

STABLE ISOTOPE DYNAMICS IN SUMMER FLOUNDER TISSUES, WITH  
APPLICATION TO DIETARY ASSESSMENTS IN CHESAPEAKE BAY

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APPROVAL SHEET

This thesis is submitted in partial fulfillment of  
the requirements for the degree of  
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## DEDICATION

To my loving wife, whose support, humor, and affection have been tireless and essential to the completion of this work.

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## PREFACE

Chapters 1 and 2 of this thesis were written as separate, independent manuscripts to be submitted for publication in peer-reviewed journals.

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## ABSTRACT

Stable isotope techniques were applied to summer flounder, *Paralichthys dentatus*, in Chesapeake Bay to elucidate the relative importance of different prey groups on the growth and productivity of this species. Prior to field application, a laboratory diet-shift study was conducted to evaluate methodological assumptions and obtain necessary isotopic parameters. Specifically, the goals of the laboratory study were to 1) determine isotopic turnover rates and fractionations of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in liver, whole blood, and white muscle and 2) estimate the relative importance of growth and metabolic processes on isotopic turnover. Groups of captive juvenile summer flounder (130-255mm total length) were monitored for up to 180 days after switching their food to a new diet with different stable isotope values. Although differences existed between carbon (C) and nitrogen (N), the rate of isotopic change was consistently ranked liver>blood>muscle for the three tissues due to increased metabolic activities of liver and blood. Half lives ranged from 9-21, 20-44, 49-73 days for liver, blood, and muscle respectively. Fractionation estimates for  $\delta^{15}\text{N}$  in muscle (range: 2.4-4.2‰) corresponded with previous research, but estimates for  $\delta^{13}\text{C}$  (range: 0.1-4.8‰) tended to be greater than the traditionally assumed values of 0-1‰. Liver and blood fractionation estimates were similar to those of muscle, differing by usually <1‰. A generalized model for predicting the time scale of isotopic turnover from growth-based turnover parameters was also developed to help evaluate assumptions of isotopic equilibrium in the field.

Information obtained from the laboratory study facilitated the use of stable isotopes as dietary tracers for wild summer flounder (138-624mm total length) in Chesapeake Bay. Summer flounder tissues (liver, blood, and muscle) and commonly consumed prey species were sampled seasonally during late spring / early summer (May-July) and fall (November) in 2006 and 2007. To account for similarity in isotopic measurements and to apply mixing models, prey species were aggregated into two trophic guilds: crustaceans (mysid shrimp, sand shrimp, mantis shrimp) and fishes (bay anchovy, juvenile sciaenids, spotted hake). Lack of  $\delta^{13}\text{C}$  differentiation among trophic guilds and summer flounder prevented the use of  $\delta^{13}\text{C}$  as a useful dietary indicator. Analysis of  $\delta^{15}\text{N}$  revealed that crustaceans comprised the majority of summer flounder diet, accounting for ~85-100% of flounder diets on average, except in spring of 2006 when fishes and crustaceans were equally represented in the diet. Summer flounder tended to occupy the same trophic level as the other fishes, suggesting more of a competitive relationship than a predatory one. However, a positive trend in  $\delta^{15}\text{N}$  with length in all tissues indicated that larger summer flounder fed at ~1 trophic level above smaller flounder. Differences in isotopic values between slow and fast turnover tissues did not reveal this ontogenetic dietary pattern at the level of the individual, because the changes in feeding were of small isotopic magnitude and occurred too gradually for reliable detection. Based on stable isotopic analysis, growth and production of summer flounder in Chesapeake Bay are highly dependent on assimilation of mysid, sand, and mantis shrimps, more so than previously expected based on stomach content research.

STABLE ISOTOPE DYNAMICS IN SUMMER FLOUNDER TISSUES, WITH  
APPLICATION TO DIETARY ASSESSMENTS IN CHESAPEAKE BAY

CHAPTER 1

TURNOVER RATES AND FRACTIONATIONS OF  $\delta^{13}\text{C}$  AND  $\delta^{15}\text{N}$  IN MUSCLE,  
BLOOD, AND LIVER TISSUES OF JUVENILE SUMMER FLOUNDER

## INTRODUCTION

Detailed information on trophic interactions is critical for understanding energy pathways within ecosystems (McConnaughey and McRoy 1979, Deegan and Garritt 1997) as well as ecosystem productivity and resilience (Chapin et al. 1997, Holmlund and Hammer 1999). Studies have generally relied on stomach contents to obtain information on trophic interactions, but stable isotope analyses have developed as another valuable tool to examine diets of organisms. The application of stable isotopes to trophic ecology relies on the fact that the mass of a consumer will be derived from the material that it consumes. Ratios of stable isotopes (typically  $^{15}\text{N}/^{14}\text{N}$  and  $^{13}\text{C}/^{12}\text{C}$ ) from consumers and their prey can be compared to establish trophic connections. Several quantitative methodologies have developed which calculate the contributions of different prey sources to a consumer using mixing models (Phillips and Gregg 2003, Fry 2006) and estimate the trophic level of a consumer within a food web (Vander Zanden and Rasmussen 1999, Post 2002). These calculations and many general conclusions from stable isotope studies are often based on two primary assumptions: 1) that sampled tissues are in equilibrium with the diet (Gannes et al. 1997), and 2) that fractionations between the material consumed and the material assimilated as tissue are known (Post 2002). However, these critical assumptions are often not validated for individual studies.

The assumption that a consumer is in isotopic equilibrium with its diet depends on the animal's isotopic turnover rate and the consistency of its dietary habits. Since body

tissues are comprised of the assimilated portion of the diet, they are reflective of the average diet for the consumer over a prior feeding period. The turnover rate of stable isotopes will determine the amount of time over which this isotopic signal is averaged. Turnover generally depends on two factors: the dilution of existing mass by new mass synthesized from recently consumed prey (i.e., growth), and the replacement or conversion of existing tissue using material synthesized from recent diet (i.e., metabolism) (Hesslein et al. 1993). Accordingly, growth and metabolic rates are the key determinants of how rapidly the isotopic signatures of consumed prey will become evident in the tissues of the consumer. If the isotopic blend of a fish's diet has been consistent relative to the time scale needed for complete isotopic turnover, then the consumer will indeed be in isotopic equilibrium with the diet. However, proper consideration of turnover rates becomes crucial in assessing diets when the consumer relies on seasonally abundant prey types (Latour et al. 2008), migrates between differing habitats (Cunjak et al. 2005), or exhibits ontogenetic shifts in feeding (Renones et al. 2002). Generally, any dietary shifts that occur within the time-scale needed for complete isotopic turnover have the potential of hindering proper interpretation of stable isotope data (O'Reilly et al. 2002).

Fractionation is the process of isotopic discrimination in which ratios of stable isotopes change in measurable amounts between two pools, or between prey and consumer in the case of trophic studies (Fry and Sherr 1984, Fry 2006). These changes in the stable isotope ratios result from the preferential excretion of  $^{14}\text{N}$  and a slight isotopic discrimination against  $^{13}\text{C}$  during respiration (Peterson and Fry 1987). For calculating source contributions or estimation of trophic position, many studies consistently assume a

fractionation of approximately 3.4‰ for  $\delta^{15}\text{N}$  and 0-1‰ for  $\delta^{13}\text{C}$ , as promoted by some studies and literature reviews (DeNiro and Epstein 1978, DeNiro and Epstein 1981, Minagawa and Wada 1984, Post 2002, Sweeting et al. 2007). However, a growing body of literature continues to characterize factors that contribute variability to fractionation values. For example, fractionation values have been shown to vary among individuals (DeNiro and Epstein 1978, 1981), species (DeNiro and Epstein 1978, 1981, Tieszen et al. 1983, Vander Zanden and Rasmussen 2001), diet (Gorokhova and Hansson 1999), tissue types (DeNiro and Epstein 1981, Hobson and Clark 1992b), and feeding mode (Vander Zanden and Rasmussen 2001).

The impacts that assumed fractionations can have on estimates of trophic level and mixing model results vary. Vander Zanden and Rasmussen (2001) contended that assumed fractionations may contribute relatively small amounts of error to trophic level calculations provided primary consumers and not primary producers are used as the baseline indicator for trophic position estimates. Post (2002), on the other hand, found trophic level estimates to be highly sensitive to fractionation values of nitrogen. For mixing models, error introduced by incorrect fractionations is dependent on the isotopic separation amongst the contributing sources (Vander Zanden and Rasmussen 2001). Consequently, in situations where prey sources have similar stable isotope ratios, errors derived from fractionation assumptions can have larger impacts on the resultant conclusions.

Potential errors introduced by neglecting isotopic turnover rates and assuming fractionation values have helped motivate more rigorous laboratory studies on turnover and fractionation (Gannes et al. 1997; Vander Zanden and Rasmussen 2001, Sweeting et

al. 2005). For fishes, many laboratory studies have focused on larval (Herzka and Holt 2000, Witting et al. 2004,) or juvenile stages (Bosley et al. 2002, Logan et al. 2006, Sakano et al. 2005, Sweeting et al. 2005, 2007), while few have examined adults (Hesslein et al. 1993, MacAvoy et al. 2001). With few exceptions, most studies have attributed changes in isotopic composition (following a diet shift) to growth as opposed to metabolic replacement of tissues. Unlike endothermic mammals and birds which generate heat through metabolism (DeNiro and Epstein 1978, 1981, Tieszen et al. 1983, Hobson and Clark 1992a), standard metabolic rates are much lower in ectothermic fishes, contributing less to isotopic turnover. With growth thought to be the primary driver of turnover in most fishes, slower growing species or age classes can require more than a year to completely equilibrate isotopically to a new diet (Hesslein et al. 1993, MacNeil et al. 2006).

Most studies measuring stable isotope ratios in fishes tend to only sample muscle tissue in larger individuals or analyze the whole body of small (e.g., larval) specimens. However, both turnover rates and fractionation can vary across body tissues of an animal (Tieszen et al. 1983, Hobson and Clark 1992a, Hobson and Clark 1992b). Mixed results have been reported for fishes; some have documented little difference in turnover rates among muscle, blood, and/or liver (Hesslein et al. 1993, MacAvoy et al. 2001, Sweeting et al. 2005) while others have suggested that turnover is notably faster in liver, than blood, than muscle (Logan et al. 2006, MacNeil et al. 2006). For example, MacNeil et al. (2006) reported it would take 166 (liver), 265 (blood), and 422 (muscle) days for the different tissues of the ocellate river stingray (*Potamotrygon motoro*) to almost completely express a new diet's isotopic signature.

Knowledge of tissue-specific turnover rates in fishes can benefit researchers in several ways. Most importantly, this type of information allows researchers to tailor the sampled tissue to the appropriate time-scale of interest depending on the objectives of an isotopic study. For example, tissues with higher isotopic turnover rates can be used to address prey utilization on shorter temporal scales (Perga and Gerdeaux 2005), to capture finer scale dietary shifts, or to study nutritional contribution of seasonally available migratory prey (MacAvoy et al. 2001). Analyzing isotopic differences among multiple tissues of an individual can also theoretically act as an “isotopic clock”, whereby the timing of previous dietary shifts can be determined based on the different turnover times of the tissues (Fry 2006, Phillips and Eldridge 2006).

The primary objective of this study was to empirically determine the turnover rates and fractionations of carbon (C) and nitrogen (N) stable isotopes in three different tissues of juvenile summer flounder, *Paralichthys dentatus*. Liver, whole blood, and muscle were examined with the hypothesis that liver and blood tissues would have faster turnover rates than muscle as shown in some other fishes (Logan et al. 2006, MacNeil et al. 2006). The secondary objective was to model the relative contributions of metabolism and growth to the process of isotopic turnover in these tissues. The motivation for this laboratory study came from a desire to assess diets of summer flounder within Chesapeake Bay, USA. As a valuable commercial and recreational species, a thorough understanding of summer flounder dietary habits is necessary for fisheries management to move towards a more ecosystem-based approach (Latour et al. 2003). Summer flounder inhabit Chesapeake Bay during the spring and summer months before migrating offshore to spawn in the fall and winter (Murdy et al. 1997). Given the residence time of

summer flounder within the Bay, traditional isotopic analysis of muscle tissue may be inappropriate to categorize which prey groups drive flounder production within the Bay. This research was thus ultimately intended to provide information for more accurate application of stable isotope techniques to study the trophic dynamics in wild summer flounder, a seasonally migrating fish.

## METHODS

### *General*

A diet-shift experiment was conducted to determine isotopic turnover rates and fractionation in juvenile summer flounder. Fish with similar isotopic signatures were switched to a new diet whose isotope signal differed from the baseline values. As individuals assimilated the new food, their isotopic signatures approached the value of that new food over time (although slightly adjusted for fractionation). Individuals were periodically sampled from the population to monitor the change in their isotopic signals.

### *Fish rearing*

A group of juvenile, age-0, summer flounder (n=72) was purchased from a commercial fish hatchery (GreatBay Aquaculture, Portsmouth, NH). These fish had been spawned from wild broodstock and raised on a similar diet of formulated feed. Hatchery fish were transported from NH to the Virginia Institute of Marine Science, VA and allowed to acclimate to an experimental tank for 3 weeks while maintaining the previous diet. A second group of juveniles (n=55) was captured from the lower Chesapeake Bay using a bottom trawl and maintained in captivity for 2-3 months on a constant diet of squid. The squid diet was shown to mimic the isotopic signature of food items regularly consumed by juvenile summer flounder in Chesapeake Bay (A. Buchheister, VIMS, unpublished data) and would thus maintain the wild fish at roughly the same isotopic

values as when they were initially captured. The hatchery (H) and wild (W) fish were treated separately throughout the experiment because of the differences in their rearing histories and initial isotopic values. The hatchery and wild groups of fish were each maintained in a 450-gallon recirculating tank with water held at average temperatures ( $\pm$  SD) of  $19.9 \pm 0.7^\circ\text{C}$  (wild fish) and  $20.4 \pm 0.7^\circ\text{C}$  (hatchery fish). Both tanks utilized the same re-circulating water, which was obtained from the York River (filtered to  $1 \mu\text{m}$ ). Average salinity in the tanks was  $26.1 \pm 0.9$ . The temperature of  $20^\circ\text{C}$  was chosen to be an average representation of bottom water temperatures of summer flounder habitat in Chesapeake Bay from May to November which ranges from about  $15\text{-}25^\circ\text{C}$ . Use of water from the mouth of the York River assured that experimental salinities were representative of the Chesapeake Bay, which averages near 25 throughout the year based on environmental monitoring data from the Chesapeake Bay Program. Fish were kept on a 13:11 hour light:dark schedule.

Two days prior to the start of the diet shift experiment, all individuals were marked with a unique coded wire tag (Northwest Marine Technology, Shaw Island, WA) inserted into the blind-side musculature, and fish were measured for total length (mm) and weight (mg) (Table 1). The minute tags did not appear to affect fish behavior and a minimal proportion of fish (2.6%) did not retain their tags (likely due to insertion error), making the coded wire tags ideal for the study. At the start of the experiment, seven fish were sacrificed from each group to obtain baseline isotopic signatures for muscle, liver, and blood tissues. The remaining fish were all switched to a constant diet of commercially-purchased krill, *Euphausia superba*, (Fishalicious Foods, Custer, WA), which was shown to have a different isotopic signature from both the wild and hatchery

groups. Fish were fed up to 5% of their body weight per day in an attempt to replicate realistic growth rates of fish in the wild (Rountree and Able 1992).

To monitor turnover of stable isotopes, 4-6 individuals were randomly sampled and sacrificed at specified time periods from each group. Towards the end of the experiment, up to 10 individuals were sampled in order to have no fish remaining in the tanks. All protocols pertaining to sampling and euthanizing of experimental fish were approved by the College of William and Mary's Institutional Animal Care and Use Committee. Fish were sampled at 7, 13, 24, 35, 49, 67, 89, 126, 155, and 180 days from the start of the experiment. Three samples of the krill diet were periodically sampled to assure consistency in the dietary isotopic signature.

#### *Processing of sampled fish*

Summer flounder selected for isotopic determination were removed from the water at least 12 hours after the previous feeding and measured for total length (mm) and weight (mg). Samples of whole blood (0.5 ml), liver, and dorsal muscle (above the pectoral fin) were taken, rinsed with de-ionized water, and dried in pre-combusted glass vials at 45°C for at least 2 days. Coded wire tags were recovered from the musculature of the fish to identify individuals. Dried tissue samples were ground, and a 0.8-1.2 mg subsample was packaged into a 4x6 mm tin capsule for stable isotope analysis.

Prepared samples were analyzed at the University of California-Davis Stable Isotope Facility using a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer. Stable isotope ratios are reported in relation to conventional standards:

$$(1) \quad \delta X = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1000$$

where X is the stable isotope of C or N, and R is the mass ratio of the heavy to light stable isotope (e.g.,  $^{15}\text{N}/^{14}\text{N}$ ) for either the sample or the standard. The conventional standards used for the analyses were Pee Dee Belmnite for C and air for N, and  $\delta X$  is reported in per mil (‰). Repeated measurements of a calibration standard indicated that instrument precision (SD) was 0.15 and 0.08‰ for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively.

### *Lipid correction for livers*

A mathematical correction was used to normalize liver  $\delta^{13}\text{C}$  values, due to the high lipid concentrations in this tissue which are known to bias  $\delta^{13}\text{C}$  values (DeNiro and Epstein 1977, Logan et al. 2008). Twelve liver samples were divided into two aliquots to determine  $\delta^{13}\text{C}$  both before lipid extraction ( $\delta^{13}\text{C}_{\text{NE}}$ ) and after lipid extraction ( $\delta^{13}\text{C}_{\text{LE}}$ ). Lipids were extracted using a solvent mixture of 65% dichloromethane and 35% methanol (Bligh and Dyer 1959) using an ASE-200 Accelerated Solvent Extractor (Dionex ®) at 80°C and 1800 psi. Following the extraction, samples were dried and analyzed for stable isotope ratios as described above. Following Logan et al. (2008), the relationship between elemental C:N (for non-extracted samples) and the change in  $\delta^{13}\text{C}$  due to extraction ( $\delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}}$ ) was evaluated using four alternative models. Akaike's Information Criterion for small sample sizes ( $\text{AIC}_c$ ) identified the McConnaughey and McRoy (1979) model as the best fit to the data (see Logan et al. 2008 for competing models). This model takes the form of:

$$(2) \quad \delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}} = D\left(\theta + \frac{3.90}{1 + 287/L}\right) \quad \text{where}$$

$$L = \frac{93}{1 + (0.246 \times \text{C} : \text{N} - 0.775)^{-1}}.$$

In equation (2), L represents the sample lipid content as a function of C:N, while D represents protein-lipid discrimination. The parameters D and  $\theta$  were estimated ( $\pm$  SE) as  $D = 6.2883 \pm 1.3519$  and  $\theta = 0.0612 \pm 0.1527$ . Equation (2) was used to adjust all liver  $\delta^{13}\text{C}$  values based on C:N. Lipid corrections were not applied to muscle and blood  $\delta^{13}\text{C}$  values due to the relatively low C:N values and limited variability across individuals (mean C:N  $\pm$  SD: muscle,  $3.40 \pm 0.13$ ; blood,  $4.12 \pm 0.39$ ; liver,  $14.54 \pm 4.68$ ).

