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Tissue turnover and stable isotope clocks to quantify resource shifts in anadromous rainbow trout

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Abstract Stable isotopes can illuminate resource usage by organisms, but effective interpretation is predicated on laboratory validation. Here we develop stable isotope clocks to track resource shifts in anadromous rainbow trout (*Oncorhynchus mykiss*). We used a diet-switch experiment and model fitting to quantify N stable isotope ($\delta^{15}N$) turnover rates and discrimination factors for seven tissues: plasma, liver, fin, mucus, red blood cells, muscle, and scales. Among tissues, diet-tissue $\delta^{15}N$ discrimination factors ranged from 1.3 to 3.4 ‰. Model-supported tissue turnover half-lives ranged from 9.0 (fin) to 27.7 (scale) days. We evaluated six tissue turnover models using Akaike's information criterion corrected for small sample sizes. The use of equilibrium tissue values was supported in all tissues and two-compartment models were supported in plasma, liver, and mucus. Using parameter estimates and their uncertainty we developed stable isotope clocks to estimate the time since resource shifts. Longer turnover tissues provided accurate estimates of time since resource switch for durations approximately twice their half-life. Faster turnover tissues provided even higher precision estimates, but only within their half-life post-switch. Averaging estimates of time since resource shift from multiple tissues provided the highest precision estimates of time since resource shift for the longest duration (up to 64 days). This study therefore provides insight into physiological processes that underpin stable isotope patterns, explicitly tests alternative models, and quantifies key parameters that are the foundation of field-based stable isotope analysis.

Keywords *Oncorhynchus mykiss* · Migration · Mixing model · Nitrogen · Discrimination factor

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Introduction

Stable isotope analysis can provide insight into ecological processes that would otherwise be difficult or impossible to detect (e.g., Koch et al. 1994; Studds et al. 2008; Newsome et al. 2010). However, experimental validation of parameters like tissue turnover and discrimination factors is important to sound ecological inference of windows of resource use and trophic interactions (Deniro and Epstein 1981; Gannes et al. 1997; Martínez del Rio and Wolf 2005). Further, many stable isotope model frameworks rest on assumptions that, if not tested, provide an uncertain foundation for field application (Martínez del Rio and Wolf 2005; Martínez del Rio and Anderson-Sprecher 2008; Boecklen et al. 2011).

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Stable isotope values of an organism provide a dynamic window into its assimilated food (Deniro and Epstein 1981; Gannes et al. 1998; Carleton and Martínez del Rio 2010). After a diet-switch, different tissues take different amounts of time to turnover to the novel diet isotopic signature due to tissue-specific rates of macromolecular synthesis and catabolism (Martínez del Rio and Wolf 2005; Carleton et al. 2008). For example, splanchnic tissues have been shown to have faster turnover rates than structural tissues (Tieszen et al. 1983; Carleton et al. 2008; Bauchinger and McWilliams 2009; Buchheister and Latour 2010). Studies of freshwater fishes have also found that turnover rates can vary across tissues (e.g., McIntyre and Flecker 2006; Church et al. 2009; Carleton and Martínez del Rio 2010). By using known turnover rates with measured isotopic values from tissues and the environment, clocks can be developed to estimate the timing of resource switches (Phillips and Eldridge 2006; Klaassen et al. 2010; Buchheister and Latour 2010), for a variety of applications such as estimating timing of migration (e.g., Oppel and Powell 2010) or settlement (e.g., Herzka et al. 2001).

How tissue turnover is modeled can affect many ecological applications of stable isotopes, including the estimation of turnover rate and discrimination factors (Martínez del Rio and Wolf 2005; Martínez del Rio and Anderson-Sprecher 2008; Kurle 2009), and may affect the estimation of the timing of resource shifts. In certain tissues turnover may be better represented by modeling multiple compartments, i.e., multiple turnover rates within a tissue, each with its own proportional contribution to the tissue's turnover (Cerling et al. 2007; Carleton et al. 2008; Martínez del Rio and Anderson-Sprecher 2008). Tissue turnover models also can differ in terms of how they incorporate end-members. For instance, data may necessitate using diet values and a discrimination factor rather than equilibrium tissue values (Martínez del Rio and Wolf 2005; Martínez del Rio and Anderson-Sprecher 2008). Alternatively, due to differences in protein content, different diet items may show diet-specific discrimination factors (McCutchan et al. 2003; Pearson et al. 2003; Caut et al. 2009). Despite these different alternative formulations for isotopic turnover, to date isotopic clocks have assumed one-compartment and used equilibrium tissue values (Phillips and Eldridge 2006; Klaassen et al. 2010). It remains unknown how model formulation affects estimates of time since diet-switch.

Stable isotopes may be especially useful to examine organisms with complex life histories and trophic roles, e.g., anadromous rainbow trout (steelhead; *Oncorhynchus mykiss*). *O. mykiss* are found in cold waters around the world (Moyle 2002) and, depending upon location, can be either an important invasive species (e.g., Cambray 2003) or an imperiled native species (e.g., Gustafson et al. 2007). Coastal estuaries play a nursery role for *O. mykiss* by providing a far greater growth potential than upper watershed rearing habitats, thereby increasing marine survival (Hayes et al. 2011). In fact, *O. mykiss* can migrate multiple times between freshwater, estuarine and marine habitats (e.g., Hayes et al. 2011). Thus, *O mykiss* lend themselves as a model system where the application of stable isotope clocks may answer important ecological questions, while simultaneously serving as a system to examine the general theory and application of stable isotopes.

In this study we developed and applied stable isotope clocks for *O. mykiss*. Previously, Church et al. (2009) quantified tissue turnover for two tissues (mucus and muscle) of *O. mykiss*. In our study, we used a controlled diet-switch experiment to investigate isotopic turnover in seven different tissues of *O. mykiss*. We also compared how the number of compartments and isotopic end-member inputs affected tissue turnover model fit and parameter estimates. Using bootstrapped resampling, we investigated how tissue turnover estimation and clock frameworks affect estimates of the time since resource switch. This paper thus examines the strengths, and limitations of using stable isotope clocks to track resource shifts.

Materials and methods

Diet switch experiment

On 6 May 2008, one hundred and twenty-eight *O. mykiss* fry with a mean mass of 0.48 g were transported from Coleman National Hatchery, California to aquaria at the National Oceanic and Atmospheric Administration Southwest Fisheries Science Center, Santa Cruz, California. Fish were held in two cylindrical tanks (1.6 m diameter, 1,500 L) with continuous flow of oxygenated 14 °C freshwater for the duration of the experiment. Fish were fed at an ad libitum rate throughout the experiment.

We chose two hatchery feeds that had different N stable isotope ($\delta^{15}N$) values. To minimize nutritional stress upon switching (Hobson and Clark 1992) the first (diet-1) and second diets (diet-2) had the same crude protein (50 %), fat (20 and 22 % respectively), and fiber (1 %) derived from similar mixed food sources of fish meal, fish oil, wheat, and a vitamin mix, but the first diet also contained poultry meals, and corn meal whereas the second diet instead contained krill meal. For approximately 180 days prior to switch, we fed all fish diet-1 (Bio-Oregon Bio Olympic Fry, $\delta^{15}N$ of food = 7.9 ± 0.2; mean ± SD) to equilibrate tissues to that diet. On 23 September 2008 (day 0), we placed eight fish representative of the experiment's size range in a separate tank to act as a control group and continued to feed them diet-1 for the duration of the

experiment. We then switched the remaining fish to diet-2 (Bio-Oregon Bio Vita Fry, $\delta^{15}N$ of food = 13.9 ± 0.1, mean ± SD). Fourteen days prior to and immediately prior to the switch on day 0, fish were sampled to establish initial $\delta^{15}N$ values for all tissues. Fish were sampled 1, 3, 7, 14, 28, 56, 121, and 210 days after switching to diet-2 to track $\delta^{15}N$ changes in tissues through time. At each sampling interval, eight fish were selected across the range of observed sizes.

