Fish scales as ecological indicators:

Empirical approaches to improve their practical application to fish ecology

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Abstract

The collection of scales is common when fish communities are sampled within research and monitoring programmes in freshwater fisheries. Although used primarily to age individual fish, there is increasing evidence of their potential for application to other ecological methods, yet there is also considerable uncertainty in how this can be achieved. Thus, the purpose of this research was to examine how the use of scales within age and growth studies can be enhanced and investigate their application to freshwater trophic ecology, with a particular focus on advancing their utilisation within stable isotope analysis (SIA).

The research used fishes of the Cyprinidae family as the focal species. Cyprinids are of substantial global, socio-economic importance as their communities are valued ecologically, commercially and recreationally. The research assessed current methodologies, highlighted extant gaps in knowledge and sought to resolve these issues. It covered work regarding the intrinsic error contained in estimating fish age from scales and improved scale application within SIA through the provision of key data that is currently lacking within the literature.

An initial invasion ecology case-study provided new insights into the growth and trophic impacts of a model native and two non-native fishes under three distinct approaches of differing spatial scale and complexity. The results revealed a range of ecological consequences for the native species from the invaders, although the extent of these was also a function of spatial scale. Additionally, a number of procedural concerns relating to the collection of fish age data and current SIA methodologies were highlighted.

The use of scales to derive estimates of the ages of fish is well established, with outputs used to address questions on aspects of fish and fisheries ecology, but the process remains prone to inherent errors. The research revealed that precision of growth estimates is significantly influenced by the sub-sampling regime applied. Where individuals are long-lived and slow-growing, sub-sampling strategies that result in few scales being analysed produced imprecise data and potentially erroneous outcomes. Additionally, uncertainty in the accuracy of ageing scales also potentially results from subjective interpretation of

scale features. A statistical model was developed to incorporate this uncertainty into analyses, using Bayesian statistics and a bootstrapping methodology, to improve age and growth rate estimates. The model successfully produced error adjusted von Bertalanffy growth parameters.

Food web and trophic analyses have traditionally been completed through stomach content analysis, but increasingly SIA is preferred, as it provides greater temporal perspectives and requires smaller sample sizes. In fish studies, dorsal muscle tissue is typically favoured, but this is often collected destructively. The research revealed that non-destructively collected tissues, such as scales, can act as a proxy for muscle and their isotopic values can be converted with minimal error when species-specific factors are used. When stable isotope data are applied to dietary studies, their use in statistical mixing models requires accurate step-wise enrichment values between diet and consumer (i.e. discrimination factors). There is considerable uncertainty in the variability of discrimination factors between species and the influence of their diet. Consequently, specific diet-tissue discrimination factors were produced for a range of cyprinid species and diet was shown to significantly affect diet-tissue discrimination. The application of species-specific values within mixing models can result in significant differences when compared with using standard values and consideration of the influence of diet needs to be made when investigating omnivorous species. The rate of turnover of carbon and nitrogen stable isotopes was also determined and variability between tissues was revealed, indicating that species- and tissue-specific half-lives should be considered when deciding upon experimental time-frames.

In summary, the research has provided substantial information targeting extant knowledge gaps relating to the application of scales from cyprinid fishes to ecological studies. Regarding fish age and growth, issues surrounding accuracy and precision of estimates has been tackled, informing researchers of the influence on precision of applying sub-sampling regimes to subsequent growth analyses and providing an original statistical tool that can improve accuracy through producing growth parameters that better reflect inherent errors in fish age data. In contributing to the use of scales in SIA, novel data have been provided that will reduce the requirement for destructive sampling of fishes and enhance present understandings of the significance of species- and tissue-specific discrimination factors and turnover rates.

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Author's declaration

I confirm that this thesis is all my own work, with the following exceptions:

Chapter 2 was partly published and was written in collaboration with Robert Britton as:

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Chapter 1. General introduction

This thesis investigates how the use of fish scales in ecological studies can be improved through developing contemporary methods and providing vital new insights that will enhance their application to important research questions. Fishes of the Cyprinidae (cyprinid) family are used as the focal species to complete these investigations, although the methodologies and techniques will be applicable more widely. Cyprinid fishes are of substantial socio-economic importance, both locally and globally, comprising the second largest family of vertebrates on earth (over 3000 species; Nelson *et al*., 2016). Their communities are valued as both a commercial crop and within recreational fisheries, as well as having considerable ecological significance due to their wide-ranging distribution in many areas of the world. The research covers topics that include how the use of scales within fish age and growth studies can be enhanced, as well as investigating their application to freshwater trophic ecology, with a particular focus on advancing their utilisation within stable isotope analysis, an increasingly applied, contemporary, ecological technique. The purpose this chapter is to introduce some basic concepts and develop the rationale for the research aim and objectives.

1.1. Estimating fish age from body structures

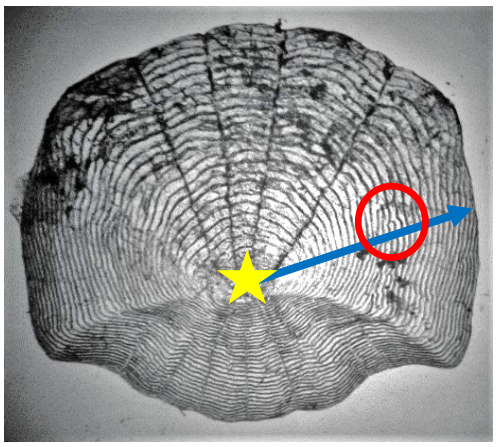

Figure 1. A chub *Squalius cephalus* scale at x 48 magnification. The red circle shows the location where the first spring circuli cuts across an incomplete winter circuli, creating an annulus. The yellow star indicates the scale focus and the blue arrow represents the scale radius (i.e., the distance from the scale focus to the scale edge).

As fish are ectothermic animals, their internal body temperature is strongly affected by the temperature of their environment (Neuheimer and Taggart, 2007) and so their growth is significantly influenced by temperature. In areas with periodic differences in temperature throughout the year, such as temperate regions, fish grow in response to seasonal patterns and fluxes in temperature (Jobling, 1997; Pörtner *et al*., 2001), with most growth occurring during the warmer summer months and relatively little growth occurring through the cooler winter period (Gotthard, 2001). Much like the ability to estimate the age of a tree through the growth rings present within its trunk, calcified structures within the body of a fish portray its growth history (Casselman, 1990). For example, as fish scales grow, ridges, known as circuli, form around the outer edge and create a concentric pattern on the scale over the course of a year (Schneider *et al*., 2000; Fig. 1). In the warmer months, the circuli are spread further apart, and in the colder months, they are closer together and may create incomplete rings and hence the pattern of spacing between circuli corresponds to the growth patterns of the fish. The first circuli of spring may 'cut across' an incomplete winter circuli, creating a distinguishable 'check' on the surface of the scale, indicating an annual marking, or annulus (Schneider *et al*., 2000; Fig. 1). Validation studies have been undertaken for a variety of species that confirm significant relationships between the growth represented on calcified structures and the actual growth rate of the fish (e.g., Leim, 1924; Casselman, 1990; Heidarsson *et al*., 2006). Whilst scales are a common structure used to age fish (Robillard and Marsden, 1996), other structures can also be used, including otoliths (Bagenal and Tesch, 1978), opercula (Baker and Timmons, 1991), vertebrae (Brown and Gruber, 1988), fin rays (Cass and Beamish, 1983) and cleithra (Casselman, 1990).

A principal difference between some of the structures used to age fish is that the collection of scales is non-lethal, enabling the individual fish to be returned to water alive after the removal of some scales, with these lost scales subsequently regenerated by the fish (Broussonet, 1786, 1789; Bereiter-Hahn and Zylberberg, 1993). In contrast, the collection of all the other structures previously mentioned is destructive, a potential issue for studies of fishes that are protected or endangered or that have conservation implications. For example, the goliath grouper *Epinephelus itajara* is listed as Critically Endangered on the IUCN (International Union for Conservation of Nature) Red List of Threatened Species (Craig, 2011) and is federally protected against fishing in the United States of America (NMFS, 2006). However, fisheries such as those in French Guiana

remain open, and age and growth analyses are required in order to assess the impacts on the sustainability of the *E. itajara* stock (Artero *et al*., 2015). Therefore, the ageing of non-lethally collected structures, such as scales, are required and have been investigated (Brusher and Schull, 2009).

The process of analysing scales to estimate fish ages is well established, with the first documentation of the method dating back to the 18th century (Jackson, 2007). Scales and otoliths are the most common structures used for ageing fish (Campana, 2001), with scales increasingly preferred due to their non-destructive collection, as outlined above. Additionally, once dried, scales are suitable for long-term storage with little degradation in quality (Al-Absy and Carlander, 1988). Nevertheless, scales can be problematic for ageing fish due to subjective interpretations of scale features, resulting in inaccuracies, with otoliths tending to provide more accurate ages (Secor *et al*., 1995; Liao *et al*., 2013). The most common method to determine ages from scales is through their direct mounting on a projecting microscope, with other methods including use of cellulose acetate impressions of scales (Smith, 1954), digitizing tablets (Frie, 1982), imaging systems and digital software that can aid in identifying annuli, such as TNPC (Traitement Numérique des Pièces Calcifiées or Numerical Treatment of Calcified Structures) software developed for the digital processing of calcified structures (Mahe *et al*., 2016). In response to the arduous techniques and outdated equipment often used, Hagen *et al*. (2001) developed an approach for scale analysis through creating a digital archive of scale images to share among researchers and applied a high-resolution digital imaging technique to extract scale measurements and counts of circuli and annuli. However, the implementation of such technologically aided ageing systems are still not widespread.

1.2. Utilisation of age estimates and growth history

In addition to age estimates, scales enable reconstruction of the growth history of each fish through the process of back-calculation (Francis, 1990; Ricker, 1992; Pierce *et al.*, 1996). This determines the growth rate of each fish, as the length at each annulus can be estimated using measurements of scale radii (i.e., the distance from the scale focus to the scale edge; Fig. 1) and fish body lengths at capture within equations such as those of Dahl–Lea (Dahl, 1907; Lea, 1910) and Fraser–Lee (Fraser, 1916; Lee, 1920). Both are used commonly and their accuracy has been validated (e.g., Klumb *et al*., 1999; Heidarsson *et al*., 2006).

The derived estimates of fish age and growth rates are important in developing understandings of the fundamental processes and factors that influence the ecology of fish populations and the biology of fisheries (Bagenal and Tesch, 1978; Francis, 1990; Coggins and Pine, 2010). These data play key roles in addressing questions on basic ecological relationships whose outputs can then be used to underpin the formulation of management strategies for fisheries specifically, and aquatic ecosystems more generally (Beardsley and Britton, 2012). The types of information that can be obtained from ageing fish scales include growth rate, age at maturity, number of spawning periods per life span, age at harvest, age class composition, abundance of year classes and longevity (Carlander, 1974). These can help indicate the productivity of aquatic ecosystems and how this can shift with environmental or management changes, such as nutrient enrichment, habitat loss and restoration (Beardsley and Britton, 2012), through, for example, comparison of growth increments for the specific years involved. The information collected from the ageing of scales can provide estimates of recruitment, mortality and maturity (Campana, 2001), that can then be used to estimate optimum yields and catch regulations in fisheries.

Fish scales are also routinely collected in response to Government policy and legislation. For example, the Water Framework Directive (WFD) is a European directive which aims to protect and improve the water environment and requires the ecological status of rivers in England to be assessed (WFD, 2000). The ecological status of the fish fauna is a key element in the way that rivers are classified, as fish are recognised as important indicators of the quality of the freshwater ecosystem. Surveys of fish populations, including cyprinid fishes, are used to assess the status of stocks which contributes to the overall status of a water body via metrics including species composition and age structure. Waters with good and high ecological status contain disturbance-sensitive species and have fish populations with age structures that indicate no or few failures in their reproduction or development, as would be indicated by the absence of age classes (WFD, 2000).

The application of these data to fisheries management has not, however, always been successful due to issues of ageing error. For example, individuals within a population of orange roughy *Hoplostehus atlanticus*, in a commercial fishery in New Zealand, were drastically under-aged in the 1980s and these ages were applied to biological models to devise appropriate fishing limits (Smith *et al*., 1995). Due to the substantial under-ageing of the structures, growth and mortality rates that were set in the model were overly

optimistic and therefore the exploitation rates that the model suggested were far higher than the population could sustain. Consequently, the population was rapidly over exploited and subsequently collapsed (Smith *et al*., 1995). The systematic under-estimation of fish age can impede understandings of recruitment variability and longevity and can also bias estimates of survivorship. For instance, a misunderstanding of cisco *Coregonus artedi* biology, driven by scale-ageing error, again through age under-estimation, was linked to the collapse of their fisheries in the Great Lakes of North America (Yule *et al.*, 2008). Increases in body length after age 8 years in this species is negligible, but individuals can survive for an additional 10 or more years (Yule *et al.*, 2008), and so the application of scales to estimate their ages is open to a high degree of error in fishes aged over 8, as annual circuli formation will be minimal and therefore annuli will be difficult to identify at the scale edge. Unfortunately, the Van Oosten (1929) method of scale ageing that was used to study the populations had not been validated for *C. artedi* and so early investigators concluded that the species were short-lived and had relatively constant recruitment levels. As a result, they too were overfished and all the populations in the Great Lakes collapsed during the 20th century (Yule *et al*., 2008). Additionally, in freshwater fish of the Cyprinidae family, Musk *et al*. (2006) revealed errors in the ageing of fish in older age groups whose growth rates had decreased following sexual maturity. This resulted in recruitment patterns being previously misinterpreted. Hence, any exclusion of data from difficult scales, primarily from older fishes, could seriously affect mortality estimates that are required to calculate optimum yields (Carlander, 1974). Therefore, the accurate ageing of fish, using body structures such as scales and otoliths, is required if such errors are not to be repeated in future.

1.3. Error and subjectivity surrounding the ageing process

There are two main categories of error that surround the process of ageing fishes from hard structures, regardless of the structure used. Firstly, process error, which is due to the difficulty of identifying annual markings on structures and secondly, interpretation error, which is the subjective element and comes from a reader's ability to distinguish true annuli from other features (Chang, 1982). Some of the difficulties that are encountered include resorption of the scale edge (Crichton, 1935) that occurs under conditions of starvation, such as the cessation of feeding due to spawning, which may be so severe that previous annuli are totally reabsorbed and cannot be discerned (Linfield, 1974).

Furthermore, annuli may not be created where the fish are so small at the first winter that the scales are only just forming; or when growth does not stop or slow down enough to disrupt the scale pattern, such as in tropical climates; or when fish growth is so slow that the scale does not grow enough to show an annulus or the annuli are so narrow that determining them is extremely difficult, such as in fish that may be old and growing very little (Carlander, 1974), as in *C. artedi* (*cf.* Section 1.2.). Moreover, markings that may appear to be annuli, but that are false and have been created during a growth year can be challenging to separate from true annuli and can occur in response to stress, sudden changes in temperature or injury (Schneider *et al*., 2000). Attempts have been made to standardise the identification of these 'false annuli' through accepting only those which can be followed all around the scale and which are a constant feature of all scales examined from any one fish. However, the periodic features in calcified structures tend to vary markedly in appearance and relative size among fish (Campana and Neilson, 1985), contributing to the difficulty of determining true annuli from false.

In a report published by the Environment Agency, it was estimated that £1.18 billion was spent on freshwater angling in England and Wales in 2005, with coarse angling responsible for £971 million of this, demonstrating the economic importance of freshwater angling activities (Radford *et al*., 2007). The Cyprinidae family is central to catch-and-release freshwater angling in England and Wales, with many species prized by recreational anglers. Consequently, a number of cyprinid fishes are farmed in hatcheries, such as roach *Rutilus rutilis*, chub *Squalius cephalus* and European barbel *Barbus barbus* (Britton *et al*., 2004a), with these fishes then stocked into wild fisheries in large numbers (Cowx and Gerdeaux, 2004; Hickley and Chare, 2004). Thus, in some locations, stocked fishes may make up a significant proportion of the population (Bašić and Britton, 2016). For stock assessment and work under the WFD, this might be problematic, as particular difficulties have been reported that specifically relate to identifying false annuli on the scales of recaptured stocked fish that spent their early life stages at a hatchery (Britton *et al.,* 2004a; Ibáñez *et al*., 2008; Britton, 2010). For example, Britton *et al*. (2004a) indicated that fish of known age, 1+, often appeared to be at least 3+ years old and, in some cases, as high as 5+ years. Subsequent work revealed that false annuli were being produced on scales in responses to husbandry changes, such as movement from indoor to outdoor tanks where a sudden temperature change would occur (Ibáñez *et al*., 2008). Hence, given all of these issues, the process of determining the ages of fishes from scales

will always retain an element of subjectivity that will contribute various degrees of error to age determinations (Campana *et al*., 1995).

In addition to the error surrounding the process of ageing fish from hard structures, errors also occur due to deviations in the true versus derived age. These errors relate to accuracy, which is a measure of the proximity of the age estimate to its true value (Kalish *et al*., 1995), and precision, which is the reproducibility of individual measurements from a structure (Campana *et al*., 1995; Kalish *et al*., 1995). The reliability of age estimates can thus be called into question where studies have failed to assess the accuracy and precision of the technique used (Campana, 2001). The issue of ageing accuracy and precision is confounded in many fish populations by individuals that are slow-growing and long-lived as this increases the probability of ageing error (Vilizzi *et al*., 2013). Furthermore, populations of cyprinid fish in temperate lowland rivers are often distinguished by populations of relatively slow-growing species, such as *S. cephalus* that comprise of individuals that can sometimes live for over 20 years (Mann, 1976; Britton, 2007). Moreover, their lengths-at-age are often characterised by significant individual variation within and between age classes (Britton, 2007). This often results from multiple spawning strategies causing variation in length at age 1 (Nunn *et al*., 2002), but with consistent annual length increments thereafter (Bolland *et al*., 2007).

Increased awareness of the intrinsic errors associated with the ageing process has triggered efforts to quantify and account for it, with studies emphasising the importance of ageing validation studies (e.g., Beamish and MacFarlane, 1983; Campana, 2001; Francis *et al*., 2010) and estimating measures of reader bias and precision (e.g., Campana *et al*., 1995, Campana, 2001). As it is possible to derive precise age estimates that are inaccurate, it is important that studies address the accuracy of their procedures before the precision of their estimates (Campana *et al*., 1995). Validation studies can help to improve the accuracy of, and confidence in, age estimates (Jackson, 2007) and consequently, there are an assortment of validation methodologies available. These range from the basic, such as mark-recapture studies using fish of known age in the wild (Shirvell, 1981), to the more complex, such as radiochemical dating (Campana, 2001) and marginal increment analysis, a method that often incorporates marking body structures with chemicals such as tetracycline (Nagiec *et al*., 1995).

Quality control procedures applied within the process of ageing fishes have also been shown to improve accuracy. Procedures currently used include the employment of a

secondary reader who re-ages a random sub-set of a scale sample (i.e. 10 %: Musk *et al*., 2006; 20 %: Kimura and Anderl, 2005) without prior knowledge of the ages estimated by the primary reader and if any disagreements are found, the scale(s) are reviewed by both readers to allow a consensus to be reached. Alternatively, Liao *et al*. (2013) performed a quantitative evaluation of ageing bias and its effects on the assessment of Atlantic striped bass *Morone saxatilis* stock by comparing the results of scale- and otolith-age data using a statistical catch-at-age model and found biases in scale ages, including a 15 % underestimate of population abundance, a 19 % overestimate of fishing mortality and that weak age 1 recruitment years appeared stronger and strong years appeared weaker. They also demonstrated that by using a relatively small sample of paired scale–otolith ages, these biases can be corrected (Liao *et al*., 2013). The ability to use statistical and graphical techniques to assess precision and bias in age estimates has been recognised (Campana *et al*., 1995), with, for example, the use of age-bias plots to identify the problems of detecting under- and over-ageing and issues where younger fish are over-aged and older fish are under-aged, or *vice versa*.

Despite all of these advances in knowledge on how ageing accuracy can be improved, the process is still subject to erroneous interpretations that remain difficult to eradicate. Errors in ageing that arise from the subjective determination of true annuli will always remain when wild fish are sampled. Consequently, despite these exhaustive efforts to reduce error, statistical methods that can incorporate aspects of ageing uncertainty into analyses, such as models based on Bayesian statistics or bootstrapping methodologies, might provide a more realistic alternative as they are able to acknowledge that some ageing error is inherent to the methods and can work with, not against, the uncertainty in order to produce adjusted and more realistic growth metrics and parameters. For cyprinid fishes, however, such a tool has yet to be developed. Furthermore, where issues of using different sample sizes to calculate population ages and growth rates occur, such as the application of different sub-sampling regimes, as are routinely applied within many stock assessment programmes, then statistical methods can be applied to determine the effect on the precision of growth data. Indeed, some studies have already been completed to quantify the effects that different sampling strategies have on the estimates of mean length-at-age of reef fishes (e.g. Goodyear, 1995) and the influence that sample size has on the precision of derived population parameters (e.g. Kritzer *et al*., 2001). However, similar information is not available for many freshwater fishes, including long-lived

cyprinid fishes in temperate freshwaters. Consequently, this remains as an outstanding knowledge gap in the ageing data derived from scales of cyprinids.

1.4. Application of fish scales to ecological studies

As scales have been collected by Government agencies and research institutions in numerous countries for many years, archives of fish scale collections are available for various freshwater systems and species. These archives have the potential to provide a valuable tool for assessing ecological changes that may have occurred over extensive temporal scales. For example, scale archives have been used to provide insights into the long-term growth performance of freshwater fishes in relation to changes in water quality (Grey *et al*., 2009; Beardsley and Britton, 2012), changing patterns of recruitment in relation to climate (Britton *et al*., 2004b) and to quantify temporal variations in Atlantic salmon *Salmo salar* smolt ages (Englund *et al*., 1999). However, many scale archives are still largely under-valued and under-utilised.

1.4.1. Stable isotope analysis

Stable isotopes of a particular atom have nuclei that contain the same number of protons but a different number of neutrons and so they differ in their nuclear mass. The ratio of naturally occurring heavy to light stable isotopes of different elements, such as carbon (^{13}C: ^{12}C) and nitrogen (^{15}N: ^{14}N), vary predictably in the environment and from resource to consumer (Fry *et al*., 1999). As stable isotopes are transferred up food chains and food webs, there is a step-wise enrichment of the heavier isotopes compared with the lighter isotopes, and hence, trophic structure can be reconstructed and trophic niche size can be analysed in order to expose the arrangement of the overall food web (Grey, 2006; Fig. 2). This can provide insights into the trophic relationships between species and their diets (Vander Zanden *et al*., 1999; Grey, 2006). Stable isotopes are measured using a mass spectrometer (Peterson and Fry, 1987) and are usually expressed in delta (δ) values, which are parts per thousand (per mille, ‰) differences from an international standard, such as carbon in the PeeDee limestone and nitrogen gas in the atmosphere (Peterson and Fry, 1987). The energy source of a consumer is indicated by the carbon stable isotope ($\delta^{13}C$), which tends to show minor changes between trophic levels (DeNiro and Epstein, 1978; Post, 2002), and trophic position by the nitrogen stable isotope ($\delta^{15}N$) (DeNiro and

Epstein, 1981; Minagawa and Wada, 1984; Post, 2002; Fig. 2). The analysis of food web structure and trophic niche size has traditionally been completed through stomach contents analysis (Hyslop, 1980; Rybczynski *et al*., 2008). However, stomach contents analysis only provides a 'snap-shot' of the diet of a fish, can require large sample sizes and is incapable of elucidating the extent to which a fish is assimilating energy from putative food resources (Pinnegar and Polunin, 1999). Thus, stable isotope analysis (SIA) has been increasingly used in ecological studies in recent times, either singularly or in addition to stomach contents analysis (Cucherousset *et al*., 2012b).

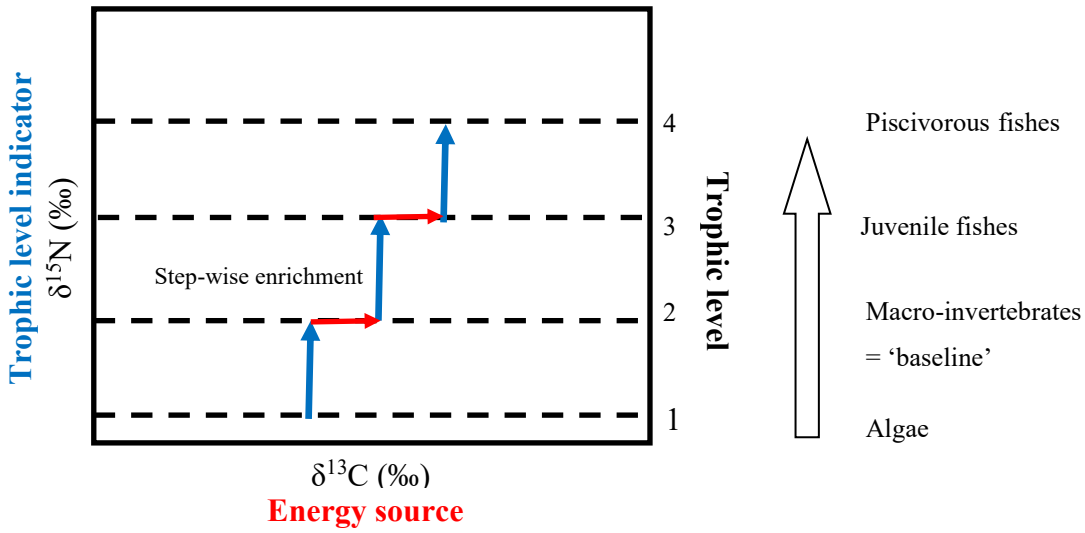

Figure 2. A stable isotope 'map' of a simplified aquatic food chain demonstrating the step-wise enrichment of carbon and nitrogen stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) that occurs between a consumer and its resources at increasing trophic levels.

The interpretation of stable isotope data is reliant upon two principal metrics; the step-wise enrichment of heavier stable isotopes from diet to consumer (Fig. 2), referred to as the discrimination factor (or fractionation, depending on the author), and the rate of isotopic change that occurs within tissues when a consumer undergoes a dietary shift, known as the turnover rate. Turnover rates are particularly important when studying the trophic ecology of mobile or migratory species or species that experience ontogenetic shifts in their diets (e.g., MacAvoy *et al*., 2001; Buchheister and Latour, 2010; Hertz *et al*., 2015), as, if tissues are sampled prior to them reaching isotopic equilibrium with the new diet then erroneous data interpretations could occur (O'Reilly *et al*., 2002). Notably, variability has been shown in turnover rates between tissues of freshwater fishes and thus

species-specific data are often necessary (e.g., McIntyre and Flecker, 2006; Church *et al.,* 2009; Carleton and Martínez del Rio, 2010). In order to accurately predict the diet of consumers, correct discrimination factors are also required. The values most commonly cited in the literature are discrimination factors of 0.4 ± 1.3 ‰ for $\delta^{13}C$ and 3.4 ± 0.98 ‰ for $\delta^{15}N$ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002). However, studies such as Mill *et al*. (2007) stress the importance of determining discrimination values for consumers on a case-by-case basis, rather than using standard values, as the factors can be affected by many aspects including age, diet quality, protein quality and content, body size, sample preparation and tissue type (Pinnegar and Polunin, 1999; Jacob *et al*., 2005; Jardine *et al*., 2005). Discrimination factors also have importance as fundamental components of the statistical mixing models that derive quantitative estimates of dietary contributions of consumer species from their isotopically distinct putative food sources (Parnell *et al*., 2010).

The application of stable isotope mixing models to diet predictions requires an understanding of their assumptions and limitations in order to provide insights into consumer-resource relationships (Phillips *et al*., 2014). They require precise estimates of diet-tissue discrimination factors (Phillips and Gregg, 2001; Bond and Diamond, 2011; Phillips *et al*., 2014), assume that the factors are constant, irrespective of the animal's biology or feeding behaviour (Mill *et al*., 2007), and that the tissues analysed are in equilibrium with the diet. In a recent review, Phillips *et al*. (2014) stated that diet-tissue discrimination factors are one of the major causes of uncertainty in using mixing models to assess diet. It is therefore vital that discrimination factors are as accurate as possible, as small variations may lead to important differences in the outputs (Ben-David and Schell, 2001) and may produce a confused or obscured dietary analysis (Pinnegar and Polunin, 1999). However, diet-tissue discrimination factors can be challenging to estimate in wild situations, due to inter- and intra-specific, temporal and spatial variation and dietary and environmental influences (Grey, 2006; Davis *et al*., 2012; Layman *et al*., 2011). Hence, *ex situ* experiments on discrimination factors and turnover rates can be completed alongside SIA in field studies in order to gain insights into the underlying ecological processes (Cucherousset *et al*., 2012b; Layman *et al*., 2011).