### *Growth-based modeling*

Changes in stable isotope ratios were modeled as a function of relative growth since the diet switch. The growth-based model predicts the isotopic value of a fish as (Fry and Arnold 1982):

$$(3) \quad \delta_{\text{WR}} = \delta_{\text{f}} + (\delta_{\text{i}} - \delta_{\text{f}})W_{\text{R}}^c,$$

where  $\delta_{\text{f}}$  is the expected isotopic value when completely equilibrated to the new diet and  $\delta_{\text{i}}$  is the initial isotopic signature prior to the diet shift. The average isotopic value for the seven fish sacrificed before the diet shift was used as the estimate for  $\delta_{\text{i}}$  in the model. The relative increase in weight of each fish ( $W_{\text{R}}$ ) is calculated as the final wet weight divided by the initial wet weight. The variable  $\delta_{\text{WR}}$  is the measured isotopic value for a fish given its increase in weight, and  $c$  is a turnover rate constant. Both  $\delta_{\text{f}}$  and  $c$  were estimated using nonlinear regression (SAS Version 9.1, SAS Institute 2002). In this model, if  $c = -1$ , growth is entirely responsible for turnover, whereas if  $c < -1$ , metabolism is contributing to turnover of the stable isotope in the tissue, with more negative values representing greater contributions by metabolism (Fry and Arnold 1982).

The fractionation estimates of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  between the diet and each tissue ( $\Delta_{\text{TISSUE}}$ ) were calculated as (Minagawa and Wada 1984):

$$(4) \quad \Delta_{\text{TISSUE}} = \delta_f - \delta_d$$

where  $\delta_d$  is the mean stable isotope signature for samples of krill diet. Standard errors for the fractionation estimate were based on errors associated with the model estimate of  $\delta_f$  ( $\text{SE}_{\delta_f}$ ) and the standard error of the mean for  $\delta_d$  ( $\text{SE}_{\delta_d}$ ):

$$(5) \quad \text{SE}_{\Delta_{\text{TISSUE}}} = \sqrt{\text{SE}_{\delta_f}^2 + \text{SE}_{\delta_d}^2}.$$

By rearranging equation (3), the amount of relative growth needed to achieve a  $\alpha$  % turnover of  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  ( $G_{w/100}$ ) was calculated as:

$$(6) \quad G_{w/100} = \exp\left(\frac{\ln(1 - \alpha/100)}{c}\right).$$

The growth-based half-life ( $G_{0.5}$ ) is obtained when  $\alpha = 50\%$  and represents the amount of growth needed for a 50% conversion between the initial and final isotopic values. The fractions of new tissue derived from growth ( $D_g$ ) and derived from metabolism ( $D_m$ ) were calculated at the midpoint between the old and new isotopic values (Witting et al. 2004):

$$(7) \quad D_g = \frac{2(G_{0.5} - 1)}{G_{0.5}}$$

$$(8) \quad D_m = \frac{(2 - G_{0.5})}{G_{0.5}}$$

However, note that the equations originally published by Witting et al. (2004) were misprinted (D. A. Witting, pers. comm.).

The Fisher-Behren's test statistic was used to test for significant differences in parameter estimates across tissues and between groups (Quinn and Deriso 1999). A t-test was used to test  $c$  against a null hypothesis of  $c = -1$  (Zar 1999).

### *Time-based modeling*

Changes in stable isotope ratios were also modeled as a function of time since the diet switch. The time-based exponential model (Hesslein et al. 1993) describes the isotope value of a fish at time  $t$  ( $\delta_t$ ) as:

$$(9) \quad \delta_t = \delta_f + (\delta_i - \delta_f)e^{-(k'+m)t},$$

where  $\delta_f$  and  $\delta_i$  are as previously defined and  $m$  is the metabolic turnover constant. The group-specific growth rate constant,  $k'$ , was estimated by fitting an exponential growth model to the available data (Ricker 1979):

$$(10) \quad W_f = W_i e^{k't},$$

where  $W_i$  is a fish's initial wet weight and  $W_f$  is the final weight when sampled on day  $t$ . Due to different initial sizes and different growth rates between groups, the  $k'$  parameter was estimated separately for the wild and hatchery groups using nonlinear regression (SAS version 9.1, SAS Institute 2002). In equation (9), if turnover is due to growth alone, then  $m = 0$ . The values of  $\delta_t$ ,  $\delta_i$ , and  $t$  were measured or calculated, and  $\delta_f$  and  $m$  were estimated using nonlinear regression.

Diet-tissue fractionations ( $\pm$  SE) were also derived from time-based model estimates of  $\delta_f$  using equations (4) and (5). The length of time needed to achieve a  $\alpha$  % turnover of  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  was calculated as (Tieszen et al. 1983):

$$(11) \quad T_{\alpha/100} = \frac{\ln(1 - \alpha/100)}{-(k' + m)}.$$

Half life ( $T_{0.5}$ ) is obtained when  $\alpha = 50\%$ .

The relative contributions of growth ( $k'$ ) and metabolism ( $m$ ) were calculated as the ratio of each parameter to the sum of the two parameters. This calculation yields the proportion of turnover attributable to growth ( $P_g$ ) and the proportion attributable to metabolism ( $P_m$ ). The Fisher-Beheren's test statistic was used to test for significant differences in parameter estimates across tissues and between groups (Quinn and Deriso 1999). A t-test was used to test  $m$  against a null hypothesis of  $m = 0$  (Zar 1999). To assess growth patterns of the wild and hatchery populations over the duration of the experiment, specific growth rates ( $k$ ) were calculated for each individual using the exponential growth model, equation (10), by substituting  $k$  for  $k'$  (Ricker 1979).

*Model fitting and selection.*

Growth- and time-based models were fitted to each tissue, for each isotope and for each group. For both types of models, the regression assumption of normality was supported by histograms of the residuals and by Kolmogorov-Smirnoff tests conducted at different levels of the independent variable. Levene's tests and residual plots indicated that homogeneity of variance was not supported for the growth- and time-based models of  $\delta^{15}\text{N}$  for muscle and liver, nor for  $\delta^{13}\text{C}$  turnover in liver. These models were weighted by the inverse of the variance of the response variable as a way to account for the heteroscedasticity in the estimation and fitting procedures (Neter et al. 1990). Due to heteroscedasticity, equation (10) was also fitted by weighted least squares, using the inverse of the  $W_f$  variance (calculated at each time period) as the weighting factor (Neter et al. 1990).

An information-theoretic (IT) approach was used to help assess the importance of metabolism and growth to the turnover process. Both growth- and time-based turnover models were re-parameterized to not allow metabolism to contribute to turnover. In this alternate parameterization, growth alone would be entirely responsible for isotopic turnover within each tissue (i.e.,  $c = -1$  in equation (3);  $m = 0$  in equation (9)). The IT approach evaluates the strength of evidence for competing models and determined whether a model parameterized for growth only performed better than a model which allowed for a metabolic contribution to turnover. Akaike's Information Criterion (corrected for small sample sizes),  $AIC_c$ , was calculated for each of the two competing models according to the equation:

$$(12) \quad AIC_c = n \times \ln\left(\frac{RSS}{n}\right) + 2K + \frac{2K(K+1)}{n-K-1}$$

where  $n$  is the sample size,  $K$  is the number of estimable parameters in the model, and  $RSS$  is the residual sum of squares as determined from the nonlinear regression (Burnham and Anderson 2002).  $AIC_c$  differences ( $\Delta AIC_c$ ) between the two competing models were calculated as:

$$(13) \quad \Delta AIC_c = AIC_i - AIC_{\min}$$

where  $AIC_i$  is the  $AIC_c$  of model  $i$ , and  $AIC_{\min}$  is the lowest  $AIC_c$  of the competing models. The best model, or the model with the most support, will have  $\Delta AIC_c = 0$ . As a general rule of thumb,  $\Delta AIC_c$  values from 0-2 indicate substantial support for the model,  $\Delta AIC_c$  from 4-7 suggest considerably less support, and  $\Delta AIC_c > 10$  indicate essentially no support for that model (Burnham and Anderson 2002).

## RESULTS

### *Growth rates*

Specific growth rates ( $k$ ) varied across individuals, across time, and between groups (Fig. 1). Overall, individual growth rates ranged from  $-0.010$  to  $0.024 \text{ d}^{-1}$ , and only three fish experienced negative growth rates, or a loss of weight. Hatchery fish tended to grow more rapidly than wild fish, exhibiting the highest  $k$  values, particularly in the first 50 days of the experiment. Group-specific growth rates ( $k'$ ) reflected this difference between groups with  $k'$  values ( $\pm$  SE) of  $8.16 \times 10^{-3} \pm 1.65 \times 10^{-4} \text{ d}^{-1}$  and  $6.51 \times 10^{-3} \pm 2.51 \times 10^{-4} \text{ d}^{-1}$  for hatchery and wild groups, respectively. Growth rates tended to increase initially in both groups before leveling off and then decreasing slightly towards the end of the experiment. The decrease in growth rates was particularly evident in the relative growth ( $W_R$ ) trends of wild fish over time (Fig. 2). Declines in individual growth rates corresponded with reduced consumption rates that were observed during the second half of the experiment, particularly for the wild fish.

### *Isotopic turnover and fractionation*

Turnover of both C and N isotopes was clearly evident in all tissues over the duration of the experiment. Isotope values of the experimental krill diet were between 5.9 and 11.9‰ removed from the mean initial isotopic signatures of the wild and hatchery fish tissues (Table 2). Initial  $\delta^{13}\text{C}$  values of hatchery and wild fish were similar,

but hatchery fish exhibited more depleted  $\delta^{15}\text{N}$  values due to the different diets on which each group was raised.

#### *Turnover and fractionation of $\delta^{13}\text{C}$*

Isotopic turnover of  $\delta^{13}\text{C}$  in summer flounder tissues was well represented by both growth- and time-based models. For a given group, turnover was most rapid in liver followed by blood then muscle, as indicated by the more negative values of  $c$  (growth-based model, Table 3) and higher values of  $m$  (time-based model, Table 4) relative to the other tissues. However, the magnitude of these parameters for liver and blood were similar, translating into small differences in isotopic trajectories by growth (Fig. 3A, 3B) or over time (Fig. 4A, 4B).

Half lives for  $\delta^{13}\text{C}$  in liver, blood, and muscle tissue were estimated as 21, 23, and 69 d for hatchery fish, and 16, 20, and 49 days for wild fish, respectively (Table 4). A 95% isotopic turnover in liver and blood could occur in as little as 69 or 89 days, whereas almost complete turnover in muscle could take up to 298 days. In terms of growth, a 50% turnover was reached in liver, blood, and muscle tissues when hatchery fish had grown 1.25, 1.37, and 3.04 times their initial size (Table 3). Wild fish needed to grow 1.11, 1.18, and 2.13 times their initial size to attain 50% turnover in liver, blood, and muscle tissues, respectively.

For blood and liver tissues, estimates of the asymptotic isotopic value,  $\delta_f$ , were relatively consistent between modeling methods and between experimental groups. Differences in  $\delta_f$  estimates between the time-based and growth-based models were relatively small (<0.6‰) for blood and liver, although the growth-based estimates tended

to be lower than the time-based estimates (Tables 3, 4). For these two tissues in any given model,  $\delta_f$  estimates between wild and hatchery groups differed by less than 1‰. Unlike liver and blood tissues, there was a high degree of variability in  $\delta_f$  estimates for muscle tissue, with differences up to ~3‰ between growth- and time-based models. Also, differences in  $\delta_f$  estimates between H and W groups were about 1.7‰ for each modeling method. The uncertainty in these estimates was reflected in the higher SE of these parameters.

Fractionation estimates for  $\delta^{13}\text{C}$  varied by tissue, group and by the model used. Liver fractionation values were the most consistent ranging from 2.8 to 3.1‰ (Tables 3, 4). Fractionation estimates for blood ranged from 2.4-3.4‰, while muscle fractionation varied from 0.1-4.8‰. Due to  $\delta_f$  values that were consistently more depleted in the hatchery group, the fractionation estimates for this group were always lower than for the wild group.

#### *Turnover and fractionation of $\delta^{15}\text{N}$*

Turnover rates of  $\delta^{15}\text{N}$  reflected the same general patterns seen for  $\delta^{13}\text{C}$  with liver rates being the fastest (Figs. 3A, 3B, 4A, 4B). Values for  $m$  were approximately one order of magnitude greater for the liver than for the other tissues (Table 4). Half life was about 10 days for liver compared to approximately 38 and 70 days for blood and muscle, respectively. Growth-based estimates of  $c$  also indicated a much more rapid turnover in liver with a doubling in weight resulting in approximately a 95% turnover to the new krill diet (Table 3). Turnover rates of  $\delta^{15}\text{N}$  for blood were intermediate between liver and muscle, with muscle tissue consistently responding the slowest to the dietary change.

Muscle tissue required over 290 days (Table 4) or over a 14-fold increase in weight (Table 3) to attain 95% of  $\delta^{15}\text{N}$  turnover, given the observed growth rates.

Estimates of  $\delta_f$  for liver were similar between time- and growth-based models, but differences between models reached 1.1-1.3‰ for blood and muscle. Differences between W and H groups (for a given tissue and method) varied, but were greatest for blood (1.6-1.7‰). Again, the magnitude of the difference between group or method estimates of  $\delta_f$  seemed to be related to the SE of the parameter estimates.

Fractionation estimates for  $\delta^{15}\text{N}$  were more variable than the estimates for  $\delta^{13}\text{C}$ . Depending on the model used and the experimental group, fractionation estimates for liver, blood, and muscle varied from 1.5-2.3‰, 1.1-3.9‰, and 2.4-4.2‰, respectively (Tables 3, 4). Fractionations of  $\delta^{15}\text{N}$  also exhibited a group-related trend with higher estimates for the wild group, due to consistently higher  $\delta_f$  values relative to the hatchery group.

#### *Metabolic contributions to turnover*

The relative importance of metabolism and growth varied by tissue, as indicated by several analytical techniques. For liver and blood, models that were parameterized to include metabolism were more supported by the data, yielding much lower  $\text{AIC}_c$  values (Tables 5, 6). The very large  $\Delta\text{AIC}_c$  of the competing model with no metabolism suggest that there is practically no evidence that growth is solely responsible for isotopic turnover in these tissues. The importance of metabolic processes to turnover in liver and blood was further indicated by the estimates of  $m$  and  $c$  being significantly different from 0 or -1, respectively (Tables 3, 4). According to the growth-based model, metabolism

accounted for over 45% of  $\delta^{13}\text{C}$  turnover in blood while metabolic contributions reached 80% for liver  $\delta^{13}\text{C}$  turnover (Table 3). Metabolic contributions to  $\delta^{15}\text{N}$  turnover remained comparable, except for blood tissues of hatchery fish which appeared to be predominantly growth-driven. Time-based models generated slightly greater estimates of metabolic contributions to isotopic turnover in blood and liver with values generally over 75% (Table 4).

In contrast to liver and blood, isotopic turnover of muscle tissue was primarily driven by the growth-related process of dilution. Values of  $\Delta\text{AIC}_c$  indicated that models accounting for growth alone performed better than, or nearly as well as, models which allowed for metabolism, with only two exceptions (Tables 5, 6). For growth-based models, no estimates of  $c$  were significantly less than -1 and growth alone accounted for over 90% of isotopic turnover in muscle (Table 3). Accordingly, time-based model estimates of  $m$  tended to be not significantly different from 0, with metabolism responsible for lower percentages of total turnover in muscle relative to blood and liver tissues (muscle  $P_m$ : 19-54%, blood and liver  $P_m$ : 48-90%; Table 4).

## DISCUSSION

### *Turnover and fractionation in tissues*

Following the experimental dietary shift, both C and N stable isotope trajectories approached the dietary signal, and were well-described by the turnover models. Tissue-specific turnover rates translated into variable amounts of time needed for tissues to reflect the new dietary material. This lends additional credence to criticisms that caution against assuming that predators are in isotopic equilibrium with their prey in field settings (Gannes et al. 1997), particularly when dealing with migratory species, or species whose diet exhibits a large amount of seasonal variability. Both of these scenarios pertain to summer flounder, confirming the utility of this laboratory study. With the observed growth rates, it was estimated that summer flounder muscle tissue may require over 300 days to be almost completely equilibrated (95% turnover) with a new diet, while liver and blood require as little as 40 and 89 days, respectively. Consequently, muscle tissue of wild summer flounder that exhibit similar growth rates to those in this study may never be fully equilibrated to their diets if the isotopic values of their prey vary through the course of their seasonal spawning migrations.

Differences in turnover rates among tissues were driven by variable metabolic contributions to the turnover process. Although the metabolic component in the turnover models incorporates all turnover processes aside from growth, protein synthesis and degradation rates in fish livers have been shown to be 1-2 orders of magnitude greater

than in muscle tissues (Smith 1981, Houlihan et al. 1988, de la Higuera et al. 1999). In birds, blood plasma proteins, synthesized in the liver, also have high turnover rates that translate into short half-lives of isotopes relative to muscle tissue (Hobson and Clark 1992a, Hobson and Clark 1993). Although some studies have shown little or no difference in isotopic turnover rates between liver and muscle of fishes (Hesslein et al. 1993, Sweeting et al. 2005, McIntyre and Flecker 2006), the majority of research on fishes supports our findings of faster liver and blood turnover relative to muscle (MacAvoy et al. 2001, Suzuki et al. 2005, Watanabe et al. 2005, Logan et al. 2006, MacNeil et al. 2006, Guelinckx et al. 2007). We found metabolic contributions up to 81% and 90% for blood and liver, comparable to the 73% and 83% contributions seen for ocellate stingrays (MacNeil et al. 2006). Isotopic turnover in muscle tissue on the other hand was predominantly mediated by growth and simple dilution, as shown for most fishes (e.g., Hesslein et al. 1993, Herzka and Holt 2000).

Within a given tissue, relative contributions of growth and metabolism to isotopic turnover differed for C and N. Most notably, turnover of  $\delta^{13}\text{C}$  was approximately twice as fast as the turnover of  $\delta^{15}\text{N}$  in blood tissues. Isotope-specific turnover rates in fish have been reported elsewhere, with turnover of C more rapid than N in tissues as seen in our study (MacAvoy et al. 2001, Suzuki et al. 2005, Guelinckx et al. 2007). Differences in turnover of isotopes within a tissue may depend on the proximate composition of that tissue, and the catabolic processes operating on each biochemical constituent of the tissue. For example, Hobson and Clark (1993) found that cellular and plasma fractions of bird blood differed dramatically with regard to  $\delta^{13}\text{C}$  turnover rates due to different metabolic activities. Just as turnover rates of a single isotope vary within the fractions of

the blood, turnover of C and N isotopes could also vary as a function of different physiological processes operating on each isotope pool. The specific mechanisms driving isotopic turnover within each tissue are still poorly understood, and our results confirm the need for considering  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  individually.

Diet-tissue fractionations varied among tissues and between isotopes, consistent with previous studies. For muscle tissue, our fractionation estimates ( $\Delta^{15}\text{N}$ : 2.4-4.2‰;  $\Delta^{13}\text{C}$ : 0.1-4.8‰) conformed to the wide range of values reported in the literature ( $\Delta^{15}\text{N}$ : ~-1 to 6‰,  $\Delta^{13}\text{C}$ : -3 to 4‰; Post 2002, McCutchan et al. 2003, Sweeting et al. 2007). However, muscle  $\Delta^{13}\text{C}$  estimates were greater than the traditionally assumed values of 0-1‰. Relatively large standard errors in muscle fractionation estimates likely contributed to this discrepancy and also to the lack of consistency in estimates between experimental groups and modeling methods. More precise fractionation estimates could have been obtained if isotopic equilibrium had been reached in blood and muscle tissues, but logistical constraints prevented extension of the experiment by the 4-6 months that would have been required for this to occur.