Sample collection and preparation

From each of the eight fish per sample we collected plasma, liver, fin, mucus, red blood cells (RBC), muscle, and scales to be analyzed for stable isotope composition. Fish were euthanized using tricaine methanesulfonate. The fork length and weight of each fish was measured. We took blood directly from the caudal vein. Blood samples were refrigerated immediately after collection (2–4 °C), then immediately centrifuged for 10 min at 3,000 r.p.m. to separate RBC from plasma, and the plasma was pipetted into a new vial. The caudal fin was removed and later subsampled (see below). An approximately 1 × 1 × 2-cm cube of muscle was cut from just below the dorsal fin, the fish was dissected and the liver was removed. Scales scraped from just below the dorsal fin were washed with high pressure deionized water in a 425-μm sieve, and then lightly agitated in the sieve under running deionized water. Fin, liver, and muscle were rinsed with deionized water. All samples stored in 1.5-ml centrifuge tubes and each fish stored in individual bags were frozen. Mucus was taken from frozen fish using methodologies adapted from Church et al. (2009). Specifically, after thawing fish for 5 min we scraped clean mucus from the dorsal area into 30-ml scintillation vials. Using deionized water mucus was diluted and filtered through a 212-μm sieve into 50-ml scintillation vials to remove foreign particles. We gave the sieve a final rinse, resulting in approximately 25 ml of clean diluted mucus samples that were then frozen.

Stable isotope analysis

All samples were freeze dried for 48 h. Plasma, mucus, and RBC were homogenized into a fine powder in the vial and liver and muscle were homogenized with mortar and pestle. Dried powder, pieces of dried caudal fin trimmed from the distal edge, or whole dried scales were placed into 5 × 9-mm tin capsules until target mass was attained (0.7 ± 0.05 mg). The $\delta^{15}N$ values and elemental composition of tissues and food were measured at the University of California, Santa Cruz on a Carlo Erba 1108 elemental analyzer coupled to a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer. Repeated samples of internal PUGel (n = 221) and acetanilide (n = 77) standards were used for calibration and quality control for our tissue and diet samples (n = 814). The international standard is atmospheric nitrogen, with precision better than 0.2 ‰ for $\delta^{15}N$ values.

Statistical approach

Modeling tissue turnover

We compared how different tissue turnover models fit our data from the laboratory diet-switch experiment (Table 1). Specifically, we examined model formulations with different approaches to end-member data by estimating either: equilibrium tissue values, single-, or diet-specific tissue discrimination factors. Further, we considered multiple-compartment frameworks for each approach of end-member data. All of these differences in isotopic incorporation can be represented in two general types of models:

Table 1 Overview of tissue turnover models compared in model selection and used to generate three different isotopic clocks

	Abbreviation	Equation(s)
Model name		
Equilibrium tissue—one compartment	ET 1	(1)
Discrimination factor—one compartment	DF 1	(2)
Equilibrium tissue—two compartment	ET 2	(1)
Discrimination factor—two compartment	DF 2	(2)
Clock name		
Single-tissue clock		(3) and (4)
Algebraic two-tissue clock		(5) and (6)
Averaged clocks		(3) and (4)

These four models were created by varying two factors. First, models differed by how they dealt with tissue isotope signatures: equilibrium tissue models did not deal with prey isotope signatures and instead modeled tissue isotope signature; in contrast, discrimination factor models used prey isotope signatures and discrimination factors. Second, models differed by the number of turnover compartments: one-compartment models use a single pool through which isotopes turn over at a single rate; in contrast, two-compartment versions allowed for the turnover to be described by two different turnover rates with different proportional contributions to the overall tissue turnover. Parameter estimates from each of the above-described tissue turnover models may be used in combination with field measurements in one of three clocks. Single-tissue clocks back-calculate the timing of resource switch using information from one tissue, whereas algebraic two-tissue clocks perform a single algebraic calculation of the timing of resource switch using data from two different tissues. Averaged clocks take the mean of two or more independently calculated single-tissue clocks

Equilibrium tissue model:

$$\delta X_t = \delta X_{\text{Post}} - (\delta X_{\text{Post}} - \delta X_{\text{Pre}})\left(pe^{-\frac{t}{\tau_1}} + (1-p)e^{-\frac{t}{\tau_2}}\right), \text{ and} \quad (1)$$

Discrimination factor model:

$$\delta X_t = (\delta X_{\text{diet}_2} + \Delta) - (\delta X_{\text{diet}_2} - \delta X_{\text{diet}_1})\left(pe^{-\frac{t}{\tau_1}} + (1-p)e^{-\frac{t}{\tau_2}}\right). \quad (2)$$

For both equations δX_t is the measured isotopic value of a given element (in this case N) for a tissue at time t. We estimated turnover rate as the average residence time (τ) or the reciprocal of the fractional incorporation rate $\tau = 1/\lambda$; with half-lives calculated as $t_{1/2} = \tau\ln(2) = \ln(2)/\lambda$ for one-compartment models (Carleton et al. 2008; Martínez del Rio and Anderson-Sprecher 2008). For each equation we modeled tissue specific isotopic turnover with both one- and two-compartment models. In essence, two-compartment models allow for the change in isotope signatures to be described by two different rates—perhaps tissue materials are being cycled through a fast and a slow pathway (e.g., the dashed line of mucus shows a faster turnover rate between day 0 and 3, and a slower turnover rate after day 3 relative to the constant turnover rate of the one-compartment solid line; Fig. S1, ESM). For two-compartment models we simultaneously estimated each compartment's average residence time, τ_1 and τ_2, and p, the proportional contribution of τ_1 to the overall turnover within a tissue at time t ($\sum p_i = 1$; Martínez del Rio and Anderson-Sprecher 2008). For one-compartment models $p = 1$. In Eq. 1 we estimated the isotopic ratio of the tissue in equilibrium with the pre-switch and post-switch diets as δX_{Pre} and δX_{Post} respectively. We refer to Eq. 1 as the 'equilibrium tissue model.' Alternatively in Eq. 2 we used measured isotopic values of the pre- and post-switch diets (as opposed to the tissue), and estimated a common discrimination factor Δ that accounts for the difference between diet and tissue. We refer to Eq. 2 as the 'discrimination factor model.' We also separately modeled tissue turnover using diet-specific discrimination factors (Pearson et al. 2003; Caut et al. 2009), combined with measured isotopic values of the pre- and post-switch diets. Interestingly, this approach returned identical estimates and SE of τ for single-compartment and p, τ_1, τ_2 for two-compartment models, as well as identical model fit and model error to modeling tissue turnover using equilibrium tissue values (Eq. 1). In this regard, both one- and two-compartment model approaches of modeling tissue turnover using single- or diet-specific discrimination factors, as well as modeling equilibrium tissue values are all represented using Eqs. 1 and 2. Considering one- and two-compartment versions of Eqs. 1 and 2 we will compare four competing models (Table 1): a one-compartment equilibrium tissue model (ET 1), a two-compartment equilibrium tissue model (ET 2), a one-compartment discrimination factor model (DF 1), and a two-compartment discrimination factor model (DF 2).

We used non-linear least squares to estimate each model's parameter point estimates and associated SE. We calculated Akaike's information criterion corrected for small sample sizes (AICc) scores to evaluate the relative support for each model and refer to parameter SEs for how well each model described the data (Burnham and Anderson 2002). We performed all analyses is R (R Development Core Team 2008).