Dorsal muscle tissue is most commonly analysed in stable isotope studies of fishes, as it most closely resembles that of the diet of the fish (Pinnegar and Polunin, 1999). Recently, however, there has been a shift towards analysing samples that can be collected

non-destructively, such as fin tissue and scales, as significant relationships between the isotopic signatures of the tissues have been revealed (e.g., Jardine *et al*., 2005; Kelly *et al*., 2006; Hanisch *et al*., 2010; Jardine *et al*., 2011; Fincel *et al*., 2012). Fish scales are effective archived biological material, since they are typically stored dry for many years without preservatives that could degrade stable isotope signals (Roussel *et al*., 2014). Thus, scale archives are now recognised as important ecological resources (Syväranta *et al*., 2008), containing information not only on age and growth rates, as previously mentioned, but that can potentially reveal trophic changes that have occurred over relatively long time-scales. Given the number of scale archives held by various museums and institutions, and the relatively few studies that have retrospectively applied them to trophic analyses (Grey, 2006; Syväranta *et al*., 2008), they largely exist as a significant, but currently under-utilised resource. This is despite archived scales having revealed spatial and temporal changes in food web dynamics in studies dating back over 20 years (e.g. Wainright *et al*., 1993) and more recently they have been applied to long-term studies on assessments of anthropogenic impacts on nutrient cycling in river ecosystems (Roussel *et al*., 2014) and responses to improvements in the water quality of a freshwater lake (Grey *et al*., 2009). One possible explanation of their under-exploitation is that the collection of samples to represent the 'baseline' (i.e., primary consumers; Fig. 2) within stable isotope analyses might be lacking or inconsistent in relation to the scale archive, although the determination of trophic interactions within and between fish species does not require this data. In addition, to enable comparison of stable isotope studies based on scales with those based on other tissues, principally dorsal muscle, investigation into the relationships of the stable isotope data of the different tissues is required (e.g. Bašić and Britton, 2015) and existing information on stable isotope tissue conversion factors is largely lacking, particularly for cyprinids, despite recent efforts (e.g. Tronquart *et al*., 2012). Consequently, the extensive application of fish scales to investigating long-term ecological changes in freshwater systems remains constrained by this substantial knowledge gap.

1.5. Assessing the ecological impacts of non-native fishes

Fishes are among the most widely introduced group of aquatic animal in the world (i.e. at least 624 species; Gozlan, 2008). Their strong association with human activities has resulted in their accidental and/or intentional release into unnatural environments, and in

the UK, the translocation of fishes of angling interest, particularly of species belonging to the Cyprinidae family, have been significant (Copp *et al*., 2005). Therefore, the tools for application to assessments of the ecological impacts of non-native fishes on recipient communities are fundamentally important.

In fish introduced outside of their native range, patterns of invasion success and impacts on resident fishes are often explained by the expression of their life-history traits, including growth, that are strongly influenced by temperature and latitude (Cucherousset *et al*., 2009; Benejam *et al*., 2009; Britton *et al*., 2010c), as well as through alterations to food web structure within the receiving communities, from, for example, increased competition for resources (Vander Zanden *et al*., 1999; Britton *et al*., 2010b). As the biological features that determine the success of an invasive species include rapid growth, which can be determined from fish scales, and a broad feeding spectrum, that can be revealed through SIA of fish tissues, along with traits such as high fecundity and high tolerance of environmental conditions (Ruesink, 2005), assessment of these characteristics can be used to evaluate the potential risk of a non-native species becoming invasive, as well as revealing any negative consequences within the native ecosystem. Indeed, the quantification of age and growth is a fundamental component in prioritising control and management operations of invasive fishes (Kwak *et al*., 2006) and SIA has been successfully used to investigate competition and predation in food webs (Peterson and Fry, 1987; Fry, 2006), thus, both scale ageing and SIA have recently been used in combination to determine the ecological effects of a non-native fish on a native species (e.g. Jackson *et al*., 2016a). Between 1999 and 2010, 80 % of studies specifically using SIA to assess the ecological impacts caused by the presence of non-native fishes were performed on cyprinids as the non-native species (Cucherousset *et al*., 2012b), highlighting the significance and relevance of the invasive threat posed by this family of fishes.

Two species of cyprinids that present strong examples of non-native or invasive fishes are common carp *Cyprinus carpio* and goldfish *Carassius auratus*. *Cyprinus carpio* is now among the most widely distributed and invasive species on earth (Vilizzi *et al*., 2015). Consequently, *C. carpio* is ranked within the top 100 of the world's worst invasive alien species (Lowe *et al*., 2000). The application of scale ageing to provide information on their invasion success has indicated their fast, temperature dependent growth rates are a key determinant that enables their rapid colonisation of warm waters, such as those in

Australia (Vilizzi and Walker, 1999; Koehn, 2004) and equatorial countries such as Kenya (Britton *et al*., 2007; Oyugi *et al*., 2011). Therefore, information on *C. carpio* age and growth rates (Jackson *et al*., 2007; Weber and Brown, 2011a), that can be obtained from their scales, can provide important ecological information on their invasions that assist formulation of subsequent management strategies, including options to suppress and/or extirpate populations that negatively influence important sport and commercial fisheries (Britton *et al*., 2011b; Yates *et al*., 2016). Additionally, *C. carpio* can potentially have strong ecological impacts on ecosystem functioning and trophic interactions (e.g., Koehn, 2004; Cucherousset and Olden, 2011), caused by, for example, direct competition for resources, as well as *C. carpio*-induced habitat alterations, that have been shown to reduce the diversity of prey and decrease the dietary niche of sympatric species (e.g. Jackson *et al*., 2012). As previously mentioned, a broad feeding spectrum can increase the success of an invasive species, and in a recent study, SIA revealed that invasive fishes, including the cyprinids *C. carpio* and *C. auratus*, occupied similar trophic niches and trophic positions (Guinan *et al*., 2015) that may be attributed to their omnivorous feeding strategies (García-Berthou, 2001; Lorenzoni *et al*., 2007), thus allowing them to maximise their feeding potential and increasing their likelihood of establishment.

Carassius auratus is not as widely distributed as *C. carpio,* but has successfully established populations throughout Europe, where it has been referred to as the worst alien fish species (Veer and Nentwig, 2015), as well as in North (Gido and Brown, 1999) and South America (Gomez *et al*., 1997), New Zealand (Rowe, 2007) and Australia (Lintermans, 2004). Similarly to *C. carpio*, the back-calculated lengths from scales of *C. auratus* have been used to determine their growth rates in order to assist in their control (Lorenzoni *et al*., 2010), however, use of SIA to expose the trophic implications of these populations is not yet widespread. A possible explanation is the notion that *C. auratus* threats relate mainly to hybridisation with native species and not from competition for resources (Hänfling *et al*., 2005; Tarkan *et al*., 2009), although this has not been fully explored and may have been overlooked. Hence, both scale ageing and SIA can be valuable tools for assessing the ecological impacts of, and trophic interactions between, non-native and native fishes.

1.6. Research aim and objectives

Sections 1.3 and 1.4 outlined several inherent issues that remain apparent in the application of scales from cyprinid fishes to ecological studies that rely on current methods to determine age and growth rate and/or perform stable isotope analysis. Thus, the primary aim of this research is to overcome these considerable information gaps through the expansion of knowledge and development of methodologies via a range of *in-* and *ex-situ* studies within freshwater systems. This is initially completed by work on an invasion ecology case-study that, whilst providing new insights into the ecological interactions of native and non-native fishes, also highlights a number of methodological concerns that relate to ageing and stable isotope analysis. The following research then aims to resolve these specific procedural problems through the acquirement of novel data and development of a new statistical modelling tool. Consequently, the research objectives (O) are to:

O1: Assess the ecological impacts on a model native fish, crucian carp *Carassius carassius*, from invasion by the non-native fishes *Carassius auratus* and *Cyprinus carpio*, using *in-* and *ex-situ* experimental contexts, with identification of gaps in knowledge and analytical tools for subsequent study (Chapter 2);

O2: Identify how different strategies of collecting scales affects the subsequent precision in the growth rate data across three species of cyprinids (Chapter 3);

O3: Determine how data from the ageing of scales can incorporate information on the uncertainty of the ages in order to account for potential errors in age estimates, using statistical modelling approaches based on bootstrapping methods (Chapter 4);

O4: Enhance the application of scales to stable isotope studies, with a view to minimising the use of dorsal muscle tissue, and thus destructive sampling, through

completing the following sub-objectives, using a range of cyprinid fishes as model species in primarily *ex situ* experimental contexts:

O4a: Assess the isotopic relationships between non-lethally collected tissues and calculate their conversion factors to muscle tissue (Chapter 5);

O4b: Determine the effect of diet composition on diet-tissue discrimination factors (Chapter 6);

O4c: Quantify carbon and nitrogen stable isotope turnover rates in a range of tissues in *Barbus barbus* (Chapter 7).

1.7. Thesis structure

The subsequent data chapters (Chapters 2 to 7) are each developed from objectives 1 to 4 (*cf.* Section 1.6). They take the form of discrete pieces of work and so are presented in that format without a generic materials and methods chapter. The final chapter (Chapter 8) will discuss the findings from Chapters 2 to 7, with recommendations to improve the utility and practical application of fish scales within ecological studies. A list of references and appendices will conclude the thesis.

1.8. Ethical considerations

The necessary ethical aspects and associated regulated scientific procedures carried out were considered in an independent ethical review committee under Bournemouth University's Home Office (HO) Certificate of Designation. All procedures (anaesthesia, PIT tagging) were completed under appropriate project licences. Permission to use the data in Chapter 3 was granted by the Environment Agency.

Chapter 2. Stable isotope and growth rate analyses suggest contrasting ecological impacts on crucian carp *Carassius carassius* from two invasive fishes

Part of this chapter was published as:

Busst, G. M. and Britton, J. R., 2015. Quantifying the growth consequences for crucian carp *Carassius carassius* of competition from non-native fishes. *Ecology of Freshwater Fish*, *24*(3), pp.489-492.

This paper covered the work reported in the tank aquaria experiments (Sections 2.3.1, 2.4.1 and 2.5.1).

2.1. Summary

Ecological consequences for native fishes arising from invasive fish include the negative impacts of increased inter-specific competition in co-existing fishes, including modified trophic positions and trophic niche sizes, and suppressed growth rates. Here, the impacts on the growth rates and trophic ecology of a model native fish, crucian carp *Carassius carassius*, from two model invasive fishes, goldfish *Carassius auratus* and common carp *Cyprinus carpio*, were tested over three spatial scales and system complexities. In tank aquaria, testing growth rate impacts for *C. carassius* of intra- and inter-specific competition revealed that inter-specific competition from *C. carpio* and *C. auratus* significantly suppressed growth rates when compared to intra-specific interactions. In pond enclosure experiments, the three species were held for 100 days in allopatric and sympatric treatments, with their trophic relationships tested using stable isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) and growth rates using incremental increases in length. These revealed that the trophic position, trophic niche size (as the isotopic niche) and somatic growth rates were similar for *C. carassius* in allopatry and in sympatry with *C. auratus*. When in sympatry with *C. carpio*, however, *C. carassius* growth rates were significantly reduced, despite a significant increase in their trophic niche size when compared to allopatry. In a

field based, non-replicated experiment, *C. carassius* and *C. carpio* were held for up to 300 days in allopatric and sympatric contexts. In sympatry, the trophic niche of *C. carassius* was increased, contrasting with *C. carpio*, which was significantly reduced, when compared to allopatry. The trophic niche of *C. carassius* was also significantly larger than *C. carpio* in sympatry. These results reveal a range of ecological consequences from the invaders for *C. carassius*, although the extent of these was also a function of spatial scale. A series of issues relating to scale ageing and the application of stable isotope analysis to fish ecological studies were also detected and discussed.

2.2. Introduction

Biological invasions can have negative and irreversible impacts on the receiving populations (Cucherousset and Olden, 2011), with their influences potentially including the adverse effects of increased inter-specific competition for resources within the recipient communities, such as reduced growth rates and abundance of native species (Rahel and Olden, 2008; Gozlan *et al*., 2010). Competitive interactions between invasive and native fishes have traditionally been demonstrated using analyses of diet composition gained from stomach contents analysis (Declerck *et al.*, 2002; Cucherousset *et al.*, 2012b), although this can require large sample sizes that are often achieved through destructive sampling (Hyslop, 1980). In recent years, the application of stable isotope analysis to invasion ecology has revealed its high utility for analysing temporal and spatial impacts of invaders on native fishes (Vander Zanden *et al*., 1999; Britton *et al*., 2010b; Remon *et al*., 2016; *cf*. Section 1.5). These include, for example, the impacts on food webs of invasive red swamp crayfish *Procambarus clarkii* (Jackson *et al*., 2012, 2016b) and the importance of terrestrial food sources for invasive European catfish *Silurus glanis* (Cucherousset *et al*., 2012a)

Competition in fish communities occurs principally through two mechanisms, interference competition and exploitative competition. The former is where the competitor uses aggression to monopolise resources and the latter is where there is no direct interaction between competitors, with the species generally having similar feeding modes and exploiting the available resources non-aggressively, thus reducing the overall resource availability for individuals (Connell, 1983). In the context of invasive fishes, as

the invader might become more numerous, exploit resources more effectively, or be a superior competitor (Ruetz *et al*., 2003), adverse effects for native fishes, such as reduced somatic growth rates and reduced fitness and population abundance, can occur (Crowl *et al.*, 1992; Martin *et al.*, 2010; Britton *et al*., 2011a; Weber and Brown, 2011b). For example, a high density population of invasive topmouth gudgeon *Pseudorasbora parva* that shared trophic space with native roach *Rutilus rutilus* resulted in the significantly suppressed growth rates of the native fish (Britton *et al*., 2010b).

Ecological theory suggests that increased competition for food resources, such as following the introduction of a non-native species, can result in the development of larger trophic niches in the competing species, through, for example, developing a broader feeding spectrum, as this reduces the intensity of the inter-specific feeding interactions (Svanbäck and Bolnick, 2007; Svanbäck *et al.*, 2008; Bolnick *et al.*, 2010). Contrarily, the niche variation hypothesis predicts that under increased inter-specific competition, populations become less generalised in their diet (Van Valen, 1965), with trophic niche sizes of the competing species reducing and, often, diverging (partitioning) (Human and Gordon, 1996; Thomson, 2004; Olsson *et al.*, 2009). Niche divergence can thus facilitate the co-existence of the non-native and native species, enabling the integration of the non-native species into the community (Guo *et al*., 2014; Jackson and Britton, 2014; Tran *et al.*, 2015). This is because divergence minimises the occurrence of inter-specific feeding interactions, through reducing the direct sharing of resources, with the underlying mechanisms likely to include specialisations in foraging microhabitat and shifts in diel activity patterns (Jones *et al.*, 2001; Amarasekare, 2003). Thus, the development of these feeding interactions can determine the outcome of introductions of non-native species (Baiser *et al.*, 2010; Jackson *et al.*, 2012), for example, through their influence on the success of establishment (Tilman, 2004) and the ecological impacts that subsequently develop in the native communities (e.g., Woodford *et al.*, 2005; Kakareko *et al.*, 2013).

Invaded freshwater ecosystems are well suited to testing hypotheses on trophic niche theory, as they provide relatively stable systems with well-defined functional groups across different trophic levels that enable shifts in trophic niches to be detected using methodologies such as stable isotope analysis (Cucherousset *et al.*, 2012b; *cf.* Section 1.4.1). However, understanding how introduced species integrate into communities can be inherently difficult, due to, for example, an absence of data in the pre-invaded phase, the complexity of multi-species fish communities involving populations with high diet

plasticity, and some potential context dependency in outcomes (Cucherousset *et al.*, 2012b; Guo *et al.*, 2012, 2014). Hence, incorporating experimental systems into studies on trophic impacts can assist the understanding of the trophic relationships in species within more complex wild scenarios, as patterns in trophic position, and trophic niche breadth and divergence, can show consistency across different spatial scales and system complexities (Tran *et al.*, 2015). In addition, the outcomes of exploitative competitive interactions involving invasive fishes can be difficult to measure in field studies due to the numerous abiotic and biotic factors that influence parameters such as fish growth rates, food availability and diet composition (Britton *et al*., 2011a). Consequently, studies that initially focus on co-habitation experiments in controlled conditions can be advantageous in helping to understand the outcomes of species' interactions in the wild (Korsu *et al*., 2009).

The crucian carp *Carassius carassius* has been described as native to the British Isles (Tarkan *et al*., 2009), primarily to South East England (Wheeler, 2000), and has been regulated as such by governmental regulatory authorities. It should be noted, however, that this has been subject to some conjecture recently following genetic analyses that suggest the species has only been present in Britain for around 600 years and so may instead be considered naturalised (Jeffries *et al*., 2016). *Carassius carassius* is a relatively small bodied (generally < 300 mm) benthic feeding species that potentially has an important role in nutrient cycling and trophic dynamics within small water bodies due to the disturbance of surface sediments during foraging (Holopainen *et al*., 1992). The species has experienced a substantial decline in the extent of its range in England in recent decades (Copp *et al*., 2008; Tarkan *et al*., 2009, 2016) and is sufficiently threatened that a 'Biodiversity Action Plan' is in place in one region of Eastern England (Copp and Sayer, 2010; Sayer *et al*., 2011). As pond-dwelling fishes are particularly sensitive to the potential effects of fish introductions (Sayer *et al*., 2011), due to the likelihood of experiencing more intense interactions than in larger systems, the principal threats to populations include introductions of its Asian congener, goldfish *Carassius auratus* (Tarkan *et al.*, 2009) and the common carp *Cyprinus carpio*, a globally invasive fish (Copp *et al.*, 2008; Jackson *et al.*, 2012; Vilizzi, 2012; *cf*. Section 1.5), as well as habitat loss, such as loss of wetland pond systems (Sayer *et al*., 2011). Both *C. auratus* and *C. carpio* are increasingly present in pond systems in England for recreational catch-and-release angling (Britton *et al*., 2010a). Whilst their impacts on *C. carassius* primarily

relate to a loss of genetic integrity in populations through hybridisation (e.g. Hänfling *et al*., 2005), there are also concerns relating to the adverse effects of increased inter-specific competition for food resources (Copp *et al*., 2008). Tarkan *et al*. (2009) found no difference in growth rates among wild allopatric populations of *C. carassius* and populations in sympatry with *C. auratus*, leading the authors to suggest that there was no evidence of impacts from competitive interactions. However, this study was based on only four wild populations of limited sample sizes and any trophic implications that may have resulted from the species being present in sympatry were not quantified. Therefore, it was concluded that further research was required for the effects to be better understood.

Consequently, the aim of this chapter was, through application of stable isotope and growth rate analyses, to determine the ecological consequences for *C. carassius* of invasions of *C. auratus* and *C. carpio* across different spatial scales that involved three experimental systems: tank aquaria, pond enclosures and wild ponds. Objectives were to: (1) use controlled co-habitation aquaria experiments, under two temperatures, to quantify the consequences for the growth rates of *C. carassius* of their feeding interactions with *C. auratus* and *C. carpio* when food resources are fixed; and (2) use pond enclosures and wild pond systems to experimentally quantify the consequences for the trophic positions, trophic niche sizes and growth rates of the fishes when in allopatric and sympatric contexts. Given the results of Tarkan *et al.* (2009), the outputs were used to test the prediction that when in sympatry, *C. carassius*, *C. auratus* and *C. carpio* will have reduced trophic niche sizes compared to allopatry, with divergence in these niches that reduces their competitive interactions, maintains their somatic growth rates and so facilitates their co-existence. A final outcome of the Chapter was also the assessment of the utility of stable isotope and growth rate analyses for investigating the ecological impacts of biological invasions in the context of non-native fishes (*cf.* Section 1.5). These aspects are discussed to identify gaps in knowledge and in analytical tools for subsequent study in the thesis.

2.3. Materials and methods

2.3.1. Tank aquaria experimental design

The initial experiment was designed to test the strength of intra- and inter-specific competitive interactions between the three fishes under controlled conditions in tank aquaria. By feeding fixed food rations on a daily basis, the growth rate responses of the fishes could be measured and tested at the end of the experiment to identify whether the strength of inter-specific competition from *C. auratus* and *C. carpio* had a stronger effect on the somatic growth rates of *C. carassius* than intra-specific competition from conspecifics.

The experimental design comprised of a control and treatments that all started with four *C. carassius*. The control was an allopatric context where four more *C. carassius* were added and two treatments were used that represented sympatric contexts, where either four *C. carpio* ('+*C. carpio*') or four *C. auratus* ('+*C. auratus*') were added, with each control and treatment replicated three times. All fish were measured (fork length, nearest mm) and weighed (to 0.01 g) at the commencement of the experiment. As water temperature has a significant and positive effect on the growth and foraging rates of these fishes (Oyugi *et al*. 2012a, b), then the experiment was performed at water temperatures of 18 and 22°C to identify whether these temperatures influenced the outcome of the interactions. These temperature increases were achieved using *in situ* water heaters and were monitored for their accuracy using temperature loggers. The experiments were completed in aquaria of 45 l volume that were arranged in columns of three shelves (one aquarium per shelf) on re-circulating systems. The fish were sourced from aquaculture and were young-of-the-year that had been pond-reared using supplementary feeding (pelletized fishmeal) and were approximately 4 months old. The experimental period lasted 35 days during which the fish were fed daily with a fixed food ration of crushed pelletized fishmeal at 2 % mean *C. carassius* body weight of the control and the treatments, as this was above maintenance but below *ad libitum*, so providing the fish with a food supply that was limited but not limiting. At the conclusion of the experiment, all of the fish were re-measured and weighed.

An issue in the experimental design was the variable starting lengths and weights of the fish, as the young-of-the-year fish species available all differed in their length ranges.

Differences in the starting lengths and weights of the initial *C. carassius* and additional fish were minimised as much as possible and a generalised linear model (GLM) was used to test their significance, as the data were not normally distributed, where length or weight was the dependent variable and treatment was the independent variable. Outputs were the mean differences in length or weight according to pairwise comparisons with Bonferroni adjustments for multiple comparisons. The differences also meant that the daily food ration, at mean 2 % body weight, differed between the control and treatments, i.e. a ration was calculated for the control at their mean 2 % body weight and then separately for the treatments at their mean 2 % body weight.

To quantify the consequences for *C. carassius* growth of the additional fish in the controls and treatments at both temperatures, the growth metrics of specific growth rate (SGR) and incremental fork length (IL) were calculated for the initial four *C. carassius* in the control and treatments. Whilst only calculating IL was an option, given the field study of Tarkan *et al*. (2009) was based only on length, the relatively short experimental period (35 days) meant that there was uncertainty as to how much growth in length would be produced by the fish in that small time-frame and so SGR, based on weight, was also calculated to complement the output. IL was determined from;

(1) $IL = (Lt + 1 - Lt) / t$

where Lt is the total initial length, $Lt + 1$ is the total final length and t is the number of days. SGR was determined from;

(2) $SGR = [((\ln Wt + 1) \ln Wt) / t] * 100$

where Wt is the total starting weight, $Wt + 1$ is the total final weight and t is the number of days.

Linear mixed effects models (LMEM) were then used to test for differences in the growth metrics between the control and treatments, and between the temperatures, where the dependent variable was IL or SGR, the treatment was the fixed factor and tank position (top, middle or bottom shelf) was the random variable. The initial models used the starting lengths or weights of the initial *C. carassius* and the additional *C. carassius*, *C. auratus* and *C. carpio* as covariates to control for the effects of the differences between the treatments. The starting lengths and weights of the initial *C. carassius* and additional fishes all had non-significant effects ($P > 0.05$) in these models and thus were removed

from the final models. The outputs of each model included the mean adjusted values of the growth metric for the control and treatments at each water temperature and their significance from pairwise comparisons with Bonferroni adjustments for multiple comparisons. All statistics were completed using IBM SPSS Statistics (version 22.0).

2.3.2. Pond enclosure experimental design

This experiment was designed to test the trophic interactions and growth consequences for the three species in allopatric and sympatric contexts. It was thus similar to the tank aquaria experiment (*cf*. Section 2.3.1), but was completed under natural conditions and over a longer time period. Consequently, the experiment comprised of three control treatments, where each of the three species was present in allopatry (n = 8), and three sympatric treatments; *C. carassius* and *C. auratus*, *C carassius* and *C. carpio*, and *C. carpio* and *C. auratus* (n = 4 of each species), with each treatment replicated three times. The fishes were sourced from aquaculture and had been pond-reared using supplementary feeding (pelletized fishmeal), and were approximately 1 year old at the start of the experiment.

The controls and treatments were set up within enclosures that sat within two large ponds that were located on an ex-aquaculture site in Southern England and were adjacent, separated by an earth bank of 2 m width. The ponds were both approximately 30 x 12 m and had consistent depths of approximately 1 m. Each enclosure comprised of an aluminium frame of 1.05 m (length) x 1.05 m (width) x 1.2 m (height) that was within a net of 7 mm^2 mesh that prevented fish movements into and out of the enclosure, but allowed the ingress and egress of macro-invertebrates. Nine enclosures were placed in each of the large ponds and the replicated treatments were located randomly across them. Space between each enclosure was at least 0.5 m to ensure they provided enclosed and independent habitats for each treatment that were identical at the commencement of the experiment. Avian predators were prevented from entering the enclosures through netting placed over the top of all enclosures (15 mm^2 mesh).

The experiment commenced in May 2014 and ran for 100 days on the assumption that this would provide adequate time for fish dorsal muscle to undergo sufficient isotopic turnover and thus indicate the trophic ecology of the fishes in the pond enclosures (Jackson *et al.*, 2013). Fish reproduction in the enclosures was prevented by using

sexually immature fish. The enclosures were placed into the ponds 7 days prior to the start of the experiment and all fish were measured (fork length, nearest mm) prior to their release. As previously mentioned, water temperature has a significant and positive effect on the growth and foraging rates of these fishes (Oyugi *et al.*, 2012a, b), so a temperature logger was placed into each of the large ponds to measure daily means and to allow testing between the adjacent ponds. On day 100, each enclosure was removed from the ponds, the fish recovered, euthanized (anaesthetic overdose, MS-222) and placed on ice. At the same time, samples of macro-invertebrates were taken from the enclosures. In the laboratory, the fish were re-measured (fork length, nearest mm) and a sample of dorsal muscle taken from the anterior region above the lateral line and below the dorsal fin, for stable isotope analysis. The macro-invertebrates were sorted into samples, where samples represented between 3 and 9 individuals per species, with triplicate samples used for each pond. These samples, and the fish dorsal muscle samples, were then dried at 60 °C to constant mass.

2.3.2.1. Stable isotope analysis

The macro-invertebrate and dorsal muscle samples were then submitted to the Cornell University Stable Isotope Laboratory, New York, USA, for analysis of $\delta^{13}C$ and $\delta^{15}N$ (Cornell University Stable Isotope Laboratory, 2016). The tissues were ground to powder, with approximately 0.5 mg weighed out into a tin cup and the actual weight recorded using a Satorius MC5 microbalance to ~1000 µg. The samples were then analysed on a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., Lakewood, NJ, USA). These were verified for accuracy against internationally known reference materials, whose values are determined by the International Atomic Energy Agency (IAEA; Vienna, Austria), and calibrated against the primary reference scales for $\delta^{13}C$ and $\delta^{15}N$ values. The accuracy and precision of the sample runs were tested after every 10 samples using a standard animal sample (mink) to compensate for possible machine drift and as a quality control measure; the overall standard deviation was 0.11 ‰ for $\delta^{15}N$ values and 0.09 ‰ for $\delta^{13}C$ values. Linearity correction was carried out to account for differences in peak amplitudes between sample and reference gases (N_2 or CO_2); the analytical precision associated with the $\delta^{15}N$ and $\delta^{13}C$ sample runs were estimated at 0.42 and 0.15 ‰, respectively. Final outputs were values of $\delta^{13}C$, as an energy source indicator

and $\delta^{15}N$, as a trophic level indicator (*cf*. Section 1.4.1; Fig. 2), expressed as their isotope ratios per mille (‰), for each individual fish and macro-invertebrate sample. The C: N ratios of the fish were below 3.5 and thus there was no requirement to correct for lipid in the $\delta^{13}C$ data (Post *et al*., 2007; Skinner *et al*., 2016).

The initial step in the analysis of the stable isotope data was to determine whether there were differences in the putative foods between the two large ponds. The focus of these analyses was macro-invertebrate groups, as these are important prey resources of the model fishes at the lengths introduced to the experimental enclosures. For example, *C. carassius* is increasingly reliant on benthic macro-invertebrates as their body size increases, with shifts away from algae and benthic planktonic resources (Penttinen and Holopainen, 1992). The macro-invertebrate groups analysed were Corixidae and Odonata, with both expected to contribute strongly to fish diet (Penttinen and Holopainen, 1992). Although Chironomidae larvae were also expected to be an important dietary component of the fishes, insufficient numbers were collected during sampling to provide triplicate samples per pond and so were not included in the analyses. For each pond, the mean values of the triplicate samples per macro-invertebrate group were calculated along with their 95 % confidence limits; where the confidence limits showed overlap in their values then the stable isotope data were interpreted as not being significantly different and so the stable isotope data of the fishes were comparable between the two large ponds without any correction.

For testing differences in $\delta^{15}N$ between treatments, the data were converted to trophic position (TP), as this has greater ecological relevance. This conversion was completed using:

$$(3)\ TP_i = [(\delta^{15}N_i - \delta^{15}N_{base}) / 3.4] + 2$$

where TP_i is the trophic position of the individual fish, $\delta^{15}N_i$ is the isotopic ratio of that fish, $\delta^{15}N_{base}$ is the isotopic ratio of the primary consumers (i.e. the 'baseline' invertebrates), 3.4 is the discrimination between trophic levels and 2 is the trophic position of the baseline organism (Post, 2002; *cf*. Section 1.4.1; Fig. 2).

