reference line at which both tissue types have identical values

Because of the isotopic differences between the asp tissues, correction factors were necessary to convert the isotope values of fins and scales to those of the muscle. The fin-to-muscle and scale-to-muscle conversion equations were all significant but their coefficients of determination varied quite widely ($R^2 = 0.22$–0.90). These results are, nevertheless, comparable with other studies that have examined isotopic relationships between tissues in single populations of various fish species (Hanisch et al., 2010; Graham et al., 2013; Cano-Rocabayera et al., 2015). Stronger inter-tissue relationships ($R^2 > 0.90$) have often been observed in studies that combined isotope data from different populations in a single regression (e.g., Sanderson et al., 2009; Syväranta et al., 2010; Inamura et al., 2012; Tronquart et al., 2012). However, such an approach raises the problem of pseudoreplication and should be avoided because it may spuriously enlarge the range of isotope data (via pooling locations with different isotopic baselines) used in regression analysis (Willis et al., 2013).

In the Želivka asp, body length usually had no effect on the isotopic differences between tissues, which may indicate that inter-tissue isotopic fractionations are fairly constant during the adult life-history stage. In contrast, in the Římov asp, the isotopic offsets between tissues were mostly related to body length. In particular, both the $\delta^{13}C$ fin–muscle and scale–muscle offsets exhibited an increase of 0.5‰ per 100 mm of SL. Changes in tissue fractionation and turnover rates during a period of rapid growth and ontogenetic diet shifts might be responsible for this body length effect. Alternatively, the observed patterns in $\delta^{13}C$ could be caused by lipid content increasing as the size of the asp increased. However, although the C:N ratio (as a proxy for lipid content) of dorsal muscle statistically significantly increased with body length of the Římov asp (linear regression: $R^2 = 0.28$, $P = 0.004$), the slope of this regression was extremely small ($b = 0.0005$), suggesting that the change in C:N ratio alone cannot explain the isotopic differences between the tissues.

The fin $\delta^{15}N$ signatures of the Želivka asp did not differ between sexes. This indicates that both males and females were feeding at the same trophic level. The fin C:N ratio also did not differ between males and females, implying a similar nutritional status for both sexes. In contrast, male and female Želivka asp were significantly distinct in the fin $\delta^{13}C$ signatures, although the difference was only 0.7‰. To decide whether this sex-related difference was caused by physiological processes connected with gonad maturation, utilisation of prey resources with distinct $\delta^{13}C$ signatures or other mechanisms will, however, require further research.

The results of this study suggest that fin and scale tissues, after adjustment, can be a useful non-lethal proxy for muscle in stable isotope studies of asp. Fin clipping is widely used as a standard marking technique in fisheries research (Guy et al., 1996). Moreover, fin clips are frequently sampled for genetic analyses (Wasko et al., 2003) while scales are commonly collected for age and growth determination (DeVries & Frie, 1996). In general, such non-lethally obtained samples can also be used profitably for examining the isotopic composition of fish. In the present study, the strength of inter-tissue relationships differed between the two asp populations. Whereas for juvenile asp of the Římov Reservoir both fins and scales were good predictors of the muscle isotope values, for mature asp of the Želivka Reservoir scales appeared to be a relatively poor predictor compared with fins. Isotopic turnover rates of fish generally decrease with body mass (Vander Zanden et al., 2015) and are slower in less metabolically active tissues (e.g., scales). Nevertheless, the study of Sinnatamby et al. (2008) has demonstrated that muscle and scale tissues equilibrated to new diets at a similar rate in fast-growing Atlantic salmon *Salmo salar* Linnaeus, 1758. It is therefore reasonable to expect that fast-growing juvenile asp of the Římov Reservoir had rapid turnover rates that differed little among the tissues, and both fins and scales thus correlated well with the muscle. In slow-growing adult asp of the Želivka Reservoir, however, scales might have a considerably lower isotopic turnover, meaning that they integrated consumer diets over a longer time period than muscle or fin tissues. Consequently, in adult fish, the differing tissue turnover rates might have caused the poor agreement observed between scale and muscle isotope values. Overall, for asp in this study, fins seemed to be a better non-lethal alternative to muscle. Also, because the preparation of fin clips for the analysis was much easier than that of scales, future non-lethal sampling for isotope studies of asp, and perhaps also other fish species, should preferably use fin clips. Nevertheless, scales can still be a useful non-lethal tissue for those future works interested in determining long-term feeding ecology.

The present study has revealed that the $\delta^{15}N$ scale–muscle offset significantly differed between the asp from Želivka and Římov Reservoirs. Whether this differing isotopic offset refers to local conditions in the two reservoirs or to different body sizes of the fish examined was, however, impossible to determine. Nevertheless, some previous studies done on other species have shown that isotopic fractionation between tissues may vary among populations of a single species (Kelly et al., 2006; Blanco et al., 2009; Graham et al., 2013). Caution should therefore be taken when applying the correction factors developed in this study to asp populations in other systems, where environmental conditions may be considerably different. For each population of interest, when possible, it is best to sacrifice a subset of individuals in order to assess relationships among tissues and determine population-specific conversion factors (Willis et al., 2013). Such development of conversion factors may need to incorporate a sufficient range of body sizes representative of the given population. In summary, although some individuals must be sacrificed for calibration, the use of fin clips and scales in stable isotope studies certainly has the potential to dramatically reduce the number of lethally sampled fish.

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