Isotopic tissue turnover can reflect rates of growth and catabolic tissue replacement (Carleton et al. 2008; Carleton and Martínez del Rio 2010). We modeled growth of our experimental diet-switch fish to investigate if variation in turnover rate could be attributed to variation in growth rate and used individual calculated growth rates $k = [\ln(W/W_o)/t]$ to determine the relative contribution of growth (k) and estimated catabolic tissue replacement (m) to isotopic tissues turnover (ESM; $1/\tau = \lambda = k + m$; Hesslein et al. 1993).

Using tissue turnover to estimate the timing of a diet switch

We present clocks derived from both tissue turnover models (Eqs. 1, 2) because these models have different end-member data inputs and thus could be applicable for different data scenarios (Table 1). Specifically, the application of clocks derived from Eq. 1 requires measured equilibrium tissue values from individuals known to have resided in each of the two specific environments (Pre and Post) for a period longer than the turnover time of the focal tissue. In contrast, the application of clocks derived from Eq. 2 requires knowledge of the isotopic diet values from each of the two environments, as well as established tissue-specific discrimination factors. We examined clocks derived from one- and two-compartment versions of Eqs. 1 and 2; however, for all tissues the two-compartment clocks were biased and less precise than the analogous one-compartment versions (Fig. S2, ESM). Therefore, we only present one-compartment clocks here. We also anticipate different clock frameworks will be better suited to different field applications and therefore examine single-, two-tissue, and averaged clocks derived from both Eqs. 1 and 2 (Table 1).

Single-tissue clocks

For single-tissue clocks we first solved each tissue turnover model for the time since diet switch (t_{est}) in a similar manner to Klaassen et al. (2010), thereby deriving

single-tissue clocks for each model. Then the model's best estimate of tissue-specific turnover rates (τ) and discrimination factors (Δ) can be used in combination with known end-members (δX_{Pre} and δX_{Post}) or (δX_{diet1} and δX_{diet2}) and a measured isotopic tissue value at time t (δX_t), to calculate t_{est}.

Single-tissue clock using equilibrium tissue values:

$$t_{\text{est}} = -\tau * \ln\left(\frac{\delta X_{\text{Post}} - \delta X_t}{\delta X_{\text{Post}} - \delta X_{\text{Pre}}}\right). \quad (3)$$

Single-tissue clock using a single discrimination factor:

$$t_{\text{est}} = -\tau * \ln\left(\frac{(\delta X_{\text{diet}_2} + \Delta) - \delta X_t}{(\delta X_{\text{diet}_2} - \delta X_{\text{diet}_1})}\right). \quad (4)$$

Algebraic two-tissue clocks

If two tissues with different turnover rates have been collected and analyzed for isotopic composition then algebraic two-tissue clocks can be used. For algebraic two-tissue clocks we first solved for ($\delta X_{\text{Post}} - \delta X_{\text{Pre}}$) for Tissue_1 and Tissue_2. Like Phillips and Eldridge (2006) and Klaassen et al. (2010), by assuming that these differences were equal among tissues within an individual, we set the remaining equation for Tissue_1 equal to the remaining equation for Tissue_2 and solved for the common time since diet switch (t_{est}). Using data from our diet switch experiment we examined this assumption and found it to generally be true within the error of parameter estimation (see "Results"; Table 2).

Table 2 Estimates ± SE of the fractional size of compartment one (p), with $p = 1$ for one-compartment models, average residence time for each compartment (τ_1 and τ_2; days), equilibrium tissue values (δX_{Pre} and δX_{Post}; ‰), discrimination factor (Δ; ‰) and τ_{mean}, where $\tau_{\text{mean}} = p\ \tau_1 + (1 - p)\ \tau_2$ (Carleton et al. 2008)

Tissue	Model	p in τ_1	τ_1	τ_2	δX_{Pre}	δX_{Post}	Δ	τ_{mean}	AICc
Fin	ET 1	1	12.9 ± 1.1	–	10.6 ± 0.1	15.0 ± 0.1	–	12.9 ± 1.1	82.9
Fin	ET 2	0.9 ± 0.2	14.3 ± 3.4	2.5 ± 6.6	10.5 ± 0.1	15.1 ± 0.1	–	13.3	86.6
Fin	DF 2	0.7 ± 0.1	19.4 ± 3	0.8 ± 0.4	–	–	1.6 ± 0.1	14.5	133.2
Fin	DF 1	1	12.4 ± 1.3	–	–	–	1.8 ± 0.1	12.4 ± 1.3	160.1
Plasma	ET 2	0.7 ± 0.1	20.0 ± 5.2	2.6 ± 1.4	10.4 ± 0.2	15.6 ± 0.1	–	14.1	103.5
Plasma	ET 1	1	11.8 ± 1.1	–	10.7 ± 0.1	15.5 ± 0.1	–	11.8 ± 1.1	113
Plasma	DF 2	0.6 ± 0.1	26.1 ± 5.7	2.1 ± 0.7	–	–	2.1 ± 0.1	16.4	116
Plasma	DF 1	1	11.0 ± 1.1	–	–	–	2.1 ± 0.1	11.0 ± 1.1	154.7
Liver	ET 2	0.6 ± 0.1	25.0 ± 7.2	2.4 ± 1.1	9.9 ± 0.2	14.9 ± 0.1	–	16.1	117.7
Liver	DF 2	0.5 ± 0.1	38.1 ± 9.5	2.1 ± 0.6	–	–	1.5 ± 0.1	21.3	132.1
Liver	ET 1	1	12.3 ± 1.5	–	10.3 ± 0.2	14.7 ± 0.1	–	12.3 ± 1.5	133.1
Liver	DF 1	1	11.1 ± 1.4	–	–	–	1.4 ± 0.1	11.1 ± 1.4	182.2
Mucus	DF 2	0.7 ± 0	49.7 ± 7.6	2.3 ± 0.9	–	–	1.3 ± 0.1	35.7	102.7
Mucus	ET 2	0.7 ± 0.1	43.1 ± 7.9	2.3 ± 1.1	9.3 ± 0.2	15.0 ± 0.2	–	32.2	102.8
Mucus	ET 1	1	27.1 ± 2.9	–	9.8 ± 0.1	14.7 ± 0.2	–	27.1 ± 2.9	122.3
Mucus	DF 1	1	34.8 ± 3.7	–	–	–	1.7 ± 0.1	34.8 ± 3.7	149.6
RBC	ET 1	1	37.6 ± 2.5	–	9.9 ± 0.1	15.3 ± 0.1	–	37.6 ± 2.5	60
RBC	DF 2	0.9 ± 0	42.7 ± 3	0.3 ± 1	–	–	1.7 ± 0.1	39.7	71.6
RBC	DF 1	1	43.0 ± 2.8	–	–	–	1.9 ± 0.1	43.0 ± 2.8	78.7
Muscle	ET 1	1	39.0 ± 3.2	–	11.2 ± 0.1	16 ± 0.1	–	39.0 ± 3.2	68.4
Muscle	DF 2	0.3 ± 0.1	657.9 ± 1,084.2	35.4 ± 6.9	–	–	3.4 ± 0.1	198.8	70.3
Muscle	ET 2	0.9 ± 0	41.6 ± 4.7	1.4 ± 2.8	11.1 ± 0.1	16.1 ± 0.1	–	39.6	71.3
Muscle	DF 1	1	63.3 ± 5.5	–	–	–	3.3 ± 0.1	63.3 ± 5.5	114.7
Scale	ET 1	1	40.0 ± 2.8	–	10.2 ± 0.1	15.3 ± 0.1	–	40.0 ± 2.8	56
Scale	DF 1	1	52.3 ± 3.8	–	–	–	2.2 ± 0.1	52.3 ± 3.8	94.2