The TP and $\delta^{13}C$ data were then used in linear mixed effects models (LMEM) to assess their differences between the species in sympatry, and to identify how the allopatric and sympatric contexts affected the trophic position of each species. Species were entered into models according to their treatments so, for example, *C. carassius* were present in models

as allopatric *C. carassius*, and in sympatry with *C. carpio* and *C. auratus*. The dependent variable was $\delta^{13}C$ or TP and treatment was the fixed factor. To correct for the inflation of the residual degrees of freedom that would otherwise have occurred if the data of each individual fish were used as true replicates, the models were fitted with enclosure as a random effect on the intercept (Tran *et al.*, 2015). Differences in $\delta^{13}C$ and TP by species and treatment were determined using estimated marginal means and pairwise comparisons with Bonferroni adjustments for multiple comparisons. A similar LMEM approach was also used to test for differences in the initial fish lengths between sympatric species and to identify any length differences for each species between allopatry and sympatry.

The $\delta^{13}C$ and $\delta^{15}N$ stable isotope data were then used to calculate the trophic niche size of the fishes and treatments. Trophic niche size was calculated using the metric standard ellipse area (SEA) and thus represented the isotopic niche. Whilst the isotopic niche is closely related to the trophic niche, it is also influenced by factors including growth rate and metabolism (Jackson *et al*., 2011). Hereafter, the term trophic niche is used throughout. Standard ellipse areas were calculated in the SIAR and SIBER packages (Jackson *et al*., 2011) in the R computing program (R Development Core Team, 2013, 2014). Standard ellipse areas are bivariate measures of the distribution of individuals in isotopic space. As each ellipse encloses ≈ 40 % of the data, they represent the core dietary breadth and thus reveal the typical resource use within a species or population (Jackson *et al*., 2011, 2012). Due to variable and generally small sample sizes ($n < 20$), then a Bayesian estimate of SEA (SEA_B) was used for testing differences in niche size between analysed groups. The Bayesian approach returns a distribution representing estimates of SEA that reflect uncertainty arising from the sampling process, with larger uncertainty associated with smaller sample sizes (Jackson *et al*., 2011). SEA_B was calculated using a Markov chain Monte Carlo simulation with 10^4 iterations for each group (Jackson *et al*., 2011; R Development Core Team, 2014; Tran *et al*., 2015). This generated a range of SEA_B probable values which can be compared in a quantitative manner, the mode of which are reported along with their 95 % confidence intervals. Where these confidence intervals did not overlap between comparator groups, the niche sizes were interpreted as being significantly different. Bayesian inference also allows a direct probabilistic interpretation of the differences in SEA_B depending on the grouping level. This can be achieved through pairwise comparisons by calculating the proportion of SEA_B that

differed between two groups and can be interpreted as a direct and robust proxy for the probability that one group is larger than the other (Jackson *et al*., 2011). The probabilities are calculated from the solutions generated by the model and the values range from 0 to 1, indicating very low (0) to very high probability (1) of one group being smaller than another (Jackson *et al*., 2011). To then enable calculation of the extent of the overlap of niches within each species, SEA_c was calculated (the subscript 'c' indicates that a small sample size correction was used), with the niche overlap determined as the extent to which the respected niches shared isotopic space (%). This overlap was calculated for each combination of species in their allopatric contexts, in order to demonstrate their potential niche overlap and enable comparison with their realised niche overlap, from the sympatric context.

To quantify the growth consequences for the fishes of the allopatric and sympatric contexts, the mean incremental fork lengths (IL) were calculated as previously defined in Eq. (1), but instead of using the total initial (Lt) and total final ($Lt + 1$) lengths of the fishes, to prevent any inaccuracies where not all individuals were recovered from an enclosure, the mean initial and mean final lengths were used instead to generate a mean IL for each species within each enclosure. This enabled the calculation of an overall mean IL for each species within each context. To test for differences in IL for each species between their allopatric and sympatric treatments, generalised linear models (GLM) were used, as the data were not normally distributed. The dependent variable was IL, the independent variable was treatment and enclosure was the random variable. Outputs were the mean adjusted IL per treatment and the significance of their pairwise comparisons with Bonferroni adjustments for multiple comparisons.

2.3.3. Wild pond experimental design

The purpose of this final experiment was to identify whether the results detected in relatively controlled conditions (i.e. tank aquaria and pond enclosures) were comparable when the fish were used in similar contexts but at larger spatial scales. As the outputs of the tank aquaria and pond enclosure experiments (Sections 2.3.1 and 2.3.2) indicated that greatest impacts on *C. carassius* were from co-habitation with *C. carpio*, then it was these two species that were focused on here. Note that due to working at larger spatial scales,

i.e. ponds of 360 m^2 area, logistics dictated that this aspect of the work was not able to use replicates of the different treatments.

This experiment used three rectangular ponds on the same ex-aquaculture site as used in Section 2.3.2; each pond was approximately 360 m^2 in area and up to 1.3 m in depth. Two ponds were used for allopatric contexts (n = 124) and one was for where the two species were in sympatry (n = 62 + 62). Prior to the commencement of the experiment, all the ponds had been drained to ensure no other fishes were present. The fish were sourced from aquaculture and had been pond-reared using supplementary feeding (pelletized fishmeal) and were approximately 2 years old. They were released into the ponds on two separate occasions. In August 2015, 12 *C. carassius* and 12 *C. carpio* were added to the sympatric pond and 24 *C. carpio* and 24 *C. carassius* were added to their respective allopatric ponds. All were measured (fork length, nearest mm) prior to their release. The numbers of fish used were relatively low due to difficulties in sourcing *C. carassius*. However, following concerns of over-winter survival, in February 2016 a second batch of fishes were sourced and 50 *C. carassius* and 50 *C. carpio* were added to the sympatric pond and 100 *C. carassius* and 100 *C. carpio* were added to their respective allopatric ponds. For these fishes, a random sub-sample of 30 fish of each species was measured (fork length, nearest mm) before release. The fishes from the first addition are subsequently referred to as the 2015 fish, and fishes from the second addition, the 2016 fish. They were consequently identifiable through their differences in length.

Similar to the experimental tank aquaria, an issue in the experimental design was the variable starting lengths of the fish as the fish species available all differed in their length ranges and, due to the large number of fish being added to the ponds, only a sub-sample was measured prior to release. It was thus unavoidable that there were differences between the mean lengths of the fishes used between the allopatric and sympatric treatments. Consequently, the differences in the starting lengths of the fish in each addition were minimised as much as possible, particularly with regard to the fishes in the sympatric pond.

At the end of the experimental period (June 2016), the fish were recaptured from each pond through the use of baited fish traps that were left within the ponds overnight and removed and emptied the following morning. From the allopatric ponds, 9 *C. carassius* and 24 *C. carpio* were recovered and from the sympatric pond, 12 *C. carassius* and 12 *C. carpio* were recovered. All fishes were euthanized (anaesthetic overdose, MS-222) and

placed on ice. At the same time, samples of macro-invertebrates were taken from each pond. In the laboratory, the fish were re-measured and a sample of dorsal muscle taken from the anterior region above the lateral line and below the dorsal fin, for stable isotope analysis. In addition, between 5 and 10 scales were also removed from the region above the muscle sample to assist in identification and separation of the 2015 and 2016 fishes. Scales were air dried before being stored in paper envelopes. The macro-invertebrates were sorted into samples, where samples represented between 3 and 5 individuals per species, with triplicate samples used for each pond. These samples, and the fish dorsal muscle samples were then dried at 60 °C to constant mass before analysis at the Cornell University Stable Isotope Laboratory, New York, USA for their stable isotopes of $\delta^{13}C$ and $\delta^{15}N$ (*cf*. Section 2.3.2.1 for details).

As per the pond enclosures, the initial analytical step was to determine whether there were significant differences in the stable isotope data of the putative food resources of the three ponds. The 95 % confidence limits showed no overlap in the stable isotope data between the ponds, indicating a significant difference in isotopic values, so in order to compare the fish data between the ponds, their data were corrected for these isotopic differences in their food sources. For $\delta^{15}N$, correction was by calculating trophic position (TP), as previously defined in Eq. (3) and for $\delta^{13}C$, correction was according to $\delta^{13}Ccorr$:

(4) $\delta^{13}Ccorr = \delta^{13}C_i - \delta^{13}C_{meaninv} / CR_{inv}$

where $\delta^{13}C_i$ is the uncorrected isotope ratio of that fish, $\delta^{13}C_{meaninv}$ is the mean invertebrate isotope ratio (i.e. the 'baseline' invertebrates; *cf*. Section 1.4.1; Fig. 2) and CR_{inv} is the invertebrate carbon range ($\delta^{13}C_{max}$ - $\delta^{13}C_{min}$: Olsson *et al.*, 2009). The corrected stable isotope data were then used in linear mixed effects models (LMEM) to assess differences in $\delta^{13}Ccorr$ and TP between the species in sympatry, and to identify how the allopatric and sympatric contexts affected the trophic position of each species. Species were entered into models according to their treatments. Thus, *C. carassius* were present in models as allopatric *C. carassius* and in sympatry with *C. carpio*. $\delta^{13}Ccorr$ or TP was the dependent variable and treatment was the fixed factor. Differences in $\delta^{13}Ccorr$ and TP by species and treatment were determined using estimated marginal means and pairwise comparisons with Bonferroni adjustments for multiple comparisons.

The corrected stable isotope data were then used to calculate the standard ellipse area (as SEA_B), representing the trophic niche for each species and treatment as previously

described, using the SIBER package (Jackson *et al.*, 2011) in the R computing program (R Development Core Team, 2013). Then, SEA_c was calculated to determine the niche overlap between the sympatric fishes to reveal the amount of actual resource sharing. In addition, this overlap was calculated for *C. carassius* and *C. carpio* in their allopatric contexts in order to demonstrate their potential niche overlap and enable comparison with their realised niche overlap, as was performed in the pond enclosure experiment.

2.4. Results

2.4.1. Tank aquaria experiments

The initial and final lengths and weights of the fishes used in the experiment are shown in Table 1 and increases in lengths and weights were apparent in the control and treatments. Outputs of the GLM testing the differences between the starting lengths and weights of the initial and additional fishes revealed there were some significant differences between the controls and treatments, with the largest difference between the mean lengths being only 11.8 ± 1.4 mm and mean weights 7.73 ± 0.69 g (Table 2). The LMEMs testing for differences in both of the growth metrics (IL, SGR) between the control and treatments, and between the temperatures, were significant ($P < 0.01$, for IL and SGR). The model results revealed that, when compared to the control, the consequences of adding *C. auratus* or *C. carpio* were significantly reduced SGR and IL for *C. carassius*, irrespective of temperature ($P < 0.01$ in all cases; Fig. 3). Whilst the addition of *C. auratus* resulted in a greater decrease in *C. carassius* growth rate than the addition of *C. carpio* at both temperatures, the differences were not significant ($P > 0.05$ in all cases; Fig. 3). In the controls at 18 and 22 °C, the difference in SGR of *C. carassius* was not significant ($P > 0.05$; Fig. 3), but IL was significantly reduced at 18 °C ($P < 0.05$; Fig. 3). In the controls, there were also no significant differences in the IL and SGR between the initial and additional *C. carassius* ($P > 0.05$ in all cases). In both treatments, differences in *C. carassius* growth rates were not significant between the temperatures ($P > 0.05$; Fig. 3). Thus, the water temperatures used did not significantly affect the magnitude of growth depression in the sympatric contexts. However, the addition of either *C. auratus* or *C. carpio* resulted in significantly depressed growth of *C. carassius* compared with the addition of conspecifics.

Table 1. Mean initial (A) and final (B) fork lengths and weights of the fishes used in the experimental tank aquaria. Errors around the means represent standard errors.

A)

Temperature (ºC)	Treatment	Initial length *C. carassius* (mm)	Initial weight *C. carassius* (g)	Initial length additional fish (mm)	Initial weight additional fish (g)
18	Control	40.38 ± 1.60	1.02 ± 0.05	41.44 ± 1.62	1.16 ± 0.03
	+*C. auratus*	51.58 ± 1.13	2.44 ± 0.09	48.50 ± 0.82	2.63 ± 0.04
	+*C. carpio*	45.75 ± 0.63	1.54 ± 0.07	56.33 ± 0.57	4.10 ± 0.07
22	Control	42.36 ± 0.92	1.08 ± 0.04	40.43 ± 0.95	1.04 ± 0.05
	+*C. auratus*	50.67 ± 0.94	2.36 ± 0.05	48.67 ± 1.05	2.76 ± 0.07
	+*C. carpio*	50.83 ± 2.15	2.57 ± 0.06	52.17 ± 0.79	3.36 ± 0.08

B)

Temperature (ºC)	Treatment	Final length *C. carassius* (mm)	Final weight *C. carassius* (g)	Final length additional fish (mm)	Final weight additional fish (g)
18	Control	43.17 ± 1.45	1.45 ± 0.04	44.07 ± 1.36	1.76 ± 0.06
	+*C. auratus*	52.42 ± 1.13	2.66 ± 0.08	54.17 ± 0.89	4.18 ± 0.10
	+*C. carpio*	47.17 ± 0.69	1.70 ± 0.08	61.00 ± 0.43	6.15 ± 0.16
22	Control	42.83 ± 0.98	1.34 ± 0.05	41.07 ± 1.00	1.25 ± 0.05
	+*C. auratus*	51.25 ± 1.16	2.37 ± 0.05	55.25 ± 1.16	4.41 ± 0.10
	+*C. carpio*	52.42 ± 2.11	2.68 ± 0.05	58.42 ± 1.08	4.84 ± 0.14

Table 2. Outputs of generalised linear models testing the differences between the controls and treatments of the mean starting fork lengths and weights of the initial *C. carassius* and the additional *C. carassius*, *C. auratus* and *C. carpio* from the tank aquaria experiment (*cf*. Table 1). Values represent mean differences according to pairwise comparisons with Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.05$. All models were significant at $P < 0.01$. Errors around the means represent standard deviations.

Treatment	Temperature (ºC)	Initial *C. carassius* length (mm)	Initial *C. carassius* weight (g)	Initial additional length (mm)	Initial additional weight (g)
Control vs. Control	18 / 22	1.0 ± 1.3	0.47 ± 0.71	1.0 ± 1.1	0.47 ± 0.69
+*C. auratus* vs. +*C. auratus*	18 / 22	0.9 ± 1.8	0.32 ± 0.71	-0.2 ± 1.6	-0.49 ± 0.69
+*C. carpio* vs. +*C. carpio*	18 / 22	-5.1 ± 1.8	-4.10 ± 0.71*	-3.5 ± 1.6	2.93 ± 0.69*
Control vs. +*C. auratus*	18 / 18	-10.2 ± 1.6*	-1.10 ± 0.71	-7.1 ± 1.4*	-1.89 ± 0.69
	22 / 22	-10.3 ± 1.6*	-1.30 ± 0.71	-8.3 ± 1.4*	-2.85 ± 0.69*
	18 / 22	-9.3 ± 1.6*	-0.79 ± 0.71	-7.3 ± 1.4*	-2.38 ± 0.69*
Control vs. +*C. carpio*	18 / 18	-4.4 ± 1.6	2.50 ± 0.71*	-7.3 ± 1.4*	-7.73 ± 0.69*
	22 / 22	-10.5 ± 1.6*	-2.00 ± 0.71	-11.8 ± 1.4*	-5.28 ± 0.69*
	18 / 22	-9.5 ± 1.6*	-1.62 ± 0.71	-10.8 ± 1.4*	-4.81 ± 0.69*
+*C. auratus* vs. +*C. carpio*	18 / 18	5.8 ± 1.8*	3.60 ± 0.71*	-0.2 ± 1.6	-5.84 ± 0.69*
+*C. auratus* vs. +*C. carpio*	22 / 22	0.8 ± 1.8	-0.51 ± 0.71	-3.7 ± 1.6	-2.90 ± 0.69*

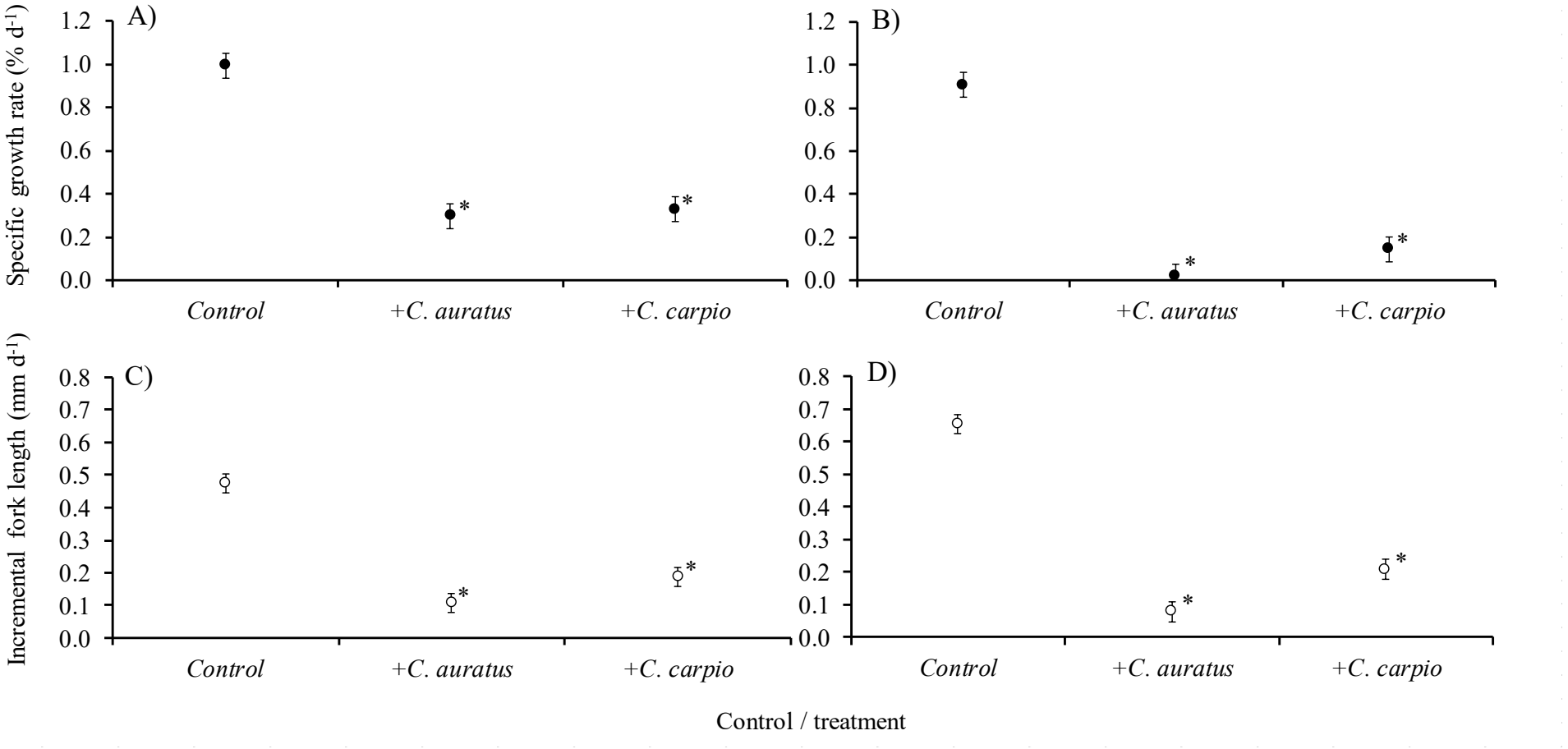

Figure 3. Comparisons between controls and treatments of mean specific growth rates of *C. carassius* at 18 °C (A) and 22 °C (B), and incremental fork length of *C. carassius* at 18 °C (C) and 22 °C (D); *difference in growth rate between the control and treatment is significant at $P < 0.05$. Error bars represent standard errors.

2.4.2. Pond enclosure experiments

The initial and final lengths of the fishes are shown in Table 3, with all fishes increasing in length over the experimental period. Although the LMEM testing the differences between the starting lengths of the species was significant ($P < 0.05$), pairwise comparisons revealed that there were no significant differences in the starting lengths of fish in any of the treatments involving the fish in sympatry, nor between the lengths of any of the species between their allopatric and sympatric treatments ($P > 0.05$; Table 3). There were also no significant differences in daily mean water temperature between the two ponds in which the enclosures were located ($F_{1,98} = 0.23$, $P > 0.05$). Mean daily temperatures were 18.3 ± 0.2 and 18.5 ± 0.4 °C across the 100 days.

At the end of the 100 days, all but four of the fish that started the experiment were recovered (96 % recovery rate), with no more than one fish absent from a replicate (Table 3). The GLMs testing the influence of treatment on the incremental growth rates of each species were significant ($P < 0.01$ in all cases). Whilst the growth rates of allopatric *C. carassius* and *C. auratus* were not significantly different to when they were present in sympatry ($P > 0.05$), their growth rates were both significantly reduced in sympatry with *C. carpio* ($P < 0.05$; Table 3). In contrast, *C. carpio* grew at a faster rate in their sympatric treatments than in allopatry, with the difference significant in sympatry with *C. carassius* ($P < 0.05$; Table 3).

Comparison of the macro-invertebrate stable isotope samples between the two large ponds revealed considerable overlaps in their 95 % confidence intervals and were thus considered similar, and so the stable isotope data could be compared between the ponds without correction (Odonata: $\delta^{13}C$: -34.98 ± 0.31 vs. -33.14 ± 2.23; $\delta^{15}N$: 3.76 ± 0.26 vs. 3.98 ± 0.12; Corixidae: $\delta^{13}C$: -31.75 ± 0.70 vs. -33.37 ± 0.91; $\delta^{15}N$: 4.26 ± 0.81 vs. 3.40 ± 0.95). Nevertheless, $\delta^{15}N$ was converted to TP for the purpose of testing the data as this has more ecological relevance. The LMEMs testing the influence of species and treatment on TP and $\delta^{13}C$ were both significant ($P < 0.01$; Table 4). When compared to their allopatric treatments, TP of *C. carassius* was significantly increased when in sympatry with *C. carpio* ($P < 0.05$). When *C. carassius* and *C. auratus* were sympatric, there were no significant differences in $\delta^{13}C$ and TP compared with their allopatric treatments ($P > 0.05$; Table 4), but for *C. carpio*, $\delta^{13}C$ and TP were significantly decreased when sympatric with both *C. carassius* and *C. auratus* when compared to their allopatric treatment ($P < 0.01$; Table 4).

The calculation of standard ellipse areas (as SEA_B and SEA_c) as a measure of trophic niche size revealed that there were changes in the niche sizes of the fishes between their allopatric treatments and when the species were in sympatry, with the direction and extent of this change being context specific. For *C. carassius*, there was minimal change in their trophic niche size between their allopatric treatment and when sympatric with *C. auratus* or *C. carpio*, based on the distribution of SEA_B (Table 5A). For *C. auratus* however, when compared to their allopatric treatment, their trophic niche size decreased slightly when in sympatry with *C. carassius* (probability of *C. auratus* niche being smaller than *C. carassius* was 0.86, compared to 0.69 in allopatry) and their trophic niches overlapped in both contexts (Table 5; Fig. 4A, B). In contrast, when sympatric with *C. carpio, C. auratus* trophic niche increased and the probability of *C. auratus* niche being smaller was reduced from 1.00 in allopatry to 0.82 in sympatry. In allopatry, *C. auratus* and *C. carpio* had significantly different trophic niches, based on the distribution of SEA_B. Whilst this suggested that their niches would not overlap in sympatry, they actually did, with *C. auratus* sharing 50 % of their trophic niche with *C. carpio* (Table 5; Fig. 4E, F). For both *Carassius* species, their niche sizes were significantly smaller than *C. carpio* in both allopatric and sympatric contexts even though *C. carpio* trophic niche size was reduced in sympatry compared with allopatry (Table 5A). For *C. carassius* and *C. carpio*, there was no trophic niche overlap in either context according to SEA_c, however, based on the distributions of SEA_B, their trophic niches were not significantly different (Table 5B; Fig. 4C, D).

Table 3. Mean initial and final fork lengths and growth rates (as mean incremental length, IL) of the fishes used in the pond enclosure experiment; *significantly different to allopatric context at $P < 0.05$, as determined from generalised linear models where enclosure was the random variable. Errors around the means represent standard errors.

Species	Context		Initial length (mm)			*n*	Final length (mm)			*n*	IL (mm d^{-1})			
C. carassius	Allopatric		61.46	±	1.27	24	76.96	±	1.70	24	0.15	±	0.02	
	Sympatric with	*C. auratus*	61.67	±	2.23	12	76.75	±	1.39	12	0.15	±	0.02	
		C. carpio	61.42	±	1.52	12	67.73	±	1.32	11	0.06	±	0.02	*
C. auratus	Allopatric		64.88	±	1.41	24	86.95	±	1.41	22	0.22	±	0.01	
	Sympatric with	*C. carassius*	64.75	±	1.34	12	85.82	±	1.46	11	0.21	±	0.01	
		C. carpio	63.92	±	1.97	12	78.10	±	1.51	10	0.14	±	0.01	*
C. carpio	Allopatric		64.08	±	1.41	24	74.50	±	1.54	24	0.10	±	0.02	
	Sympatric with	*C. carassius*	57.58	±	1.65	12	75.08	±	1.28	12	0.18	±	0.02	*
		C. auratus	59.92	±	2.02	12	75.17	±	2.56	12	0.16	±	0.02	

Table 4. Outputs and significance of the final linear mixed effects models testing the difference in carbon stable isotope ($\delta^{13}C$) and trophic position (TP) between the species across the pond enclosure experiment, where enclosure was the random effect on the intercept. Values represent mean differences according to pairwise comparisons with Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.01$.

Final models:				
$\delta^{13}C$ ~ species x experimental treatment (AIC = 430.87; log likelihood = 428.87; $P < 0.01$)				
Trophic position ~ species x experimental treatment (AIC = -5.14; log likelihood = -9.14; $P < 0.01$)				
Pairwise comparison			Difference in $\delta^{13}C$	Difference in TP
Allopatric *C. carassius*	vs.	*C. carassius* sympatric with *C. auratus*	0.402	0.031
		C. carassius sympatric with *C. carpio*	1.248	0.414 *
Allopatric *C. auratus*	vs.	*C. auratus* sympatric with *C. carassius*	0.178	0.023
		C. auratus sympatric with *C. carpio*	0.034	0.223
Allopatric *C. carpio*	vs.	*C. carpio* sympatric with *C. carassius*	1.810 *	0.313 *
		C. carpio sympatric with *C. auratus*	1.783 *	0.312 *
C. carassius in sympatry with *C. auratus*			0.986	0.204
C. carassius in sympatry with *C. carpio*			3.580 *	0.457 *
C. carpio in sympatry with *C. auratus*			1.577	0.07

Table 5. Mean carbon stable isotope ($\delta^{13}C$), trophic position (TP) and trophic niche sizes (as standard ellipse areas; SEA_B with 95 % confidence intervals (CI) and SEA_c) of the fishes used in the pond enclosure experiment (A) and probabilities that trophic niche sizes (as SEA_B) are smaller, whether trophic niche sizes are significantly different (as indicated by no overlap in SEA_B 95 % CI's) and percentage overlaps of trophic niches (as SEA_c) in allopatric and sympatric contexts (B). Errors around the means represent standard errors.