Few studies have directly estimated fractionations for liver and blood in fishes. Liver estimates for  $\Delta^{15}\text{N}$  ranged from 0-3.5‰ in two studies on the European sea bass (Sweeting et al. 2007) and the salt marsh mummichog (Logan et al. 2006), corresponding with our estimates of 1.5-2.3‰. Watanabe et al. (2005) demonstrated a fractionation of approximately -0.3‰ for  $\Delta^{13}\text{C}$  of Japanese flounder liver, which was considerably lower than our estimates of 2.8-3.1‰. Our estimates for liver (lipid-normalized) and blood fractionation contribute to the few studies available on these tissues. To our knowledge, our results include the first explicit estimates of isotopic fractionation of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$

for fish blood. The tissue-specific fractionations we report are influenced by the different biochemical constituents of the tissues (e.g., amino acids, proteins, lipids), which have been shown to differ in their own fractionations (DeNiro and Epstein 1977, Macko et al. 1987, McClelland and Montoya 2002). Also, dietary components when assimilated are not necessarily allocated equally across all tissues, contributing to tissue-specific fractionations (Gannes et al. 1997).

Overall, each of the experimental groups (wild and hatchery) yielded similar relative conclusions regarding tissue turnover rates, but rates were consistently higher for wild fish (despite slower growth) and fractionations were consistently lower for hatchery fish. These patterns could be related to a suite of factors, including differences in initial sizes, growth rates, maturity schedules, physiology (Graham and Farrell 1992), environmental histories, and genetic diversity. The differences between groups and the potential influence of these factors are reminders that natural populations of summer flounder likely exhibit variability in both isotopic turnover and fractionation. In light of the variability between hatchery and wild fish, it may be more prudent for field studies on summer flounder to rely on parameter estimates derived from the wild group, but we acknowledge the possibility that isotope dynamics may differ between laboratory and field settings (Vander Zanden and Rasmussen 2001).

Both time- and growth-based turnover models captured the patterns of isotopic changes experienced by the experimental populations, supporting the mechanistic foundations of the models in which somatic growth and metabolic tissue replacement drive isotopic turnover (Fry and Arnold 1982, Hesslein et al. 1993). However, the time-based model relies on a single growth parameter value intended to be representative of

the entire population, and it is assumed to be constant through time (Tarboush et al. 2006). Summer flounder in our study experienced large variability in individual growth rates and irregular growth trajectories. This variability in growth rates of individuals likely contributed to a large portion of the residual error in the time-based model. Not surprisingly, time-based model residuals plotted against individual growth rates consistently showed a slight negative relationship, indicating that faster growing fish experienced greater turnover after a given amount of time than slower individuals.

Relative to the growth-based model, time-based model results may not be as applicable to situations that deviate from the experimental conditions. Time-based turnover rates have been shown to differ by temperature and feeding rates, due to the different growth rates elicited by these environmental conditions (Bosley et al. 2002, Witting et al. 2004, Watanabe et al. 2005). Turnover rates derived from growth-based models, on the other hand, do not drastically suffer from these problems because any variability in growth rates are inherently accounted for by the relative growth variable,  $W_R$  (Bosley et al. 2002, Witting et al. 2004, Watanabe et al. 2005). Consequently, turnover parameters derived from growth-based models may be more suitable in field conditions where temperatures or growth rates differ from the laboratory setup from which results were generated.

#### *Application to field studies*

Knowledge of tissue-specific turnover rates will enable researchers to select tissues suitable for the time-scale of the inferences to be made in field studies utilizing stable isotopes. To avoid making an erroneous assumption that a species is in isotopic

equilibrium with its diet, it is important to be able to predict approximately how long it takes for different tissues to equilibrate to the isotopic signature of the organism's diet. The half-lives calculated from the time-based turnover models may be inadequate for such predictions if field conditions (and consequently growth rates) differ from the laboratory experiment, as described above. Here, we propose a slight reformulation of the growth-based turnover model, equation (3), to translate turnover rate constants to the more tangible unit of time for field applications.

Growth can be adequately described with an exponential model (equation (10); Ricker 1979), and this can be substituted for  $W_R$  in the growth-based model, equation (3), yielding:

$$(14) \quad \delta_{WR} = \delta_f + (\delta_i - \delta_f)e^{ktc}$$

For a given percentage of complete turnover ( $\alpha$ ),  $\delta_{WR}$  can be re-written as

$$(15) \quad \delta_{WR} = \frac{\alpha}{100}(\delta_f - \delta_i) + \delta_i$$

For example, a 50% turnover between the initial and final isotope values would equate to  $\alpha = 50\%$  and  $\delta_{WR}$  would be half way between  $\delta_i$  and  $\delta_f$ . After substituting equation (15) into equation (14) and rearranging the terms, the following model can be obtained:

$$(16) \quad t_{\alpha/100} = \frac{\ln(1 - \alpha/100)}{kc}$$

where  $t_{\alpha/100}$  is the time needed for  $\alpha\%$  of turnover to be completed. The growth-based model fits presented in this paper provide the turnover rate constants,  $c$ , for three summer flounder tissues. Assuming that  $c$  is relatively constant across different specific growth rates ( $k$ ), then the time needed for a given amount of turnover to occur (for a specified growth rate) can be modeled using equation (16).

Plotting this model over a range of realistic growth rates for juvenile summer flounder (Rountree and Able 1992) using turnover estimates of  $\delta^{15}\text{N}$  (Table 3) emphasizes the differences in time scales represented by various tissues (Fig. 5). For example, given a growth rate of  $0.01\text{ d}^{-1}$ , muscle tissue requires approximately 60 days for 50% turnover, whereas liver tissue will have turned over 90% in the same amount of time. Differences in turnover times among tissues are greatest at lower growth rates, suggesting that tissue selection is particularly important for researchers working on slower growing animals. This model also highlights the important role that growth rates have on mediating the time that is necessary for isotopic equilibrium to be achieved.

The assumption that  $c$  is constant across different growth rates was supported by the lack of a relationship between growth-based  $\delta^{15}\text{N}$  model residuals and individual growth rates,  $k$  (ranging from approximately  $0 - 0.024\text{ d}^{-1}$ ). Also, work on larval and juvenile flatfish did not reveal significant differences in  $c$  between temperature treatments, which altered growth rates (Bosley et al. 2002, Witting et al. 2004). We did discover a slight negative relationship between growth-based model  $\delta^{13}\text{C}$  residuals and individual  $k$ , suggesting that consistency of  $c$  across growth rates may vary by isotope.

To our knowledge, no studies have directly examined the relationship between individual growth rates or temperature on the consistency of growth-based turnover estimates. Research in this area may provide crucial information to address applicability of laboratory turnover rates and equation (16) to the field. However, until more work is conducted, this model may be a useful tool for researchers that are unable to conduct the necessary laboratory studies prior to a field-based investigation using stable isotopes. Using equation (16), researchers can estimate expected turnover times given information

on the growth rate and turnover rate constants (known or assumed) for their species of interest. These estimates will allow researchers to better evaluate whether certain tissues will be in isotopic equilibrium with sampled prey in the field.

### *Conclusion*

In summary, increased metabolic activities in liver and blood of summer flounder appear to be driving faster isotopic turnover rates in these tissues relative to muscle. Specifically turnover rates of tissues were ranked: liver>blood>muscle. However, the degree to which these tissues differ depends on the isotope in question. The estimates of tissue-specific turnover rate and fractionation will aid field-based studies of summer flounder by allowing researchers to evaluate the validity of assuming that the fish are in isotopic equilibrium with their prey. A generalized model for predicting the time necessary for a certain percentage of turnover (given growth and turnover rate parameters) is presented to aid researchers. Continued research on isotope dynamics in fish tissues and the factors mediating turnover rates will help reduce errors in field applications of stable isotopes. Knowledge of tissue-specific turnover rates and fractionations will allow researchers to select from a suite of alternative tissues to answer scientific questions operating on a variety of different time-scales. Use of tissues with faster turnover rates may be of particular benefit to research focusing on highly migratory fishes or species whose diets exhibit a high degree of seasonal variability, as is the case for summer flounder.

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**Table 1.** Initial lengths and weights of hatchery (H) and wild (W) summer flounder sampled for stable isotope analysis.

Group	n	Total length (mm)				Wet weight (g)			
		Mean	SD	Min	Max	Mean	SD	Min	Max
H	66	176	19	130	230	58.8	18.3	26.3	124.9
W	48	215	25	131	255	102.0	33.2	29.3	185.3

**Table 2.** Mean initial isotopic values and standard deviations (SD) for tissues of hatchery (H) and wild (W) summer flounder and for the experimental krill diet.

Group	Tissue	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$	
		Mean	SD	Mean	SD
H	Blood	-18.08	0.16	10.60	0.42
	Liver	-16.84	0.07	9.59	0.36
	Muscle	-17.48	0.12	12.01	0.15
W	Blood	-18.16	0.38	14.49	0.60
	Liver	-16.35	0.42	14.51	0.21
	Muscle	-17.48	0.37	15.55	0.57
Krill	Whole	-27.18	0.87	3.71	0.48

**Table 3.** Parameter estimates and calculations for the growth-based model of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  turnover in summer flounder tissues. Abbreviations and parameter estimates are as follows: H – hatchery fish; W – wild fish;  $\delta_f$  – final, asymptotic isotopic value (‰); c – turnover constant;  $\Delta$  – fractionation between krill diet and each tissue (‰);  $G_{0.5}$  – growth-based half life;  $G_{0.95}$  – amount of relative growth needed to reach 95% turnover;  $D_g$  – proportion of turnover attributed to growth;  $D_m$  – proportion of turnover attributed to metabolism;  $F_g$  – fraction of complete turnover attained by the end of the experiment, based on growth. Turnover constants, c, that are significantly different from -1 are marked with a \*.

Isotope	Tissue	Group	$\delta_f$		c		$\Delta$		$G_{0.5}$	$G_{0.95}$	$D_g$	$D_m$	$F_g$
			Est.	SE	Est.	SE	Est.	SE					
$\delta^{13}\text{C}$	Liver	H	-24.42	0.04	-3.095*	0.161	2.8	0.1	1.25	2.6	0.40	0.60	0.99
		W	-24.13	0.09	-6.532*	0.859	3.1	0.1	1.11	1.6	0.20	0.80	1.00
	Blood	H	-24.84	0.27	-2.204*	0.265	2.4	0.3	1.37	3.9	0.54	0.46	0.97
		W	-23.91	0.28	-4.123*	0.674	3.3	0.3	1.18	2.1	0.31	0.69	1.00
	Muscle	H	-27.07	1.26	-0.624*	0.119	0.1	1.3	3.04	121.9	1.34	-0.34	0.63
		W	-25.23	1.15	-0.919	0.202	2.0	1.1	2.13	26.0	1.06	-0.06	0.69
$\delta^{15}\text{N}$	Liver	H	5.41	0.10	-4.023*	0.854	1.7	0.1	1.19	2.1	0.32	0.68	1.00
		W	5.99	0.02	-7.319*	0.943	2.3	0.0	1.10	1.5	0.18	0.82	1.00
	Blood	H	4.82	0.45	-1.093	0.161	1.1	0.5	1.89	15.5	0.94	0.06	0.82
		W	6.51	0.49	-2.085*	0.283	2.8	0.5	1.39	4.2	0.57	0.43	0.93
	Muscle	H	6.10	0.48	-0.939	0.158	2.4	0.5	2.09	24.3	1.04	-0.04	0.77
		W	6.64	0.79	-1.121	0.213	2.9	0.8	1.86	14.5	0.92	0.08	0.77

**Table 4.** Parameter estimates and calculations for the time-based model of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  turnover in summer flounder tissues.

Abbreviations and parameter estimates are as follows: H – hatchery fish; W – wild fish;  $\delta_f$  – final, asymptotic isotopic value (‰); m – metabolic turnover constant ( $\text{d}^{-1}$ );  $\Delta$  – fractionation between krill diet and each tissue (‰);  $k'$  – group-specific growth rate ( $\text{d}^{-1}$ );  $T_{0.5}$  – time-based half life (d);  $T_{0.95}$  – time needed to reach 95% turnover (d);  $P_g$  – proportion of turnover attributed to growth;  $P_m$  – proportion of turnover attributed to metabolism;  $F_t$  – fraction of complete turnover attained by the end of the experiment, based on time. Metabolic turnover constants, m, that are significantly different from 0 are marked with a \*.

Isotope	Tissue	Group	$\delta_f$		m		$\Delta$		$k'$	$T_{0.5}$	$T_{0.95}$	$P_g$	$P_m$	$F_t$
			Est.	SE	Est.	SE	Est.	SE						
$\delta^{13}\text{C}$	Liver	H	-24.36	0.04	0.0255*	0.0016	2.8	0.1	0.00816	21	89	0.24	0.76	1.00
		W	-24.26	0.07	0.0370*	0.0033	2.9	0.1	0.00651	16	69	0.15	0.85	1.00
	Blood	H	-24.26	0.21	0.0218*	0.0039	2.9	0.2	0.00816	23	100	0.27	0.73	1.00
		W	-23.77	0.22	0.0273*	0.0047	3.4	0.2	0.00651	20	89	0.19	0.81	1.00
	Muscle	H	-24.08	0.46	0.0019	0.0014	3.1	0.5	0.00816	69	298	0.81	0.19	0.84
		W	-22.39	0.39	0.0075*	0.0026	4.8	0.4	0.00651	49	213	0.46	0.54	0.92
$\delta^{15}\text{N}$	Liver	H	5.18	0.10	0.0668*	0.0083	1.5	0.1	0.00816	9	40	0.11	0.89	1.00
		W	5.91	0.08	0.0581*	0.0063	2.2	0.1	0.00651	11	46	0.10	0.90	1.00
	Blood	H	5.97	0.29	0.0076*	0.0027	2.3	0.3	0.00816	44	190	0.52	0.48	0.94
		W	7.57	0.31	0.0145*	0.0027	3.9	0.3	0.00651	33	143	0.31	0.69	0.98
	Muscle	H	6.52	0.47	0.0021	0.0019	2.8	0.5	0.00816	67	291	0.79	0.21	0.84
		W	7.91	1.04	0.0030	0.0021	4.2	1.0	0.00651	73	315	0.68	0.32	0.82

**Table 5.** Comparisons of  $AIC_c$  between growth-based turnover models that either allow for a metabolic contribution to isotopic turnover (model 1) or do not allow for metabolism (model 2). Abbreviations and symbols are as follows: H – hatchery fish; W – wild fish;  $AIC_c$  – Akaike’s Information Criterion corrected for small sample sizes;  $\Delta AIC_c$  –  $AIC_c$  differences. The best of the two models for a given isotope, tissue, and group is represented by the lowest  $AIC_c$  or  $\Delta AIC_c = 0$ .

Isotope	Tissue	Group	n	$AIC_c$		$\Delta AIC_c$	
				Model 1	Model 2	Model 1	Model 2
$\delta^{13}C$	Liver	H	58	-9.0	127.7	0.00	136.73
		W	40	-7.6	79.2	0.00	86.77
	Blood	H	58	-20.8	1.1	0.00	21.94
		W	41	-2.0	25.6	0.00	27.58
	Muscle	H	58	-89.0	-82.7	0.00	6.31
		W	41	-64.4	-66.6	2.17	0.00
$\delta^{15}N$	Liver	H	58	5.9	35.3	0.00	29.37
		W	40	-1.7	128.8	0.00	130.53
	Blood	H	58	-88.3	-90.2	1.87	0.00
		W	41	-23.0	-9.6	0.00	13.42
	Muscle	H	58	35.8	33.8	2.05	0.00
		W	41	0.6	-1.4	1.95	0.00

**Table 6.** Comparisons of  $AIC_c$  between time-based turnover models that either allow for a metabolic contribution to isotopic turnover (model 1) or do not allow for metabolism (model 2). Abbreviations and symbols are as follows: H – hatchery fish; W – wild fish;  $AIC_c$  – Akaike’s Information Criterion corrected for small sample sizes;  $\Delta AIC_c$  –  $AIC_c$  differences. The best of the two models for a given isotope, tissue, and group is represented by the lowest  $AIC_c$  or  $\Delta AIC_c = 0$ .

Isotope	Tissue	Group	n	$AIC_c$		$\Delta AIC_c$	
				Model 1	Model 2	Model 1	Model 2
$\delta^{13}C$	Liver	H	59	10.3	166.0	0.00	155.76
		W	42	13.0	112.8	0.00	99.73
	Blood	H	59	4.6	47.6	0.00	42.97
		W	44	-6.3	43.5	0.00	49.80
	Muscle	H	59	-56.7	-57.0	0.38	0.00
		W	44	-26.0	-16.7	0.00	9.29
$\delta^{15}N$	Liver	H	59	45.6	144.9	0.00	99.29
		W	42	16.6	164.0	0.00	147.47
	Blood	H	59	-26.7	-18.6	0.00	8.06
		W	44	-4.3	31.0	0.00	35.29
	Muscle	H	59	35.4	34.8	0.61	0.00
		W	43	15.7	15.9	0.00	0.20

**Fig. 1.** Specific growth rates calculated for individual summer flounder for both hatchery (open triangles) and wild (filled circles) groups. Estimated group-specific growth rates ( $k'$ ) for hatchery ( $8.16 \times 10^{-3} \text{ d}^{-1}$ ) and wild ( $6.51 \times 10^{-3} \text{ d}^{-1}$ ) groups represented by dashed and solid lines, respectively.

**Fig. 2.** Relative growth ( $W_R$ ) for individual summer flounder from both hatchery (open triangles) and wild (filled circles) groups. Estimated growth trajectories for each group were based on group-specific growth rates,  $k'$ , (hatchery group – dashed line; wild group – solid line).

**Fig. 3.** Changes in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of summer flounder tissues as a function of relative growth ( $W_R$ ) after a diet switch. Circles denote data for wild fish (A, C) and triangles represent hatchery fish (B, D). Data and growth-based model fits are shown for muscle (white symbols, dotted line), blood (gray symbols, dashed line), and liver (black symbols, solid line). The straight, dashed line indicates the mean value for the krill diet.

**Fig. 4.** Changes in mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of summer flounder tissues as a function of time (days) since the diet switch. Circles denote data for wild fish (A, C) and triangles represent hatchery fish (B, D). Data (mean  $\pm$  SD) and time-based model fits are shown for muscle (white symbols, dotted line), blood (gray symbols, dashed line), and liver (black symbols, solid line). The straight, dashed line indicates the mean for the krill diet.

**Fig. 5.** Projected time to 50% (A) and 90% (B) turnover of  $\delta^{15}\text{N}$  for summer flounder muscle (dotted line), blood (dashed line), and liver (solid line), based on equation (16) and estimated c parameters for wild fish.

Figure 1.