Estimates are shown for the seven *Oncorhynchus mykiss* tissues using either ET 1 or ET 2 (Eq. 1), or DF 1 or DF 2 (Eq. 2). Tissues are ordered from fastest turnover to slowest, and models are ranked by Akaike's information criterion for small sample sizes (*AICc*) score within each tissue, with lowest AICc scores being the best supported by the data

The ET 2, two-compartment model for red blood cells (*RBC*), and both two-compartment models for scale failed due to singular Hessians. For other abbreviations, see Table 1

Algebraic two-tissue clock using equilibrium tissue values: derived from Eq. 1 with Tissue$_1$ and Tissue$_2$

$$t_{\text{est}} = \frac{\ln\left(\frac{\delta X_{\text{Post}_1} - \delta X_{t_1}}{\delta X_{\text{Post}_2} - \delta X_{t_2}}\right)}{\left(\frac{1}{\tau_1} - \frac{1}{\tau_2}\right)}. \quad (5)$$

Algebraic two-tissue clock using a single discrimination factor: derived from Eq. 2 with Tissue$_1$ and Tissue$_2$

$$t_{\text{est}} = \frac{\ln\left(\frac{\left(\delta X_{\text{diet}_2} + \Delta\right) - \delta X_{t_1}}{\left(\delta X_{\text{diet}_2} + \Delta\right) - \delta X_{t_2}}\right)}{\left(\frac{1}{\tau_1} - \frac{1}{\tau_2}\right)}. \quad (6)$$

Averaged clocks

We also propose the approach of averaging independent estimates of t_{est} from multiple tissues. We refer to this approach as using 'averaged clocks.' In this approach, we simply calculate two (or more) single-tissue clocks independently using Eqs. 3 or 4 and take the mean value of t_{est}. We hypothesize that this approach should provide more precise values of t_{est} by averaging across potential tissue-specific biases.

Boot-strapped resampling

We used a bootstrapping routine to investigate how parameter uncertainty affects error in calculating t_{est}. To do this we took simultaneous random draws of δX_{Pre} and δX_{Post}, and τ from the estimated multivariate normal defined by the parametric variance–covariance matrix of each model fit. Associated values of δX_t were drawn from a normal distribution produced from our model fitting. We iterated this 10,000 times for each true time (t_{true}) to produce sets of parameter estimates to be used for each different clock's calculation. Thus, we produced distributions ($n = 10{,}000$) of t_{est} for each clock derived from the same parameter sets at each t_{true} and therefore each clock is comparable at each time step. We produced the median and the 95 % prediction intervals from each clock's resampled distributions of t_{est}. Prediction intervals delineate the area within which 95 % of all future calculated t_{est} will occur considering variability from measurement, environmental conditions, or individual physiology (Ott 1993). Thus, more precise clocks will have narrower prediction intervals and more accurate clocks will have median t_{est} values closer to the 1:1 line of observed to expected values.

Results

Following the diet switch, δ^{15}N tissue values changed towards that of the second diet, asymptotically approaching equilibrium levels of the second diet (Fig. 1). At each sampling period, data are clustered tightly around the model fit, indicating low variation among individuals in tissue turnover. Furthermore, tissue turnover data fall evenly and tightly along the 1:1 line of observed versus expected indicating a good model fit (Fig. S3, ESM). Differences between δX_{Post} and δX_{Pre} among tissues were within ranges of error for most tissues but were slightly different than the difference in δX_{diet2} and δX_{diet1} suggesting that different diets were associated with different discrimination factors (Table 2). We also had control fish that did not undergo a diet switch—differences in mean δ^{15}N tissue values between control fish and pre-switch fish were within individual variability (Deniro and Epstein 1981) ranging from 0.12 to 0.65 ‰. Further, all tissues appeared to reach equilibrium with the second diet by the end of the experiment (Fig. 1).

Growth for our experimental fish was best described by a specialized von Bertalanffy growth model, also known as the Richards model (Fig. 2; ESM) $\{\text{Mass}_t = 557 \times [1 - 0.78\ \exp(-0.00595 \times \text{Day})]^{2.27}\}$ (Richards 1959; Essington et al. 2001). We found relationships between variability of individual growth rate and individual turnover rate in muscle, fin, mucus, and liver (ESM). Calculated growth rates $\{k = [\ln(W/W_o)/t]\}$ varied among individuals from 0.014 to 0.034 with a mean of 0.024 $\times$ day^{-1}. When we used measured growth (k) and estimated catabolic tissue turnover (m) to model isotopic turnover ($\lambda = 1/\tau$; $\lambda = k + m$; Hesslein et al. 1993) we found that catabolism contributed more to turnover for faster turnover tissues. Specifically, the estimated percent contributions of catabolism to isotopic tissue turnover were: fin (68 %), plasma (68.3 %), liver (65.7 %), mucus (32 %), RBC (6.6 %), muscle (6.1 %), and scale (0.7 %) (Table S1, ESM).

Turnover models

Isotope turnover in different tissues was best described by different models (Table 2). In particular, different tissues were supported by one- versus two-compartment models. Specifically, fin, RBC, muscle, and scale tissues were better supported by a one-compartment equilibrium tissue model (Eq. 1; Table 2). In contrast, AICc scores supported two-compartment models for plasma, liver, and mucus. Tissue isotopes generally were modeled best with the use of equilibrium tissues rather than a general discrimination factor. For instance, plasma and liver were best represented by the two-compartment equilibrium tissue model (Eq. 1; Table 2). Mucus was best represented by a two-compartment discrimination factor model (Eq. 2) with the two-compartment equilibrium tissue model (Eq. 1) having a near identical AICc score (Table 2). Aside from mucus' virtually equal support for two-compartment versions of