A)

Species	Context		*n*	Mean $\delta^{13}C$ (‰)	Mean TP	SEA_B (‰^{2}) (95 % CI range)	SEA_c (‰^{2})
C. carassius	Allopatric		18	-25.76 ± 0.34	4.65 ± 0.05	1.39 (0.87 - 2.38)	1.35
	Sympatric with	*C. auratus*	12	-25.36 ± 0.42	4.69 ± 0.06	1.62 (0.85 - 2.98)	1.49
		C. carpio	11	-24.51 ± 0.44	5.07 ± 0.06	1.18 (0.63 - 2.23)	1.43
C. auratus	Allopatric		18	-26.52 ± 0.34	4.46 ± 0.05	1.21 (0.78 - 2.00)	1.23
	Sympatric with	*C. carassius*	11	-26.34 ± 0.44	4.48 ± 0.06	0.96 (0.55 - 1.91)	1.17
		C. carpio	10	-26.49 ± 0.46	4.68 ± 0.07	1.86 (0.92 - 3.64)	1.75
C. carpio	Allopatric		18	-26.28 ± 0.34	4.92 ± 0.05	3.31 (2.02 - 5.30)	3.45
	Sympatric with	*C. carassius*	12	-28.09 ± 0.42	4.61 ± 0.06	2.77 (1.54 - 5.38)	3.03
		C. auratus	12	-28.06 ± 0.42	4.61 ± 0.06	2.77 (1.52 - 5.18)	3.15

B)

Species	Context	SEA_B smaller	SEA_B 95 % CI	SEA_c overlap (%)
C. carassius vs. *C. auratus*	Allopatric	0.31 vs. 0.69	overlap	35 vs. 38
	Sympatric	0.14 vs. 0.86	overlap	27 vs. 34
C. carassius vs. *C. carpio*	Allopatric	0.99 vs. 0.01	overlap	no overlap
	Sympatric	0.98 vs. 0.02	overlap	no overlap
C. carpio vs. *C. auratus*	Allopatric	0.00 vs. 1.00	no overlap	no overlap
	Sympatric	0.18 vs. 0.82	overlap	28 vs. 50

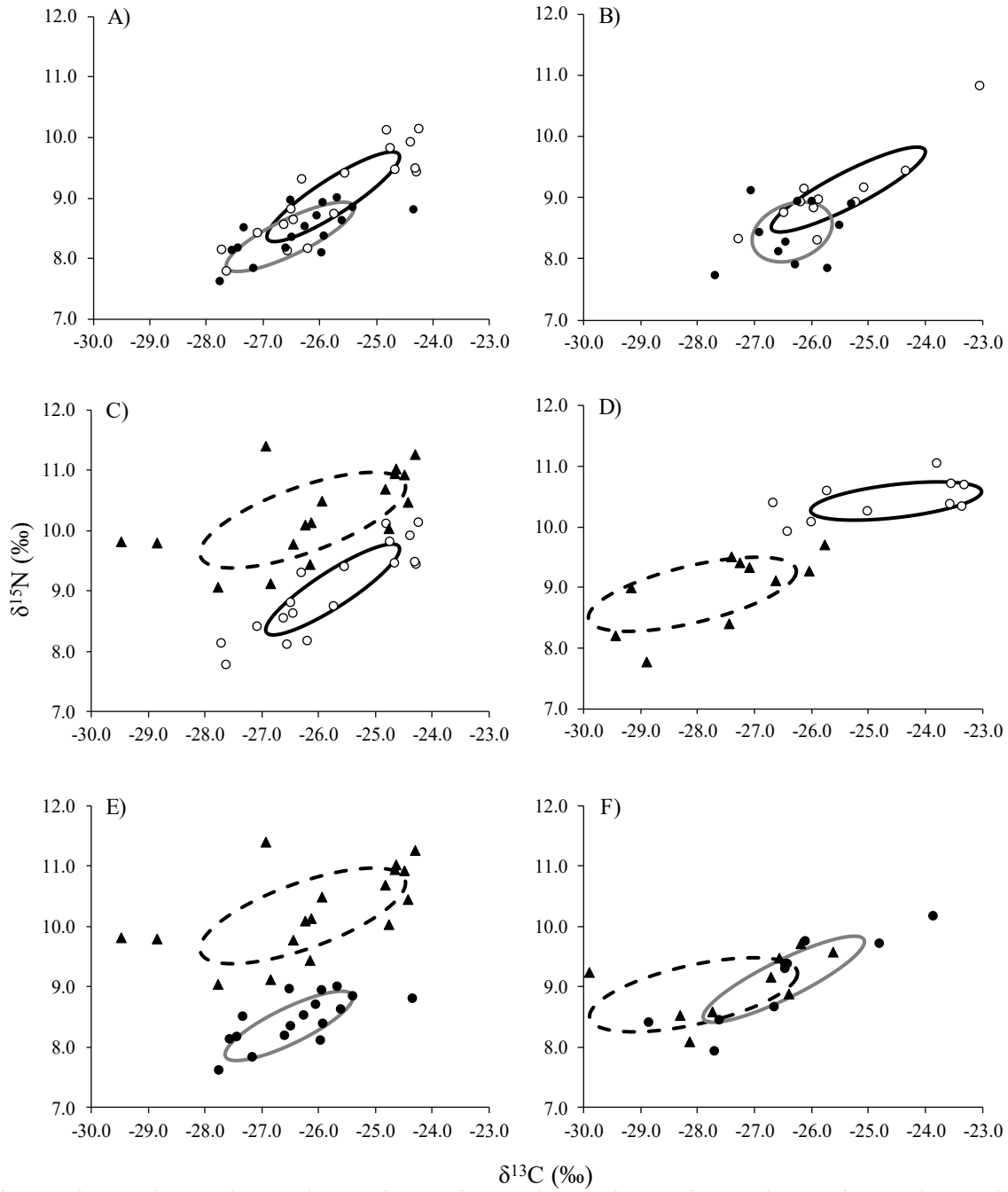

Figure 4. Stable isotope data and trophic niches (as standard ellipse area, SEA_c) of allopatric *C. carassius* and *C. auratus* (A); sympatric *C. carassius* and *C. auratus* (B); allopatric *C. carassius* and *C. carpio* (C); sympatric *C. carassius* and *C. carpio* (D); allopatric *C. carpio* and *C. auratus* (E) and sympatric *C. carpio* and *C. auratus* (F); ○ stable isotope data for *C. carassius*; ● stable isotope data for *C. auratus* and ▲ stable isotope data for *C. carpio*. Black solid lines mark the trophic niche of *C. carassius*; grey solid lines the trophic niche of *C. auratus* and black dashed lines the trophic niche of *C. carpio*.

2.4.3. Wild pond experiments

The initial and recovered lengths of the fishes are shown in Table 6. As only a sub-set of the 2016 fishes were measured before allocation to the ponds and as the fishes were not identifiable individually, IL was not calculated. However, observation of the scales collected from the fishes, in addition to their recaptured lengths, allowed identification of any fishes present that were released in 2015. There were 6 allopatric *C. carpio* recaptured in June 2016 that were identified as being from the 2015 release, as these fish were significantly larger than the other allopatric *C. carpio* (177.0 ± 8.8 mm vs. 118.8 ± 9.7 mm; Table 6B; GLM; $P < 0.01$) and not only were these fish different in length, but they were also isotopically distinct from the allopatric *C. carpio* added in 2016, suggesting an ontogenetic dietary shift (Table 7A; Fig. 5).

Comparison of the macro-invertebrate stable isotope samples between the ponds revealed overlaps in the 95 % confidence intervals for $\delta^{13}C$ between the three ponds, however for $\delta^{15}N$, the 95 % confidence interval of one pond did not overlap with the other two and thus was considered as being significantly different (Odonata: $\delta^{13}C$: -30.27 ± 0.89 vs. -30.26 ± 0.72 vs. -29.89 ± 0.51; $\delta^{15}N$: 4.65 ± 0.69 vs. 4.64 ± 0.52 vs. 6.50 ± 0.59). Consequently, all of the stable isotope data was corrected in order to allow comparison across all the ponds.

The LMEMs testing the influence of species and treatment on $\delta^{13}Ccorr$ and TP were both significant ($P < 0.01$; Table 8). For *C. carassius*, when compared to their allopatric treatment, TP was slightly increased and $\delta^{13}Ccorr$ slightly decreased when in sympatry with *C. carpio* (Table 7A), but these changes were not significant (Table 8). For *C. carpio*, when compared to their allopatric treatment, $\delta^{13}Ccorr$ decreased when in sympatry with *C. carassius* ($P < 0.01$; Table 7A), with the difference being significant for the *C. carpio* added in 2016 (Table 8). In allopatry, the difference in TP between *C. carassius* and *C. carpio* was not significant. In sympatry, however, they were significantly different, with TP of *C. carassius* increasing and *C. carpio* decreasing ($P < 0.01$; Tables 7A, 8). The difference between *C. carassius* and *C. carpio* in $\delta^{13}Ccorr$ remained significant in both allopatric and sympatric contexts ($P < 0.05$; Table 8), with both species decreasing in $\delta^{13}Ccorr$ in sympatry when compared to allopatry (Table 7A).

The calculation of standard ellipse areas (as SEA_B and SEA_c) as measures of the size of the trophic niche revealed that there were changes in both species between their

allopatric and sympatric contexts. For *C. carassius*, their trophic niche size was larger when in sympatry with *C. carpio* than in allopatry and there was some overlap in the 95 % confidence intervals of SEA_B (Table 7). For *C. carpio*, however, their trophic niche size was significantly larger in allopatry than sympatry (Table 7A; Fig. 5). Additionally, outputs from the allopatric treatments predicted that the trophic niche areas would be significantly different and so would not overlap between the 2016 *C. carassius* and 2016 *C. carpio* when in sympatry (based on distributions of SEA_B), and this prediction was accurate (Table 7; Fig. 5). Furthermore, the trophic niche of *C. carassius* was significantly smaller than *C. carpio* when these fishes were allopatric, but in sympatry, the trophic niche size of *C. carassius* increased, becoming significantly larger than *C. carpio* (switching the probability of having a smaller trophic niche from 1.00 to 0.00) (Table 7B; Fig. 5).

Table 6. Mean initial (A) and recovered (B) fork lengths of the fishes used in the wild pond experiment. All fishes from the 2015 addition were measured before allocation into the experimental pond, but in 2016, only a sub-set of 30 fish from each species were measured before allocation and therefore the sympatric and allopatric contexts cannot be specifically applied to this data. Errors around the means represent standard deviations.

A)

Addition	Context	Species	*n*	Initial length (mm)
2015	Sympatric	*C. carassius*	12	103.08 ± 5.28
		C. carpio	12	118.67 ± 4.12
	Allopatric	*C. carassius*	24	107.46 ± 6.53
		C. carpio	16	124.00 ± 5.19
2016		*C. carassius*	30	135.20 ± 7.01
		C. carpio	30	97.30 ± 9.21

B)

Addition	Context	Species	*n*	Recovered length (mm)
2015	Sympatric	*C. carassius*		
		C. carpio		
	Allopatric	*C. carassius*		
		C. carpio	6	177.00 ± 8.76
2016	Sympatric	*C. carassius*	12	141.17 ± 10.99
		C. carpio	12	144.67 ± 6.80
	Allopatric	*C. carassius*	9	133.33 ± 5.79
		C. carpio	16	118.81 ± 9.73

Table 7. Mean corrected carbon stable isotope values (δ^{13}CCorr), trophic position (TP) and trophic niche sizes (as standard ellipse areas; SEA_B with 95 % confidence intervals (95 % CI) and SEA_c) of the fishes used in the wild pond experiment (A) and the probability that the trophic niche size (as SEA_B) of *C. carassius* is smaller than *C. carpio*, whether the trophic niche sizes are significantly different (as indicated by no overlap in SEA_B 95 % CI's) and the percentage overlap of their trophic niches (as SEA_c) in allopatric and sympatric contexts (B). Errors around the means represent standard errors.

A)

Context	Species	*n*	Mean δ^{13}Ccorr	Mean TP	SEA_B (‰^{2}) (95 % CI)	SEA_c (‰^{2})
Allopatric	2016 *C. carassius*	9	0.97 ± 0.14	3.26 ± 0.05	0.07 (0.04 - 0.15)	0.06
	2015 *C. carpio*	6	1.72 ± 0.17	3.07 ± 0.06	0.14 (0.06 - 0.35)	0.23
	2016 *C. carpio*	16	4.49 ± 0.11	3.18 ± 0.04	0.26 (0.16 - 0.45)	0.38
Sympatric	2016 *C. carassius*	12	0.76 ± 0.12	3.43 ± 0.04	0.16 (0.08 - 0.29)	0.09
	2016 *C. carpio*	12	1.34 ± 0.12	3.12 ± 0.04	0.03 (0.02 - 0.05)	0.05

B)

Species	Context	SEA_B smaller	SEA_B 95 % CI	SEA_c overlap (%)
2016 *C. carassius* vs. 2015 *C. carpio*	Allopatric	0.94 vs. 0.06	overlap	no overlap
2016 *C. carassius* vs. 2016 *C. carpio*	Allopatric	1.00 vs. 0.00	no overlap	no overlap
	Sympatric	0.00 vs. 1.00	no overlap	no overlap

Table 8. Outputs and significance of the final linear mixed effects models testing the differences in corrected carbon stable isotopes (δ^{13}CCorr) and trophic position (TP) between the species across the wild pond experiment. Values represent mean differences according to pairwise comparisons with Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.01$.

Final models:

δ^{13}Ccorr ~ species x experimental treatment (AIC = 69.84; log likelihood = 67.84 ; $P < 0.01$)

Trophic position ~ species x experimental treatment (AIC = -30.94 ; log likelihood = -32.94 ; $P < 0.01$)

Pairwise comparison			Difference in δ^{13}Ccorr	Difference in TP
Allopatric *C. carassius*	vs.	*C. carassius* sympatric with 2016 *C. carpio*	0.21	0.17
	vs.	Allopatric 2015 *C. carpio*	0.75 *	0.19
	vs.	Allopatric 2016 *C. carpio*	3.52 *	0.08
Allopatric 2015 *C. carpio*	vs.	Allopatric 2016 *C. carpio*	2.77 *	0.11
	vs.	2016 *C. carpio* sympatric with *C. carassius*	0.38	0.05
Allopatric 2016 *C. carpio*	vs.	2016 *C. carpio* sympatric with *C. carassius*	3.15 *	0.06
C. carassius in sympatry with 2016 *C. carpio*			0.58 *	0.31 *

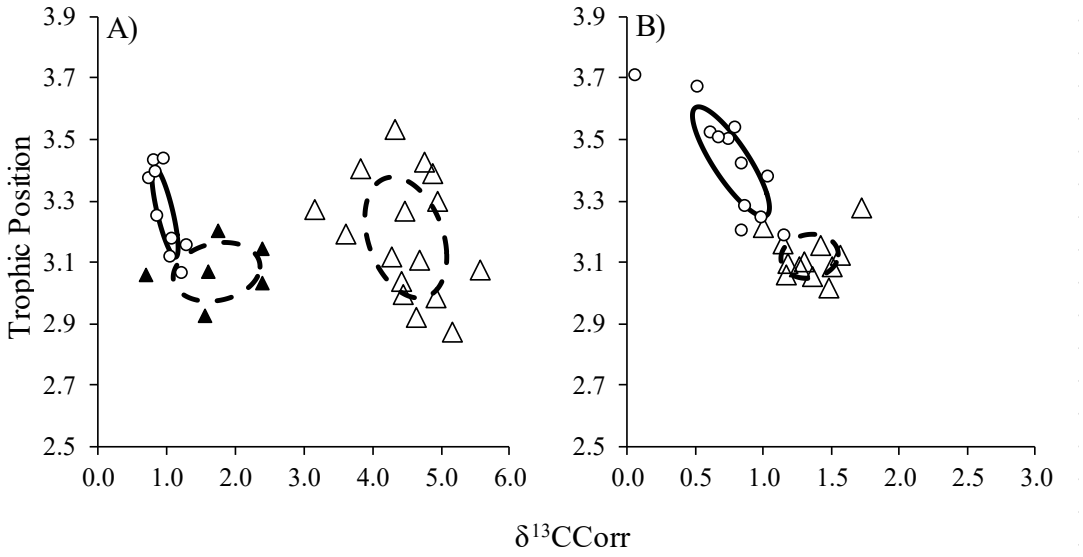

Figure 5. Corrected stable isotope data (as δ^{13}CCorr and trophic position, TP) and trophic niches (as standard ellipse area, SEA_c) of allopatric *C. carassius* and *C. carpio* (A) and sympatric *C. carassius* and *C. carpio* (B); ○ corrected stable isotope data for *C. carassius*; ▲ corrected stable isotope data for *C. carpio* added in 2015 and Δ corrected stable isotope data for *C. carpio* added in 2016. Black solid lines mark the trophic niche of *C. carassius* and black dashed lines the trophic niche of *C. carpio*.

2.5. Discussion

The principal aim of this Chapter was to reveal the ecological consequences for *C. carassius*, as a model native fish, arising from introductions of invasive *C. auratus* and *C. carpio* by comparing their trophic interactions and somatic growth rates under controlled and wild conditions, and sympatric and allopatric contexts. The three approaches used in the Chapter revealed some significant impacts on the somatic growth rates and trophic interactions of *C. carassius*, but these outcomes varied according to the condition and context. The results from each approach are discussed in turn in Sections 2.5.1 to 2.5.3.

2.5.1. Co-habitation tank aquaria

The experimental outputs from the co-habitation aquaria suggest that when *C. carassius* is sympatric with either *C. auratus* or *C. carpio* and food resources are restricted, then unless there is the possibility of resource partitioning (Guo *et al*., 2014), inter-specific competition is likely to occur and could result in depressed *C. carassius* somatic growth

rates. The mechanism of this competition was exploitative, as no antagonistic interactions were observed during the daily feeding, although this was not quantified as an output. Although the variability of the intensity of intra-specific competition under different feeding regimes and fish abundances was not measured, *C. carassius* growth is known to be negatively impacted in allopatric contexts when under conditions of limited food supply (Paszkowski *et al*., 1990; Holopainen *et al*., 1997).

An issue with experimental co-habitation studies is that outcomes do not always match field observations, as the spatial experimental constraints can result in unnaturally intense interactions, and there is a lack of complexity compared with natural situations (Korsu *et al*., 2009). Indeed, there was a marked difference between the intense competitive interactions from *C. auratus* in the experimental conditions here, which significantly reduced *C. carassius* growth, to the studies of Tarkan *et al*. (2009) and Copp *et al*. (2010), which found no differences in wild *C. carassius* growth rates between allopatric and sympatric contexts. This difference might relate to the experimental conditions of the tank aquaria providing a limiting food supply, whereas in wild conditions, the sympatric species might either have been resource partitioning (Guo *et al*., 2014) or sharing food resources that were unlimited, and so growth rates were not constrained. In combination, this suggests that negative growth consequences for *C. carassius* from inter-specific competition with non-native fishes in natural conditions are likely to only be incurred where their food resources are limiting and resource partitioning is not possible. Consequently, in order to remove the potential effects of a limited food supply due to the fixed feeding in the aquaria and to explore the competitive feeding interactions of the species under more natural conditions, the additional pond enclosure and wild pond experiments were implemented where an increased spatial scale and the availability of natural food resources added system complexity and reduced controlled parameters. Additionally, the pond experiments provided the opportunity to explore trophic interactions via the use of stable isotope analysis to assess whether resource partitioning or convergence was realised between the species in allopatric and sympatric contexts.

2.5.2. Experimental pond enclosures

In the tank aquaria experiments, the food resource and the feeding rate was fixed on a daily basis and so the fish had no option but to share these resources. This was in contrast

to the pond enclosure experiment where natural food resources were available as the enclosures were placed *in situ* within larger pond systems and their mesh size enabled the ingress of macro-invertebrate communities. The results from the pond enclosure experiment revealed that when the congenic *C. carassius* and *C. auratus* were in sympatry, there were minimal and non-significant shifts in their trophic positions and somatic growth rates when tested against their allopatric treatments. This was contrary to the prediction developed from the results of the tank aquaria experiment in which strong divergence in their niches would develop when they were in sympatry. Instead, there were considerable increases in trophic position and trophic niche size of both *C. carassius* and *C. auratus* when they were in sympatry with *C. carpio*, although this shift was insufficient for their growth rates to be similar to levels measured in their allopatric treatments. Moreover, for *C. auratus*, this increase in trophic niche size in sympatry resulted in some niche convergence with *C. carpio*, with this not predicted from the allopatric treatment where their niches were in significantly different isotopic space. Again, this conflicted with the prediction that sympatry would result in decreased niche sizes and niche divergence. Hence, the results of their allopatric and sympatric treatments in pond enclosures provided no evidence that the presence of *C. auratus* impacted the trophic niche size, trophic position and growth rate of *C. carassius,* aligning with Tarkan *et al*. (2009), who also found no differences in *C. carassius* growth rates between allopatric populations and populations sympatric with *C. auratus* in the wild. These results are despite the outcome from the tank aquaria revealing that their competitive interactions were asymmetrical, with *C. carassius* as a poor competitor in *C. auratus* presence when food availability was restricted.

As congeners, *C. carassius* and *C. auratus* are functionally similar, suggesting that sharing food resources could result in high competitive interactions that, if asymmetric, could result in depressed growth rates of *C. carassius*. However, the growth rates of both species were similar between their allopatric and sympatric contexts, suggesting that although they were occupying similar niches, their food resources were not limiting or were at least sufficient to maintain their somatic growth rates. This meant there was no requirement for developing, for example, larger niche sizes in sympatry to reduce the intensity of their interactions (Svanbäck and Bolnick, 2007; Svanbäck *et al.*, 2008; Bolnick *et al.*, 2010). This contrasts with Copp *et al*. (2010) who found feral *C. auratus* growth rates were greatest in sympatry with *C. carassius*, whereas *C. carassius* growth

was similar in allopatry and sympatry. Copp *et al*. (2010) also found differences in body condition and fecundity of the fishes, with higher values in both for *C. carassius* in sympatry. Co-habitation may thus lead *C. carassius* to allocate greater growth in weight, although here only growth in length was assessed.

By contrast, *C. carpio* had a strong trophic influence on both *Carassius* fishes in sympatry, with their presence resulting in significant increases in *Carassius* trophic position and trophic niche size. These results were contrary to the niche variation hypothesis (Van Valen, 1965) that predicted that the *Carassius* niche sizes would reduce in *C. carpio* presence due to the competitive effects resulting in greater diet specialisation (Van Valen, 1965). Nevertheless, the significant decrease in *C. carassius* and *C. auratus* growth rates when in sympatry with *C. carpio*, despite their increases in trophic niche size and trophic position, indicated that these shifts were insufficient to maintain their growth rates at levels observed in their allopatric treatments.

2.5.3. Experimental wild ponds

Given the outputs of the pond enclosure experiment, co-habitation of *C. carassius* and *C. carpio* in wild ponds was predicted to result in trophic niche divergence between the species. Indeed, this prediction was supported, with no evidence that the trophic niches of the two fishes overlapped in either allopatry or sympatry. The outcomes of the trophic interactions between these species were thus broadly similar to those of the pond enclosures, but also with some important differences.

In the pond enclosure experiment, *C. carpio* had a strong influence on both *Carassius* species in sympatry, with their presence resulting in significant increases in *Carassius* trophic position and niche size. In the wild ponds, *C. carassius* did increase in trophic position when sympatric, but this change was not statistically significant. However, the trophic niche size of *C. carassius* was significantly increased and, as in the enclosures, this result was contrary to the niche variation hypothesis that predicted that the *C. carassius* niche size would reduce in *C. carpio* presence due to the competitive effects resulting in greater diet specialisation (Van Valen, 1965). In fact, in the wild ponds the opposite was apparent, with *C. carpio* niche size significantly reduced when compared to allopatry and significantly smaller than *C. carassius* in sympatry, indicating a switch to a more specialised diet. Even though *C. carassius* and *C. carpio* occupied significantly

different trophic niches, with no niche sharing indicated in either context, significant changes were detected in the trophic niche sizes of both fishes in response to their co-habitation, with this potentially reducing the intensity of any inter-specific interactions (Svanbäck and Bolnick, 2007; Svanbäck *et al.*, 2008; Bolnick *et al.*, 2010). A possible explanation for these patterns in niche size alterations between the two approaches is that in the wild ponds, the fish were able to utilise both littoral and open water habitats, whereas in the enclosures, these were all located in the open water habitats and so the fishes might have had restricted access to a wide range of food resources.

Some significant differences in the corrected stable isotope data were detected between the *C. carpio* fishes added in 2015 (300 days) and those added in 2016 (125 days). Trophic diversifying effects have been recorded among members of a single species in response to competition, where individuals may mitigate the effects of intra-specific competition by switching to use alternative resources not used by conspecifics (Svanbäck and Bolnick, 2007), which may explain this difference. Additionally, as immature fishes rapidly develop and grow they are able to feed on progressively larger and a broader range of prey, and are thought to alter their resources and ecological niches to heighten their chances of survival through their early life stages (King, 2005). *Cyprinus carpio* demonstrate major dietary shifts through ontogeny from newly hatched larvae through development into juvenile stages and adulthood (Vilizzi, 1998; Nunn *et al*., 2007; King, 2005). For example, they have been shown to shift from feeding on zooplankton, such as copepod nauplii, in larval stages (Nunn *et al*., 2007), to broadening their diet and feeding on a range of microfauna and small macro-invertebrates, such as Chironomidae larvae, as juveniles (King, 2005). The length differences between the 2015 and 2016 cohorts may hence have promoted this division in resources use, via intra-specific competition and/or through selecting different prey due to ontogenetic dietary changes encouraged by their differences in size, and so the resulting isotopic position of the allopatric 2016 *C. carpio* fish may have been different if the 2015 fish were not present.

2.5.4. Ecological consequences for C. carassius *from invasive fishes*

Tarkan *et al*. (2011) suggested that *C. carassius* may be adversely affected under the conditions of climate change predicted for South East England (Hulme *et al*., 2002), as these circumstances are expected to exacerbate the potential impact of non-native *C.*

auratus, given their recruitment success is positively correlated with temperature (Morgan *et al*., 2004). Indeed, in the tank aquaria SGR and IL of *C. carassius* was more suppressed by the presence of *C. auratus* at the higher temperature. Additionally, Britton *et al.* (2010a) suggested that the establishment and subsequent invasion of *C. carpio* would benefit substantially from the predicted warming temperatures of 2050 in England and Wales. *Carassius carassius* might also derive some benefit from the warmer climatic temperatures predicted, as the onset of their growth season might be earlier in the year and last for longer periods (Ruiz-Navarro *et al*., 2016a, b). Nevertheless, *C. carpio* and *C. auratus* would then also have an earlier seasonal onset of growth and therefore an advantage over *C. carassius* in having greater opportunity to monopolise food availability, to grow larger more rapidly and therefore intensify inter-specific competition.

Cyprinus carpio is a highly invasive fish at a global scale and has caused negative impacts across a range of ecological indicators (e.g., Lougheed *et al.*, 1998; Parkos *et al.*, 2003; Koehn, 2004; *cf.* Section 1.5). Regarding their trophic consequences, Jackson *et al.* (2012) reported that invasive *C. carpio* reduced the trophic position of another invasive species, the red swamp crayfish *Procambarus clarkii*, through *P. clarkii* shifting their diet to avoid resource sharing with *C. carpio* and thus minimising their competitive interactions. Tran *et al.* (2015) revealed that when *C. carpio* were in sympatry with topmouth gudgeon *Pseudorasbora parva*, *P. parva* also reduced their niche size and their trophic position. In the pond experiments, however, these outcomes were not apparent; rather than decreased niche size and divergence from *C. carpio*, as per *P. parva* and *P. clarkii*, the *Carassius* species both increased their niche size and trophic position, and in the case of *C. auratus*, this resulted in niche convergence with *C. carpio*, something not predicted from allopatric treatments. Given that this was insufficient to maintain their growth rates at levels observed in their allopatric treatments in the pond enclosures then it suggests that for native populations of *C. carassius*, negative consequences will be incurred from introductions of *C. carpio* in terms of their feeding and growth rates, whereas the negative consequences from invasive *C. auratus* appear to be primarily related to the loss of genetic integrity through hybridisation (Hänfling *et al*., 2005; *cf.* Section 1.5). It should be noted, however, that *C. carassius* hybrids with *C. carpio* do also occur. Sayer *et al*. (2011) observed *C. carassius* hybrids with *C. auratus* and *C. carpio* in 20 % of the 51 ponds they studied and in some cases the fish assemblage was dominated

by hybrids, suggesting that the *C. carassius* populations were in the process of being extirpated through this process. Therefore, hybridisation threats from *C. carpio* should not be disregarded in any conservation efforts and should be considered in conjunction with the ecological impacts that can develop, as revealed here.

2.5.5. Issues with stable isotope analysis and growth rate analyses resulting from the experiments

Whilst the experiments were successful in revealing some trophic and growth consequences arising from the interactions of the three fishes, there were also a number of potentially confounding issues apparent throughout that could not be easily solved using current knowledge in the literature. These are outlined below.

Stable isotope turnover, the rate of change of stable isotopes that occurs within tissues when a consumer changes its diet, is affected by multiple factors that include the type of tissue, the body size of the consumer and their growth rate (Martínez del Rio *et al*., 2009; Weidel *et al*., 2011; Thomas and Crowther, 2014; Vander Zanden *et al*., 2015). Thus, when a dietary shift in a consumer species occurs, the stable isotopes within the tissues gradually change to approach the isotopic values of the new diet. Therefore, it is important that sufficient time passes so that they fully equilibrate before being used within trophic studies to avoid any misinterpretation (*cf.* Section 1.4.1). The pond enclosure experiment ran for 100 days and the 2016 fishes in the wild pond experiment were left for 125 days on the basis that this would be an adequate time-frame to allow muscle tissue to undergo isotopic turnover and so indicate the trophic ecology of the fishes (Jackson *et al.*, 2013). However, variability in turnover rates has been demonstrated between tissues of freshwater fishes (e.g., McIntyre and Flecker, 2006; Church *et al.,* 2009; Carleton and Martínez del Rio, 2010) and although there have been relatively fast turnover rates (< 3 months) reported for fish muscle tissue (Buchheister and Latour, 2010; Jardine *et al*., 2011), turnover rates specifically relating to the cyprinid fishes used in the Chapter are currently lacking from the literature.

General equations are available to facilitate the estimation of turnover rates where specific values are unavailable (e.g., Buchheister and Latour, 2010; Thomas and Crowther, 2014; Vander Zanden *et al.,* 2015), however, the validity and wider applicability of these equations have yet to be demonstrated beyond the studies they were

generated within. Xia *et al*. (2013a, b) measured turnover in the cyprinid grass carp *Ctenopharyngodon idellus*, of starting mass 52.7 ± 0.7 g, and estimated $\delta^{15}N$ turnover in muscle tissue to be 68 days (Xia *et al*., 2013b) and 53 days for $\delta^{13}C$ (Xia *et al*., 2013a). If these estimates are similar to the turnover rates occurring within the dorsal muscle tissues of the fishes used here, and as consumers are generally considered to have equilibrated to their food resources in four to five half-lives, i.e. 94 to 97 % isotopic replacement in their tissues (Hobson and Clark, 1992), then for $\delta^{15}N$, 100 days would only be 1.5 half-lives and for $\delta^{13}C$, 1.9 half-lives. Note, however, that turnover rates are influenced by body size and metabolic rates, and the starting mass of the fish used by Xia *et al*. (2013a, b) were considerably higher than those used here. In entirety, this suggests there is an outstanding requirement for knowledge on stable isotope turnover rates in cyprinid fishes.