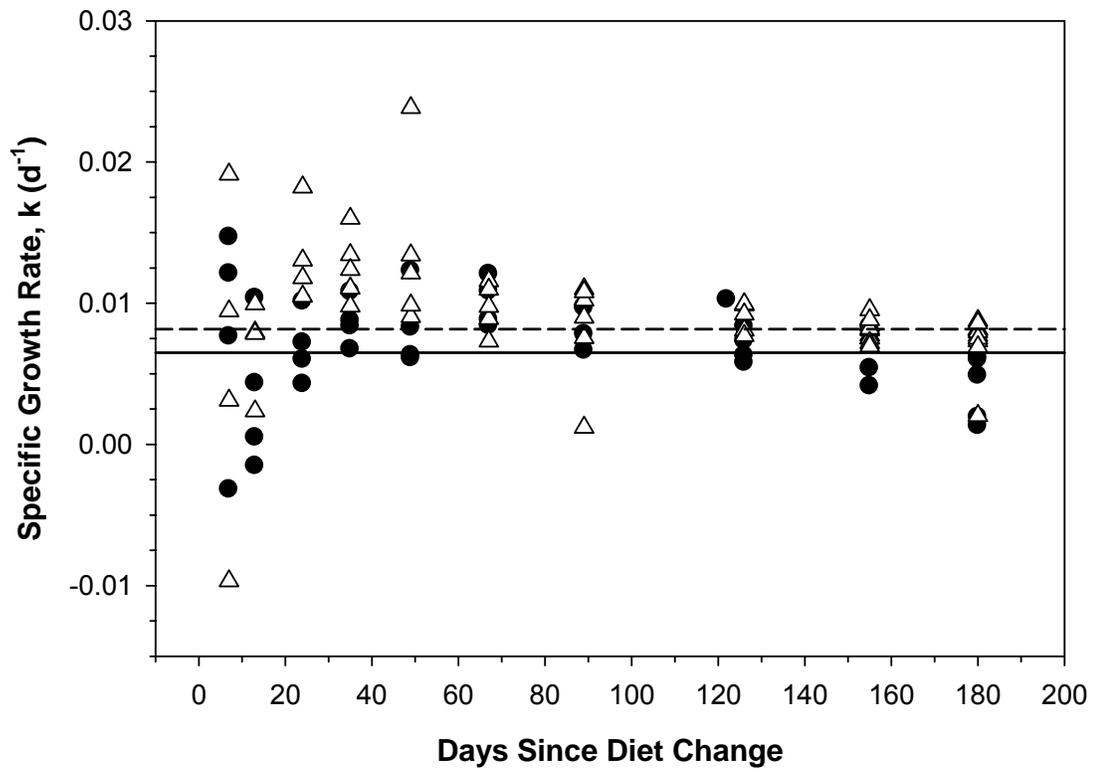


Figure 2.

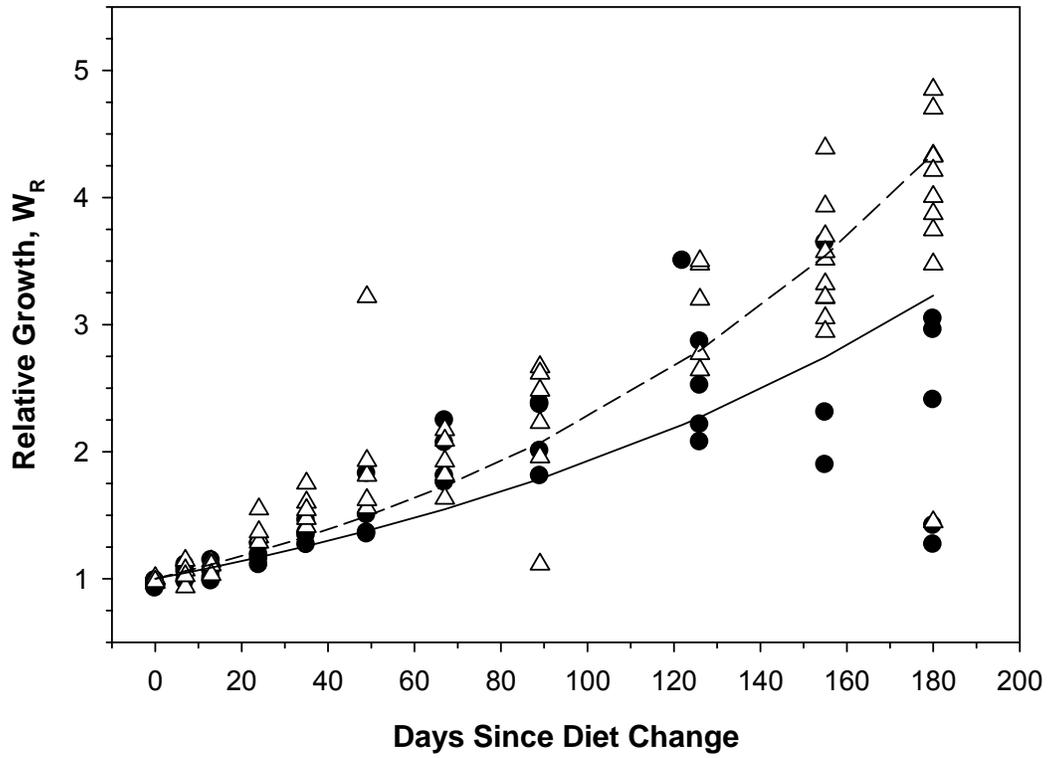


Figure 3.

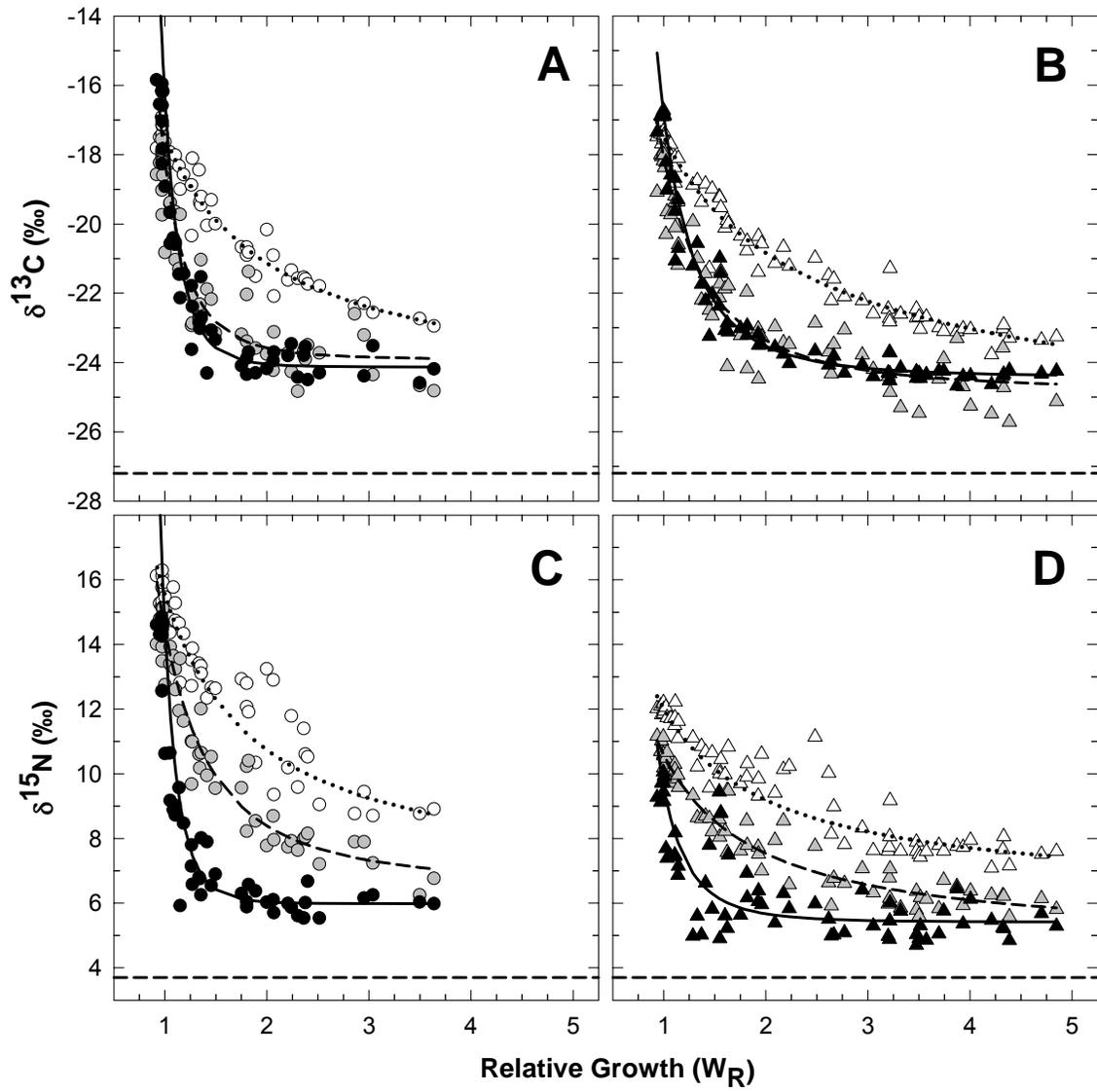
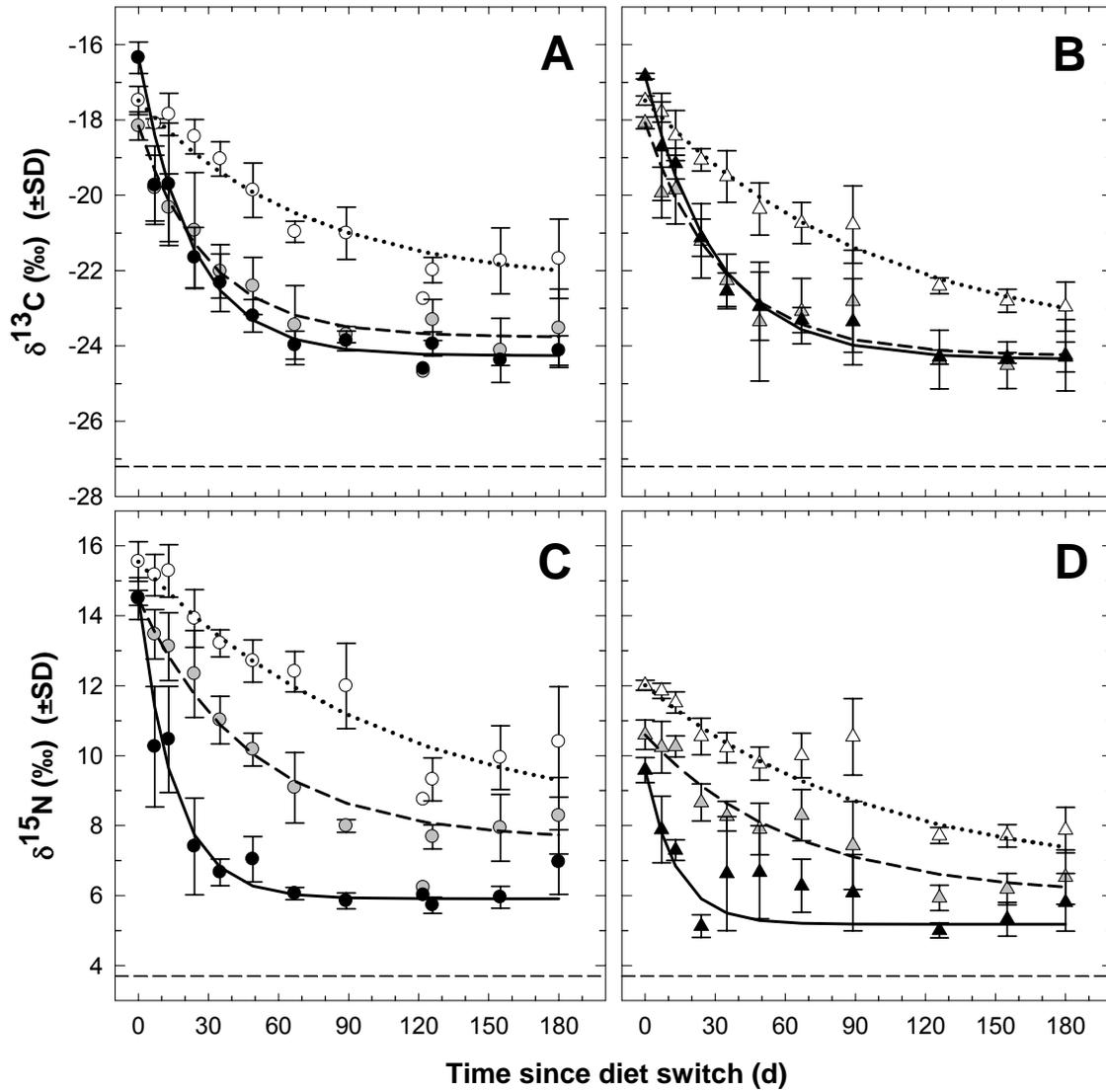
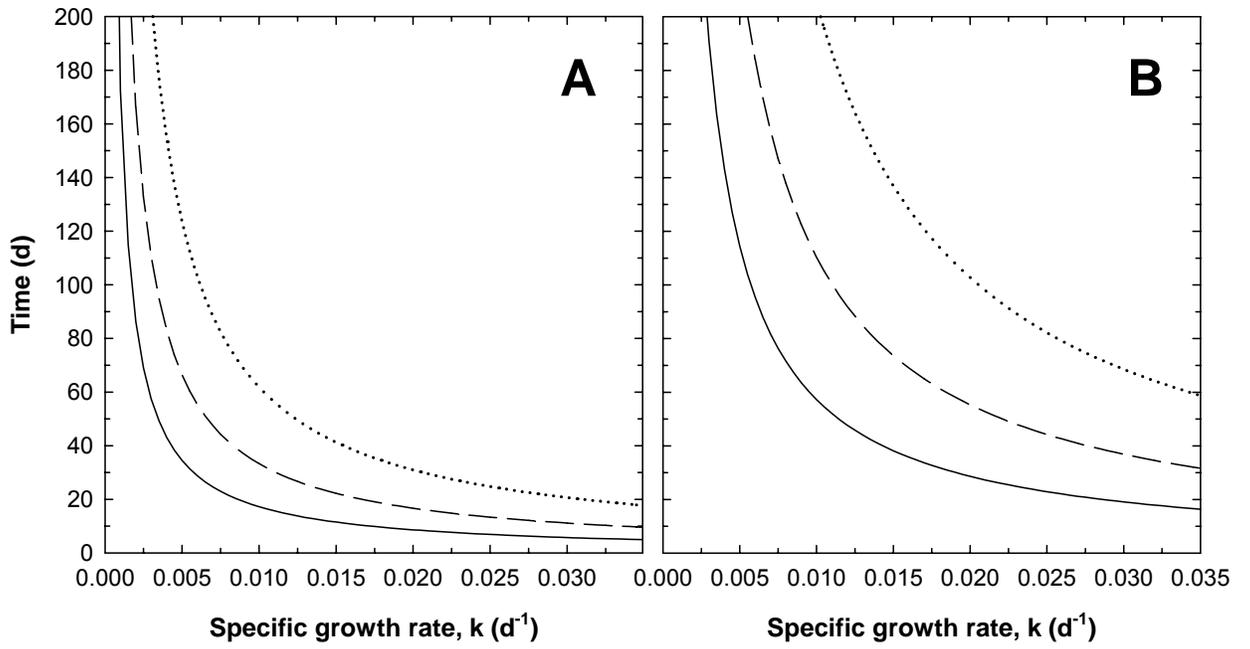


Figure 4.



**Figure 5.**



CHAPTER 2

STABLE ISOTOPE ANALYSIS OF SUMMER FLOUNDER DIETS IN  
CHESAPEAKE BAY

## INTRODUCTION

The summer flounder, *Paralichthys dentatus*, is an important commercially and recreationally fished species within the Northwest Atlantic Ocean. As a valued economic resource found from Nova Scotia to Florida, summer flounder receives a lot of management attention (Terceiro 2002). As managers attempt to move towards more ecosystem-based approaches to fisheries management, accurate understanding of trophic interactions for this (and other) species is needed (Latour et al. 2003). Although stomach content analyses have generally been used to elucidate trophic relationships, stable isotopes have emerged as a valuable tool to compliment these traditional techniques.

Dietary studies involving stable isotope ratios (e.g.,  $\delta^{15}\text{N}$ , or the ratio of  $^{15}\text{N}$  to  $^{14}\text{N}$  relative to a standard) rely on the fact that an organism's tissue will be derived from its prey, consequently reflecting the isotopic signatures of the consumed prey. The rate at which prey are assimilated into the consumer's tissue generally depends on the growth rate of the consumer and the metabolic activity of its tissues (Hobson and Clark 1992, Hesslein et al. 1993). As a result, the consumer's tissue will be a time-integrated dietary representation on a scale of weeks to months, unlike stomach contents that reflect feeding on the order of hours. Although stomach content analysis generally provides information with greater taxonomic resolution, prey types that are quickly digested and evacuated may be under-represented with this method (Hyslop 1980). Additionally, fish with empty stomachs contribute no taxonomic information to the characterization of its diet.

Within the Chesapeake Bay mainstem, stomach content analyses revealed that summer flounder consume primarily small crustaceans (particularly mysid shrimp, sand shrimp, and mantis shrimp) and small fishes (bay anchovy, weakfish, Atlantic croaker) (Latour et al. 2008). With increasing size, the trophic breadth of the diet also increases by including a greater proportion of fishes and larger prey. Diets were also shown to vary significantly with season, reflecting seasonal fluctuations in presence and abundance of different prey groups (Latour et al. 2008). Despite this level of dietary information, it remains unclear how each prey group contributes to actual somatic growth. Stable isotopes can complement these stomach content assessments by providing additional time-integrated information about which prey groups are most assimilated and thus most responsible for driving summer flounder growth and production.

The Chesapeake Bay is one of the primary nursery habitats for juvenile summer flounder, responsible for rearing a large fraction of new recruits to the total coastal population (Able and Kaiser 1994). Summer flounder larvae, advected from offshore spawning waters, settle and metamorphose in the estuarine waters from November to April (Olney and Boehlert 1988). Food web productivity of Chesapeake Bay and other estuaries facilitates fast growth during spring and summer, allowing individuals to attain 230-300 mm total length by the fall, with many reaching sexual maturity in this time (Able et al. 1990, Szedlmayer et al. 1992, Bonzek et al. 2007). Adults also utilize Chesapeake Bay habitats from spring to fall after migrating from the offshore spawning regions (Murphy et al. 1997). The reliance of both juveniles and adults on estuarine food sources emphasizes the vital role Chesapeake Bay plays in regulating growth and

productivity of summer flounder and the importance of understanding trophic interactions in the Bay.

Although often not addressed, isotopic evaluations of diet commonly assume that isotopic signatures of prey groups remain relatively consistent. But, temporal or spatial variability in prey isotopic signatures can confound diet assessments, impairing the conclusions drawn. Within Chesapeake Bay,  $\delta^{15}\text{N}$  of primary producers and lower trophic levels has been shown to vary over space and time (Montoya et al. 1990, Hagy 2002), partly due to differences in the biogeochemical cycling of nitrogen (N) and the relative importance of nitrification and denitrification (Horrigan et al. 1990). Additionally,  $\delta^{13}\text{C}$  tends to exhibit regional variability along the salinity gradient of the mainstem owing to the relative importance of depleted carbon (C) from freshwater and terrigenous sources versus enriched C from marine phytoplankton (Hagy and Boynton 1998, Zimmerman and Canuel 2001). As a migratory fish, summer flounder habitat-use patterns vary seasonally as individuals move into and out of the Bay, exposing them to areas that may differ isotopically. During the period of estuarine residency, movements vary greatly by individual; some individuals exhibit a high degree of site fidelity for up to 4 months, while others appear to be highly mobile (Fabrizio et al. 2007). Consequently, the combination of summer flounder movements with the shifting isotopic background created by temporal and spatial isotopic heterogeneity of lower trophic levels can prohibit accurate diet assessments using stable isotopes.