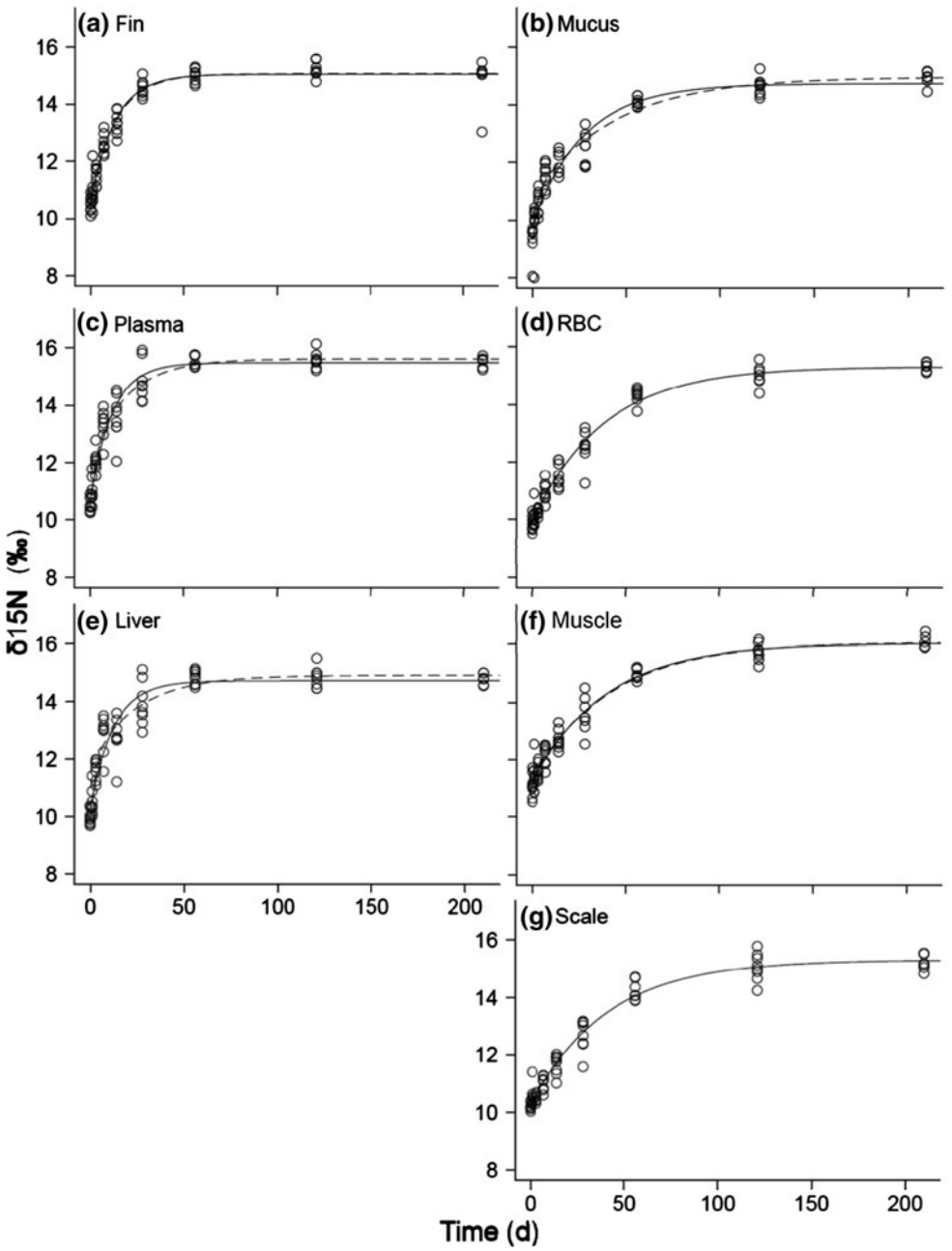

Fig. 1 $\delta^{15}N$ tissue turnover for *Oncorhynchus mykiss* **a** fin, **c** plasma, **e** liver, **b** mucus, **d** red blood cells (*RBC*), **f** muscle, and **g** scale, in order of faster (**a**, **c**, **e**) to slower (**b**, **d**, **f**, **g**) turnover tissue. Model fits of one- (*solid line*) and two-compartment (*dashed line*) equilibrium tissue models (Eq. 1) are shown

both Eqs. 1 and 2, all tissues were best supported by either a one- or two-compartment equilibrium tissue model (Eq. 1) rather than models using discrimination factor (Table 2). The resulting implication is that different diet items had different discrimination factors (Table 2). While the data supported different models, there was tight coherence of estimated tissue-specific parameters across models (Table 2; Fig. 3). For all tissues we found a linear relationship between average residence times of one- versus two-compartment versions of the equilibrium tissue value model (Eq. 1; $\tau_{2\text{-comp}} = 2.8 + 0.98 \times \tau_{1\text{-comp}}$, $r^2 = 0.97$). Both within- and among-model variation in turnover rates were low for fast turnover tissues and increased slightly for longer turnover tissues (Table 2; Fig. 3).

Parameter estimates

Model estimates revealed that different tissues were characterized by dramatically different turnover rates (Table 2; Fig. 3). Fast turnover tissues with their AICc-supported average residence times are fin (12.9 days), plasma (14.1 days), and liver (16.1 days) (Table 2; Fig. 3). Slower turnover tissues and their AICc-supported average residence times include mucus (35.7 days), RBC (37.6 days), muscle (39.0 days), and scale (40.0 days) (Table 2; Fig. 3).

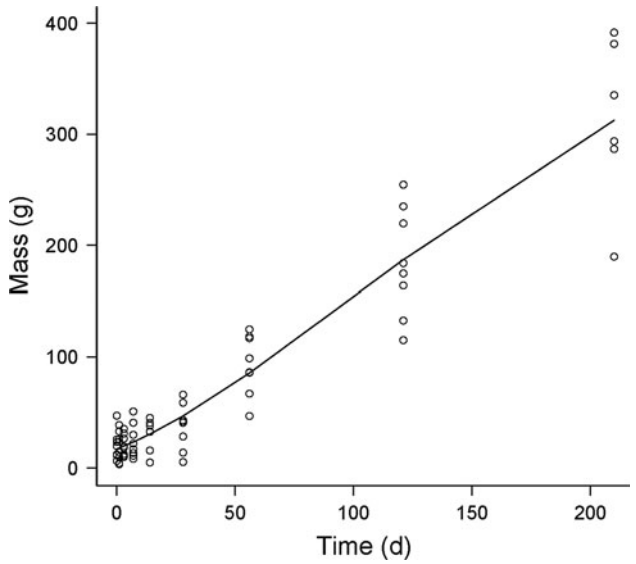

Fig. 2 Mass (g) of *O. mykiss* sampled 0–200 days during our diet-switch experiment and best fit of a "specialized" von Bertalanffy growth equation: $\text{Mass}_t = 557 \times [1 - 0.78 \exp(-0.00595 \times \text{Day})]^{2.27}$

Best-supported δ^{15}N diet-tissue discrimination factors were fin (1.6 ‰), plasma (2.1 ‰), liver (1.5 ‰) for fast turnover tissues, and mucus (1.3 ‰), RBC (1.7 ‰), muscle (3.4 ‰), and scale (2.2 ‰) for slower turnover tissues.

Stable isotope clocks

Bootstrapped prediction intervals allowed the quantification of when different tissues yielded high precision (narrow prediction intervals) and accurate (closest to true value) values of t_{est}. In general, values of t_{est} were more accurate immediately after the switch. As time since diet switch increased, prediction intervals widened, representing decreasing precision in predicting t_{est}. Through time, as values of δX_t approached values of δX_{Post}, models had increasing chances of returning values of log(0) in the numerator of Eqs. 3–6, and thereby began to fail to provide t_{est} (dashed lines; Figs. 4, 5). For all tissue clocks, median t_{est} began to underestimate t_{true} after leaving this reliable period of t_{est} [defined as the range of t_{est} for which no values of log(0) were returned in 10,000 iterations] (solid lines, Fig. 4). This bias increased with time. Furthermore, outside of this reliable range of t_{est} the prediction intervals drifted from the 1:1 or the median t_{est} and were skewed wider towards over-estimating t_{est} for each tissue (Fig. 4).

Model selection influenced the accuracy, precision, and range of inference of single-tissue clocks. One-compartment equilibrium tissue clocks (Eq. 3) consistently returned the most precise and accurate values of t_{est} over the longest period of inference (Fig. S2, ESM). For each tissue, median t_{est} from this clock was within 0.5 days of actual time since diet switch within the period of reliable estimates of t_{est} (solid lines, Fig. 4) and prediction intervals remained narrower than for other model frameworks (Fig. S2, ESM). One-compartment discrimination factor clocks (Eq. 4) performed better than any two-compartment clock and only slightly worse than one-compartment equilibrium tissue clocks (Eq. 3; Fig. S2, ESM). For all tissues, two-compartment clocks provided approximately the same duration of reliable estimates of t_{est} as one-compartment clocks, but prediction intervals were wider throughout. Median t_{est} for two-compartment clocks tended to over-estimate t_{true} soon after the switch and then under-estimate t_{true} later (Fig. S2, ESM). Due to the bias and inaccuracies of t_{est} using two-compartment clocks we compare the results from single-tissue, algebraic two-tissue, and averaged clocks derived from the one-compartment equilibrium tissue turnover model (Eq. 1) in the following paragraphs.