Another factor critical to the interpretation of stable isotope data is the isotopic discrimination (Martínez del Rio and Wolf, 2005), which is the step-wise enrichment that occurs between trophic levels, amongst consumers and their resources (Boecklen *et al*., 2011; Section 1.4.1; Fig. 2). The commonly cited value of 3.4 ± 0.98 ‰ for $\delta^{15}N$ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002), was applied here to calculate the trophic positions of the fishes in the pond experiments. However, studies increasingly suggest that discrimination can vary between species, ages, diets and tissues (Brush *et al.*, 2012; Locke *et al.*, 2013). Thus, the use of 3.4 ‰ in the calculation of their trophic positions could be inappropriate and misleading. Indeed, Sweeting *et al*. (2007a) suggest that a nitrogen discrimination of 3.15 ‰ could be used for fishes generally, although Mill *et al.* (2007) recorded a discrimination of 5.25 ‰ for ^{15}N, demonstrating the variation that exists among fishes. Application of the correct discrimination factor is particularly relevant within this Chapter for the comparisons between the allopatric and sympatric contexts where the *Carassius* fishes shifted their trophic positions in response to the presence of *C. carpio*. However, if the discrimination factors of these fishes are indeed species-specific, then the current interpretation of this data may be misguided and thus may require some adjustment.

Dorsal white muscle is the typical tissue used within trophic studies of fishes (Pinnegar and Polunin, 1999; Perga and Gerdeaux, 2005) and hence it was the tissue of choice for the stable isotope analyses completed here (*cf.* Section 1.4.1). This resulted in the destructive sampling of all the fishes. This is common practise for sample collection, as

many species are unsuitable for the use of biopsy plugs that are able to excise muscle tissue without the animal being sacrificed (Tronquart *et al*., 2012). This supposed requirement for destructive sampling to harvest appropriate tissues limits the utility of this method in research programmes on threatened or protected species and in situations where non-destructive sampling is necessary, such as in mark-recapture and tracking studies (Sanderson *et al*., 2009; Tronquart *et al*., 2012; Huang *et al*., 2013). Recently, there has been a shift towards more sustainable non-lethal sampling and the use of tissues such as fin and scales that can be removed and the fish returned alive (e.g., Jardine *et al*., 2005; Kelly *et al*., 2006; Hanisch *et al*., 2010; Jardine *et al*., 2011; Tronquart *et al*., 2012). As significant differences and strong correlations in isotopic signatures have been detected between fish tissues, there exists a requirement for the conversion of data to muscle values. Currently there exist few studies on discrimination factors of cyprinid fishes (although see Tronquart *et al*., 2012) and none that provide tissue-conversion equations for scales, for which stable isotope data in general are limited. This paucity of information is currently limiting the progression of the use of such non-lethally sampled tissues that would benefit the study of cyprinids specifically as well as other fishes.

The prediction tested in the Chapter was designed from Tarkan *et al*. (2009), whose growth analyses were based on small sample sizes, that might have impacted precision (e.g. Kritzer *et al*., 2001), and there was no quantification of ageing error via validation methods, with this potentially impacting ageing accuracy (e.g., Beamish and MacFarlane, 1983; Campana, 2001; Francis *et al*., 2010). Thus, there were potential confounds in that study that require resolution more generally. Whilst work has been completed on the effects of different sampling strategies on mean length-at-age estimates (e.g. Goodyear, 1995) and sample size on the precision of population parameters of reef fishes (Kritzer *et al*., 2001), similar information is not available for temperate cyprinid fishes (*cf.* Section 1.4.1), such as those used within the Chapter.

Consequently, the subsequent data chapters explore how the precision and accuracy of fish scale ageing could be improved in ecological studies, and how non-destructive sampling, such as the collection of fish scales, could be utilised for stable isotope analysis within trophic investigations. This would require knowledge on isotopic conversion factors between different tissues, species- and tissue-specific discrimination factors between the fish and their prey items, and the time taken for different fish tissues to reach isotopic equilibrium with their new diet, i.e. the turnover rate.

Chapter 3. Precision of the age-length increments of three cyprinid fishes: effects of fish number and sub-sampling strategy

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3.1. Summary

Scale ageing provides the basis of calculations on fish growth and productivity, and so it is important to understand how the number of fish aged and the scale sub-sampling strategies used can affect the precision of growth estimates. Here, their effects were tested on the precision of estimates of mean age-length increments from populations of roach *Rutilus rutilus*, dace *Leuciscus leuciscus* and chub *Squalius cephalus* from river fish communities in Eastern England. Regarding the number of fish analysed in each age group, for each species and mean length-increment-at-age, significant relationships were detected between sample size (n) and the coefficient of variation of the mean ($CV\overline{x}$), and the mean length-increment ($\overline{x}$) and measured variance (s^2). These enabled calculation of the number of scales required for producing a mean length-increment-at-age according to $n = a\ \overline{x}^{b-2}\ CV\overline{x}^{-2}$. The number of scales required increased substantially as precision increased, but with little variation between species per age category. Ageing between 7 and 12 scales per age group would thus provide estimates at 10 % precision. As the ages of fish are not known in advance of scale ageing, however, then the effect of scale sub-sampling regime on precision was also tested using randomised strategies of 10 fish per 5 mm length category, 5 per 5 mm, 3 per 5 mm, 10 per 10 mm, 5 per 10 mm and 3 per 10 mm. These were randomly applied to the datasets and the consequences of the reduction in the number of scales for precision was determined using $CV\overline{x} = a^{0.5}\overline{x}^{(b/2)-1}n^{-0.5}$. When compared to no sub-sampling, ageing 3 fish per 10 mm always significantly reduced data precision whereas ageing 10 fish per 5 mm never significantly reduced precision. These outputs can thus be applied to the design of fish sampling protocols where age and growth

estimates are required, with the randomised sub-sampling strategy likely to be the most useful for this.

3.2. Introduction

Estimates of fish growth rates are important for understanding the fundamental processes and factors that influence fish and fisheries biology (Bagenal and Tesch, 1978; Francis, 1990; Coggins and Pine, 2010). These data play key roles in addressing questions on basic ecological relationships whose outputs can then be used to underpin the management of fisheries specifically and aquatic ecosystems more generally (Beardsley and Britton, 2012). The accuracy and precision of ageing fish from bony structures has received a lot of research attention, with studies emphasising the importance of ageing validation studies (Beamish and MacFarlane, 1983; Campana, 2001; Francis *et al*., 2010) and estimating measures of reader bias and precision (Campana *et al*., 1995, 2001; *cf.* Section 1.3). Issues have also been highlighted in the consequences of inaccurate and imprecise ageing of bony structures, whether arising through reader error (Chang, 1982) or through difficulty of estimating age from that structure (*cf*. Section 1.3), such as for recaptured stocked fish that spent their early life stages on a culture site (Britton *et al*., 2004a; Britton, 2010). A further consideration is the effect of sampling strategy and sample size on the precision and accuracy of age-length parameter estimates (Goodyear, 1995; Garner, 1997). For example, Kritzer *et al*. (2001) revealed that the growth parameters of four reef fishes reached precisions of 10 % when 75 fish were aged per population (*cf.* Section 1.3).

Populations of cyprinid fish in temperate lowland rivers are often characterised by relatively slow-growing species comprising individuals that can sometimes live for over 20 years (Cragg-Hine and Jones, 1969; Mann, 1973, 1974, 1976; Britton, 2007; Britton *et al*., 2013). Their lengths-at-age are often characterised by significant individual variation within and between age groups (Britton, 2007). This often results from variation in length at age 1, through multiple spawning strategies, as observed in chub *Squalius cephalus* (Nunn *et al*., 2002), but with consistent annual length increments thereafter (Bolland *et al*., 2007). In England and Wales, these populations of cyprinid fishes also have high recreational value for catch and release angling (e.g., Cowx and Broughton, 1986; Hickley and Chare, 2004; Britton *et al*., 2013; *cf.* Section 1.3). Thus, stock

assessment exercises of these fisheries that involve the production of age and growth estimates are reliant on using scales, for their collection is non-destructive (Britton, 2007). Whilst work has been completed on the effects of different sampling strategies on mean length-at-age estimates (e.g. Goodyear, 1995) and sample size on the precision of population parameters of reef fishes (e.g. Kritzer *et al*., 2001), similar information is not available for temperate cyprinid fishes present in recreational river fisheries. Consequently, the aim of this Chapter was to investigate the precision of mean age-length increments produced by scale ageing according to scale sample size and scale sampling strategy for populations of three cyprinid fishes. Outputs can be used subsequently for designing fisheries research programmes and stock assessment exercises. The objectives of the Chapter were thus to determine for each species: (1) the effect of the number of scales aged on the coefficient of variation ($CV\overline{x}$) of the mean age-length increment estimates; the relationships between the mean age-length increment estimates and measured variance; and the maximum number of fish per age group required to be aged from scales to produce a length increment estimate at a specified level of precision (according to $CV\overline{x}$); and (2) the effect of randomised scale sampling strategies (hereafter referred to as sub-sampling strategies) on the number of scales aged per strategy and the precision of the mean annual length increments.

3.3. Materials and methods

3.3.1. Fish age and growth data

The age and growth data used throughout the Chapter were generated from riverine fish population monitoring surveys completed during summer periods between 2005 and 2006 in Eastern England. Sampled by electric fishing or seine netting (depending on river characteristics), the surveys mainly comprised catches of roach *Rutilus rutilus*, dace *Leuciscus leuciscus* and *S. cephalus*, and were assumed to capture representative samples of these species at lengths above 80 mm (approximately > 1 year old for these species). Following their capture, the fish were identified to species, measured (fork length, nearest mm) and between 3 and 5 scales removed from the area of the body below the base of the dorsal fin and above the lateral line and stored in a paper envelope for subsequent analysis. The fish were then returned alive to the water.

In the laboratory, the scales were aged on a projecting microscope (at x 48 magnification) and the quality control procedure described in Britton *et al*. (2013) was utilised. This meant that all scales were aged by a single primary reader and then a sub-set of 10 % of the scales were chosen at random and read by a secondary reader who had no prior knowledge of the primary readers' age estimates. If any disagreements between readers were found, the scale(s) were re-viewed by both in order to reach a consensus. Following ageing, the distances from the scale focus to the scale edge, and focus to each annulus, were measured from one scale per individual fish to enable back-calculated lengths-at-age to be determined (*cf.* Section 1.1; Fig. 1). This was completed using the scale proportional method, derived from the 'scale proportional' hypothesis (Francis, 1990):

$$(1)\ f(L_i) = (S_i / S_c)\, f(L_c)$$

where, L is the fish length, S is the scale radius, the subscript *c* indicates those values at capture, the subscript *i* indicates those values at formation of the *i*th annulus and *f* is the mean scale radius for fish at length L, determined from regression of S and L. Given the relatively high inter-population variation in the back-calculated lengths-at-age (*cf.* Hickley and Dexter, 1979; Britton, 2007), rather than using estimated lengths in subsequent analyses, annual length increments were used as these were less influenced by the fish starting length at the beginning of the growth year.

3.3.2. Effects of the number of fish aged on precision

To determine the effects of the number of fish aged on precision, back-calculated length-at-age data were utilised from 43 sampled fish populations, each from a different river in Eastern England, with each being surveyed only once. The initial analyses indicated sample sizes (*n*) were highest for length increments produced between the age of 1 and 2 years (hereafter referred to as the length-increment at age 1) and tended to decrease with age (*cf.* Section 3.4). For *R. rutilus* and *S. cephalus*, the number of fish used at each age remained relatively high up to the age of 7 years (i.e. the length-increment at age 6) but reduced thereafter, with this being age 5 years (i.e. length-increment at age 4) for *L. leuciscus*. Thus, the increments produced between age 1 and 6 were determined for relevant populations of *R. rutilus* and *S. cephalus*, and 1 and 4 for *L. leuciscus*. Note that the first growth increment (0 to 1 year) was not used as these can be difficult to compare

between years and populations due to, as previously mentioned, annual variability in spawning times in temperate environments that impacts the length of the first growth season (Nunn *et al*., 2002; Bolland *et al*., 2007).

For each age per population and species, the following metrics were determined: number of fish aged (n), mean body length-increment ($\overline{x}$), standard error (SE), variance (s^2) and coefficient of variation. The coefficient of variation that was calculated and used in subsequent analyses was the coefficient of variation of the mean ($CV\overline{x}$), determined from (SE/$\overline{x}$), rather than the coefficient of variation of samples (SD/$\overline{x}$, where SD is the standard deviation). This is because the coefficient of the mean is a function of the mean body length-increment-at-age ($\overline{x}$), the number of scales used to produce the mean (n) and the variance (s^2) in the length increments used to produce the mean (Cyr *et al*., 1992). Thus, for a given sample, $CV\overline{x}$ can be adjusted by changing values of n and s^2, so providing the ability to determine n according to s^2 and $CV\overline{x}$ (Cyr *et al*., 1992).

The first step was to determine the mean length-increment-variance relationship from (Cyr *et al*., 1992; Garner, 1997):

(2) $s^2 = a\overline{x}^b$

where the coefficients a and b were determined by least squares linear regression using the $\log_{10}$-transformed relationship of mean length and variance and following this, unbiased values of parameter a were de-transformed. In combination, this provided the coefficients a and b for use in estimating $CV\overline{x}$ according to (Cyr *et al*., 1992):

(3) $CV\overline{x} = a^{0.5}\ \overline{x}^{(b/2)-1}\ n^{-0.5}$

where the rearrangement enabled the calculation of the number of scale samples required to give a mean length-increment-at-age according to changing levels of $CV\overline{x}$ (i.e. precision) (Cyr *et al*., 1992; Garner, 1997) from:

(4) $n = a\ \overline{x}^{b-2}\ CV\overline{x}^{-2}$

For presentation purposes, precision levels (i.e. $CV\overline{x}$) were then multiplied by 100 to enable precision to be expressed as percentages (i.e. $CV\overline{x}$ values of 0.01 to 0.10 convert to 1 to 10 %). The mean lengths used in Eq. (4) were the range of mean-length-increments-at-age derived from the populations of the species. The required numbers of

scales displayed for an age group and species was thus the maximum calculated for that age according to the mean length-increment range.

3.3.3. Effects of random scale sub-sampling strategies on precision

To determine how randomised scale sub-sampling strategies influence the subsequent precision of the mean annual length-increment estimates, the following strategies were tested: 10 fish per 5 mm length category, 5/5 mm, 3/5 mm, 10/10 mm, 5/10 mm and 3/10 mm. These strategies were applied to a sub-set of the dataset used to test the effects of scale sample size (Table 9). A pre-requisite of their selection was that sub-sampling had not been applied during their collection in the field. These data were also supplemented by data for each species from the River Wensum, Norfolk, which was sampled in 1983, 1986, 1991, 1994, 2005 and 2006 (Table 9). The rationale for this was that these samples provided increased variation in overall sample numbers, lengths-at-age and age range for each species across the six sampling occasions. For each population used, the data used from each individual fish were length-at-capture, estimated age and estimated final annual length-increment.

For each fish population, the first step was to generate and store a random number for each individual fish. The fish were then sorted by length and separated into their sub-sampling increments, i.e. either 5 mm length classes (e.g. 51 to 55 mm, 56 to 60 mm etc.) or 10 mm length classes (e.g. 51 to 60 mm, 61 to 70 mm etc.). Within these increments, the fish were sorted again, this time by their random number (in ascending order). It was the output of this final sorting that was used to apply the sub-sampling strategy. For example, to apply 3/10 mm, the three fish selected per 10 mm length-increment were those with the three lowest random numbers. The subsequent output was the number, age and the final annual body length-increment for all of the fish in the original sample, and then the number, age and final annual body length-increment of the sub-sampled fish in each sub-sampling strategy. The sub-sampled data for each population was then sorted to provide the number and length-increment of fish per age group and sub-sampling strategy. These data were then applied to Eq. (3) to provide the $CV\overline{x}$ for each age according to the original sample and each sub-sampling strategy. To compare how $CV\overline{x}$ changed according to each sub-sampling strategy, a generalised linear model (GLM) was constructed for each species, as the data were not normally distributed. The dependent

variable was $CV\overline{x}$, the independent variable was scale sampling strategy and the covariate was age, as it was apparent that $CV\overline{x}$ was variable according to age. Model outputs were the mean adjusted values (for age) of $CV\overline{x}$ per scale-sampling strategy and the significance of their pairwise comparisons with Bonferroni adjustments for multiple comparisons. All statistical analyses were completed using IBM SPSS Statistics (version 22.0).

Table 9. The number (*n*), length and age range of the fish samples used for testing the effects of sub-sampling scales on the precision of mean length-increment estimates.

	R. rutilus			*L. leuciscus*			*S. cephalus*		
River	*n*	Length range (mm)	Age range (years)	*n*	Length range (mm)	Age range (years)	*n*	Length range (mm)	Age range (years)
Stour	293	61 - 321	1 - 9	174	72 - 241	1 - 7	111	57 - 487	1 - 11
Blackwater	258	60 - 350	1 - 9	121	83 - 260	1 - 7			
C & B Canal*	305	60 - 293	1 - 11						
Colne	340	61 - 335	2 - 14	113	53 - 218	1 - 7	197	80 - 482	2 - 18
Gipping	276	56 - 319	2 - 12				156	60 - 522	2 - 15
Waveney	307	24 - 218	1 - 5	103	54 - 180	1 - 5	64	45 - 529	1 - 14
Wensum (1983)	150	60 - 364	1 - 13	65	84 - 265	1 - 7	184	87 - 510	1 - 16
Wensum (1986)	75	77 - 335	1 - 11	64	72 - 260	1 - 9	94	174 - 500	3 - 14
Wensum (1991)	108	54 - 376	1 - 12	56	106 - 258	2 - 7	331	59 - 521	1 - 17
Wensum (1994)	124	46 - 347	1 - 10	89	62 - 243	1 - 6	254	52 - 515	1 - 18
Wensum (2005)	63	71 - 321	1 - 14	81	71 - 252	1 - 7	73	75 - 537	1 - 17
Wensum (2006)	145	67 - 309	1 - 10	68	82 - 247	1 - 6	86	67 - 551	1 - 15

*Chelmer and Blackwater Canal

3.4. Results

3.4.1. Effects of the number of fish aged on precision

There was a general decrease in the annual body length-increments with age in the three species (Table 10). Coefficients of variation of the mean length-increments by age varied between 0.01 and 0.13 (Table 10), with $CV\overline{x}$ significantly increasing as sample sizes decreased (Table 11). The relationships of mean length-increment and measured variance were also significant (Table 12). Use of the de-transformed *a* and *b* coefficients from these relationships within Eq. (4) enabled the number of scales, and therefore individuals, required per age and species to provide an estimate of mean length-increment at different levels of precision. This revealed a substantial increase in the maximum number of scales required as precision increased (Table 13). For example, for *R. rutilus* at 10 % precision, a maximum of beween 7 and 10 scales were required, increasing to between 26 and 49 scales at 5 % (Table 13A). Between the species, the maximum number of scales required at each age and level of precision was similar; at 10 % between 7 and 10 scales were also required for *L. leuciscus*, and between 10 and 12 for *S. cephalus* (Table 13).

3.4.2. Effects of scale sub-sampling strategy on precision

When the sub-sampling strategies were applied to each dataset, their initial consequence was to significantly reduce the number of fish used in subsequent analyses when compared to the original sample (GLM, $P < 0.01$ in all cases; Fig. 6). As the fish increased in length with age, this affected the number of fish captured at that age, with no populations where there were age groups with more than four fish above the age of 7 years for *R. rutilus* and 6 years for *L. leuciscus*. For *S. cephalus*, the maximum age where there was more than four fish per age group was also 7 years except for two populations where this was 11 years. In all models, the effect of age as a covariate on the number of fish aged was significant ($P < 0.01$).

The GLM revealed that sub-sampling always decreased the precision of the mean annual length-increments when compared to the original data (as indicated by increasing values of $CV\overline{x}$), but this reduction was only significant for some sub-sampling regimes ($P < 0.01$; Fig. 7). This was species-dependent; for *R. rutilus*, only 10/5 mm was not significantly different to the original data ($P > 0.05$, Fig. 7A), for *L. leuciscus* 3/10 mm

was the only significantly different sub-sampling strategy ($P < 0.01$; Fig. 7B), and for *S. cephalus*, 3/5 mm, 5/10 mm and 3/10 mm were all significantly different to the original data ($P < 0.01$; Fig. 7C). In all models, the effect of age as a covariate on precision was significant ($P < 0.01$).

Table 10. The number of populations of *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C) per age category used to determine the effects of scale sample size on the precision of mean annual body length-increments and their range in number (*n*), mean length-increment, standard error (SE) and the coefficient of variation of the mean ($CV\overline{x}$).

A)

Age category	Populations	*n*	Mean length-increment (mm)	SE	$CV\overline{x}$
1	43	4 - 424	27 – 52	0.5 - 2.8	0.01 - 0.10
2	28	4 - 319	24 – 46	0.6 - 5.3	0.01 - 0.10
3	28	4 - 319	21 – 51	0.7 - 4.3	0.01 - 0.10
4	23	4 - 231	18 – 40	0.8 - 5.4	0.02 - 0.11
5	20	4 - 147	18 – 37	0.9 - 3.9	0.02 - 0.12
6	12	4 - 103	18 – 30	1.3 - 4.1	0.02 - 0.11

B)

Age category	Populations	*n*	Mean length-increment (mm)	SE	$CV\overline{x}$
1	33	4 - 201	43 - 65	0.7 - 3.6	0.01 - 0.09
2	19	4 - 173	33 - 51	0.8 - 3.2	0.02 - 0.09
3	16	4 - 127	26 - 40	1.1 - 4.6	0.02 - 0.12
4	15	4 - 101	20 - 35	0.7 - 2.9	0.02 - 0.12

C)

Age category	Populations	*n*	Mean length-increment (mm)	SE	$CV\overline{x}$
1	26	4 - 264	33 - 61	0.8 - 9.6	0.01 - 0.09
2	16	4 - 264	30 - 60	0.9 - 6.0	0.01 - 0.11
3	16	4 - 239	34 - 59	0.8 - 6.1	0.02 - 0.12
4	15	4 - 227	31 - 58	0.9 - 6.6	0.02 - 0.12
5	13	4 - 215	31 - 53	0.9 - 4.8	0.02 - 0.13
6	13	4 - 190	26 - 48	0.9 - 4.7	0.03 - 0.13

Table 11. Outputs of regressions (power) of the relationships between the number of fish aged in a population and the corresponding coefficient of variation of the mean length-increment ($CV\overline{\boldsymbol{x}}$) per species and age category for populations of *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C).

A)

Age category	R^2	ANOVA
1	0.82	$F_{1,41} = 187.2$, $P < 0.01$
2	0.79	$F_{1,26} = 118.8$, $P < 0.01$
3	0.87	$F_{1,26} = 201.3$, $P < 0.01$
4	0.86	$F_{1,21} = 199.3$, $P < 0.01$
5	0.73	$F_{1,18} = 44.1$, $P < 0.01$
6	0.76	$F_{1,10} = 25.1$, $P < 0.01$

B)

Age category	R^2	ANOVA
1	0.79	$F_{1,31} = 124.3$, $P < 0.01$
2	0.71	$F_{1,17} = 56.2$, $P < 0.01$
3	0.61	$F_{1,14} = 27.8$, $P < 0.01$
4	0.59	$F_{1,13} = 15.7$, $P < 0.01$

C)

Age category	R^2	ANOVA
1	0.83	$F_{1,24} = 92.4$, $P < 0.01$
2	0.72	$F_{1,14} = 85.4$, $P < 0.01$
3	0.91	$F_{1,14} = 107.2$, $P < 0.01$
4	0.89	$F_{1,13} = 91.6$, $P < 0.01$
5	0.71	$F_{1,11} = 39.4$, $P < 0.02$
6	0.89	$F_{1,11} = 31.3$, $P < 0.01$

Table 12. Outputs of linear regressions of the relationships between the age category of $\log_{10}$(mean length-increment) and $\log_{10}$(measured variance) per species for populations of *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C), where predicted variance (s^2) = $a\overline{x}^b$.

A)

Age category	R^2	ANOVA	a	b
1	0.65	$F_{1,41} = 14.4$, $P < 0.01$	0.13	1.59
2	0.61	$F_{1,26} = 18.8$, $P < 0.01$	0.16	1.49
3	0.59	$F_{1,26} = 23.8$, $P < 0.01$	0.14	1.51
4	0.60	$F_{1,21} = 9.1$, $P < 0.01$	0.12	1.47
5	0.63	$F_{1,18} = 22.8$, $P < 0.01$	0.16	1.39
6	0.45	$F_{1,10} = 5.1$, $P < 0.05$	0.25	1.38

B)

Age category	R^2	ANOVA	a	b
1	0.69	$F_{1,31} = 27.3$, $P < 0.01$	0.07	1.72
2	0.49	$F_{1,17} = 4.1$, $P < 0.05$	0.11	1.68
3	0.51	$F_{1,14} = 7.8$, $P < 0.01$	0.16	1.52
4	0.53	$F_{1,13} = 8.9$, $P < 0.01$	0.10	1.69

C)

Age category	R^2	ANOVA	a	b
1	0.66	$F_{1,24} = 12.4$, $P < 0.01$	0.28	1.44
2	0.62	$F_{1,14} = 10.2$, $P < 0.01$	0.20	1.51
3	0.61	$F_{1,14} = 9.9$, $P < 0.01$	0.19	1.49
4	0.63	$F_{1,13} = 11.2$, $P < 0.01$	0.13	1.53
5	0.49	$F_{1,11} = 9.4$, $P < 0.02$	0.25	1.49
6	0.51	$F_{1,11} = 11.3$, $P < 0.01$	0.18	1.56

Table 13. The maximum number of fish (*n*) required per age category for estimates of mean length-increment according to precision levels (as defined by $CV\overline{\boldsymbol{x}} \times 100$), where the maximum was the highest estimate from the mean length-increment range in Table 10 for populations of *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C).

A)			$CV\overline{x} \times 100$				
Age category	*a*	*b*	1	2.5	5	7.5	10
1	0.31	1.59	803	128	32	14	8
2	0.33	1.49	653	104	26	12	7
3	0.42	1.51	945	151	38	17	9
4	0.45	1.47	973	156	39	17	10
5	0.58	1.39	995	159	49	18	10
6	0.63	1.38	1050	168	42	19	10

B)			$CV\overline{x} \times 100$				
Age category	*a*	*b*	1	2.5	5	7.5	10
1	0.19	1.72	663	106	27	12	7
2	0.26	1.68	849	136	34	15	8
3	0.51	1.52	1068	171	43	19	11
4	0.25	1.69	988	158	40	18	10

C)			$CV\overline{x} \times 100$				
Age category	*a*	*b*	1	2.5	5	7.5	10
1	0.71	1.44	1002	160	40	18	10
2	0.58	1.51	1096	175	44	19	11
3	0.63	1.49	1043	167	42	19	10
4	0.52	1.53	1035	166	41	18	10
5	0.61	1.49	1059	172	42	19	11
6	0.51	1.56	1216	195	49	22	12

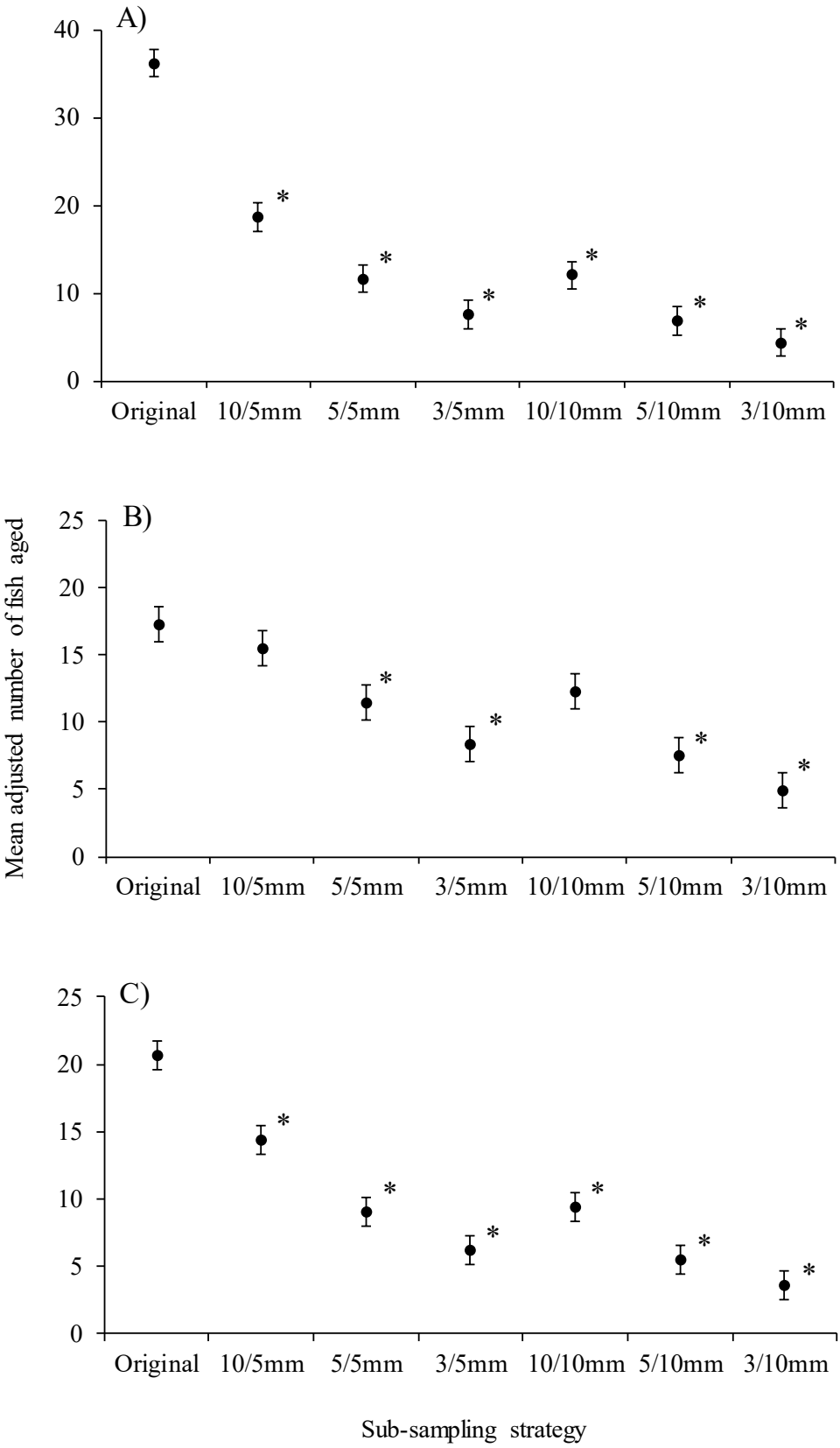

Figure 6. The mean adjusted number of fish aged per scale sub-sampling strategy for *R. rutilus* (A), *L. leuciscus* (B), and *S. cephalus* (C); values were derived from the generalised linear models where the effect of age on the number of fish aged was the covariate; 'original' represents the scale sample with no sub-sampling applied; *significantly different to the original at $P < 0.01$. Error bars represent standard errors.