One way to address potential problems associated with this variability is to sample tissues that integrate the diet over different time periods. Recent work, examining tissue-specific turnover rates and fractionations in summer flounder, revealed that turnover was

fastest in liver, followed by blood then by muscle likely due to differing metabolic activity in the tissues (Chapter 1). Based on average growth rates of flounder in the wild (1.5% d<sup>-1</sup>; Rountree and Able 1992),  $\delta^{15}\text{N}$  would equilibrate to the dietary signal in ~40 (liver), 75 (blood), and 135 days (muscle) in each of the summer flounder tissues analyzed (Chapter 1). Turnover of  $\delta^{13}\text{C}$  would be similar, if not faster for liver and blood tissues relative to  $\delta^{15}\text{N}$  (Chapter 1). Liver and blood tissues thus have the potential for alleviating problems relating to the assumption of constant isotopic signatures of prey because diets are integrated over shorter time periods. Few studies have examined multiple tissues in fish for diet assessments due to the lack of basic isotopic turnover and fractionation information on tissues other than muscle (Gaston and Suthers 2004, Estrada et al. 2005, MacNeil et al. 2005). The laboratory study on summer flounder facilitates a more accurate application of the stable isotope techniques in the field using multiple tissues, which can improve the resolution of the diets and help alleviate the complexities introduced by fish movement and temporal heterogeneity of prey  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

The objective of this study was thus to utilize the new information on tissue-specific turnover and fractionations of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  to 1) characterize summer flounder diets in the mainstem of Chesapeake Bay and 2) explore ontogenetic patterns in food habits. This isotopic evaluation of flounder diet complements previous stomach content data by demonstrating which prey groups are actually being assimilated and contributing to growth. Despite the prominent role of Chesapeake Bay in the life histories of many fishes, few studies have applied stable isotope techniques to address diets of higher trophic levels in this estuary. We demonstrate the utility of isotopic approaches while also

highlighting the assumptions and complications associated with applying stable isotopes to migratory fish species that inhabit dynamic environments.

## METHODS

### *Sample collection*

Summer flounder and commonly consumed prey items were collected from the Virginia portion of Chesapeake Bay where flounder distribution tends to be concentrated (Murphy et al. 1997, Latour et al. 2008) (Fig. 1). Samples were primarily obtained from the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP) bottom trawl survey (for description of trawl procedures and gear, see Latour et al. 2003). Samples of smaller fishes and invertebrates were augmented with collections from the VIMS Juvenile Finfish and Blue Crab Trawl Survey, which uses a smaller, finer-mesh net (for description of trawl procedures and gear, see Fabrizio and Tuckey 2008). In cases where samples from the VIMS Juvenile Trawl Survey were required, they were obtained from similar locations and time periods as the ChesMMAP collections. When possible, stations with both flounder and prey samples were selected, but generally, stations were chosen to represent a broad area within Chesapeake Bay to characterize flounder diets on the population level.

Common prey taxa were identified based on previous research examining stomach contents of summer flounder within the study area. Typical prey items included mysid shrimp (primarily *Neomysis americana*), sand shrimp (*Crangon septemspinosa*), mantis shrimp (*Squilla empusa*), bay anchovy (*Anchoa mitchilli*), weakfish (*Cynoscion regalis*), spotted hake (*Urophycis regia*), spot (*Leiostomus xanthurus*), and Atlantic croaker

(*Micropogonias undulatus*), which together comprised ~83% of flounder diets by weight in Chesapeake Bay (Latour et al. 2008). Collections of prey species were restricted to sizes found in the stomach contents of summer flounder (Table 1, Latour et al. 2008). Given difficulties in obtaining mysid shrimp in November 2007, freshly consumed specimens were collected from stomachs of flounder captured in September and October of that year (Grey et al. 2002).

For larger fish prey, large samples of white muscle were excised from the musculature below the first dorsal fin and frozen. Smaller fishes and invertebrates were frozen whole. For summer flounder, ~1ml of whole blood was withdrawn from the caudal vein and placed in a sterile vial, and individuals were subsequently processed for length, wet weight, sex, and maturity stage. Otoliths were also removed for sectioning and aging in the laboratory. Small samples of liver and white muscle (from the ocular side above the pectoral fin) were excised and bagged individually. All flounder tissue samples were immediately frozen for later analysis. Preservation by freezing was selected due to its minimal effect on  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (Kaelher and Pakhomov 2001, Sweeting et al. 2004). All collection protocols were approved by the College of William and Mary's Institutional Animal Care and Use Committee.

Collections were made during different time periods to characterize seasonal dietary patterns and account for temporal variability in isotopic signatures resulting from migration or fluctuations in isotopic signatures of prey. Samples were collected in 2006 and 2007 during two seasonal periods corresponding with the middle and later parts of flounder residency of Chesapeake Bay. Samples collected in May, June, or July represented the spring/early summer and are referred to as "Spring" samples, while

samples from November are termed “Fall”. In 2006, sampling extended further south than in 2007 (Fig. 1).

### *Stable isotope analysis*

In the laboratory, smaller samples (~1-2g) of white muscle were taken from each fish sample collected in the field. Invertebrate samples were measured for length and processed whole. For mysid shrimp, multiple individuals were aggregated to obtain sufficient mass for stable isotope analysis. All muscle, liver, and whole-body samples were rinsed with de-ionized water, dried at 50°C and ground using a mortar and pestle. Blood samples were dried and ground in their storage vials. Inorganic carbonates found in exoskeletons of the crustacean prey were removed with added drops of 10% HCl (Pinnegar and Polunin 1999). Dry subsamples (1±0.2mg) of all tissues were packaged in tin capsules and analyzed at the University of California-Davis Stable Isotope Facility using a Europa Hydra 20/20 continuous flow isotope ratio mass spectrometer. Stable isotope ratios are reported in relation to conventional standards:

$$(1) \quad \delta X = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1000$$

where X is either  $^{13}\text{C}$  or  $^{15}\text{N}$ , and R is the mass ratio of the heavy to light stable isotope ( $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ ) for either the sample or the standard. The conventional standards used for the analyses were Pee Dee Belmnite for C and air for N.  $\delta X$  is reported in per mil (‰). Repeated measurements of a calibration standard indicated that instrument precision (SD) was 0.29‰ and 0.12‰ for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively. The mass ratio of elemental C and N (C:N) was also obtained for each analyzed sample.

A mathematical correction was used to normalize liver  $\delta^{13}\text{C}$  values, due to the high lipid concentrations in this tissue which are known to bias  $\delta^{13}\text{C}$  values (DeNiro and Epstein 1977, McConnaughey and McRoy 1979). Previous laboratory work on summer flounder established a conversion between elemental C:N and the bias on  $\delta^{13}\text{C}$  introduced by not removing lipids (Chapter 1). This conversion equation followed McConnaughey and McRoy (1979) and Logan et al. (2008), and allowed a lipid-adjusted  $\delta^{13}\text{C}$  ( $\delta^{13}\text{C}_{\text{adj}}$ ) to be calculated as:

$$(2) \quad \delta^{13}\text{C}_{\text{adj}} = \delta^{13}\text{C}_{\text{raw}} + D\left(\theta + \frac{3.90}{1 + 287/L}\right) \quad \text{where}$$

$$L = \frac{93}{1 + (0.246 \times \text{C:N} - 0.775)^{-1}}.$$

In equation (2), L represents the sample lipid content as a function of C:N, D represents protein-lipid discrimination, and  $\delta^{13}\text{C}_{\text{raw}}$  is the  $\delta^{13}\text{C}$  of a liver sample whose lipids were not extracted. The parameters D and  $\theta$  ( $\pm$  SE) were estimated for summer flounder as  $D = 6.2883 \pm 1.3519$  and  $\theta = 0.0612 \pm 0.1527$  (Chapter 1). All liver  $\delta^{13}\text{C}$  values were normalized for lipid content based on sample C:N using equation (2). Lipid corrections were not applied to muscle and blood  $\delta^{13}\text{C}$  values due to the relatively low C:N values (Table 1; Post et al. 2007), limited variability across individuals, and presumably small effects on  $\delta^{13}\text{C}$  (Kiljunen et al. 2006).

### *Data analysis*

Contributions of prey to summer flounder diets were assessed graphically and calculated using mixing models. Summer flounder were classified as either age-0 or older (age-1+) to examine ontogenetic changes in diet as found in stomach contents (Latour et

al. 2008). Prey groups were separated into two trophic guilds that captured the major differences in stable isotopic signatures (Phillips and Gregg 2005, McIntyre et al. 2006). Prey were classified as either crustacean or fish, alleviating problems associated with similarity in prey isotopic signatures and inability to calculate unique solutions to mixing models due to an excess of sources. IsoError, a two-source mixing model that accounts for variability in both prey and predator isotope values, was used to calculate the mean contribution ( $\pm$ SE) of each trophic guild to summer flounder diets (Phillips and Gregg 2001). To apply the mixing models, summer flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were first adjusted to account for fractionation, defined as changes in isotopic values occurring during physiological processing of consumed material. Tissue- and isotope-specific fractionations of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (represented as  $\Delta^{13}\text{C}$  and  $\Delta^{15}\text{N}$ , respectively) were obtained from a laboratory diet shift experiment on summer flounder (Chapter 1), and fractionations were subtracted from the flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values. The fractionations were based on growth-based turnover models of wild fish, with values of 2.0‰ (muscle), 3.3‰ (blood), and 3.1‰ (liver) for  $\delta^{13}\text{C}$  and values of 2.9‰ (muscle), 2.8‰ (blood), and 2.3‰ (liver) for  $\delta^{15}\text{N}$  (Chapter 1). Where possible, the mixing model was applied to each summer flounder tissue for each combination of year, season, and age group.

A multivariate analysis of variance (MANOVA) was used to test for significant year and season effects on mean isotopic values ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) of each trophic guild. MANOVA assumptions of normality and homogeneity of variance were upheld based on residual analysis, Kolmogorov-Smirnoff tests, and Levene's tests. For each trophic guild, the following *a priori* comparisons were tested using the Wilks' Lambda statistic at an  $\alpha$ -level of 0.05: season effects were tested within each year, and year effects were tested

within each season (SAS version 9.1, SAS Institute 2002). MANOVA tests on summer flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  included age as an additional binary factor, in which flounder were designated as either age-0 or older (age-1+).

Dietary shifts that occur within the time frame of the slowest turnover tissue, can be reflected by differences in isotopic values between tissues (MacNeil et al. 2005, Fry 2006). For each individual, fractionation-adjusted  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for muscle were subtracted from fractionation-adjusted blood and liver values to examine recent shifts in flounder feeding. For example, a recent dietary shift towards feeding on prey with enriched  $\delta^{15}\text{N}$  would manifest as a positive difference between liver and muscle tissues due to the faster turnover of the liver. Mean differences ( $\pm$  SE) between tissue pairs were calculated and paired t-tests were used to test for significant differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . For each tissue, regression analysis was also used to test for significant relationships between stable isotope measurements and fish length.

## RESULTS

A total of 59 summer flounder were analyzed for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , with most individuals ranging from 138-478 mm and 0-3 years of age. However, three outliers reached 599-624 mm with ages of 4-7 years. Lengths of age-0 fish averaged ~150mm less than age-1+ individuals, but the variability in the older fish was much greater (Table 1). A total of 32 females, 26 males, and 1 fish of unknown sex were sampled. Larger individuals were predominantly females corresponding with the skewed sex ratio of age-1+ summer flounder in the Middle Atlantic Bight and Chesapeake Bay (Morse 1981, Bonzek et al. 2007). Blood and liver samples were not collected from summer flounder in 2006, and individual prey species were not represented in every season/year combination mostly due to seasonal trends in species abundances (Table 1).

### *Stable isotope composition of summer flounder and prey*

Grouping of summer flounder prey groups into two trophic guilds (crustaceans and fishes) was supported by isotopic separation between the guilds, particularly for  $\delta^{15}\text{N}$  (Fig. 2). Fishes generally exhibited  $\delta^{15}\text{N}$  values approximately 3‰ greater than crustaceans, conforming to the traditional assumption of an ~3.4‰ shift per trophic level (Post 2002, Sweeting et al. 2007).  $\delta^{13}\text{C}$  was not a useful dietary indicator due to similarity and overlap of prey  $\delta^{13}\text{C}$  values and due to the high degree of variability of flounder values

that often extended beyond the constraints of prey  $\delta^{13}\text{C}$  (e.g., Fig. 2D). Consequently, mixing models were only applied using  $\delta^{15}\text{N}$  values.

After being adjusted for fractionation, isotopic values of summer flounder resembled the values of the crustacean prey, suggesting a greater dependence on this lower trophic guild. Mixing model results, using only  $\delta^{15}\text{N}$ , indicated that the proportion of crustaceans in the diets of summer flounder approached 100% in the fall for both age-0 and age-1+ fish (Table 2). On average, fishes accounted for 15% or less of the total diet during fall, but standard errors for the estimates ranged from 8-19%. During spring of 2006, age-1+ flounder appeared to assimilate equal amounts of fishes and crustaceans, but this is confounded by the relatively slow turnover of muscle and the potential variability in prey isotopic values. Highly depleted  $\delta^{15}\text{N}$  values of flounder tissues in spring of 2007 prevented the use of the mixing model in this season (Fig. 2C, Table 2) but the raw  $\delta^{15}\text{N}$  values placed age-0 summer flounder at a similar trophic level to the crustaceans and age-1+ fish as intermediate between the fishes and invertebrates (Fig. 2C).

#### *Temporal variability in stable isotopes*

Isotopic values of trophic guilds were not temporally consistent, exhibiting seasonal patterns that differed by year. In 2006, crustacean  $\delta^{13}\text{C}$  became more depleted while  $\delta^{15}\text{N}$  became more enriched from spring to fall, but the opposite pattern was observed in 2007 (Table 3). The prey fishes experienced a similar depletion of  $\delta^{13}\text{C}$  and enrichment of  $\delta^{15}\text{N}$  from spring to fall during 2006, but seasonal differences in isotope signatures of fishes were not significant in 2007 ( $F=2.69$ ,  $p>0.05$ ). Seasonal isotopic differences within a trophic guild were relatively small and typically less than 1.4‰ in

magnitude; however, standard deviations for group means reached 1.7‰ due to inter- and intra-species variability.

Isotope means of trophic guilds also tended to vary by year, with most MANOVA tests of interannual difference by season yielding significant results ( $p < 0.05$ , Table 3). Only the isotopic means of prey fishes in spring were found to be similar between 2006 and 2007. Combined, the lack of consistent seasonal and interannual patterns in prey stable isotopes can complicate isotopic diet assessments by creating a shifting isotopic background upon which summer flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  must be pinpointed.

Temporal differences in summer flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were primarily driven by depleted  $\delta^{15}\text{N}$  of age-0 individuals in spring of 2007. These values were significantly lower than age-1+ fish caught at the same time ( $p < 0.05$ ), evident in MANOVA tests of all sampled tissues (Table 4). Increases in  $\delta^{15}\text{N}$  for age-0 fish tissues from spring to fall (Fig. 2, Table 2) also led to significant seasonal differences for muscle, blood, and liver of age-0 individuals (Table 4). The significant MANOVA test between age groups in fall 2006, was due to differences in  $\delta^{13}\text{C}$  as opposed to  $\delta^{15}\text{N}$  (Fig. 2).

#### *Summer flounder tissue differences*

Sampling of multiple summer flounder tissues in 2007 was intended to mitigate problems associated with the temporal variability found in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of prey groups. Given that isotopic turnover rates for the sampled tissues rank liver > blood > muscle (Chapter 1), differences in isotopic signatures between tissues should reflect temporal changes in diets once corrected for fractionation. Blood  $\delta^{13}\text{C}$  values were consistently more than 1.5‰ depleted relative to muscle values (Fig. 3B). Although this could

potentially indicate more recent feeding on  $\delta^{13}\text{C}$ -depleted prey, this was not supported by the liver tissue which has the fastest turnover rate (Fig. 3). The consistency in the depleted blood phenomenon across seasons and ages and the absence of the signal in the liver tissue suggests these depleted values are likely the result of a bias pertaining to the applied  $\delta^{13}\text{C}$  fractionations for blood.

Excluding blood  $\delta^{13}\text{C}$ , isotopic differences between tissues were small (typically  $<1\text{‰}$ ). Although some differences were significant, error in fractionation estimates and isotopic measurements contribute some uncertainty to the results. There was a stronger signal indicating that age-1+ fish in fall of 2007 may have recently begun feeding on more  $\delta^{15}\text{N}$  depleted prey, in contrast to the broader ontogenetic trends in  $\delta^{15}\text{N}$  (see below). In general, however, summer flounder tissues reflected consistent feeding primarily on crustaceans in fall of 2007 (Table 2), without drastic changes in feeding through the previous few months based on the turnover rates of the different tissues.

#### *Ontogenetic trends in $\delta^{13}\text{C}$ , $\delta^{15}\text{N}$ , and C:N*

Positive relationships between  $\delta^{15}\text{N}$  and summer flounder length indicate ontogenetic changes in feeding towards higher trophic level prey as flounder grow (Fig. 4). This effect was most pronounced in 2007, manifesting itself in all sampled tissues and most evident in the spring sampling. Assuming  $\delta^{15}\text{N}$  fractionations of 2.3-2.9‰ for the sampled tissues (Chapter 1), larger individuals were feeding at approximately one trophic level above small young-of-the-year fish. This pattern was responsible for significant differences in isotopic values between age-0 and age-1+ fish in spring of 2007 for all tissues (Table 4). Larger individuals exhibited  $\delta^{15}\text{N}$  values similar to or slightly greater

than those of prey fishes (Fig. 4). For each tissue, linear regressions of  $\delta^{15}\text{N}$  on flounder length were significant ( $p < 0.05$ ) using pooled 2007 data. Removing three outlier fish  $> 350\text{mm}$ , slopes were not significantly different among tissues (slope estimates  $\pm$  SE: muscle,  $0.0156 \pm 0.0026 \text{‰ mm}^{-1}$ ; blood,  $0.0197 \pm 0.0024 \text{‰ mm}^{-1}$ ; liver,  $0.0152 \pm 0.0026 \text{‰ mm}^{-1}$ ). In 2006, however, there was not a significant relationship between  $\delta^{15}\text{N}$  and length, possibly due to the lack of age-0 representation in the spring season, when size and dietary differences may be most pronounced between age classes.

Relationships between  $\delta^{13}\text{C}$  and fish length were not observed, except in 2006 where there was a slight trend of more enriched  $\delta^{13}\text{C}$  in muscle tissue of larger fish (Fig. 5). Positive relationships between  $\delta^{13}\text{C}$  and salinity (Fry and Sherr 1984, Hagy and Boynton 1998) could indicate that in 2006 smaller fish were feeding in less saline waters near the rivers. However, the general lack of strong relationships corresponds with the similarity of  $\delta^{13}\text{C}$  among prey species and flounder, indicating that the basal organic matter sources remained relatively constant through the sizes examined.