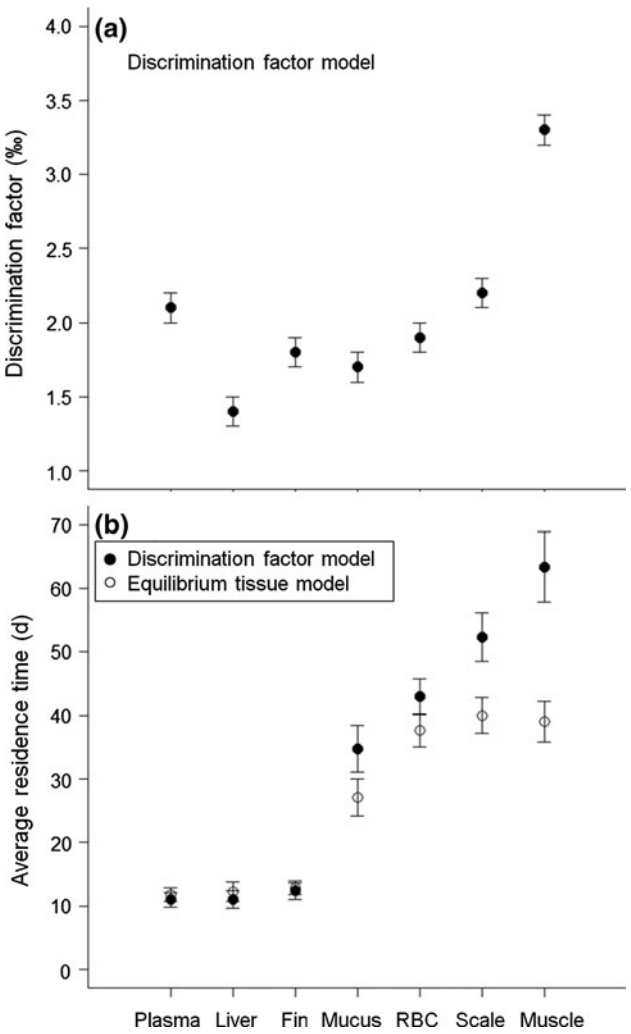

Fig. 3 *O. mykiss* one-compartment **a** discrimination factors (Δ) Eq. 2, and **b** average residence times (τ) based on equilibrium tissues (Eq. 1, *open circles*) and diet and discrimination factors (Eq. 2, *closed circles*); *error bars* are SE

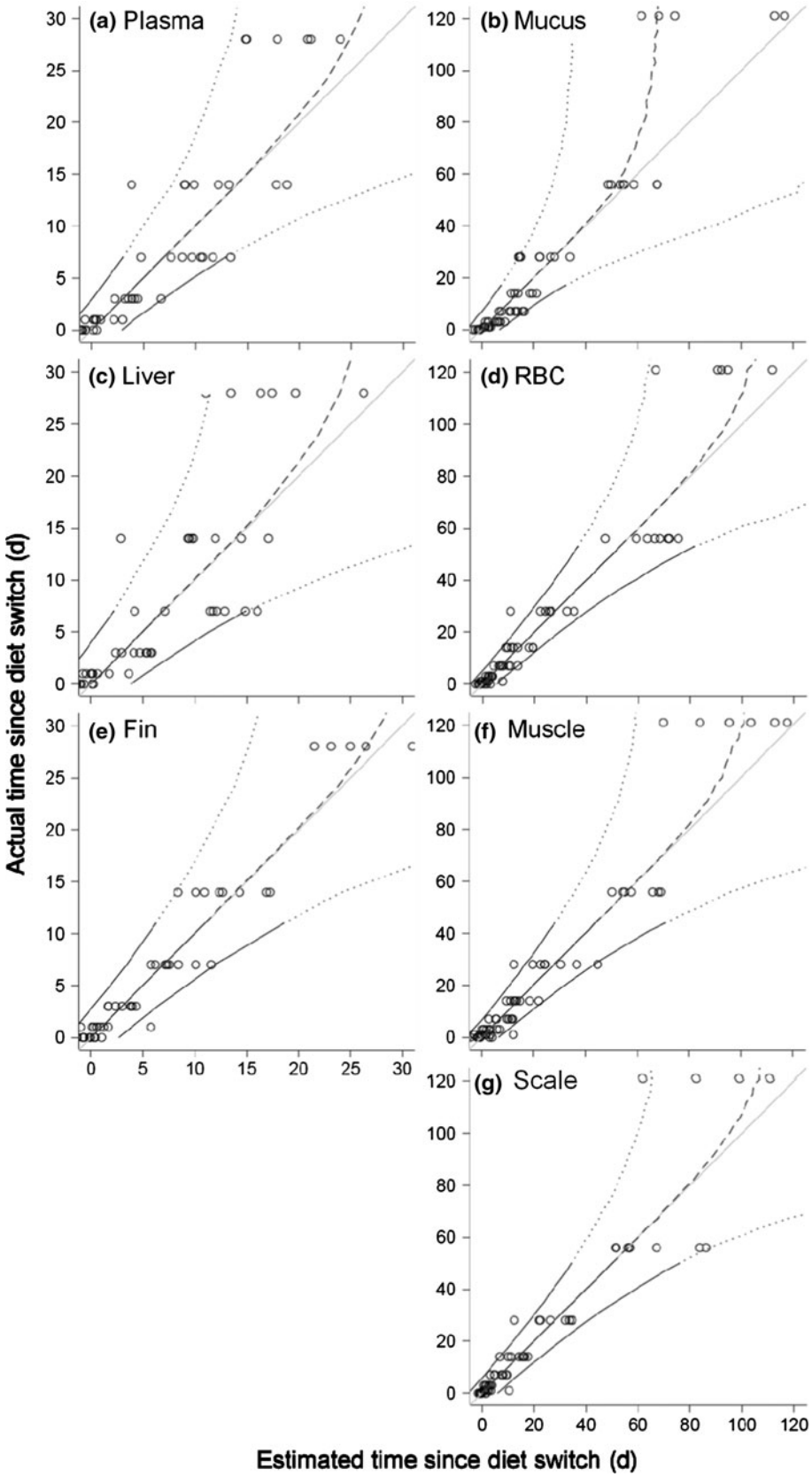

Fig. 4 Estimated time (t_{est}) vs. actual time (t_{actual}) for one-compartment equilibrium single-tissue clocks (Eq. 3) in order of faster (**a**, **c**, **e**) to slower (**b**, **d**, **f**, **g**) turnover tissue. To better represent each clock, *axes* for panels are scaled differently. *Data points* are calculated t_{est} from our diet-switch fish. Lines are bootstrapped 95 % prediction intervals (*dotted*) and median t_{est} (*dashed*). *Solid portions* of the *lines* represent t_{est} for which no log(0) was returned, *dashed* or *dotted portions* represent t_{est} for which at least one log(0) was returned. The 1:1 *line* (*gray solid line*) is shown for reference