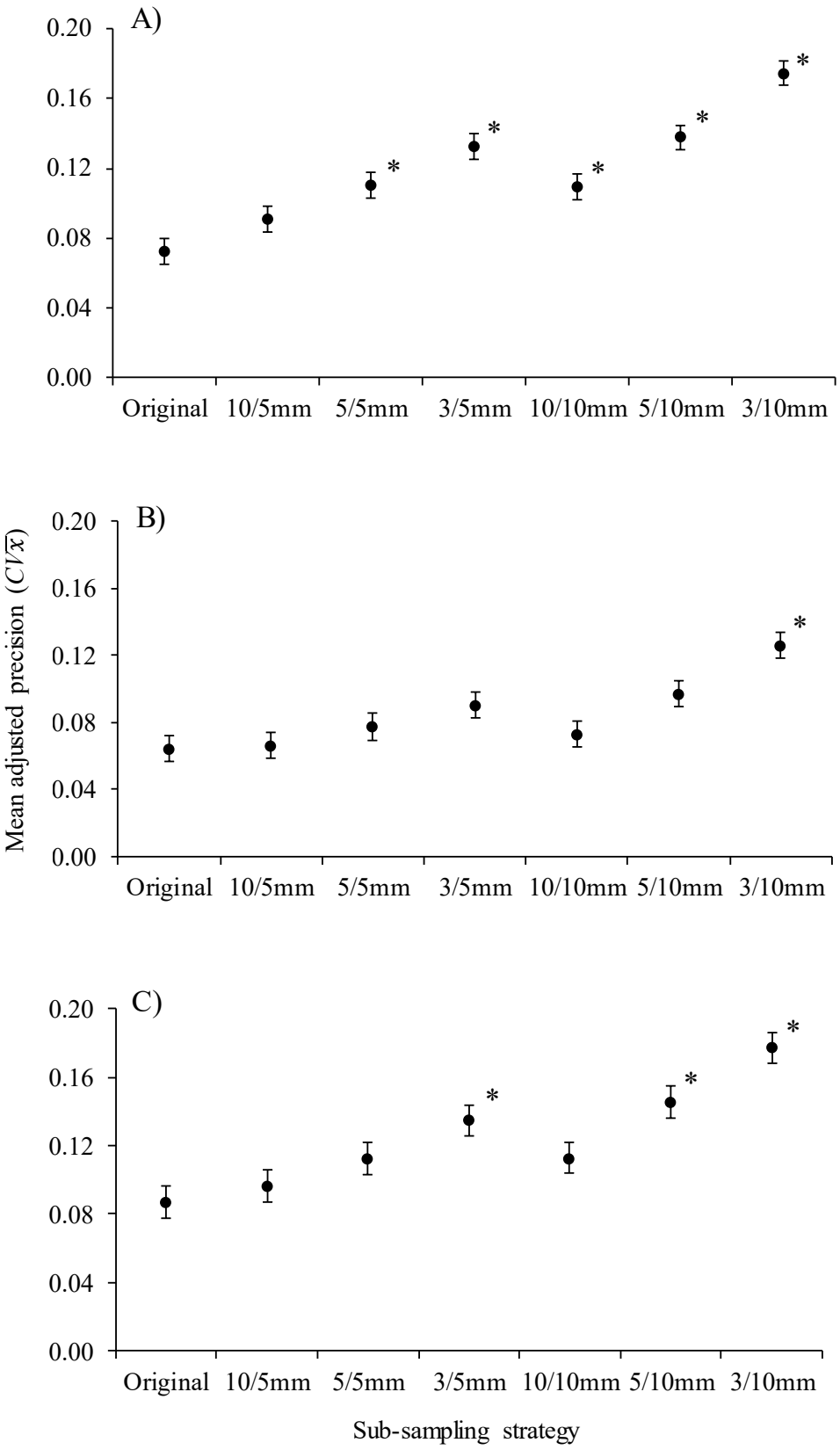

Figure 7. The mean adjusted precision ($CV\bar{x}$) of estimated mean length-increment per scale sub-sampling strategy for *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C); values were derived from the generalised linear models where the effect of age on precision was the covariate; 'original' represents the scale sample with no sub-sampling applied; *significantly different to the original at $P < 0.01$. Error bars represent standard errors.

3.5. Discussion

Scale sample size had substantial effects on the precision of body length-increment estimates at age, with increased precision in samples that aged greater numbers of scales, and therefore fish. Randomised scale sub-sampling strategies had significant consequences for the precision of subsequent length-increment estimates at age. The sub-sampling regimes which caused the greatest reductions in the number of scales aged resulted in the highest losses of precision.

The outputs of the effects of the numbers of scales aged have utility in the analysis of archived scale records when retrospective age and growth analyses are to be completed (*cf*. Section 1.4). The outputs suggest that achieving 10 % precision for the mean length-increment estimates for the majority of age groups present in riverine populations of these fishes should be broadly achievable in most studies and would thus require between 7 and 12 fish to be aged per age group. Obviously, where increased precision is necessary, the number of fish scales analysed would be increased accordingly. Note that when users apply this option to the ageing of their scale samples, it is recommended that between 7 and 12 fish are aged in each age class, where feasible, rather than ageing a total of between 7 and 12 older fish and relying on the back-calculated lengths of these fish to satisfy the precision estimates in the lower age groups. This should then avoid issues in populations where 'Lee's phenomenon' is apparent, i.e. the older fish are slower growing (Lee, 1912).

Arguably, of the two methods utilised here, it is the outputs of the effects of the randomised scale sub-sampling that has the greatest utility to fish biologists. This is because they can be applied to both the scenario outlined above, given that the ages of fish are not known in advance of their analysis, and also in the design of new fish population sampling programmes and protocols. Application of a single, specific scale sub-sampling strategy is not being recommended, as this should be determined in relation to the overall sampling objectives, the resources available and the species concerned. However, where very precise data are required but sub-sampling is necessary then the outputs suggest the sub-sampling strategy applied should be selection of a random 10 fish per 5 mm, at least for *R. rutilus*.

Throughout the Chapter, length-increments were used to derive the precision estimates. This contrasts to other studies, such as Kritzer *et al*. (2001), who used von

Bertalanffy growth parameters in conjunction with bootstrapping methodologies that provided simulated sample sizes between 25 and 1000. Here, with up to 43 riverine populations available per species and with sample numbers per population available of up to 424 fish, there was arguably little requirement for data simulation in this manner. The sample data were of sufficient size to enable generation of the precision estimates, with the underpinning calculations from Eq. (2) indicating significant relationships between the variables for each age and species. Moreover, length-increments at age were used in preference to von Bertalanffy growth model parameters, in part this was due to Živkov *et al*. (1999) who suggested that for many populations, 'L infinity' ($L\infty$), the asymptotic length at which growth is zero, has little biological value as growth rates do not approach the asymptote. In addition, use of length-increments at age enables a wider range of growth analyses to be completed and hypotheses to be tested without preventing subsequent use of the von Bertalanffy growth model where appropriate.

The cyprinid fish species used here are capable of life spans of at least 8 years (*L. leuciscus*), with individual *S. cephalus* in some populations living to at least 20 years old (Mann, 1976; Britton, 2007; Beardsley and Britton, 2012). Here, however, the maximum age used was 7 years old (i.e. the length-increment produced between age 6 and 7 years). The only exception was for sub-sampling *S. cephalus* where two populations used some 11 year old fish in the sub-sampling analysis. The minimal presence and use of older fish in the analyses was for two reasons. Firstly, with increasing age, the number and sample size of populations suitable for inclusion was reduced, and thus the addition of more age groups may have resulted in decreased significance of the underlying relationships of the parameters. Furthermore, the 16 age-length increments used across the three species in the scale number analyses revealed broadly similar requirements at 10 % precision, and so could arguably be considered as being representative of samples from all ages across their populations. Secondly, there remains an underlying question of ageing accuracy and issues of error in relation to the process of scale ageing (*cf*. Section 1.3). Although a quality control procedure was used during the ageing and measuring of the scales (*cf*. Section 3.3.1), separate validation studies were not completed, as per the recommendations of Beamish and MacFarlane (1983) and Campana (2001). The ability to accurately age scales from cyprinid fishes tends to decrease with age (Musk *et al*., 2006), with this also observed in other species (e.g. Kimura and Lyons, 1991). Thus, had age groups above those used been included here then it is likely that there would have

been increased proportions of incorrectly aged scales, and thus incorrect length-increment estimates, within the calculations. It should be noted, however, that as per Kritzer *et al*. (2001), the purpose of this Chapter was not to assess this ageing accuracy, only to provide estimates of precision according to data collected from a process where ageing accuracy was not quantified. Nevertheless, the output that ageing between 7 and 12 fish per age group was required to produce a mean length-increment at a 10 % level of precision, across the three species, was consistent with Kritzer *et al*. (2001) who suggested ageing of 7 to 10 fish per age group was also suitable for estimating a variety of population parameters for reef fishes at 10 % precision.

In summary, the effect of sub-sampling scales during the sampling of populations of cyprinid fishes can impact the precision of mean length-at-age data that are subsequently produced. Correspondingly, where sub-sampling strategies are utilised, or indeed small sample sizes are collected in field studies, due to, for example, low capture efficiency, then this has potential to impact the precision of the subsequent growth data. Given the relatively low sample sizes in Tarkan *et al.* (2009), outlined in Chapter 2, then these might have influenced the precision of the data used to compare the growth performance of *C. carassius* and *C. auratus*.

Chapter 4. Development and application of a simple method to incorporate uncertainty in fish age estimates into growth rate analyses

4.1. Summary

Errors in fish ages estimated from hard structures such as scales and otoliths occur through issues of precision and accuracy. Uncertainty in the estimates of ages tends to increase with fish age and length. In combination, these result in inherent errors in growth rates analyses, such as those from the von Bertalanffy growth function (VBGF) and thus could impact management decisions based on these data. Here, a simple method was developed to incorporate this uncertainty in age estimates into growth rate analyses to produce more robust VBGF parameters. Using scales collected from riverine populations of chub *Squalius cephalus*, dace *Leuciscus leuciscus* and roach *Rutilus rutilus*, age estimates were derived and assigned confidence ratings of 1 (certain) to 3 (most uncertain). The VBGF parameters of $L\infty$, k and t_0 were then determined. For each age per species, the uncertainty levels were translated into distributions (%) of probable ages around the age estimate. These were then used in a bootstrapping procedure to randomly generate a new age from a normal distribution that produced adjusted VBGF parameters ($L\infty$-$_{\text{adjusted}}$, k-$_{\text{adjusted}}$ and t_0-$_{\text{adjusted}}$). Across the three fishes, ageing uncertainty increased with fish age, with significant non-linear relationships. Comparison of the original (uncertainty omitted) versus adjusted (uncertainty included) VBGF parameters revealed some significant differences, with general patterns of higher $L\infty$-$_{\text{adjusted}}$ and lower k-$_{\text{adjusted}}$ than the original estimates, suggesting that these were produced from under-aged fish. These adjusted VBGF parameters also impacted length-at-age estimates, with shifts toward slower growth rates. The development of this simple method based on bootstrapping procedures should provide a highly useful ecological tool that works with uncertainty in scale age estimates, and potentially other hard structures. Here, its application to populations of riverine fishes revealed that it produced adjusted VBGF parameters that better reflect the uncertainty in the original data. In doing so, it should enable improved management decision-making in fish and fisheries ecology.

4.2. Introduction

Estimating the ages and growth rates of fish populations is fundamental to studying aspects of their ecology (Bagenal and Tesch, 1978; Allen and Hightower, 2010; Beardsley and Britton, 2012). Data on the ages of individual fish provides insights into fish population demographics such as age structure and longevity, and dynamic rate functions including growth, recruitment and mortality that regulate fish populations (Ricker, 1975). Quantifying dynamic rate functions generally requires the use of data on the ages of fish that have been estimated from hard structures such as scales, otoliths and fin rays, especially in temperate regions (Beamish and Macfarlane, 1983; Britton *et al*., 2004a). Thus, obtaining accurate and precise age estimates is a pre-requisite for understanding the ecology of fish populations and their response to exploitation and management actions (Britton *et al*., 2004a; Ibáñez *et al*., 2008).

Within species, energy allocation for somatic growth is traded-off against other life-history parameters, such as reproduction (Lester *et al*., 2004; Shuter *et al*., 2005). This is particularly true for fishes which have indeterminate growth (Charnov and Berrigan, 1991). The von Bertalanffy growth function (VBGF) (von Bertalanffy, 1938) utilises the interactions between life-history parameters to model growth and is an important component of many population growth rate analyses (Pardo *et al.*, 2013; Rogers-Bennett and Rogers, 2016). Calculation of the VBGF is often reliant upon an accurate description of the lengths-at-age of fish within a population, yet estimates of fish ages from hard structures are often accompanied by errors that can have substantial effects on subsequent analyses (Campana, 2001; *cf.* Section 1.3). These ageing errors relate to accuracy, which is a measure of the proximity of the age estimate to its true value, and precision, which is the reproducibility of individual measurements from a structure (Campana *et al*., 1995; Kalish *et al*., 1995). There are instances worldwide where ageing error, usually through under-estimation of age, has contributed to the mismanagement of a fish stock (*cf*. Section 1.2). For example, estimates of productivity were over-estimated and longevity under-estimated in the orange roughy *Hoplostethus atlanticus* fishery of New Zealand, resulting in overfishing and a collapse in population numbers (Smith *et al*., 1995; Tracey and Horn, 1999; *cf*. Section 1.2).

A number of studies have identified issues of error regarding the accuracy and precision of ageing fish from structures such as scales and otoliths (e.g., Beamish and Macfarlane, 1983, 1995; Ibáñez *et al*., 2008). These issues include poor readability and inconsistent annulus formation that can cause incorrect age estimates (Ibáñez *et al*., 2008; Quist *et al*., 2012; *cf*. Section 1.3). Validation exercises help minimise these errors, thus improving the accuracy and reducing uncertainty in the age estimates (Jackson, 2007; *cf*. Section 1.3). Validation methodologies include using mark-recapture of fish of known age and marginal increment analysis that often incorporates marking body structures with chemicals such as tetracycline (Shirvell, 1981; Nagiec *et al*., 1995; *cf*. Section 1.3). In general, the results of validation exercises indicate that both accuracy and precision decrease with the size and age of fish, with these issues often difficult to eliminate completely given the inherent subjective nature of the age determination process (Musk *et al*., 2006). Beamish and Macfarlane (1983, 1995) stressed the importance of considering and accounting for the inherent error in fish age estimates in order to improve ecological evaluations and management decision-making.

The need to incorporate uncertainty into fish age estimates obtained from hard structures has led to the development of various techniques. For example, Richards *et al*. (1992) used classification matrices to describe the relationship between observed and true ages within a statistical framework by estimating the probability of assigning a particular age to fish, given its true age, and their classification distributions tended to increase with age, highlighting the issues of ageing older fish. Alternatively, use of confidence-ranking systems, which represent graded levels of uncertainty, have been shown to improve age and growth estimates as they enable data to be used only from those individuals for which the age is associated with the highest readability and precision (e.g., Koch *et al*., 2008, 2009, Spiegel *et al*., 2010; Watkins *et al*., 2015a, b). Uncertainty generally increases as age increases and this has been demonstrated in many fish species, including shovelnose sturgeon *Scaphirhynchus platorynchus* (Koch *et al*., 2008), common carp *Cyprinus carpio* and mountain whitefish *Prosopium williamsoni* (Watkins *et al*., 2015b). In age estimates of bowfin *Amia calva*, precision between readers was only 100 % for the fish that had been assigned the highest confidence ratings (Koch *et al*., 2009). In addition, ageing error can be incorporated into growth models, for example, Cope and Punt (2007) applied a random effects modelling framework to VBGF parameters and found that the results were more accurate and precise than traditional non-linear techniques. Whilst the

issues of ageing error in older fishes and reader bias were not resolved in that study, it suggests that incorporating levels of uncertainty that capture ageing bias and ageing errors into growth modelling techniques has potential for resolving some of these outstanding issues.

The aim of this Chapter was thus to develop a simple method to incorporate uncertainty from age estimates taken from subjective interpretations of hard structures, such as scales, into the process of fish ageing and age analyses, to provide more realistic estimates of growth rates and growth parameters. The objectives were to: (1) identify, through literature review, the extent of uncertainty across studies that quantified error in estimating fish ages from hard structures; (2) develop a statistical model that incorporates identified levels of uncertainty into estimates of fish growth rates; and (3) apply this method to datasets of three temperate freshwater cyprinid fishes; chub *Squalius cephalus*, dace *Leuciscus leuciscus* and roach *Rutilus rutilus*.

4.3. Materials and methods

4.3.1. Literature review on the uncertainty of estimating fish age from hard structures

To obtain information regarding the range of uncertainty from existing studies, a literature review was performed using ISI Web of Science and the following search terms (cyprinid*, *S. cephalus*, *L. leuciscus*, *R. rutilus,* along with; age validation, age estimates, accuracy, precision), combined with manual searches of references cited within these articles. The uncertainty surrounding age estimates produced by reading scales from members of the Cyprinidae family were the focus of the review, although information from studies that used other calcified structures and other fishes was also considered. The common themes that arose from the review formed the basis of the procedure that was developed to incorporate inherent ageing error into age estimates.

4.3.2. Datasets of three temperate freshwater fishes

The research was based on age data generated from scales of *S. cephalus, L. leuciscus* and *R. rutilus* that were obtained from 19 rivers across England. These populations had

been sampled between 2012 and 2014. The sites were 100 m stretches, sampled using electric fishing, with all captured fish identified to species level, measured (fork length, to nearest mm) and between 3 and 5 scales removed from the anterior region above the lateral line and below the dorsal fin and stored in paper envelopes. These scales were aged by a single primary reader using a projecting microscope at × 48 magnification. Validation of the estimated ages was provided by a proportion (10 to 25 %) of the scales per population being randomly selected and aged by a secondary reader, without knowledge of the primary readers age estimate, if any disagreement was found, the scale(s) were re-viewed by both in order to reach a consensus (Musk *et al*., 2006; *cf.* Section 3.3.1). Information on the date of sampling, species and length of each fish was available to both readers.

For each age estimated, a confidence ranking was also assigned, whereby three levels of uncertainty were used, 1 to 3, where 1 was 'most certain' and 3 was 'least certain'. Following the derivation of an age estimate, one scale per fish was measured for the distance from the scale focus to the first, second and last annulus (*cf*. Section 1.1; Fig. 1). Using the scale proportional method (Francis, 1990; *cf*. Section 3.3.1), the lengths at age 1, 2 and the last annulus were then estimated by back-calculation. This enabled the length-increment between age 1 and 2 years to be determined. The length at the last annulus ensured that subsequent analyses could compare the lengths of the captured fish without bias from their time of sampling, such as fish captured early and late in the growth season. The length-increment between age 1 and 2 years was used as a growth rate metric. As in Chapter 3 (*cf.* Section 3.3.2), this was in preference to the length-increment produced in the first growth year (i.e. between age 0 and 1 years) to avoid issues relating to variability in spawning times with *S. cephalus* being a 'fractional spawning' species, i.e., they ripen successive batches of eggs within a season, contrasting with 'total spawning' where a single batch is shed in a short period, or 'protracted spawning' where a single batch is spawned over an extended period (Nunn *et al*., 2002; Bolland *et al*., 2007).

The relationship between the estimated ages and their uncertainty levels was identified by fitting linear, quadratic and cubic non-linear regressions (*i*; 1-3) to the data from each species. This used the *lmtest* function in the R computing program (R Core Development Team, 2013). The best-fitting model was selected by minimising the small-sample, bias-corrected form of the Akaike information criterion (AICc) through the *AICcmodavg* package. The model with the smallest AICc value ($AICc_{min}$) was thus selected as the most

appropriate model. According to Burnham and Anderson (2002), models with an Akaike difference (Δi = AICci – AICc$_{min}$) of more than 10 have essentially no support and might be omitted from further consideration, whereas models with $\Delta i < 2$ have substantial support. To then determine how the body length-increment between age 1 and 2 years influenced the uncertainty of the age estimate of that fish, the increments were rounded to the nearest 5 mm and the mean level of uncertainty calculated for each species and each 5 mm incremental interval. The mean level of uncertainty was then plotted against the growth increment for each species to reveal any relationships.

4.3.3. Incorporating uncertainty into estimates of fish growth rates

The age estimates and their uncertainty levels, for each species, were then used to develop a simple method that would incorporate uncertainty into growth rate analyses via statistical modelling and bootstrap methodologies. The first step was to translate each age estimate and its level of uncertainty into a distribution (%) of probable ages surrounding the age estimate. For example, for a fish estimated to be 4 years old, the distribution that could actually be 3, 4 or 5 years old was determined according to its level of uncertainty, i.e. 30 % of the fish could actually be 3 years old, 60 % 4 and 10 % 5, with this informed by the literature review and author opinion. To convert these distributions into a form for use statistically, a list of 100 individual ages (each age representing 1 percent from the distribution of probable ages) for each age and uncertainty level was generated. A 'best estimate' age (BEA) was then calculated through taking the mean of each set of 100 ages along with the standard deviation (SD). This was repeated for each species, generating a BEA and its associated SD for each estimated age and level of uncertainty.

The next step was to generate growth rate parameters through application of the von Bertalanffy growth function (VBGF) (von Bertalanffy, 1938). The VBGF is a logarithmic function that describes change in body size over time for a wide range of taxa and is commonly applied to fishes (Chen *et al*., 1992; Frisk *et al*., 2001). The three-parameter model was used (Beverton and Holt, 1957):

$$L_t = L_\infty (1 - e^{-k(t - t0)})$$

where t is the time period, L is the length of the fish (mm), L_∞ is the asymptotic length (the maximum theoretical size that a species will grow towards), k is the growth coefficient (the rate at which growth approaches the asymptotic length) and t_0 is the size-

at-age zero which equates to the *y*-intercept. For each fish population per species, the VBGF growth parameters of $L\infty$, k and t_0 were generated. Initially, this was completed without inclusion of the uncertainty levels to generate the growth parameters for the original data set ($L\infty$-$_{original}$, k-$_{original}$ and t_0-$_{original}$) using the *nls* function in R (*cf.* Appendix 1: Step 1, for R code).

The VBGF growth parameters were then generated with inclusion of the levels of uncertainty to provide a set of adjusted parameters ($L\infty$-$_{adjusted}$, k-$_{adjusted}$ and t_0-$_{adjusted}$). These were generated by applying the BEA and SD for each estimated age and uncertainty level to a bootstrapping procedure. For every estimated age and uncertainty level in the original data, its corresponding BEA and SD were used to randomly generate a new age from a normal distribution using the *rnorm* command. From these new, randomly generated ages, estimates for the three VBGF parameters were obtained. This process was conducted 1000 times (following the procedure in Crawley, 2005), producing an output of adjusted VBGF parameters (*cf.* Appendix 1: Step 2, for R code). Data for fishes assigned age estimates above those for which the distributions of probable ages were produced were excluded prior to analyses. The outputs from this procedure were the three growth parameters for the original data set and a set of 1000 adjusted growth parameters from the bootstrapped modelling procedure, from which an adjusted mean and upper and lower 95% confidence limits were obtained.

4.3.4. Relationships between the original and adjusted VBGF parameters

The values for $L\infty$ and k generated for all populations from the original data sets were plotted against the means of their adjusted values (i.e., $L\infty$-$_{original}$ vs. $L\infty$-$_{adjusted}$ and k-$_{original}$ vs. k-$_{adjusted}$). Deviation from the equivalence line demonstrated a shift in parameter estimates. Linear regressions were performed to determine whether a significant shift had occurred using the 95 % confidence interval (CI) range of the b coefficient, significant deviation was indicated when the range failed to cross 1.0 (Sheath *et al*., 2015). Data points that were deemed biologically irrelevant were removed prior to analyses (e.g. $L\infty$ for *R. rutilus* > 500 mm), as these anomalies suggest issues with the original data set, for example, that the sampled fish have not started slowing down their growth sufficiently to produce an exponential growth curve and thus enable robust calculation of the

parameters. $L\infty$ was then plotted against k for the original and adjusted data. All statistical analyses were completed using IBM SPSS Statistics (version 22.0).

4.3.5. Effects of adjusted data on growth outputs

The VBGF parameter values generated from the original data from each population were used to estimate age-at-length data for the range of ages used within the model (i.e., for *S. cephalus* aged 1 to 15 years, *L. leuciscus* aged 1 to 10 years and *R. rutilus* aged 1 to 13 years). This was completed via their input into the von Bertalanffy growth equation. In order to capture the full extent of variety within the adjusted VBGF parameter values, the upper and lower 95 % CI for the mean of $L\infty$-$_{adjusted}$, k-$_{adjusted}$ and t_0-$_{adjusted}$ was used to generate the corresponding upper and lower estimated lengths for each age. Therefore, for each species and population, there were three length estimates for each age in the age range; the estimated length from the original data and the upper and lower estimated lengths from the 95 % CI from the adjusted data. These estimated age-at-length values were then used to produce growth curves for comparison. In addition to producing growth curves for the individual populations, a single plot containing the data from all populations was also produced for each species to enable comparison of general themes.

4.4. Results

4.4.1. Literature review on age uncertainty

A total of 17 peer reviewed papers were sourced on fish age and growth rate errors. Of these, none provided data specifically on *L. leuciscus*, although the majority provided information for other cyprinid species (*cf.* Appendix 2). In relation to *S. cephalus* and *R. rutilus*, Mann (1973, 1976) noted that their scales were difficult to read from fish over 10 years old due to close annuli formation on the scale edge. Musk *et al*. (2006), performing an age precision exercise on *R. rutilus* scales, found that agreement in age estimates significantly decreased with fish age. Across all the species reviewed, general themes were that: (*i*) accuracy, precision and certainty in producing age estimates from calcified structures decreases with fish age; (*ii*) ageing accuracy was highest in younger fishes; (*iii*) older fish were more likely to be under-aged than over-aged, with this increasing with

fish age; (*iv*) reader certainty in assigned age estimates and agreement between readers decreased with fish age; and (*v*) scales became more difficult to read with age due to crowded annuli on scale margins and therefore scales might only be appropriate for ageing younger, smaller and/or immature fishes (*cf*. Appendix 2).

These outputs were used to develop the ranked uncertainty levels that were applied subsequently during the ageing of scales. As reader uncertainty is a function of the precision of age estimates (e.g., Koch *et al*., 2008, 2009, Spiegel *et al*., 2010; Watkins *et al*., 2015a, b), for each species and scale age estimate, dependent on meeting clearly defined criteria (Table 14), an uncertainty level was assigned. It was these levels of uncertainty that were then translated into distributions of probable ages for *S. cephalus* aged 1 to 15, *L. Leuciscus* aged 1 to 10 and *R. rutilus* aged 1 to 13 (Table 15).

Table 14. Criteria used to assign levels of uncertainty to scales from populations of *S. cephalus*, *L. leuciscus* and *R. rutilus* from 19 rivers in England (adapted from Spiegel *et al*., 2010).

Level of uncertainty	Guidelines for assigning level of uncertainty
1: Certain	• Annuli easy to identify. • No disagreement between scales. • Cutting over present for majority of annuli. • Annuli exhibit tightly packed circuli.
2: Some uncertainty	• Scale disagreement of a maximum of 1 year. • Cutting over apparent on the majority of annuli.
3: Most uncertain	• Annuli difficult to identify. • Disagreement between scales ≥ 2 years. • Some annuli exhibit cutting over. • False annuli present. • Annuli do not exhibit tightly spaced circuli.