Ratios of C to N are generally considered to be an indicator of lipid content in tissues (McConnaughey and McRoy 1979, Post et al. 2007). Within a tissue, N is primarily found in proteins, whereas lipids contain little N and C predominates. Consequently, high C:N tends to reflect lower protein content and higher lipid content. Across fish sizes, C:N remained relatively constant for muscle and blood tissues. However, C:N of liver tissues demonstrated a clear pattern with fish length. Liver C:N of smaller individuals remained low until fish reached a length of approximately 250mm, at which point C:N began to increase (Fig. 6). This C:N range of 5-14 represents approximately a 20-60% lipid content in summer flounder liver tissue (A. Buchheister,

VIMS, unpublished data), and the point of increase corresponded with individuals attaining sexual maturity (Fig. 6).

## DISCUSSION

### *Chesapeake Bay food web*

Few studies have examined stable isotopes in Chesapeake Bay fishes, limiting the comparisons to be drawn with our results. However, the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of the sampled species support the relative trophic positions of these groups in the generalized food web (Baird and Ulanowicz 1989, Hagy 2002). Given the low trophic fractionation associated with C,  $\delta^{13}\text{C}$  is commonly used to identify sources of primary production (Peterson and Fry 1987). The range of  $\delta^{13}\text{C}$  of all samples in this study (approximately -22 to -16) was consistent with a primarily phytoplankton-dominated source of C to the food web (Fry and Sherr 1984). The high degree of overlap in  $\delta^{13}\text{C}$  values across species implies that the sampled prey are reliant on similar organic matter sources, making C a poor dietary tracer in this study. The greater fractionation values of  $\delta^{15}\text{N}$  allow  $\delta^{15}\text{N}$  measurements to be indicators of trophic level within food webs (Peterson and Fry 1987). Excluding spring of 2007, prey fishes exhibited  $\delta^{15}\text{N}$  that were on average 2.7-2.9‰ greater than the crustacean guild, a difference indicative of approximately one trophic level. This corroborates stomach content work showing the predominance of mesozooplankton (particularly mysid shrimp) in the diets of bay anchovy (Hartman et al. 2004), juvenile weakfish (Latour et al. in review), and spotted hake (Steimle et al. 2000). Spot and Atlantic croaker, are more benthic and a greater contribution of amphipods, copepods, and annelids to their diets may explain their tendency towards intermediate  $\delta^{15}\text{N}$  values

between crustaceans and the other more pelagic fishes (Stickney et al. 1975, Nemerson and Able 2004). The values of  $\delta^{15}\text{N}$  for our sampled prey also agreed with the sparse isotopic data available for these species in Chesapeake Bay or similar environments (bay anchovy – Hagy and Boynton 1998, Litvin and Weinstein 2003; weakfish – Litvin and Weinstein 2003; sand shrimp – Douglass 2008).

Estimates of trophic level for our sampled organisms correspond with previous research. Trophic levels calculated from stable isotope data rely on measurements of a baseline organism, ideally a primary consumer that integrates temporal variability in  $\delta^{15}\text{N}$  of autotrophs (Post 2002). Samples of blue mussel (*Mytilus edulis*), a filter-feeding bivalve, collected in fall of 2007 yielded  $\delta^{15}\text{N}$  values ( $\pm\text{SD}$ ) of  $9.76 \pm 0.32\text{‰}$  ( $n=8$ ). Using this as a baseline for trophic level 2.0 and assuming  $\Delta^{15}\text{N} = 3.4\text{‰}$ , crustaceans in this time period can be classified as occupying trophic level 2.9, compared to trophic level 3.8 for the fishes (Post 2002). Hagy (2002) found nearly identical mean ( $\pm\text{SD}$ ) trophic levels for mysid shrimp (2.91), bay anchovy ( $3.69 \pm 0.43$ ), and YOY weakfish ( $3.92 \pm 0.74$ ), although estimates varied spatially and temporally. Overall, the isotopic results of the sampled prey groups correspond with trophic relationships based on stomach content research and support the sparse stable isotope data available for these organisms in Chesapeake Bay.

#### *Summer flounder diets and stable isotope dynamics*

Stable isotope assessments of summer flounder diets for smaller individuals agreed with those based on stomach contents for Chesapeake Bay flounder. For individuals <225mm, mysid, sand, and mantis shrimps dominated stomach contents by weight (79% -

Latour et al. 2008), corresponding with our mixing model estimates of mean crustacean contributions to age-0 diets (86-100%). The proportion of fishes in the stomach contents of summer flounder increased with body size, becoming the dominant prey group for flounder >375mm (Latour et al. 2008). Ontogenetic changes in summer flounder feeding can also occur at smaller sizes (<60mm, Burke 1995) and on the continental shelf (Link et al. 2002). In this study, an ontogenetic pattern in summer flounder feeding was evidenced by positive relationships between  $\delta^{15}\text{N}$  and fish length for all tissues, showing that larger flounder consumed more  $\delta^{15}\text{N}$ -enriched prey that presumably occupied higher trophic levels. Feeding on fishes by larger summer flounder would have been indicated by tissue  $\delta^{15}\text{N}$  values greater than those of prey fishes, but average  $\delta^{15}\text{N}$  for age-1+ flounder were not significantly greater (ANOVA,  $p>0.05$ ), except in spring of 2006. However, some individuals appeared to assimilate appreciable amounts of fishes, driving their  $\delta^{15}\text{N}$  over one standard deviation above the mean of the prey fishes (Fig. 4).

Despite the ontogenetic pattern in flounder  $\delta^{15}\text{N}$ , mixing models could not directly support increased reliance on prey fishes for larger and older individuals. The ontogenetic isotopic trend was primarily driven by samples collected in spring of 2007 when significant differences in isotopic values were found between age groups. However, depleted flounder  $\delta^{15}\text{N}$  values prevented application of mixing models in this season. According to  $\delta^{15}\text{N}$  values in this season, age-0 flounder occupied a trophic level more consistent with the sampled invertebrates with minimal feeding on fishes. This would imply that summer flounder were feeding on trophic levels lower than mysid and sand shrimps, including smaller zooplankton such as copepods, but there is no stomach content evidence that summer flounder (of the sizes examined here) feed on such prey (Grimes et

al. 1989, Rountree and Able 1992, Latour et al. 2008). Consequently, the sampled prey may not have been isotopically representative of consumed organisms due to regional or temporal variability in  $\delta^{15}\text{N}$ , creating the disparity between flounder  $\delta^{15}\text{N}$  and that of the prey. This possibility is supported by the unusually small  $\delta^{15}\text{N}$  separation (1.09‰) between the crustacean and fish trophic guilds and the higher variability in crustacean  $\delta^{15}\text{N}$  in the spring of 2007.

Contributions of fishes and crustaceans to the diets of summer flounder would be mediated by interannual and seasonal variability in prey abundance and availability. For example, spring of 2006 exhibited the highest contribution of fishes to the diet of age-1+ fish (50%), while there was minimal feeding on fishes in spring of 2007. Analysis of ChesMMAAP diet data for these two years (following Latour et al. 2008) showed that the proportion of fishes by weight in the flounder (250-375mm) diets was ~20-45% higher in 2006 than in 2007 for fish collected in March, May, and July (R. J. Latour, VIMS, unpublished data). Although indices of relative abundance for most prey fishes were not significantly different between 2006 and 2007 in Chesapeake Bay (Fisher-Behren's tests,  $p > 0.05$ ; Durell and Weedon 2007, Fabrizio and Tuckey 2008), prey populations can fluctuate annually. Changes in relative prey availability and competition amongst predators can modify feeding patterns and may help explain the dietary differences between years. Interestingly, by fall in each year, consumption of fishes prevailed according to stomach contents of flounder (250-375mm), but this was not reflected by stable isotope data, even by the liver with the fastest isotopic turnover.

Stable isotopes indicate that crustaceans play a dominant role in the diet of summer flounder across the sizes sampled. Low percentages of fishes in mixing model results and

mostly non-significant  $\delta^{15}\text{N}$  differences between summer flounder and the other fishes, suggest more of a competitive relationship instead of a predatory one among the sampled fishes. Although some individuals exhibited  $\delta^{15}\text{N}$  greater than bay anchovy and other prey fishes, on average age-1+ flounder assimilated a greater proportion of crustaceans than expected based on stomach contents (Latour et al. 2008). Despite temporal variability in flounder diets, the majority of flounder productivity was driven by mysid shrimp and other crustacean prey. The crustacean trophic guild thus plays an important role in energy transfer through the food web as a critical link for juvenile fishes as well as larger summer flounder. Mysid shrimp in particular may be most responsible for flounder productivity given their preponderance in summer flounder stomachs (Latour et al. 2008); however, the presented isotopic data could not clearly distinguish among the mysid, sand, and mantis shrimps to support this.

The discrepancy between stable isotope and stomach content analyses (Latour et al. 2008) could result from several different scenarios. In 2007, the prevalence of fishes in the summer flounder stomach contents was greatest in July and November (R. J. Latour, VIMS unpublished data). Tissues sampled for isotopes in fall of 2007 may not have had sufficient time to reflect appreciable assimilation of fishes during this time if growth rates were low. Alternatively, the discrepancy between methodologies could result from under-representation of crustaceans in stomach contents due to differences in prey digestibility, assimilation efficiency, and evacuation rates (Hyslop 1980). Although mysid shrimp are smaller and slightly less energy dense than bay anchovy and other fishes (Wang and Houde 1994, Lankford and Targett 1997, A. Z. Horodysky, VIMS, unpublished data), their small sizes and high surface to volume ratios can facilitate digestion and evacuation

(Lankford and Targett 1997, Andersen 1999). Success rates of capture are also presumably greater when feeding on mysid shrimp than on bay anchovy, potentially offsetting the foraging costs associated with eating greater numbers of individuals to equal the mass of one anchovy. Consequently, feeding on the mysid shrimp may represent a more energetically profitable strategy for summer flounder; however, more directed research is needed to address this possibility.

Trends in liver C:N of summer flounder potentially reflected different energy allocation strategies through ontogeny. Liver C:N, a proxy for lipid content (McConnaughey and McRoy 1979, Post et al. 2007), remained low for juveniles and then increased as summer flounder began to mature at ~250 mm (Bonzek et al. 2007). This pattern could result from a preferential allocation of energy towards somatic growth by juveniles as a means to maximize the benefits associated with larger body sizes (Sogard 1997). Once individuals begin to mature, energy may be diverted away from somatic growth towards lipid storage in the liver in preparation for the energetic demands of gamete production and spawning. Trends of C:N in muscle and blood were likely not found because these tissues do not have the same energy storage capacity as livers in fishes (Hoar and Randall 1969).

In this study, all three tissues captured the same ontogenetic trend of  $\delta^{15}\text{N}$  with size, while also producing similar diet estimates. Regression slopes for  $\delta^{15}\text{N}$  and length were not significantly different among tissues, and each tissue reflected a diet primarily comprised of crustaceans. Given the positive relationship between  $\delta^{15}\text{N}$  and length, we expected to see enriched  $\delta^{15}\text{N}$  values for the faster turnover tissues which better-represent an individual's more recent diet while the fish was slightly larger due to growth. This was

not seen in the blood nor the liver. Instead,  $\delta^{15}\text{N}$  values for blood and liver were slightly depleted relative to muscle. The lack of the ontogenetic signal within an individual's tissue likely results from the slow increase in  $\delta^{15}\text{N}$  with increasing size. Age-0 fish grew ~100mm during the 4-month period between spring and fall samplings in 2007, and this equates to an approximate increase in  $\delta^{15}\text{N}$  of 1.5 ‰ (Fig. 4). This small change in  $\delta^{15}\text{N}$  would be accrued gradually, and any small differences in  $\delta^{15}\text{N}$  among tissues could be obscured by isotopic noise introduced by fractionation and measurement errors in the tissues and by isotopic variability in prey. Similarity in  $\delta^{15}\text{N}$  across tissues indicates that the dietary changes exhibited by summer flounder occur gradually as their trophic breadth expands with size. Also, there was no evidence of drastic, punctuated changes in diets or utilization of isotopically distinct prey in other habitats.

#### *Temporal and spatial variability in stable isotopes*

Temporal variability in stable isotope values of prey is a potential source of error in isotopic diet analyses, one that is primarily mediated through bottom up effects (Peterson and Fry 1987, Cloern et al. 2002). Changes in environmental conditions (e.g., runoff, upwelling, temperature, salinity) can alter nutrient pools and biogeochemical cycling patterns, which can consequently modify the isotopic values of autotrophs and be transferred up the food web. Although significant, seasonal and yearly differences in  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  within trophic guilds were typically small (<1.4‰). These small differences could result from trophic averaging whereby isotopic variability in lower trophic levels are minimized as those signals are integrated and assimilated over longer time scales (O'Reilly et al. 2002). Additionally, temporal differences in trophic guild isotopic

signatures were likely influenced by the different representative species that were actually sampled in a given season. Individual isotopic variability within each species also contributed to fluctuations in the means and standard deviations of trophic guild estimates of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ .

Spatial variability in prey stable isotope values is another factor that can introduce error into diet estimation, particularly when dealing with a migratory species such as the summer flounder. Within Chesapeake Bay and other estuaries, the most consistent spatial trend involves a positive relationship between  $\delta^{13}\text{C}$  values and salinity over large salinity ranges (0-25) (Fry and Sherr 1984, Hagy and Boynton 1998). However, sampling only occurred in bottom waters with salinity  $>15$ , resulting in no significant trend in flounder  $\delta^{13}\text{C}$  with latitude or salinity (linear regression,  $p>0.05$ ). Spatial differences in  $\delta^{15}\text{N}$  are controlled by complex biogeochemical processes that operate on more localized scales (Cifuentes et al. 1988, Horrigan et al. 1990, Montoya et al. 1990). Consequently, impacts of spatial isotopic variability on summer flounder diet assessments are hard to predict, particularly given that some summer flounder can exhibit large movements while residing in Chesapeake Bay (Fabrizio et al. 2007).

Both temporal and spatial factors may have contributed to the lack of congruity in  $\delta^{15}\text{N}$  between summer flounder and their prey in spring of 2007. These factors also contributed to variability in isotopic values found within and between sampled species. However, the similarity in dietary evaluations among fast- and slow-turnover tissues and the relative consistency of trophic guild stable isotope measurements suggests that such variability did not alter the major conclusions of the study.

### *Fractionation issues*

Applied fractionation values used in this study represented the best available science, but contributed to some error in the results. As recommended (Gannes et al. 1997), fractionation values for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  fractionations were empirically determined for the same species, same tissues, and similar body sizes (Chapter 1). However, the consistent depletion of  $\delta^{13}\text{C}$  in flounder blood relative to muscle and lack of such a signal in the liver suggested that the applied  $\delta^{13}\text{C}$  fractionation (3.3‰) may have contributed to this bias, highlighting a common source of error in stable isotope analyses. Aside from the factors controlled for (species, tissue, size), fractionation values can vary by diet (Gorokhova and Hansson 1999), feeding mode (Vander Zanden and Rasmussen 2001), and growth rate (Trueman et al. 2005). Additionally, laboratory fractionations tend to be larger than field-derived values (Vander Zanden and Rasmussen 2001) as appeared to be the case with the blood. Accordingly, relatively minor errors in tissue fractionation estimates could have prevented detection of the small expected  $\delta^{15}\text{N}$  differences among tissues resulting from the ontogenetic trend in  $\delta^{15}\text{N}$ . Errors in the applied fractionation values would also affect mixing model estimates, particularly since isotopic separation between trophic guilds was small (Vander Zanden and Rasmussen 2001). However, the  $\delta^{15}\text{N}$  fractionation estimates used in the mixing models did not deviate greatly from previous research, unlike  $\delta^{13}\text{C}$  fractionations (Post 2002, Sweeting et al. 2007). Also, fractionation errors would not alter the major conclusions due to the similarity of unadjusted flounder  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  with those of the other sampled fishes, which indicate that most of the sampled fishes feed at the same trophic level.

### *Future work and conclusions*

Despite the prevalence of stable isotope applications in the ecological literature, relatively few dietary studies have concurrently examined multiple tissues of fishes in the field (Gaston and Suthers 2004, Estrada et al. 2005, MacNeil et al. 2005, Perga and Gerdeaux 2005). In our study, tissue differences were within the noise levels of the ambient isotopic environment, but confirmed a relative consistency in dietary trends. Any small isotopic differences among tissues resulting from gradual ontogenetic changes in feeding were obscured by measurement and fractionation errors. Future studies using multiple tissues could benefit from exploring dietary changes that involve isotopic changes of greater magnitude that facilitate detecting isotopic differences among tissues. Examples include predation of anadromous fishes by riverine piscivores (Garman and Macko 1998, MacAvoy et al. 2001), long migrations of pelagic fishes (Estrada et al. 2005), consumption of prey whose isotopic values change greatly throughout the year (Perga and Gerdeaux 2005), and ontogenetic dietary shifts between more isotopically distinct prey. Research that focuses on isotopic turnover and fractionation of different tissues (e.g., Logan et al. 2006, MacNeil et al. 2006, Guelinckx et al. 2007) will aid application of multiple tissues to fishes in field settings. However, the factors that influence fractionations need to be better understood to minimize errors and reduce the isotopic differences that can be accurately detected among tissues. Also, within the Chesapeake Bay mainstem where phytoplankton may be the primary source of organic matter, future research may benefit by using additional stable isotopes (e.g.,  $\delta^{34}\text{S}$ ) to better distinguish amongst prey groups.

In conclusion, stable isotopic analysis of multiple summer flounder tissues revealed a primary dietary reliance on crustacean prey, including mysid, mantis, and sand shrimp. Similar  $\delta^{13}\text{C}$  composition of flounder and potential prey groups prevented use of this isotope as a suitable dietary tracer, but  $\delta^{15}\text{N}$  demonstrated that summer flounder generally occupied trophic levels that mirrored other small fishes (bay anchovy and juvenile sciaenids). These results were in general agreement with diets based on stomach contents (Latour et al. 2008), although importance of crustaceans may have been under-represented in stomach contents of larger flounder. We support recommendations that stable isotope methods are best when applied in conjunction with additional techniques (Cloern et al. 2002, Fry 2006). Combination of stomach content and stable isotope analyses provide both taxonomic specificity and integrative information on assimilation. Together, these methods characterize diets of fishes more comprehensively and would be of greater benefit to resource managers and other researchers.

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**Table 1.** Sampling information for summer flounder and other collected taxa, including numbers of samples, mean C:N ( $\pm$  SD), and mean length ( $\pm$  SD). \*Note: Individual lengths were not measured for mysid shrimp, but sizes ranged ~5-15mm.

Group	Species	Tissue	Age	Number of samples				C:N	Length (mm)
				Spring '06	Fall '06	Spring '07	Fall '07		
Fish prey									
	Atlantic croaker	muscle	--		6		8	3.2 $\pm$ 0.2	62 $\pm$ 15
	bay anchovy	muscle	--	9	8	8	9	3.4 $\pm$ 0.3	61 $\pm$ 8
	spot	muscle	--	8	8	6		3.4 $\pm$ 0.4	133 $\pm$ 16
	spotted hake	muscle	--	4				3.8 $\pm$ 0.2	155 $\pm$ 12
	weakfish	muscle	--		4	6	6	3.2 $\pm$ 0.2	97 $\pm$ 34
Crustacean prey									
	mantis shrimp	whole	--	6	4	6	6	3.9 $\pm$ 0.3	100 $\pm$ 20
	mysid shrimp	whole	--		2	9	9	3.7 $\pm$ 0.2	*
	sand shrimp	whole	--	9	8	6	4	3.7 $\pm$ 0.2	29 $\pm$ 7
Predator									
	summer flounder	muscle	0		8	8	12	3.2 $\pm$ 0.2	228 $\pm$ 47
			1+	14	7	5	5	3.1 $\pm$ 0.3	376 $\pm$ 108
		blood	0			6	12	3.8 $\pm$ 0.1	229 $\pm$ 54
			1+			5	5	3.8 $\pm$ 0.1	335 $\pm$ 70
		liver	0			7	12	5.4 $\pm$ 1	227 $\pm$ 53
			1+			5	5	8.2 $\pm$ 2.6	335 $\pm$ 70

**Table 2.** Mixing model results for contribution of fish and crustacean prey to summer flounder diets (mean  $\pm$  SE) based on  $\delta^{15}\text{N}$  (mean  $\pm$  SD). Contribution to diet was calculated for each level of summer flounder age and tissue. "--" denotes model solutions whose 95% confidence intervals were not between 0 and 100.