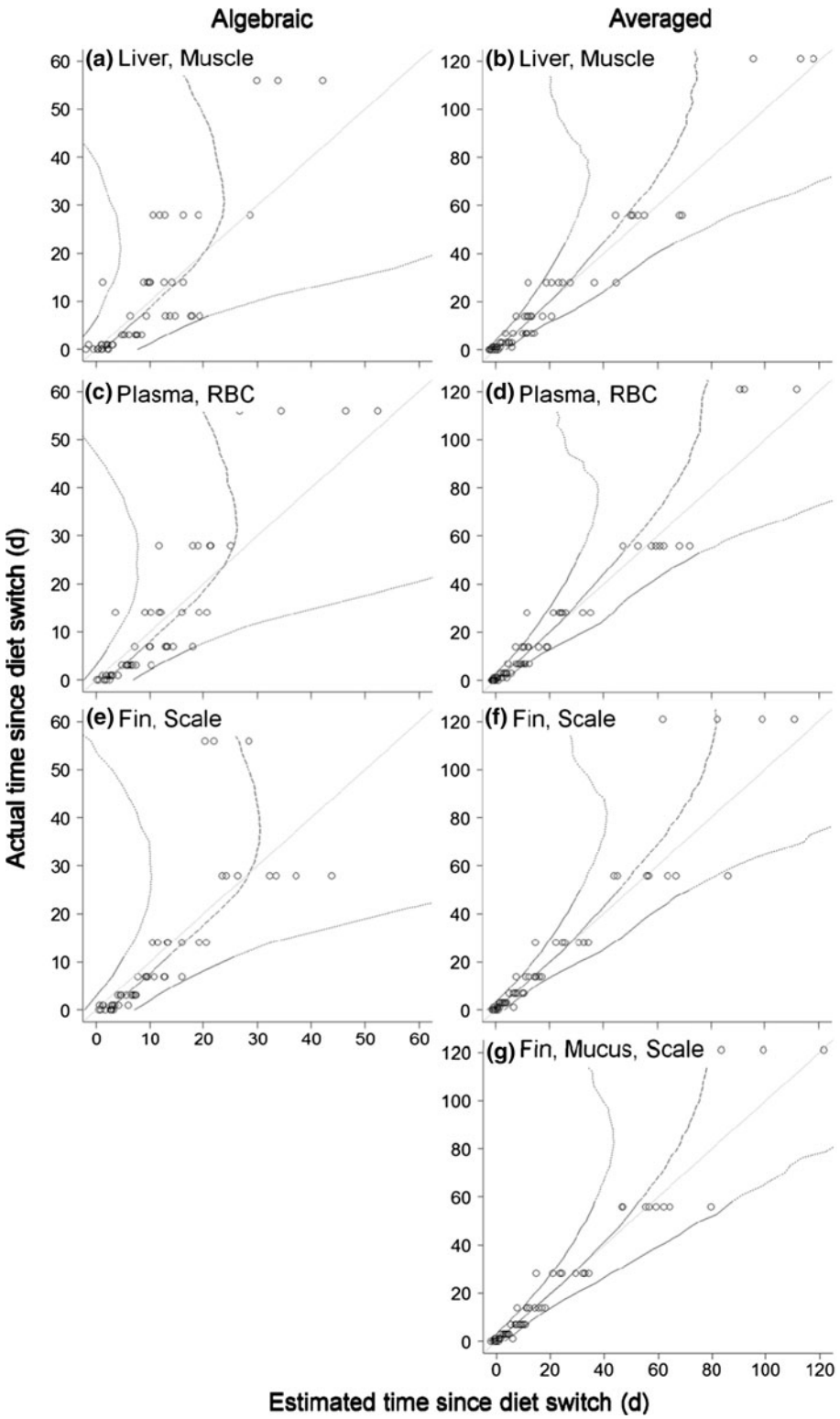

Fig. 5 Estimated time (t_{est}) vs. actual time (t_{actual}) for one-compartment equilibrium algebraic two-tissue (**a**, **c**, **e**) and averaged clocks (**b**, **d**, **f**, **g**) for four tissue combinations. To better represent each clock, *axes* for panels are scaled differently. *Data points* are calculated t_{est} from our diet-switch fish. *Lines* are bootstrapped 95 % prediction intervals (*dotted*) and median t_{est} (*dashed*). *Solid portions* of the *lines* represent t_{est} for which no log(0) was returned, *dashed* or *dotted portions* represent t_{est} for which at least one log(0) was returned. The 1:1 *line* (*gray solid line*) is shown for reference

Single-tissue clocks

Single-tissue clock performance varied across tissues with different turnover rates. For the faster turnover tissues of plasma, liver, and fin, Eq. 3 provided reliable t_{est} for approximately the same duration as the half-life of the element (for example, approximately 8.2, 8.6, and 9 days, respectively, for these fast turnover tissues; Fig. 4). During this reliable period, median t_{est} was within 0.1 days of t_{true} for fast turnover tissues. Mucus, a medium turnover rate

tissue, also returned reliable t_{est} approximately the same duration as its half-life (18.8 days), with median t_{est} within 0.2 days of t_{true} (Fig. 4). The longer turnover tissues of RBC, muscle, and scale returned reliable t_{est}, with the median value of t_{est} within 0.5 days of t_{true} for approximately twice their half-life (ca. 52, 50, and 55 days, respectively, for RBC, muscle, and scale; Fig. 4). Faster turnover tissues returned the narrowest prediction intervals for a duration post-switch equal to their half-lives; however, longer turnover tissues were more precise from that point on (Fig. 4).

Algebraic two-tissue clocks

Algebraic two-tissue clocks were also more precise soon after the diet switch, but prediction intervals widened through time. For all algebraic two-tissue clocks the reliable period of t_{est} was the same duration as that of the shortest single-tissue clock of the tissue combination, and prediction intervals widened dramatically outside of this reliable prediction period (Fig. 5a, c, e). In general, prediction intervals were wider than those of either single-tissue clocks of the pair, but were narrower than the longer turnover clocks soon after switch. Within the reliable period of t_{est}, algebraic two-tissue clocks over-estimated t_{true} by approximately 2 days, yet eventually underestimated t_{true} (Fig. 5a, c, e). Of the algebraic two-tissue clocks, the fin-scale clock was the most precise returning the narrowest prediction intervals for the longest period of reliable inference (11 days, solid lines; Fig. 5e).

Averaged clocks

Of all approaches, averaged clocks provided the most precise values of t_{est} over the longest period of time. Using multiple tissues potentially increased the reliable range of t_{est} in that if one tissue returned a value of log(0) another tissue may not. Prediction intervals were at least as narrow as the most precise tissue in the combination for at least as long as the tissue with the longest period of reliable t_{est}. All tissue combinations presented here returned median t_{est} within 1 day of t_{true} for the same duration as the shortest turnover clock of the pair (Fig. 5b, d, f, g). After this time, median t_{est} began to underestimate t_{true} even within the period of reliable estimates, apparently pulled down by the shorter turnover tissue. Nevertheless, averaged clocks provided reliable values of t_{est} for up to 64 days post-switch, with median t_{est} within 6 days of t_{true} at this time, and the narrowest prediction intervals throughout (e.g., fin-mucus-scale; Fig. 5g).

Discussion

In this study, we performed a laboratory diet-switch experiment on juvenile *O. mykiss* to illuminate patterns of isotopic turnover. These data allowed accurate estimations of discrimination factors and turnover rates which varied among tissues for a wide range of use in *O. mykiss* ecology. Using an information theoretic approach we compared how six competing tissue turnover models represented this diet-switch for each of seven tissues. We found consistent support for using equilibrium tissue value, but support for one- or two-compartments differed among tissues. Furthermore, by comparing several different isotope clocks, we revealed that the number of compartments affected values of τ_{mean} and t_{est} more than whether equilibrium tissue values or diet and a discrimination factor were used (Table 2; ESM). One-compartment equilibrium tissue clocks (Eqs. 3, 5) consistently returned the most precise and accurate values of t_{est} (ESM) supporting previous assumptions of turnover modeling and clocks (Martínez del Rio and Wolf 2005; Phillips and Eldridge 2006; Klaassen et al. 2010; Buchheister and Latour 2010). Slow turnover tissues like scale quantified resource switches over a longer window of time, while faster turnover tissues like fin provided more precise estimates of the timing of a recent switch. Like previous studies we found single-tissue clocks provided the most accurate and precise values of t_{est} relative to algebraic two-tissue clocks (Phillips and Eldridge 2006; Klaassen et al. 2010) but that averaged clocks provided more precise t_{est} over an extended range of inference.