Table 15. Examples of how ageing uncertainty was incorporated into age estimates using distributions of probable ages (%) from scales of *R. rutilus* (A), *L. leuciscus* (B) and *S. cephalus* (C).

A)

Age estimate (years)	Level of uncertainty	Estimated distribution of probable ages
1	1	1: 95%; 2: 5%
	2	1: 80%; 2: 20%
	3	1: 70%; 2: 15%; 0: 10%; 3: 5%
3	1	3: 90%; 4: 10%
	2	3: 75%; 4: 13%; 2: 12%
	3	3: 65%; 4: 13%; 2: 12%; 1: 5%; 5: 5%
6	1	6: 70%; 5: 10%; 7: 20%
	2	6: 55%; 5: 15%; 7: 20%; 8: 10%
	3	6: 40%; 5: 15%; 7: 25%; 8: 15%; 4: 5%;
9	1	9: 60%; 8: 10%; 10: 20%; 11: 10%
	2	9: 50%; 8: 10%; 10: 20%; 11:15%; 7: 5%
	3	9: 35%; 8: 15%; 10: 25%; 11: 15%; 7: 5%; 12: 5%

B)

Age estimate (years)	Level of uncertainty	Estimated distribution of probable ages
1	1	1: 95%; 2: 5%
	2	1: 80%; 2: 20%
	3	1: 70%; 2: 15%; 0: 10%; 3: 5%
3	1	3: 90%; 4: 10%
	2	3: 75%; 4: 13%; 2: 12%
	3	3: 60%; 4: 15%; 2: 15%; 1: 5%; 5: 5%
6	1	6: 75%; 5: 10%; 7: 15%
	2	6: 60%; 5: 15%; 7: 20%; 8: 5%
	3	6: 45%; 5: 15%; 7: 20%; 8: 15%; 4: 5%
9	1	9: 65%; 8: 5%; 10: 20%; 11: 10%
	2	9: 55%; 8: 7%; 10: 20%; 11: 15%; 7: 3%
	3	9: 40%; 8: 10%; 10: 25%; 11: 15%; 7: 5%; 12: 5%

Table 15 *continued*

C)

Age estimate (years)	Level of uncertainty	Estimated distribution of probable ages
1	1	1: 95%; 2: 5%
	2	1: 80%; 2: 20%
	3	1: 70%; 2: 15%; 0: 10%; 3: 5%
4	1	4: 80%; 3: 10%; 5: 10%
	2	4: 60%; 3: 20%; 5: 20%
	3	4: 40%; 3: 20%; 5: 20%; 2: 10%; 6: 10%
8	1	8: 55%; 7: 15%, 9: 20%; 10: 15%
	2	8: 45%; 7: 15%; 9: 20%; 10: 15%; 6: 5%
	3	8: 30%; 7: 20%; 9: 25%; 10: 15%; 6: 10%
12	1	12: 30%; 11: 10%; 13: 35%; 14: 25%
	2	12: 20%; 11: 10%; 13: 35%; 14: 30%; 15:5%
	3	12: 15%; 11: 5%; 13: 35%; 14: 30%; 15: 10%; 16: 5%

4.4.2. Age data and uncertainty of age estimates

Across the three species, data from 4702 fishes were used: 1241 *S. cephalus* (age estimates: 1 to 20 years), 1007 *L. leuciscus* (1 to 10 years), and 2454 *R. rutilus* (1 to 13 years). Lengths-at-last-annulus varied between 48 and 532 mm for *S. cephalus*, 24 and 275 mm for *L. leuciscus*, and 36 and 335 mm for *R. rutilus*. All fishes revealed a similar non-linear relationship between estimated age and uncertainty level, with uncertainty in age estimates increasing with age (Fig. 8). Mean uncertainty per age and per species ranged between 1.1 and 3.0 for *S. cephalus*, 1.1 and 2.5 for *L. leuciscus* and 1.1 and 2.3 for *R. rutilus* and in all species, low uncertainty levels were apparent in ages up to 5 years (Fig. 8). For *S. cephalus,* all ages estimated as 15 years or older had the highest level of uncertainty. Analysis of the relationships between age and uncertainty revealed that for *S. cephalus*, the cubic function produced the lowest AICc with the quadratic and linear functions having very little support (Δi = 29.23 and 31.18, respectively; Table 16). For *L. leuciscus* and *R. rutilus*, the quadratic function was the best model fitted and had the lowest AICc, with the cubic function having some support but Δi = > 2 in both cases (Table 16).

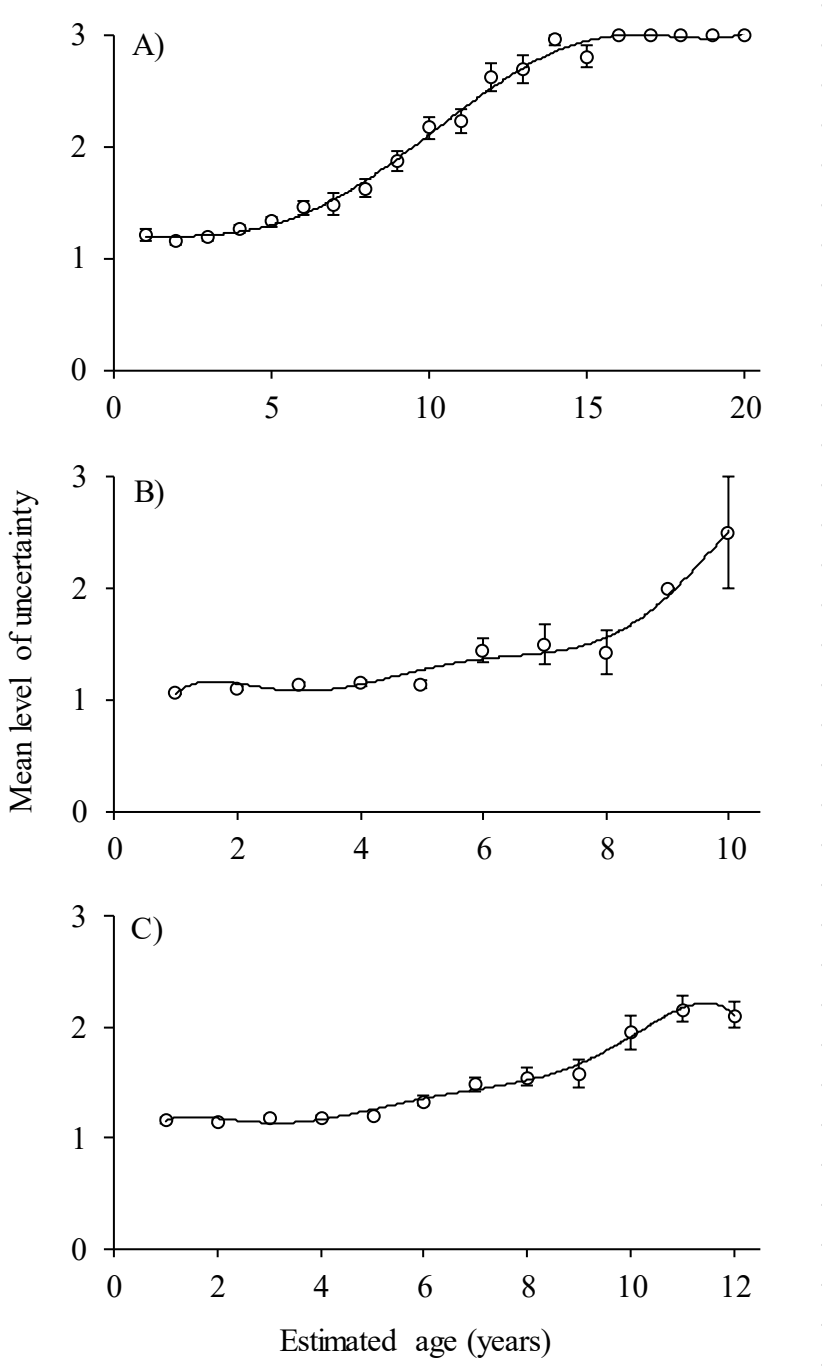

Figure 8. Age estimates versus mean level of uncertainty assigned during the scale ageing process for *S. cephalus* (A), *L. leuciscus* (B) and *R. rutilus* (C). Error bars represent standard errors.

Table 16. For each species and for each candidate model the small-sample bias-corrected form of Akaike's information criterion (AICc), Akaike differences (Δi) and weights (wi).

Species	Model	AIC_c	Δ_i	w_i (%)
S. cephalus	Cubic	-32.15	0.00	100
	Quadratic	-2.91	29.23	0
	Linear	-0.97	31.18	0
L. leuciscus	Quadratic	2.20	0.00	82
	Cubic	5.67	3.47	14
	Linear	8.27	6.07	4
R. rutilus	Quadratic	-17.51	0.00	80
	Cubic	-14.67	2.84	19
	Linear	-7.33	10.18	0

4.4.3. Length-increment versus level of uncertainty in age estimates

The annual growth increments produced in the second year of the fishes (between age 1 and 2 years) ranged between 10 and 80 mm for *S. cephalus*, 10 and 75 mm for *L. leuciscus* and 10 and 70 mm for *R. rutilus* (Fig. 9). Both *S. cephalus* and *R. rutilus* revealed a 'U' shaped pattern between this growth increment and their mean level of uncertainty, indicating that uncertainty in age estimates was higher for fishes with growth increments that were lower or higher than expected; increment < 30 mm and > 65 mm for *S. cephalus* (Fig. 9A) and increment < 20 mm and > 60 mm for *R. rutilus* (Fig. 9C). For *L. leuciscus*, the uncertainty was highest for fishes with a lower growth increment (< 25 mm), but not for higher (Fig. 9B).

4.4.4. Relationships between the VBGF parameters

Linear regression of the original versus adjusted values of $L\infty$ revealed that the 95 % confidence interval (CI) of b failed to cross 1 for both *S. cephalus* and *R. rutilus*, indicating significant deviation from equivalence (Table 17; Fig. 10A, E). This was not apparent for *L. leuciscus* (Table 17; Fig. 10C). Linear regression of the original versus adjusted values of k revealed that the 95 % CI of b failed to cross 1 in *L. leuciscus* and *R. rutilus* (Table 17; Fig. 10D, F). This was not apparent for *S. cephalus* (Table 17; Fig. 10B). Plots of $L\infty$ versus k revealed similar significant relationships across the fishes, with $L\infty$ increasing as k decreased, with the interaction between the VBGF parameters remaining consistent between the original and adjusted data for *S. cephalus* and *R. rutilus* (Fig. 11A, C), but not for *L. leuciscus* (Fig. 11B).

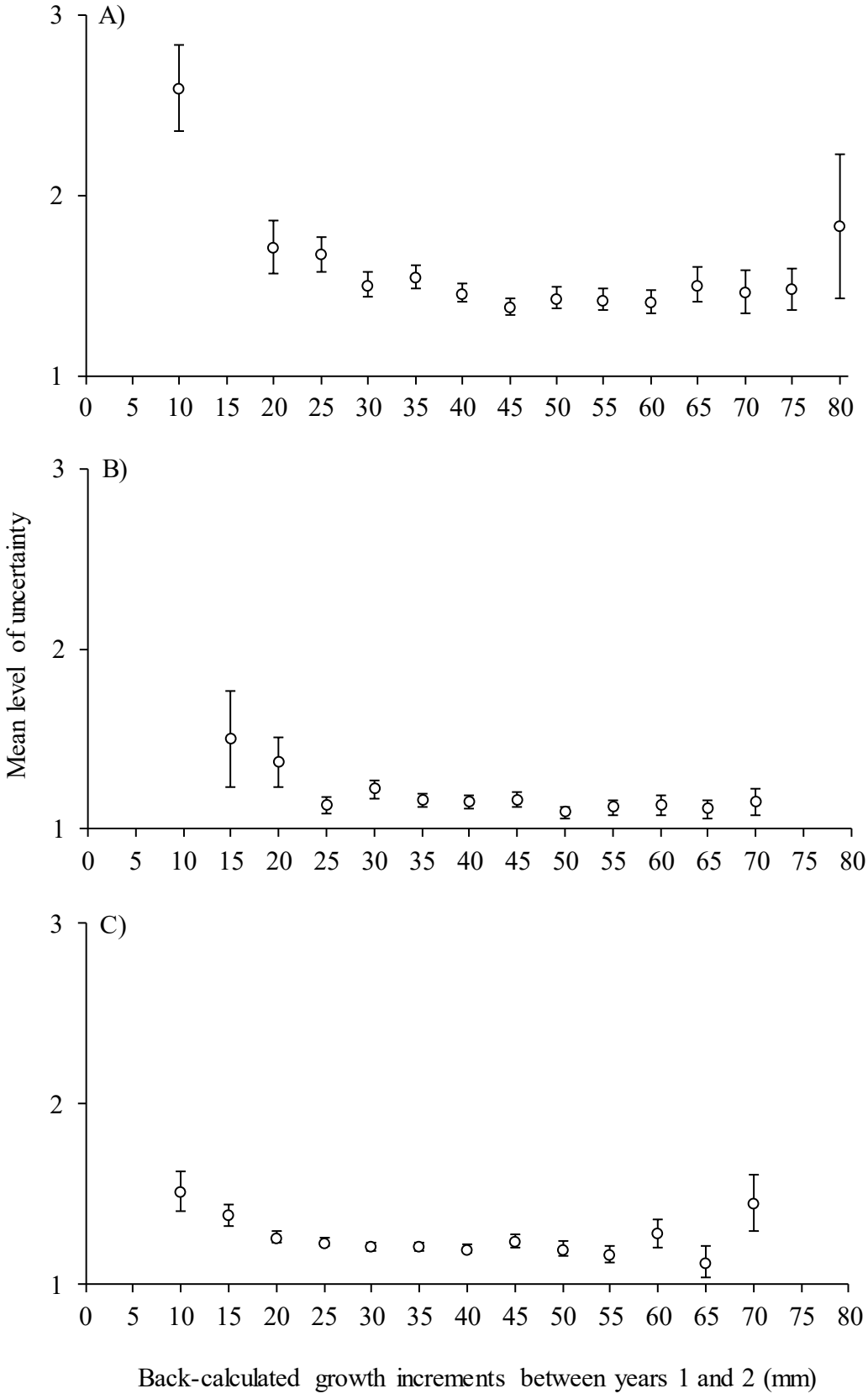

Figure 9. Back-calculated growth increments (between years 1 and 2) versus mean level of uncertainty assigned during the scale ageing process for *S. cephalus* (A), *L. leuciscus* (B) and *R. rutilus* (C). Error bars represent standard errors.

Table 17. Outputs of linear regressions of the von Bertalanffy growth parameters (VBGF); $L\infty$ and k, between their original (uncertainty omitted) and adjusted (uncertainty incorporated) values; *significant deviation from the equivalence line.

Species	VBGF parameters	R^2	F	P	b: 95 % confidence interval	
S. cephalus	$L\infty$: original vs. adjusted	0.95	259.88	< 0.001	0.70 - 0.92	*
	k: original vs. adjusted	0.94	195.75	< 0.001	0.77 - 1.05	
L. leuciscus	$L\infty$: original vs. adjusted	0.89	50.55	< 0.001	0.69 - 1.42	
	k: original vs. adjusted	0.87	40.16	< 0.001	0.40 - 0.90	*
R. rutilus	$L\infty$: original vs. adjusted	0.98	470.21	< 0.001	1.11 - 1.38	*
	k: original vs. adjusted	0.95	154.62	< 0.001	0.60 - 0.88	*

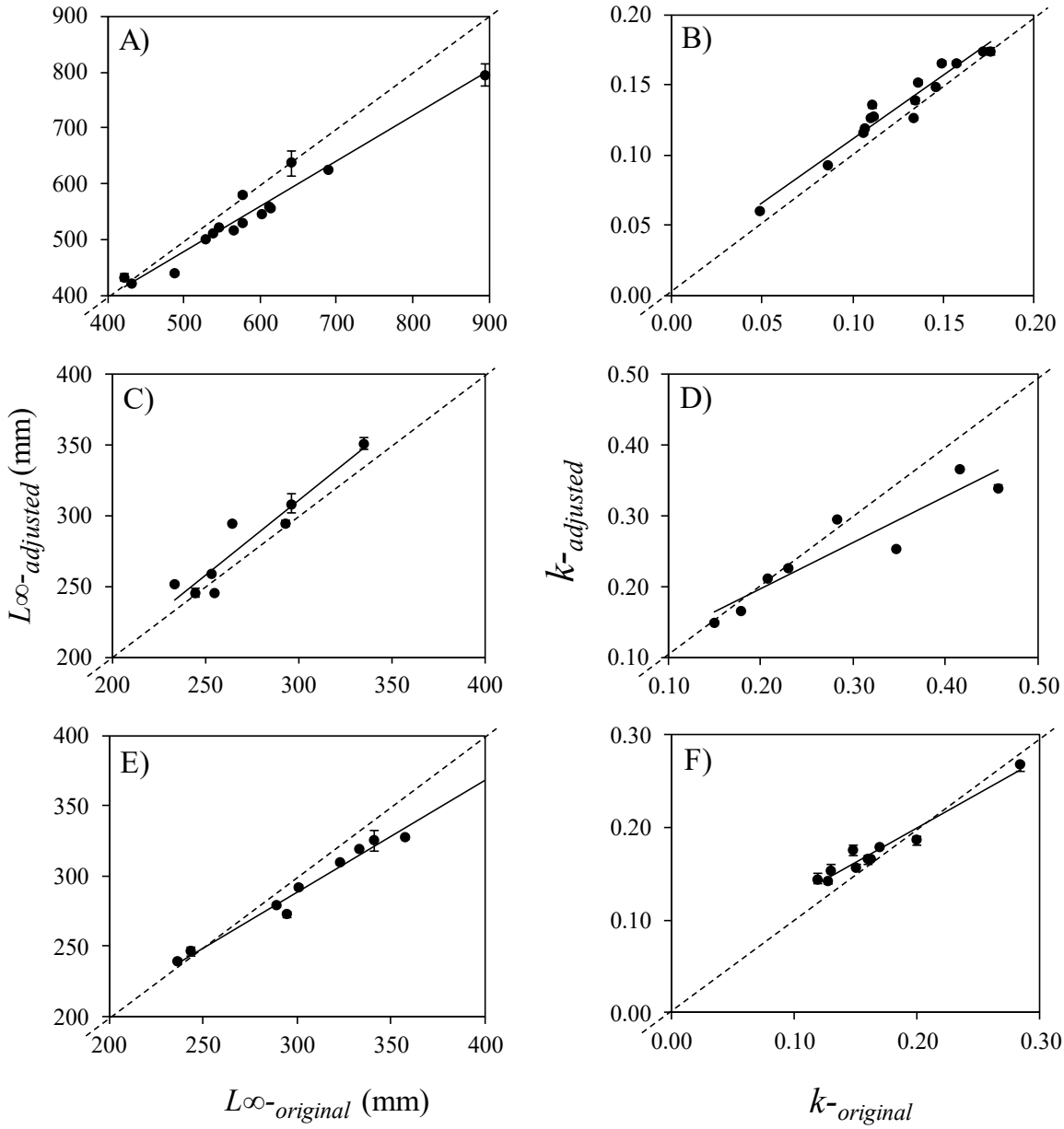

Figure 10. Bias plots between original estimates (uncertainty omitted) and their mean adjusted values (uncertainty incorporated) of $L\infty$ and k for populations of *S. cephalus* (A and B, respectively), *L. leuciscus* (C and D) and *R. rutilus* (E and F); solid lines represent linear regressions, dashed lines represent the equivalence line. Error bars represent 95 % confidence limits.

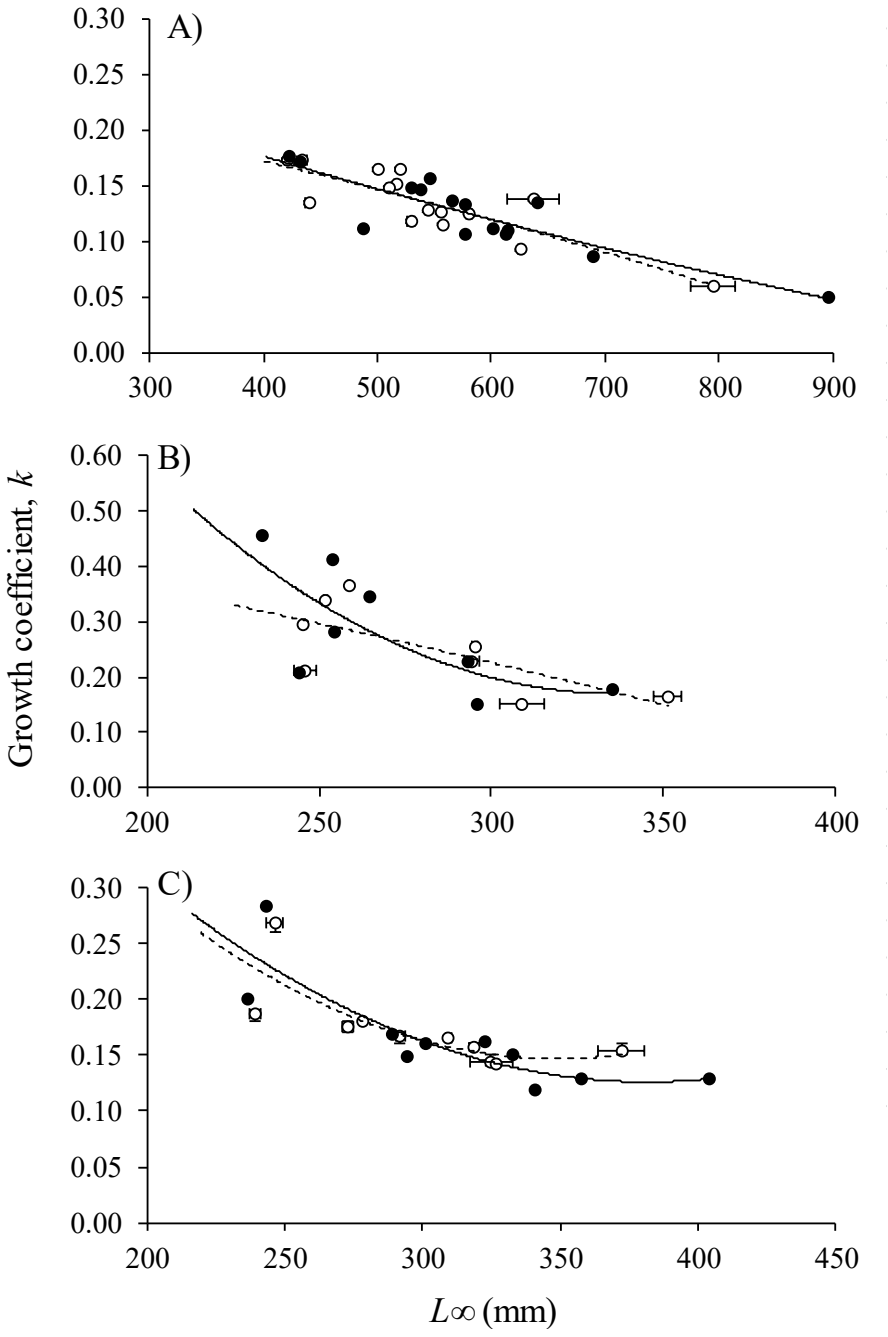

Figure 11. Relationship between $L\infty$ and k for the original estimates (uncertainty omitted) and their mean adjusted (uncertainty incorporated) values for populations of *S. cephalus* (A), *L. leuciscus* (B) and *R. rutilus* (C); solid lines and filled circles represent the original data, dashed lines and open circles represent the adjusted data. Error bars represent 95 % confidence limits.

4.4.5. Effects on length-at-age estimates

Comparison of the original versus adjusted length-at-age data produced from the VBGF equation revealed that in the adjusted data, the mean lengths-at-age were generally reduced in the older fish in all three species (Fig. 12). When plotted by population, these patterns were apparent for most populations of *S. cephalus* and *R. rutilus* (e.g. Fig. 13A, G) and one population of *L. leuciscus* (Fig. 13D). However, 75 % of *L. leuciscus* populations showed the opposite pattern, with the adjusted data increasing the mean lengths-at-age across the entire age range of the fishes (e.g. Fig. 13F), and this opposing pattern was also seen in a single population of *S. cephalus* and *R. rutilus* (Fig. 13C, I). Out of the 19 river populations used in the analyses, two populations of *S. cephalus*, one population of *L. leuciscus* and four populations of *R. rutilus* show the original data growth curve siting within the upper and lower 95 % confidence limit curves of the adjusted data (Fig. 13B, E, H).

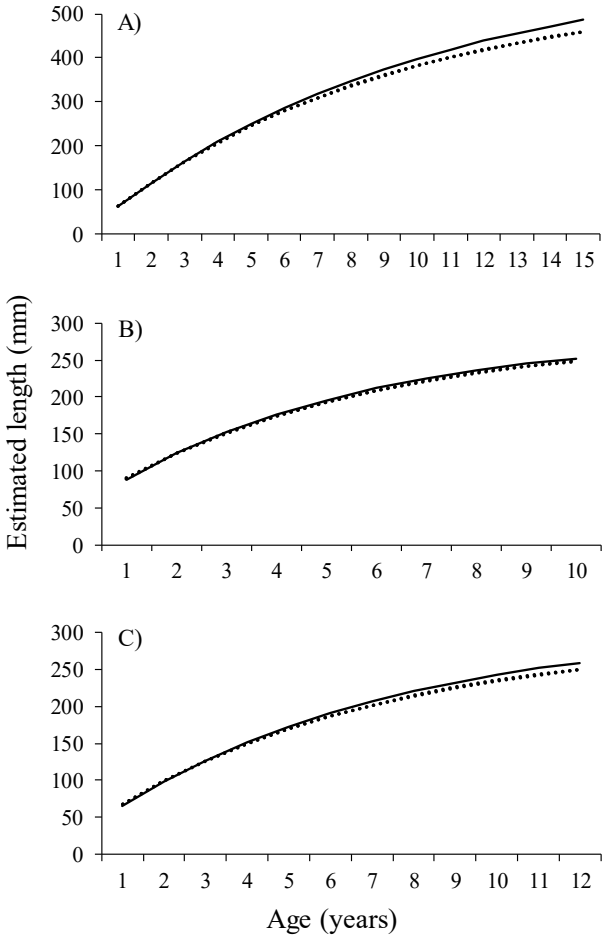

Figure 12. Length-at-age growth curves for combined populations of *S. cephalus* (A), *L. leuciscus* (B) and *R. rutilus* (C) generated from the von Bertalanffy growth parameters. Solid lines represent the original data (uncertainty omitted) and dashed lines represent the upper and lower 95 % confidence range for the adjusted data (uncertainty incorporated).

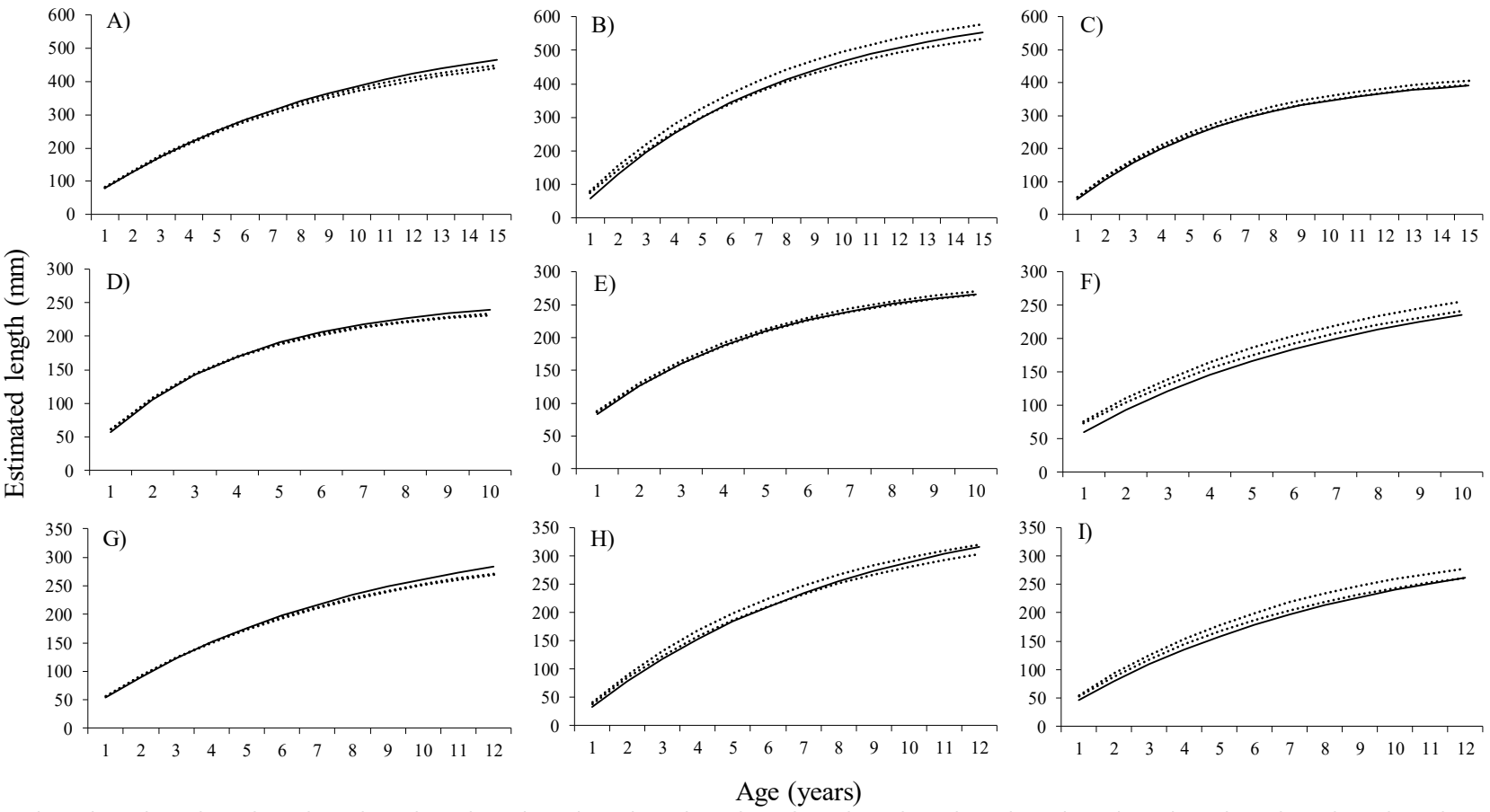

Figure 13. Length-at-age growth curves for selected populations of *S. cephalus* (A: River Lee; B: River Cray; C: River Great Stour), *L. leuciscus* (D: River Colne; E: River Wey; F: River Can) and *R. rutilus* (G: River Thames; H: River Old Bedford; I: River Medway) generated from the von Bertalanffy growth parameters. Solid lines represent the original data (uncertainty omitted) and dotted lines represent the upper and lower 95 % confidence range for the adjusted data (uncertainty incorporated).