Time Period	Age	Tissue	Summer Flounder		Prey - Fishes			Prey - Crustaceans		
			$\delta^{15}\text{N}^*$	n	$\delta^{15}\text{N}$	n	% in diet	$\delta^{15}\text{N}$	n	% in diet
Spring 2006										
	1+	muscle	13.8 $\pm$ 0.8	14	15.1 $\pm$ 1.2	21	50 $\pm$ 10	12.4 $\pm$ 0.6	15	50 $\pm$ 10
Fall 2006										
	0	muscle	13.8 $\pm$ 1.4	8	16.5 $\pm$ 1.5	26	1 $\pm$ 19	13.8 $\pm$ 0.6	14	99 $\pm$ 19
	1+	muscle	14.1 $\pm$ 0.4	7	16.5 $\pm$ 1.5	26	9 $\pm$ 8	13.8 $\pm$ 0.6	14	91 $\pm$ 8
Spring 2007										
	0	muscle	11.3 $\pm$ 0.2	8	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
		blood	10.4 $\pm$ 0.4	6	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
		liver	10.8 $\pm$ 0.5	7	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
	1+	muscle	12.8 $\pm$ 0.9	5	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
		blood	12.4 $\pm$ 0.8	5	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
		liver	12.4 $\pm$ 0.6	5	15 $\pm$ 1.1	20	--	14 $\pm$ 1.1	21	--
Fall 2007										
	0	muscle	13.3 $\pm$ 1	12	15.8 $\pm$ 0.8	23	14 $\pm$ 12	12.9 $\pm$ 0.9	19	86 $\pm$ 12
		blood	12.9 $\pm$ 1	12	15.8 $\pm$ 0.8	23	-3 $\pm$ 12	12.9 $\pm$ 0.9	19	103 $\pm$ 12
		liver	13.1 $\pm$ 0.7	12	15.8 $\pm$ 0.8	23	5 $\pm$ 10	12.9 $\pm$ 0.9	19	95 $\pm$ 10
	1+	muscle	13.4 $\pm$ 0.5	5	15.8 $\pm$ 0.8	23	15 $\pm$ 11	12.9 $\pm$ 0.9	19	85 $\pm$ 11
		blood	13.3 $\pm$ 0.5	5	15.8 $\pm$ 0.8	23	13 $\pm$ 10	12.9 $\pm$ 0.9	19	87 $\pm$ 10
		liver	12.2 $\pm$ 0.4	5	15.8 $\pm$ 0.8	23	-27 $\pm$ 12	12.9 $\pm$ 0.9	19	127 $\pm$ 12

\*Note - summer flounder  $\delta^{15}\text{N}$  values were adjusted for fractionation (see methods).

**Table 3.** Mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for trophic guild samples collected in the Chesapeake Bay mainstem. For each trophic guild, significant MANOVA results ( $p < 0.05$ ) are indicated by different letters for the following comparisons: season effect tested in each year, year effect tested within each season.

Trophic guild	Time period	n	$\delta^{13}\text{C}$		$\delta^{15}\text{N}$		MANOVA
			Mean	SD	Mean	SD	
Crustacean							
	Spring 2006	15	-18.66	0.41	12.41	0.59	a
	Fall 2006	14	-19.28	1.15	13.81	0.58	b
	Spring 2007	21	-19.17	0.61	13.95	1.11	c
	Fall 2007	19	-18.17	0.93	12.95	0.90	d
Fishes							
	Spring 2006	21	-18.63	0.73	15.11	1.23	a
	Fall 2006	26	-19.97	1.66	16.48	1.53	b
	Spring 2007	20	-18.29	1.34	15.04	1.14	ac
	Fall 2007	23	-18.97	0.89	15.81	0.83	c

**Table 4.** F-statistics and p-values for pairwise comparisons following a MANOVA of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measured for three summer flounder tissues. Comparisons were only tested for the specified levels of year, season, or age. Significant p-values ( $p < 0.05$ ) indicated by \*, highly significant p-values ( $p \leq 0.001$ ) indicated by \*\*.

Comparison	Year	Season	Age	F-statistic			p-value		
				Muscle	Blood	Liver	Muscle	Blood	Liver
2006 vs. 2007									
	--	spring	1+	4.2			0.02*		
	--	fall	0	2.8			0.07		
	--	fall	1+	1.5			0.24		
spring vs. fall									
	2006	--	1+	0.4			0.67		
	2007	--	0	15.7	19.8	33.5	<0.01**	<0.01**	<0.01**
	2007	--	1+	1.1	2.0	0.3	0.35	0.16	0.76
age-0 vs. age-1+									
	2007	spring	--	5.7	9.1	11.0	0.01*	<0.01**	<0.01**
	2006	fall	--	8.1			<0.01**		
	2007	fall	--	0.6	3.0	5.6	0.58	0.07	0.01*

**Fig. 1.** Sampling locations for summer flounder and common prey types within Chesapeake Bay. Symbol shape indicates sampling year (square – 2006, circle – 2007). Symbol color represents season of capture (gray – spring, black – fall).

**Fig. 2.** Biplots of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for summer flounder and other sampled species collected in spring 2006 (A), fall 2006 (B), spring 2007 (C), and fall 2007 (D). Fractionation-adjusted isotopic values (mean  $\pm$  SD) of individual summer flounder tissues (white – muscle, black – blood, gray – liver) plotted for age-0 fish (circles) and age-1+ fish (triangles). For reference, raw summer flounder tissue means (i.e., not adjusted for fractionation) are also plotted with smaller symbols and no error bars. Squares indicate means ( $\pm$ SD) of prey species (black – fishes, white – crustaceans), labeled as follows: ba – bay anchovy, cr – Atlantic croaker, ha – spotted hake, ma – mantis shrimp, my – mysid shrimp, sa – sand shrimp, sp – spot, we – weakfish.

**Fig. 3.** Mean differences ( $\pm$ SE) in  $\delta^{15}\text{N}$  (A) and  $\delta^{13}\text{C}$  (B) between tissues sampled from individual age-0 and age-1+ summer flounder collected in spring and fall of 2007. After adjusting for fractionation effects, stable isotope values of muscle were subtracted from blood (black bars) and also from liver (gray bars). Values significantly different from zero are noted with an asterisk.

**Fig. 4.** Individual summer flounder  $\delta^{15}\text{N}$  values for muscle (A), blood (B), and liver (C) plotted by total length (mm). Symbol shapes represent season of capture (circle – spring, square – fall) and color indicates year (white – 2006, black – 2007). For reference,  $\delta^{15}\text{N}$

of prey fishes collected in fall 2007 are plotted as mean (dashed line)  $\pm$  one standard deviation (dotted line).

**Fig. 5.** Individual summer flounder  $\delta^{13}\text{C}$  values for muscle (A), blood (B), and liver (C) plotted by total length (mm). Symbol shapes represent season of capture (circle – spring, square – fall) and color indicates year (white – 2006, black – 2007).

**Fig. 6.** C:N values for individual summer flounder liver tissue collected in 2007 plotted by total length (mm). Color indicates maturity status of individuals (white – immature, black – mature).

Figure 1.

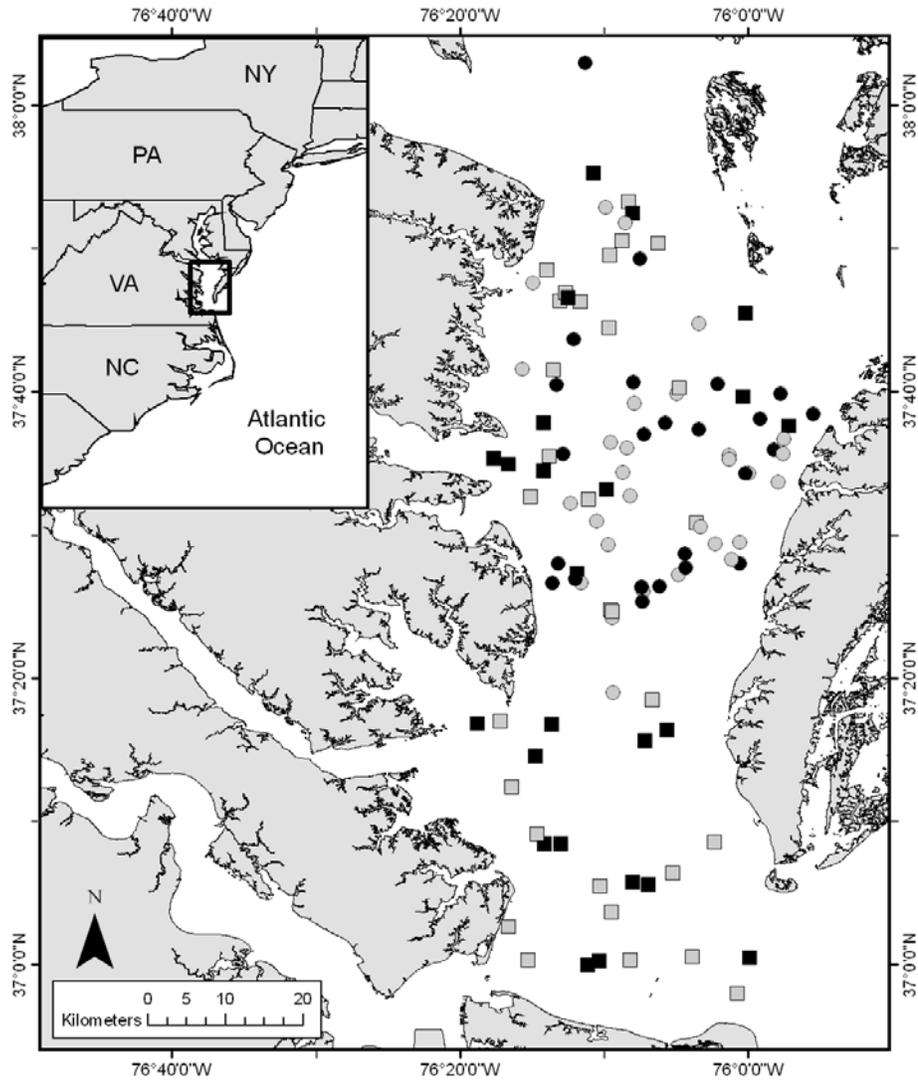


Figure 2.

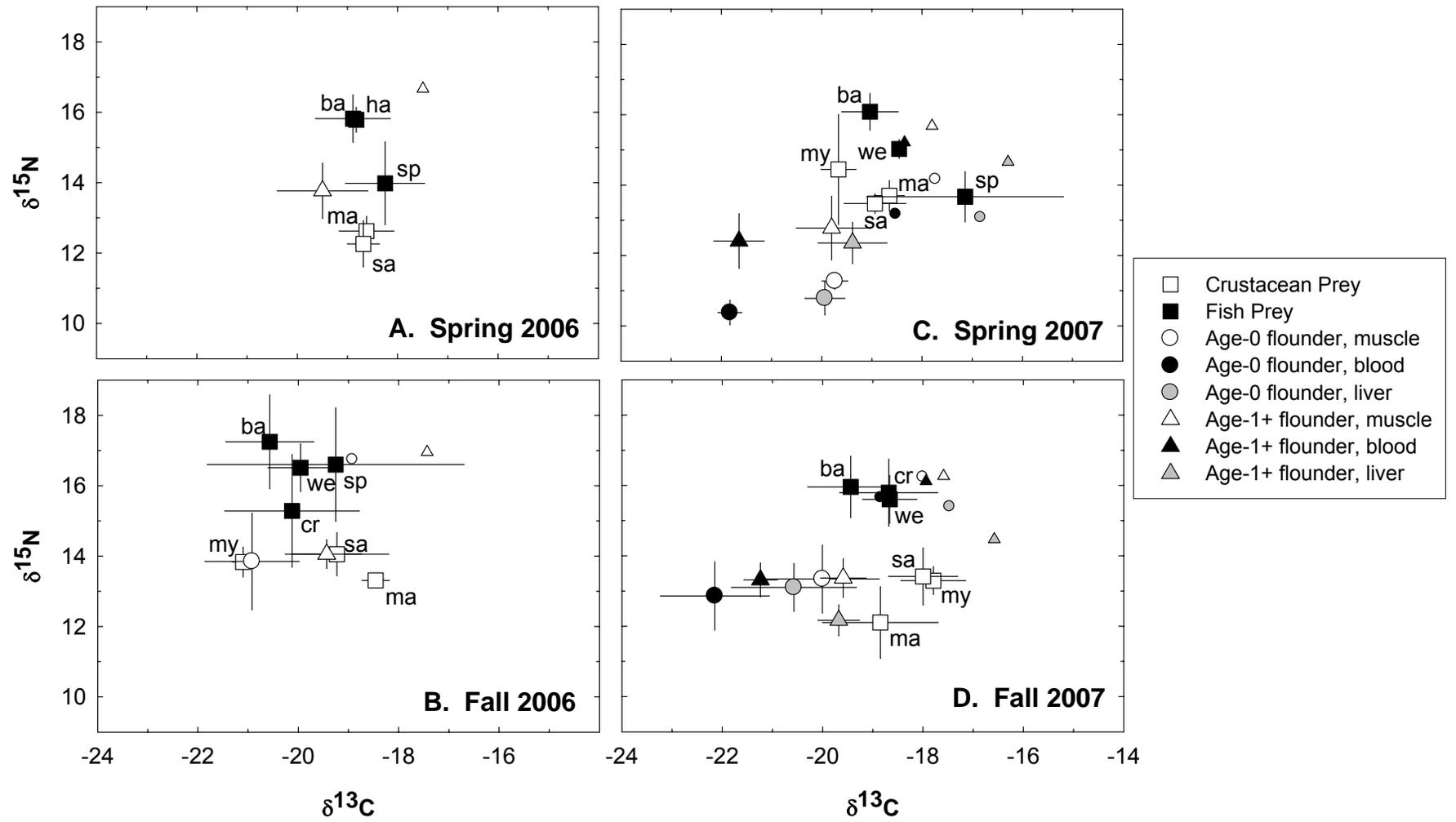


Figure 3.

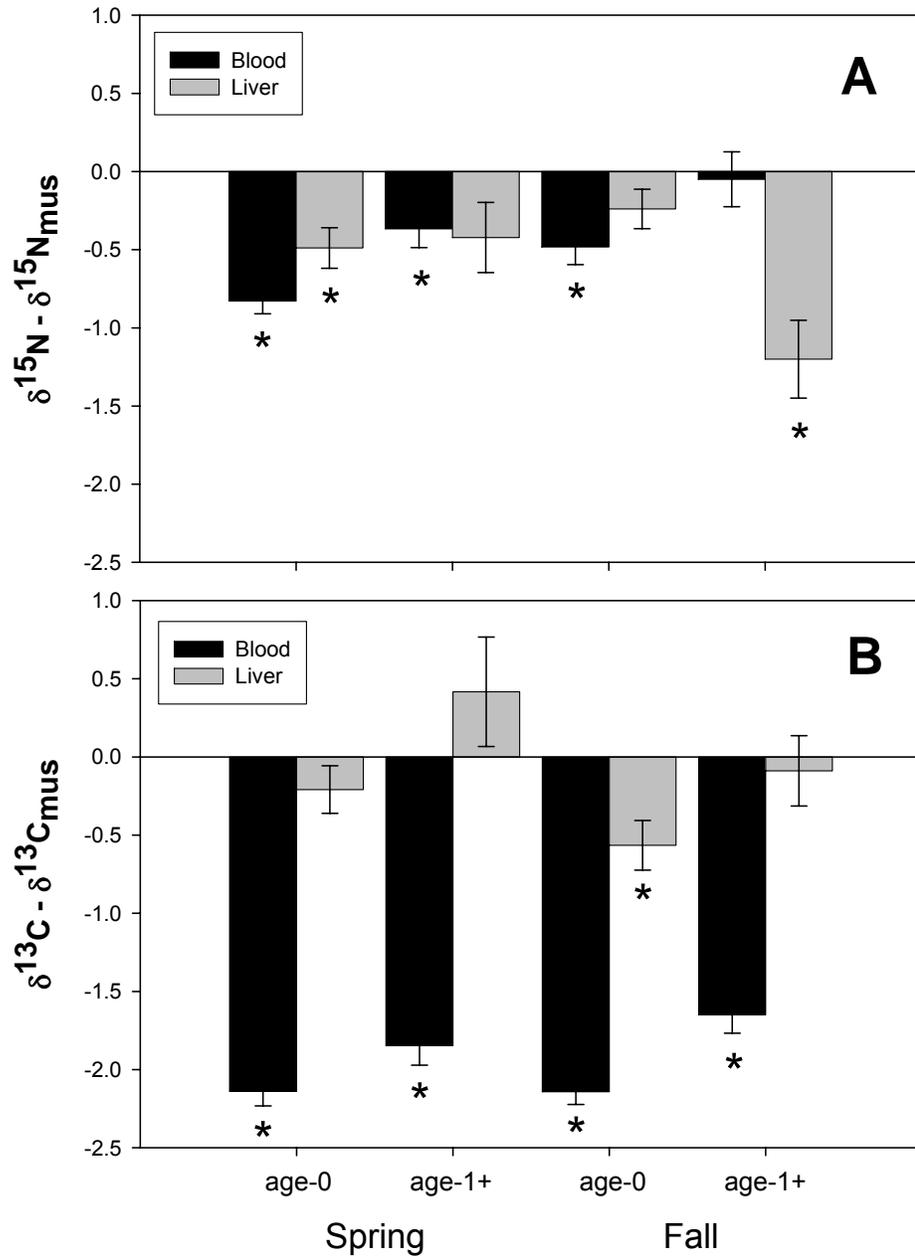


Figure 4.