The remaining discussion comprises two major sections. In the first section we discuss patterns of isotopic tissue turnover and how physiology might influence those dynamics. In the second section we discuss the advantages, disadvantages, and limitations of different tissues and clocks in estimating the timing of resource switches.

Isotope dynamics and physiology

Different tissues often exhibit different diet-tissue discrimination values and turnover rates (e.g., McCutchan et al. 2003; Bauchinger and McWilliams 2009). Our data allowed precise estimation of $\delta^{15}N$ discrimination factors which varied among *O. mykiss* tissues from 1.3 ‰ for mucus to 3.4 ‰ for muscle (Table 2; Fig. 2). Our estimated discrimination factors were within the range of values previously described for *O. mykiss* tissues of muscle, liver, and mucus (Pinnegar and Polunin 1999; McCutchan et al. 2003; Church et al. 2009). We also found that tissues such as plasma, liver, and fin had faster turnover rates than did tissues such as RBC and muscle. This relative ranking of tissue turnover rate is similar to results from studies of other fish, birds, and mammals (Tieszen et al. 1983; MacAvoy et al. 2005; Podlesak et al. 2005; McIntyre and Flecker 2006; Guelinckx et al. 2007; Carleton et al. 2008; Bauchinger and McWilliams 2009; Kurle 2009; Buchheister and Latour 2010). Intriguingly, our

estimates of turnover rates were approximately 71 % faster for muscle and 48 % faster for mucus (Table 2) than estimates from a previous *O. mykiss* isotope diet-switch study (Church et al. 2009). These differences in turnover estimates could be driven by extrinsic factors such as environmental parameters or experimental design (Martínez del Rio and Wolf 2005; Logan et al. 2006; McIntyre and Flecker 2006) or intrinsic population-level differences in rates of growth and catabolic tissue replacement (Carleton and Martínez del Rio 2010).

Rates of growth and catabolism have each been shown to influence isotopic tissue turnover (Carleton and Martínez del Rio 2010). Using methods from Hesslein et al. (1993) we found growth to be the primary determinant of isotopic turnover rate in more structural tissues, a pattern common to growing ectotherms (Martínez del Rio et al. 2009). However, our results from the remaining tissues add to a growing literature showing the importance of catabolism to turnover in growing poikilothermic fishes (Herzka et al. 2001; Logan et al. 2006; McIntyre and Flecker 2006; Carleton and Martínez del Rio 2010). Given that growth may influence turnover rates, it is tempting to try to correct for growth when comparing studies or applying laboratory-based parameters. However, Carleton and Martínez del Rio (2010) found complex relationships between tissue, ration, and resulting proportional contributions of growth and catabolism to isotopic turnover. Therefore, predicting or correcting for differences in turnover is not simple (Boecklen et al. 2011), and we reiterate Carleton and Martínez del Rio's (2010) suggestion to use caution when applying laboratory-based estimates to wild populations. For example, prediction intervals around our estimates of time since diet-switch were based on bootstrapped individual variability; however, this uncertainty does not include potential population or environmental differences.

Our study found that different tissues had apparently different fundamental patterns of turnover. Two-compartment turnover models were best supported for *O. mykiss* plasma, liver, and mucus, while one-compartment models were more supported in fin, RBC, muscle, and scale. Other studies have found support for the number of compartments to vary among tissues in birds and mammals (Carleton et al. 2008; Bauchinger and McWilliams 2009; Kurle 2009). While there is growing mathematical support for multiple compartments, there is still limited understanding of the physiological mechanisms underlying this phenomenon (Martínez del Rio and Anderson-Sprecher 2008; Boecklen et al. 2011).

Estimates of turnover and discrimination factors were relatively consistent among different model formulations (Table 2; Fig. 3). For example, we found a linear relationship between turnover rates estimated from one- and two-compartment versions of an equilibrium tissue model (τ_2-compartment $= 2.8 + 0.98 \times \tau_1$-compartment, $r^2 = 0.97$), similar to that found by Carleton et al. (2008) for birds (τ_2-compartment $= 3.54 + 0.96$ τ_1-compartment, $r^2 = 0.98$). In other words, at least for fish and birds, two-compartment models generally provide average residence times that are approximately 3 days longer than one-compartment models (Table 2). Estimated discrimination factors were relatively insensitive to the number of compartments used in turnover modeling, supporting the generality of these estimates.

Isotopic clocks in practice

Turnover model selection somewhat affected the accuracy, precision and range of inference of single-tissue clocks. For example, two-compartment models provided tissue turnover rates approximately 3 days longer than one-compartment models, a similar degree to which two-compartment clocks over-estimated t_{true} (Fig. S2, ESM). While two-compartment models best described isotopic turnover for some tissues, we found that one-compartment clocks still provided relatively precise and accurate estimates of the timing of resource switches. Thus, while there is clear evidence of complexities in isotopic turnover, the more simple model still provides adequate predictions of resource switches (Martínez del Rio and Wolf 2005). Furthermore, whether equilibrium tissue values or diet values and a discrimination factor were used had minimum effect on τ and resulting t_{est} (ESM), implying a versatility in clocks to available end-member data.

We found algebraic two-tissue clocks to be less precise and accurate than single-tissue clocks from either tissue of the pair, similar to previous studies (Phillips and Eldridge 2006; Klaassen et al. 2010). The reduced precision of algebraic two-tissue clocks relative to other clocks is likely because more parameters and their associated error are necessary to calculate t_{est}. Furthermore, the assumption that the difference in end-members ($\delta X_{Post} - \delta X_{Pre}$) is equal among tissues (Phillips and Eldridge 2006; Klaassen et al. 2010) is only loosely met (Table 2), thereby creating additional error around t_{est}. Regardless, algebraic two-tissue clocks may be of particular use for some applications because they use the relative rather than absolute turnover rates of two tissues, and because the Pre switch end-member is not necessary to generate t_{est}.

Isotopic clocks from tissues had different strengths and limitations (Figs. 4, 5). Longer turnover tissues such as scale provide the most useful single-tissue clocks by reliably providing the most precise and accurate t_{est} over the largest window of inference (up to approximately 55 days post-switch). Using faster turnover tissues like fin can complement these results by providing more precise t_{est} if the switch was more recent, for example, up to

approximately 13 days post-switch for fin. These quantifications of the effective time windows of different tissues inform the design of a field sampling regime to pinpoint the timing of resource switches. For example, by using an averaged fin-mucus-scale clock, samples would only need to be collected roughly every 9 weeks rather than the analogous 8 weeks if using a clock derived from scale alone (Figs. 4, 5).

In this study we parameterized and formulated stable isotope clocks to estimate the timing of resource shifts of *O. mykiss*. The parameters generated in this paper will hopefully provide a strong base for future stable isotope studies of this common fish (see Deniro and Epstein 1981; Gannes et al. 1997; Martínez del Rio and Wolf 2005). More generally, through model fitting and an information theoretic approach, this study provides a glimpse into the physiological underpinnings of isotope dynamics. Furthermore, through exploring the strengths and limitations of different clock formulations, this study illustrates how estimates of resource switch timing are affected by clock formulations as well as the tissue turnover models used to derive them. Stable isotope studies will be the most effective when they can link laboratory, theory, and field-based insights (Martínez del Rio et al. 2009; Layman et al. 2012).

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