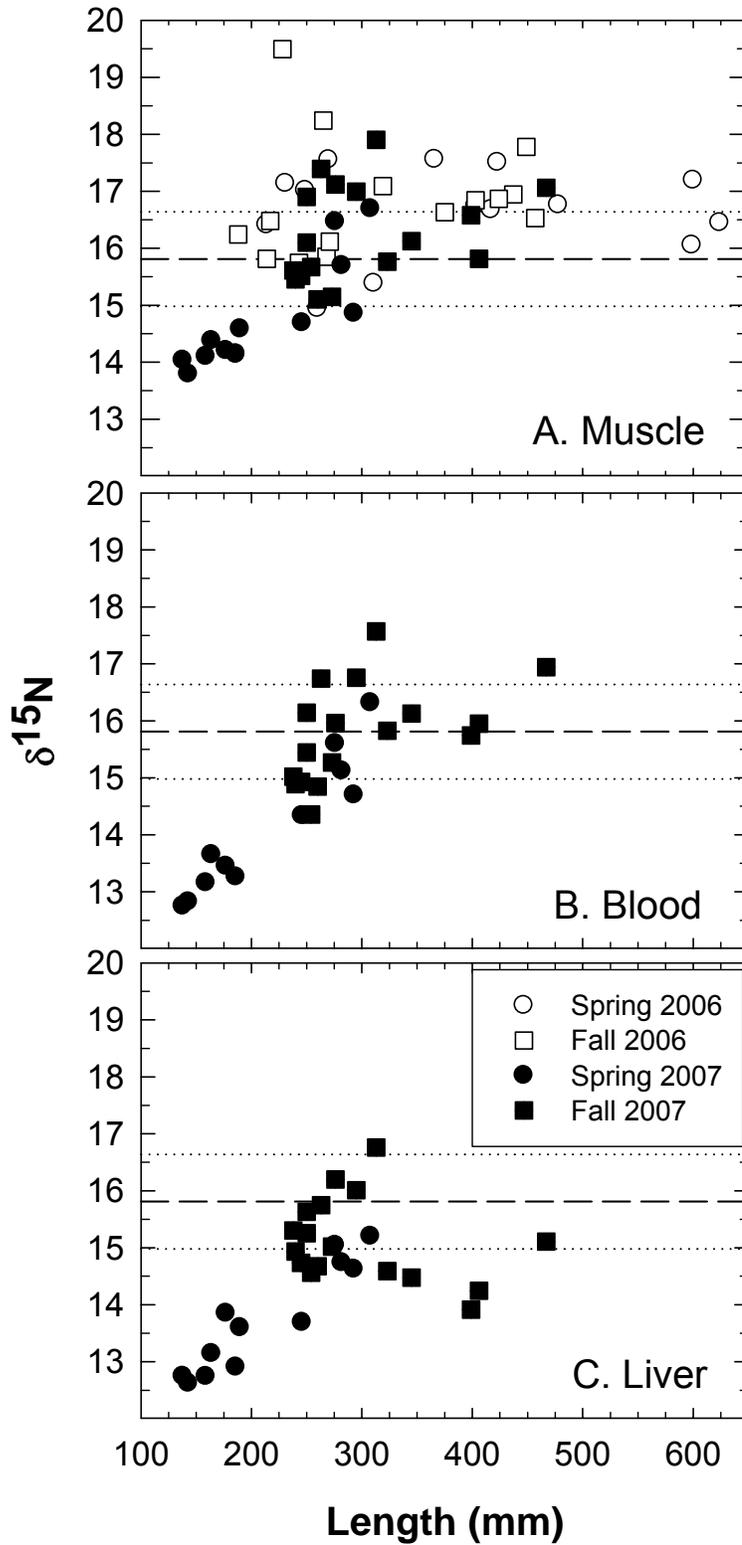
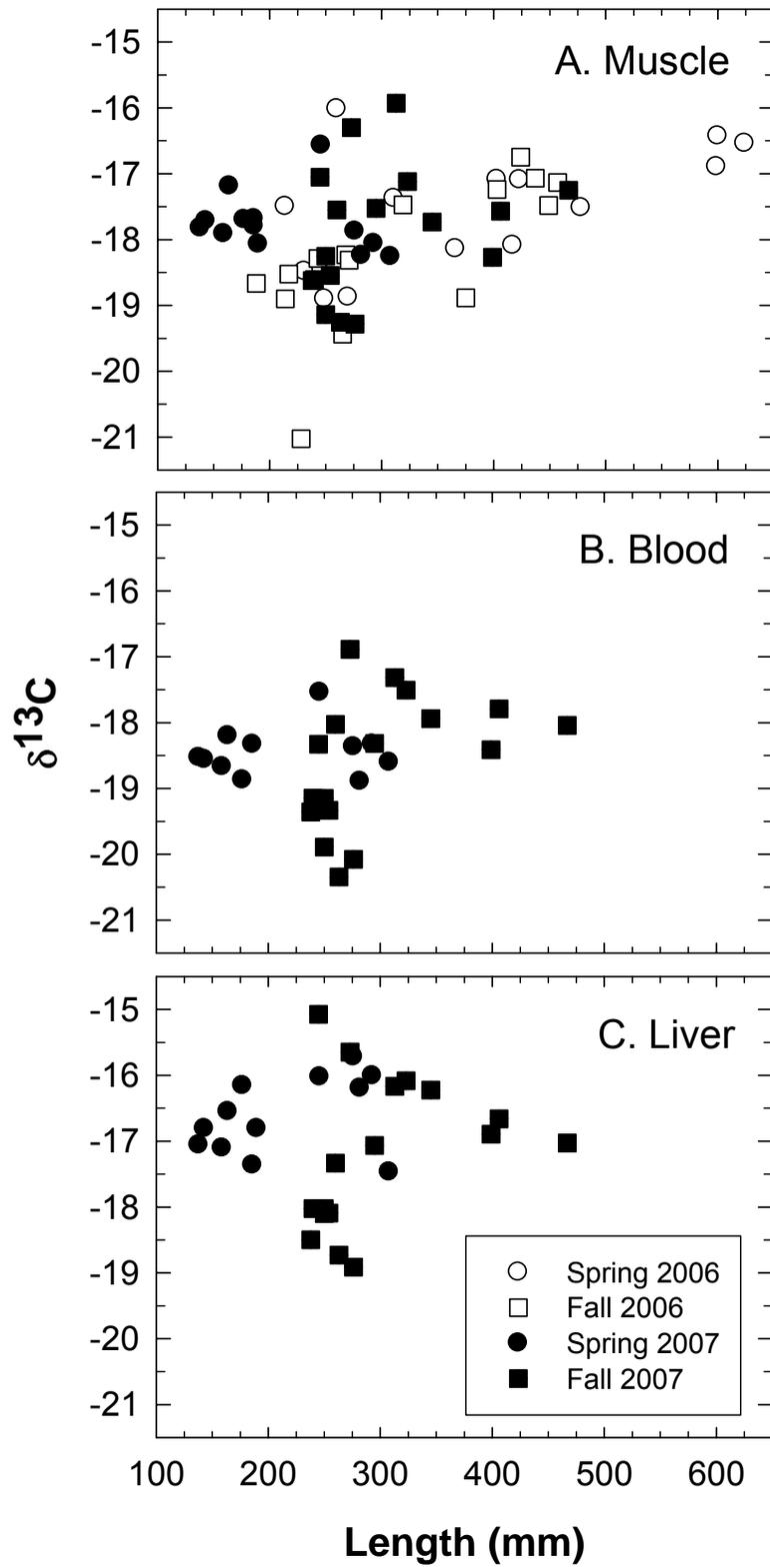
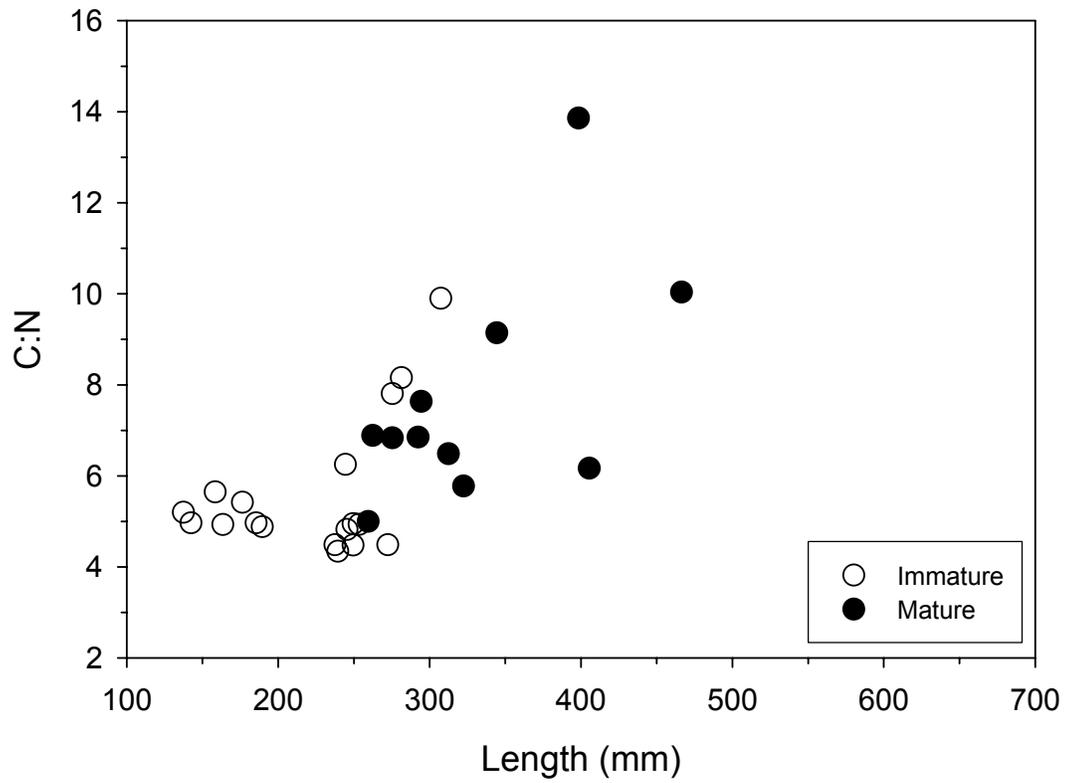


Figure 5.



**Figure 6.**



## APPENDIX

### *Lipid correction for liver $\delta^{13}\text{C}$*

Liver  $\delta^{13}\text{C}$  was corrected for lipid content using the methodology described in Chapter 1. Using twelve of the collected liver samples,  $\delta^{13}\text{C}$  was analyzed both before and after lipid extraction. In addition to the methodology described in the text (Chapter 1), lipid extract samples were concentrated to 1 ml (Zymark Turbo Vap 500). The weight of each total lipid extract was determined gravimetrically to calculate the percent lipid content of each sample. Following Logan et al. (2008), four competing models were used to describe the relationship between the bias created by lipid extraction,  $\delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}}$ , where LE represents samples that had lipids extracted and NE represents samples that did not. The following models were fit to the data (the numbering of equations mimics the numbering used in Logan et al. 2008):

$$(1) \quad \delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}} = D\left(\theta + \frac{3.90}{1 + 287/L}\right) \quad \text{where}$$

$$L = \frac{93}{1 + (0.246 \times C : N - 0.775)^{-1}}.$$

$$(1a) \quad \delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}} = \frac{a \times C : N + b}{C : N + c}$$

$$(2) \quad \delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}} = P - \frac{P \times F}{C : N}$$

$$(3) \quad \delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}} = \beta_0 + \beta_1 \ln(\text{C}:\text{N})$$

Each of these four models describes the relationship between the raw C:N and the bias introduced by not extracting lipids. The following parameters were estimated using nonlinear regression: D and  $\theta$  (model 1); a, b, and c (model 1a); P and F (model 2);  $\beta_0$  and  $\beta_1$  (model 3). For explanations of each model, refer to Logan et al. (2008). The information theoretic approach was used to identify the model that is best supported by the data by calculating  $\text{AIC}_c$  (Burnham and Anderson 2002). Under this framework, the model with the lowest  $\text{AIC}_c$  is the best model of the set.

The percent lipid content of liver samples had a positive relationship with the C:N of the samples, supporting the notion that C:N is a suitable proxy for lipid content (Fig. A1). All four models captured the trends in  $\delta^{13}\text{C}_{\text{LE}} - \delta^{13}\text{C}_{\text{NE}}$  (Fig. A2). Model 1 had the lowest  $\text{AIC}_c$ , but model 2 was nearly indistinguishable from it with regards to  $\text{AIC}_c$  (Table A1). For simplicity, only model 1 was used to correct  $\delta^{13}\text{C}$  of all liver samples for lipid content (i.e. model averaging was not employed). The parameters for this model were estimated ( $\pm\text{SE}$ ) as  $D = 6.2883 \pm 1.3519$  and  $\theta = 0.0612 \pm 0.1527$  (Table A2).

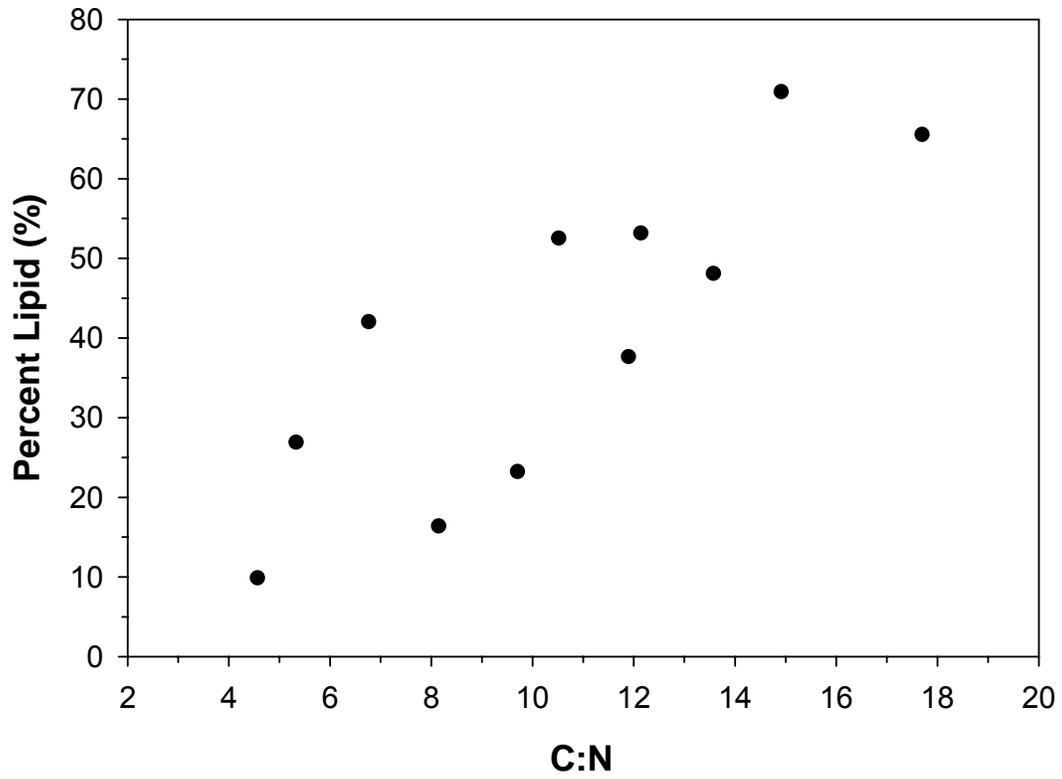
**Table A1.** Model statistics for the competing models used to describe the relationship between liver C:N and the bias in  $\delta^{13}\text{C}$  introduced by not extracting lipids. Abbreviations and symbols are as follows: RSS – residual sum of squares for the model; N – sample size; K – number of parameters (including the error term);  $\text{AIC}_c$  – Akaike’s Information Criterion corrected for small sample sizes;  $\Delta\text{AIC}_i$  –  $\text{AIC}_c$  differences;  $w_i$  – model weights, or the probability that each model is the best model of the set.

Model	RSS	N	K	$\text{AIC}_c$	$\Delta\text{AIC}_i$	$w_i$
1	4.778	12	3	-2.050	0.000	0.411
1a	4.537	12	4	2.042	4.093	0.053
2	4.789	12	3	-2.022	0.028	0.405
3	5.787	12	3	0.249	2.299	0.130

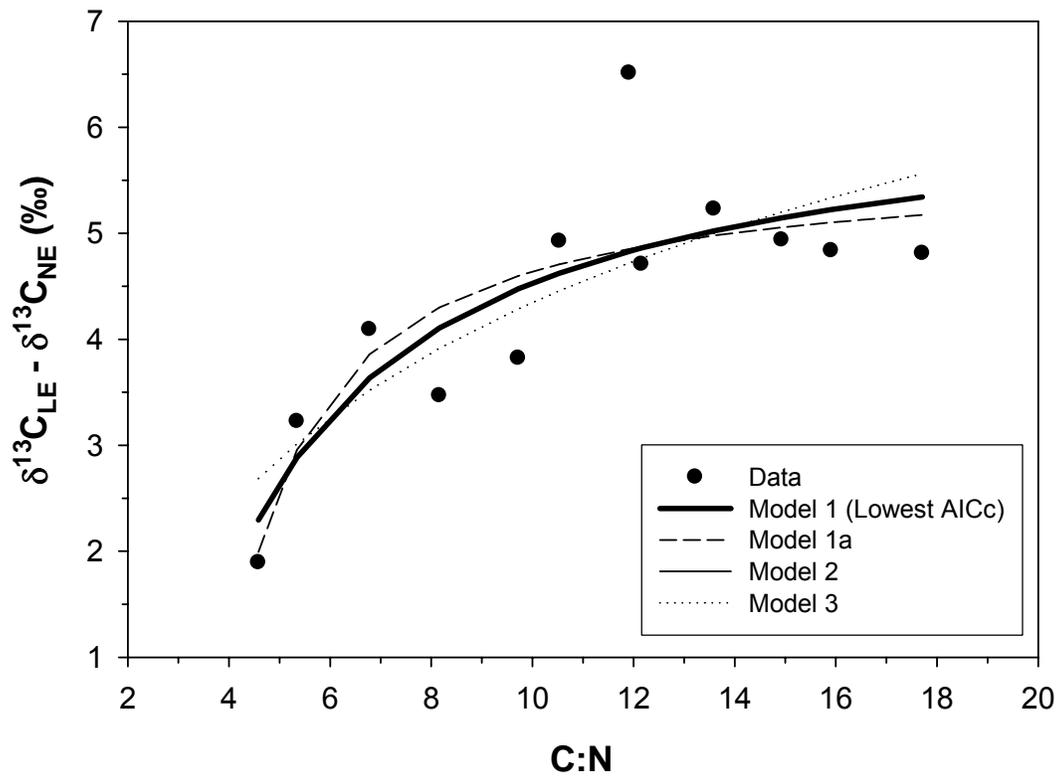
**Table A2.** Parameter estimates and standard errors (SE) for liver  $\delta^{13}\text{C}$  lipid-correction models.

Model	Parameter	Estimate	SE
1	D	6.2883	1.3519
1	$\theta$	0.0612	0.1527
1a	a	5.6965	0.7851
1a	b	-21.7933	3.0509
1a	c	-2.4176	2.0722
2	P	6.4081	0.4821
2	F	2.9370	0.4410
3	$\beta_0$	-0.5532	1.2458
3	$\beta_1$	2.1277	0.5299

**Figure A1.** Percent lipid content of liver samples plotted by raw C:N.



**Figure A2.** Relationship between C:N and  $\delta^{13}\text{C}$  bias introduced by not extracting lipids from liver samples. Bias in  $\delta^{13}\text{C}$  was calculated as the difference between  $\delta^{13}\text{C}$  for lipid-extracted samples (LE) and non-lipid extracted samples (NE). Model fits are indicated by different lines, but model 2 is obscured by the thick line of model 1.



## VITA

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Born in Abington, Pennsylvania on 19 March 1980. Graduated from Wissahickon High School in Ambler, Pennsylvania in 1998. Graduated *magna cum laude* from Duke University with a Bachelor of Science in Biology and a concentration in Marine Biology. Worked as a research fishery biologist for NOAA's Alaska Fisheries Science Center based in Seattle, Washington from 2002-2005. Entered the Master of Science program at the School of Marine Science, Virginia Institute of Marine Science, at The College of William and Mary in 2005.