4.5. Discussion

The development and application of this simple method based on using the uncertainty in age estimates within bootstrapping procedures was successful in incorporating sources of errors from scale ageing into subsequent growth rate analyses. Age estimates and uncertainty levels from the three cyprinids revealed strong patterns of uncertainty increasing with estimated fish age, with this providing the basis for subsequent analyses. The adjusted VBGF growth parameters and subsequently calculated lengths-at-age showed some deviation from the original and generally suggested an issue of under-ageing older fish, likely due to the annual growth increments of those fishes being minimal, thus creating difficulty in estimating the age from scales where annuli are stacked close to the scale edge.

Scales are frequently used to study and monitor freshwater fish populations given that their non-lethal collection is advantageous over more destructive methods such as fin ray sectioning and otolith removal (*cf*. Section 1.1). However, estimating fish age by counting annuli can result in large biases and uncertainties due to the combination of process and interpretation errors (Beamish and McFarlane, 1995; Campana, 2001; *cf*. Section 1.3). Both error types affect the accuracy and precision of fish population demographics and dynamic rate functions, such as growth and mortality, which are required for population modelling. Although advances have been made to incorporate different types of error when fitting growth models, such as the VBGF, only a few studies have attempted to quantify errors in age estimates (but see Dortel *et al*., 2013; Richards *et al.,* 1992) and many still fail to consider ageing error in their analyses. For example, stochastic environmental fluctuations (Prajneshu and Venugopalan, 1999), individual variation in VBGF growth parameters (Pilling *et al.,* 2002) and transient variation in growth rates (Webber and Thorson, 2016) have been considered, but all assumed that age estimates were accurate, potentially confounding the outputs of the studies. In this Chapter, a simple method was developed to allow incorporation of the uncertainty of a scale reader in the age estimates they assign to provide more realistic evaluations of growth rates and growth parameters.

Uncertainty levels are a useful tool that provide an easy method to include error into age estimates, as uncertainty is significantly and negatively related to precision (e.g.,

Koch *et al*., 2008, 2009, Spiegel *et al*., 2010; Watkins *et al*., 2015a, b). Uncertainty in age estimates is also significantly related to fish age, with Spiegel *et al*. (2010) reporting that precision decreased as age increased. Similarly, here, levels of uncertainty increased with fish age. Furthermore, the uncertainty in age estimates remained low for fishes up to 5 years, with Quist *et al*. (2007) also finding that agreement between ageing structures was high for cyprinids up to age 5. Likewise, Horka *et al*. (2010) found significant deviation between estimated and known ages of European grayling *Thymallus thymallus* for fish of over 5 years. This general pattern of uncertainty increasing and precision decreasing with fish age may be widely applicable. However, the interaction between uncertainty and estimated age is likely to be species-specific, given that the relationship was best described by two different non-linear functions in the three cyprinid species studied.

If data generated from the subjective process of ageing hard structures is to be used in population management, maintenance and conservation, then it can be argued that rather than being disregarded, it should be integral to the subsequent analyses. Issues have been encountered previously when ageing error has been overlooked, such as attempts to relate recruitment success to environmental factors (Myers and Drinkwater, 1989) and the phenomenon of 'ageing drift' (Frear and Cowx, 2003) which is where a single, strong year class identified in a survey early in its life becomes spread over several year classes in later surveys as their ages become more difficult to estimate. Incorporating error through building uncertainty levels into the VBGF model resulted in significant shifts in $L\infty$ and k for all three species, indicating that ageing uncertainty has consequences for growth rate analyses. Indeed, under- or over-estimation of growth parameters can have dramatic implications for fisheries management (*cf*. Section 1.2 regarding populations of orange roughy *Hoplostethus atlanticus* and the cisco *Coregonus artedi*). Pardo *et al*. (2013) demonstrated how this can occur using a simple yield-per-recruit model, where an 8 % underestimate of t_0 resulted in a 20 % increase in k compared to the true value, leading to a 20 % increase in yield-per-recruit biomass when compared with calculations based on true t_0.

In the three cyprinid fishes used here, outputs from the bootstrapping procedure suggested that there was both significant under- and over-estimation of $L\infty$ and k in the original data sets. Given that VBGF growth parameters are strongly correlated with each other (Pilling *et al*., 2002; Britton, 2007; Ruiz-Navarro *et al*., 2016b), any bias in the estimation of k is likely to impact the estimation of $L\infty$. Indeed, for all three species $L\infty$

and *k* were negatively correlated, as has been previously detected (Britton, 2007). When applied to generating length-at-age curves, the adjusted data generally reduced mean lengths-at-age, particularly in the older fish, reiterating that uncertainty and precision are correlated with age. At the population level though, the changes were species-specific, for example, the majority of *L. leuciscus* populations showed the opposite pattern where the adjusted data increased mean lengths-at-age, possible owing to the lower levels of uncertainty present in the older ages when compared to the other species.

There are alternative methods that also incorporate error into age estimates. Cope and Punt (2007) included error in their growth models by treating true age as a random effect. The outcomes from the literature review suggest, however, that there are non-random associations between estimated age and true age, and thus treating true age as a random effect will fail to capture this bias in age reading and that ageing error may be skewed for older individuals (Campana, 2001). Other studies utilise repeat readings of the same structure to produce error estimates or error matrices based on precision (e.g., Richards *et al*., 1992; Candy *et al*., 2012; Dortel *et al*., 2013). This is, however, resource- and time-consuming. Although it is argued that the method developed here represents an improvement on these other processes, one issue of subjectivity remains around translating each age estimate and uncertainty into an age distribution. Though the distributions were based on literature review wherever possible, they also required some additional input and correspondingly the 'best estimates of age' might have some inherent error. Whilst this could be quantified through long-term validation exercises using mark-recapture, the time-frames required are likely to be unrealistic for most studies. An alternative is to disregard all age estimates assigned the highest uncertainty levels or where scales have been used to age the largest fish. This would help reduce ageing error, however, these larger, older fish can be an important component of the fish population, including for catch-and-release angling and assessments of ecological status under the Water Framework Directive (WFD, 2000; *cf.* Section 1.2).

In summary, the method developed here and applied to three cyprinid fishes provides a simple process to capture the uncertainty in the age estimates of scales, and potentially from other structures. By working with the inherent uncertainty, analyses of population growth rate parameters and lengths-at-age can thus be more accurately estimated, ensuring that the subsequent growth analyses can be used within ecological studies and applied to fisheries management with more confidence.

Chapter 5. Stable isotope signatures and trophic-step discrimination factors of fish tissues collected as non-lethal surrogates of dorsal muscle

A version of this chapter was published as:

Busst, G. M, Bašić, T. and Britton, J. R., 2015. Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non-lethal surrogates of dorsal muscle. *Rapid Communications in Mass Spectrometry*, *29*(16), pp.1535-1544.

5.1. Summary

Dorsal white muscle is the standard tissue analysed in fish trophic studies using stable isotope analyses. As muscle is usually collected destructively, scales and fin tissue can be used as non-lethal surrogates; hence, the utility of scales and fin tissue as proxies for muscle tissue was examined. The muscle, fin and scale $\delta^{13}C$ and $\delta^{15}N$ values from 10 species of cyprinid fishes were compared. The fish comprised of samples from the wild and samples from tank aquaria that were held for 120 days and fed a single food resource. Relationships between stable isotope ratios of muscle, fin and scales were examined for each species and for the entire dataset, with the efficacy of four methods of predicting muscle isotope ratios from fin and scales being tested. The discrimination factors between the three tissues of the laboratory fishes and their food resource were then calculated and applied to Bayesian mixing models to assess their effect on fish diet predictions. The isotopic data of the three tissues per species were distinct, but were significantly related, enabling estimations of muscle values from the two surrogates individually. Species-specific equations provided the least erroneous conversions of scale and fin tissue stable isotope ratios to muscle (errors < 0.6 ‰). The discrimination factors for $\delta^{15}N$ were in the range obtained for other species, but were often higher for $\delta^{13}C$. Their application to data from two fish populations in mixing models resulted in significant alterations in diet predictions. In summary, scales and fin tissue can both be strong surrogates of dorsal muscle in food web studies as they can provide estimates of muscle values within an acceptable level of error when species-specific methods are used. Their derived

discrimination factors can also be applied to models predicting the composition of fish diets from $\delta^{13}C$ and $\delta^{15}N$ values.

5.2. Introduction

Stable isotope analysis (SIA) is an important tool in food web ecology, with applications such as investigating trophic structure and detecting variations in trophic niche size (e.g., Finlay *et al*., 2002; Post, 2002; Layman *et al*., 2005; Jackson *et al*., 2012). SIA takes advantage of natural variations in naturally occurring stable isotope ratios of ^{13}C to ^{12}C ($\delta^{13}C$ values) and ^{15}N to ^{14}N ($\delta^{15}N$ values) (*cf.* Section 1.4.1) and has been applied to, for example, impact assessments of biological invasions (Cucherousset *et al*., 2012b) and highlighting responses of populations to bioremediation and long-term changes in water chemistry (Grey *et al*., 2009; Roussel *et al*., 2014).

Stable isotope analysis also has ecological application in providing an alternative fish dietary analysis tool to stomach contents analyses (Cucherousset *et al*., 2012b), although their outputs can be conflicting due to fundamental differences in their methodology (Locke *et al*., 2013). Stomach contents analysis tends to require high temporal and/or spatial sampling, and relatively high numbers of individuals for processing (Hyslop, 1980). By contrast, the application of stable isotope data from focal fish species and their putative food resources to Bayesian mixing models tends to use relatively small sample sizes to predict diet composition (Jackson *et al*., 2011). This approach has a higher resolution and can, for example, indicate important dietary differences between species (Guo *et al*., 2014), locations (Bašić *et al*., 2015), and seasons (Brush *et al*., 2012). These models are based on isotopic discrimination factors and so require accurate estimates of diet-tissue discrimination (Phillips and Gregg, 2001; Bond and Diamond, 2011; Phillips *et al*., 2014). They generally assume that the discrimination factors are constant, irrespective of the biology of the focal species or its feeding behaviour (Mill *et al*., 2007), enabling use of standard values or those derived for other species. Isotopic discrimination can, however, be affected by species, age, diet quality, body size, sample preparation and tissue type (e.g., Jacob *et al*., 2005; Brush *et al*., 2012; Mill *et al*., 2013). Indeed, diet-tissue discrimination factors have been described as a major source of uncertainty in applying mixing models to predict diets (Phillips *et al*., 2014).

Dorsal white muscle is the standard tissue used in fish trophic studies (Pinnegar and Polunin, 1999; Perga and Gerdeaux, 2005), despite it often resulting in destructive sampling, as many species are inappropriate for the use of biopsy plugs that can remove muscle tissue without sacrificing the animal (Tronquart *et al*., 2012). This limits its utility in research programmes on threatened species and in work where non-destructive sampling is necessary, such as in mark-recapture and tracking studies (Sanderson *et al*., 2009; Tronquart *et al*., 2012; Huang *et al*., 2013). Correspondingly, recent work has focused on the use of other tissues for stable isotope analysis whose collection is non-destructive, such as scales and fin tissue, with subsequent conversion of their data into muscle values through regression relationships and correction factors, as significant differences in isotopic signatures have been detected between fish tissues (Jardine *et al*., 2005; Kelly *et al*., 2006; Hanisch *et al*., 2010; Jardine *et al*., 2011; Tronquart *et al*., 2012).

The aim of this Chapter was to develop stable isotope conversion and discrimination factors for different fish tissues, and to assess their influence on dietary analyses. Ten species of the Cyprinidae family of freshwater fishes were used, enabling variability between closely related species and general patterns to be assessed and identified. Presently, no studies are known on discrimination factors of cyprinid fishes, and, whilst Tronquart *et al*. (2012) provide tissue-conversion equations for fin tissue and muscle for some of the species used from populations in France, similar equations were not produced for scales, for which data remain relatively scarce. This is despite scales being collected widely in research programmes of cyprinid fishes and used within ecological and stable isotope studies (e.g., Sterner and George, 2000; Jones and Waldron, 2003; Britton, 2007; Bašić *et al*., 2015; *cf.* Section 1.4). The objectives were thus to: (1) quantify differences in $\delta^{13}C$ and $\delta^{15}N$ values in white dorsal muscle tissues and compare them with values from scales and fin tissue for a range of freshwater fishes; (2) determine the differences in the $\delta^{13}C$ and $\delta^{15}N$ values and discrimination factors of these fishes in relation to a single food resource; and (3) apply these discrimination factors to stable isotope mixing models to assess their influence on dietary predictions.

5.3. Materials and methods

5.3.1. Fish species, tissues and stable isotope analysis

Ten cyprinid fishes were used: European barbel *Barbus barbus*, goldfish *Carassius auratus*, crucian carp *Carassius carassius*, common carp *Cyprinus carpio*, dace *Leuciscus leuciscus*, fathead minnow *Pimephales promelas*, topmouth gudgeon *Pseudorasbora parva*, roach *Rutilus rutilus*, chub *Squalius cephalus* and tench *Tinca tinca*. The tissues used were white dorsal muscle, removed from the anterior region of each fish above the lateral line and below the dorsal fin, proportions of pelvic fins ('fin clip') and scales, removed from the region above the dorsal muscle tissue sample.

For the SIA, fin clips and dorsal muscle were rinsed with distilled water prior to drying and scales were lightly cleaned with distilled water to remove mucus. The outer portion of the scales were removed and used, as this represents the most recent growth and thus stable isotope values are representative of the recent diet of the fish (Grey *et al*., 2009; Bašić *et al*., 2015). All the samples were oven dried at 60 °C to constant mass prior to analysis. Lipids were not extracted from samples, as the C: N ratios were < 3.5 %, indicating low lipid content and thus lipid extraction or normalisation would have little effect on the $\delta^{13}C$ values (Post *et al*., 2007). The tissues were then analysed at the Cornell University Stable Isotope Laboratory, New York, USA, for $\delta^{13}C$ and $\delta^{15}N$ (*cf*. Section 2.3.2.1 for details) and the $\delta^{13}C$ and $\delta^{15}N$ data were provided as ‰.

5.3.2. Relationships between $\delta^{13}C$ and $\delta^{15}N$ values among wild fish tissues

The relationships between the three tissues in their $\delta^{13}C$ and $\delta^{15}N$ data were determined for samples of *L. leuciscus*, *P. parva*, *R. rutilus*, *S. cephalus* and *T. tinca* that were available as frozen samples collected (time frozen: 1 to 6 months) from a range of inland waterbodies in Southern England. These had been collected using back-mounted electric fishing (Smith Root LR-24; Smith-Root, Vancouver, WA, USA). When compared with Tronquart *et al*. (2012) ($n = 466$), the overall sample size ($n = 47$) and the species-specific sample sizes ($n = 5 – 21$) were relatively low, a result of only using samples that were already available from other research programmes. This meant that no fish were lethally sampled for completing this objective specifically. In each case, the fishes were defrosted and samples of dorsal muscle, pelvic fin and scales removed and prepared for SIA (*cf*.

Section 2.3.2.1). The initial test was to determine whether the stable isotope ratios of fin and muscle, and scales and muscle, were significantly different. This was completed through paired Wilcoxon tests, as the data were not normally distributed (Shapiro-Wilk test). To then test whether fish lengths had significant effects on the stable isotope ratios of their tissues, linear regressions were completed.

The next step was to develop predictive linear models for fin and muscle, and scales and muscle, using each species separately and then combined within a general model. The robustness of these linear models was tested in two ways. Firstly, due to the relatively small sample sizes used for some species, a *post hoc* power analysis was completed for each model. These assessed whether the slope b was identical to a fixed value of b (b = 0; i.e. b_0), where the null hypothesis was $b - b_0 = 0$. These tests were completed using G*Power software (version 3.1). Where the *post hoc* power was above 0.8, the model was considered robust and no bootstrapping was required to improve the model fit. Secondly, for each model, the $\delta^{13}C$ and $\delta^{15}N$ values of muscle were predicted from scale and fin tissue for each individual fish and its error expressed as its difference from the observed value, i.e. its residual. This testing generally followed Tronquart *et al*. (2012), although the limited size of the dataset meant that unlike Tronquart *et al*. (2012), a subset of the data could not be used solely for testing the models and so the data that was used to construct the models was also used for its testing. Four methods were compared: (1) muscle isotope ratios were used directly as the fin or scale isotope ratio values; (2) muscle isotope ratios were predicted for each individual species from fin and scale values using species-specific linear models; (3) muscle isotope ratios were predicted for each species from fin and scale values using the linear model derived for data for all species (i.e. the general model); and (4) muscle isotope ratios were predicted for each species from fin values using the general linear models derived for 14 European freshwater fishes by Tronquart *et al*. (2012):

Muscle $\delta^{13}C = 0.82 * \text{fin } \delta^{13}C - 5.89$;

Muscle $\delta^{15}N = 1.01 * \text{fin } \delta^{15}N + 0.74$.

Differences in the residuals of each of the four methods were then tested using generalised linear models (GLMs), as the data were not normally distributed (Shapiro-Wilk test), where the residuals were the dependent variable, methods were the independent variable and species was the covariate. To determine whether the mean residuals of methods 1, 3

and 4 were significantly different to the mean residuals of method 2 (the species-specific method), linearly independent pairwise comparisons with Bonferroni adjustments for multiple comparisons were used.

5.3.3. Relationships between $\delta^{13}C$ and $\delta^{15}N$ values and discrimination factors in laboratory fish

The relationships between the three tissues in their $\delta^{13}C$ and $\delta^{15}N$ data were determined for *B. barbus*, *C. auratus*, *C. carassius*, *C. carpio*, *P. promelas* and *S. cephalus* that were sourced from aquaculture. The fish used were age 0+ and 50 to 60 mm in length. The exception was *P. promelas*, where the fish were 30 to 35 mm in length. Sample sizes for each species were between 8 and 14 individuals, and each species was held separately in tanks of 45 l at 20 °C on recirculating systems. The fish were fed daily *ad libitum* on a fixed diet of crushed pelletized fishmeal (45 % protein, 10 % fat, 1.4 % crude fibre, 5.8 % ash and 1.1 % total phosphorus). A single, homogenous batch was used throughout the holding period and no other sources of food were available to the fish. The fish were held for 120 days, over which time the length increase of each fish was at least 20 mm; thus, considerable somatic growth had occurred relative to their starting length. This increase in body size meant that sufficient isotopic turnover in the tissues should have occurred to enable the $\delta^{13}C$ and $\delta^{15}N$ values to reflect their fixed diet (Perga and Gerdeaux, 2005).

Following the SIA the initial test determined whether the isotope ratios of the different tissues were significantly different (paired Wilcoxon tests). Then the conversion factors, as the mean differences in $\delta^{13}C$ and $\delta^{15}N$ values between muscle and fin, and muscle and scales, were determined for each species (i.e. the species-specific method: method 1) and then all species (i.e. the general method: method 2), and used to predict muscle values for each fish from their fin and scale values, with the residuals then expressed as the difference from the observed muscle value. Differences in the residuals of the two methods were tested using GLMs, as the data were not normally distributed (Shapiro-Wilk test); residuals were the dependent variable, methods were the independent variable and species was the covariate, with the differences in the residuals determined using pairwise comparisons with Bonferroni adjustments for multiple comparisons.

Following this, the $\delta^{13}C$ and $\delta^{15}N$ data for each species were tested for the extent of their discrimination (Δ) between each tissue and the food resource using GLMs, again as

the data were not normally distributed (Shapiro-Wilk test); the dependent variable was the $\delta^{13}C$ or $\delta^{15}N$ value and the independent variables were the food resource and the tissue type (muscle, fin or scales). Mean differences between the food resource and each tissue per species were tested using estimated marginal means and pairwise comparisons with Bonferroni adjustments for multiple comparisons, with the latter providing the species-specific discrimination factors through the mean difference between the food resource and each tissue type.

To determine the effect of the discrimination factors on predictions of fish diet using Bayesian mixing models, data were used from a population of two cyprinid fishes; *S. cephalus* and *B. barbus* from a small tributary of the River Great Ouse in Cambridgeshire (52°19'39" N; 0°06'57" W), of maximum width 8 m and depth 1.5 m. The occurrence of angling at the site was minimal and thus the fish were likely to have a relatively natural diet rather than being reliant on angler bait (Perga and Gerdeaux, 2005). Sampling was completed in August 2014 by boat-mounted, generator-powered (2.5 kVA) electric fishing (Electracatch International, Killiney, Ireland). A maximum of 5 scales were removed from each fish from the anterior region above the lateral line and below the dorsal fin after recording their fork length (nearest mm). At the conclusion of fish sampling, putative food resources were also collected; these included small fishes, such as European bullhead *Cottus gobio*, and macro-invertebrates, such as *Gammarus pulex.* For the small fishes, a minimum of three individuals were collected. For macro-invertebrates, the same number of samples was collected, but with each sample comprising 3 to 6 individuals. In the laboratory, the scales and putative food resource samples were prepared and analysed individually for their $\delta^{15}N$ and $\delta^{13}C$ values (*cf.* Section 2.3.2.1). These stable isotope data then were applied to Bayesian mixing models to determine the relative contribution of each putative food resource to the diet of the each individual *B. barbus* and *S. cephalus*. The models were run using the SIAR package in the R computing program (Parnell *et al*., 2010; R Development Core Team, 2013). Putative food resources with similar isotope ratio values were combined *a priori*, while respecting the taxon and functional affiliation of the individual species (Phillips *et al*., 2005), Gammaridae and water louse *Asellus aquaticus* were combined and applied to the model as Arthropods and unidentified species of snail and mussels as Molluscs. The initial model (model 1) was run using 'standard' discrimination factors commonly cited in the literature, i.e., 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.3 ‰ for $\delta^{13}C$ (DeNiro and

Epstein, 1981; Minagawa and Wada, 1984; Post, 2002; Perga and Gerdeaux, 2005), and was then re-run using the species-specific discrimination factors derived here for scales (model 2). The model outputs were the predicted proportions of the contribution of each putative food resource to the diet of each fish (ranging from 0 to 1). Differences in model outputs were tested by comparing these individual proportions between the two models using independent samples *t*-tests, with separate analyses performed for each species and between each category of putative food resource. All statistical analyses were completed using IBM SPSS Statistics (version 22.0).

5.4. Results

5.4.1. Relationships between $\delta^{13}C$ and $\delta^{15}N$ values in wild fish

The differences in the $\delta^{13}C$ and $\delta^{15}N$ values between fin and muscle and scales and muscle of the five species were significant ($P < 0.01$ in all cases). The effects of fish lengths on the $\delta^{13}C$ and $\delta^{15}N$ values were non-significant in all cases ($P > 0.05$), other than for the $\delta^{13}C$ values for *P. parva* ($R^2 = 0.38$; $F_{1,19} = 5.85$, $P < 0.05$). The linear relationships between $\delta^{13}C$ and $\delta^{15}N$ among the tissues were significant in all cases, including the relationships for the combined data for all the species of wild fishes ($P < 0.05$; Table 18). The GLMs testing differences in the residuals of each conversion method were significant for both $\delta^{13}C$ and $\delta^{15}N$ values (Table 19), with the species-specific linear equations generally resulting in the lowest mean residual values (usually < 0.60 ‰ for $\delta^{13}C$ and < 0.30 ‰ for $\delta^{15}N$; Fig. 14), whereas the general linear equation for the dataset produced estimates of ≤ 0.80 ‰ for $\delta^{13}C$ and ≤ 0.50 ‰ for $\delta^{15}N$ of muscle (Fig. 14). The mean residuals produced by conversions using the general equations of Tronquart *et al*. (2012) were both higher than the species-specific and general linear equations produced here (Table 19).

Table 18. Number and mean fork length of wild fishes and outputs and *post hoc* power statistic of linear regressions between the stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values of pelvic fin and white dorsal muscle, and scales and white dorsal muscle tissue.

Species	Mean length (range) (mm)	Tissue	Stable isotope	R^2	F	P	Regression coefficients		*Post hoc* power
							a	*b*	
L. leuciscus	180	Fin	$\delta^{13}C$	0.99	710.69	<0.001	0.00	0.59	1.00
n = 5	(105 - 225)		$\delta^{15}N$	0.94	49.20	<0.010	-0.09	1.02	1.00
		Scales	$\delta^{13}C$	0.99	400.70	<0.001	13.58	1.59	1.00
			$\delta^{15}N$	0.89	23.14	<0.050	-1.91	1.25	1.00
P. parva	55	Fin	$\delta^{13}C$	0.88	135.09	<0.001	-1.86	0.96	1.00
n = 21	(38 - 77)		$\delta^{15}N$	0.84	96.44	<0.001	4.21	0.62	1.00
		Scales	$\delta^{13}C$	0.88	139.08	<0.001	-6.51	0.73	1.00
			$\delta^{15}N$	0.90	175.02	<0.001	2.52	0.78	1.00
R. rutilus	79	Fin	$\delta^{13}C$	0.94	92.66	<0.001	-9.88	0.73	1.00
n = 8	(65 - 99)		$\delta^{15}N$	0.66	11.66	<0.050	-1.93	1.20	0.90
		Scales	$\delta^{13}C$	0.63	10.09	<0.050	-15.48	0.51	0.90
			$\delta^{15}N$	0.88	44.34	<0.010	-0.20	1.10	1.00
S. cephalus	222	Fin	$\delta^{13}C$	0.76	9.54	0.05	-10.96	0.64	0.80
n = 5	(204 - 245)		$\delta^{15}N$	0.93	38.60	<0.010	-1.18	1.13	1.00
		Scales	$\delta^{13}C$	0.83	14.41	<0.050	-1.00	1.05	0.90
			$\delta^{15}N$	0.88	21.02	<0.050	-2.77	1.33	1.00
T. tinca	126	Fin	$\delta^{13}C$	0.86	36.44	<0.050	3.42	1.19	1.00
n = 8	(85 - 150)		$\delta^{15}N$	0.82	27.26	<0.050	0.69	0.93	1.00
		Scales	$\delta^{13}C$	0.82	32.39	<0.050	0.87	1.06	1.00
			$\delta^{15}N$	0.85	33.39	<0.050	1.22	0.89	1.00
All species	101	Fin	$\delta^{13}C$	0.94	687.78	<0.001	0.24	1.05	1.00
n = 47	(38 - 245)		$\delta^{15}N$	0.95	915.42	<0.001	1.69	0.88	1.00
		Scales	$\delta^{13}C$	0.87	294.80	<0.001	-0.37	1.02	1.00
			$\delta^{15}N$	0.91	434.77	<0.001	2.76	0.81	1.00

Table 19. Outputs of generalised linear models testing differences in error residuals between muscle conversion methods for stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values for five cyprinid species sampled from wild populations; *difference in error between method and method 2 (the species-specific method) is significant at $P < 0.05$ (*cf.* Section 5.3.2 for details of methods). Overall model significant at $P < 0.01$ and the effect of species as a covariate was significant ($P < 0.05$). Errors around the means represent standard errors.

		Method			
$n = 47$	Wald x^2	1	2	3	4
$\delta^{13}C$ Fin: muscle	15.401	1.19 ± 0.10*	0.65 ± 0.10	0.81 ± 0.10	1.02 ± 0.10
$\delta^{13}C$ Scale: muscle	16.561	1.31 ± 0.11*	0.70 ± 0.11	1.14 ± 0.11*	
$\delta^{15}N$ Fin: muscle	27.231	0.49 ± 0.04*	0.27 ± 0.04	0.30 ± 0.04	0.53 ± 0.04*
$\delta^{15}N$ Scale: muscle	54.371	0.90 ± 0.06*	0.33 ± 0.06	0.46 ± 0.06	

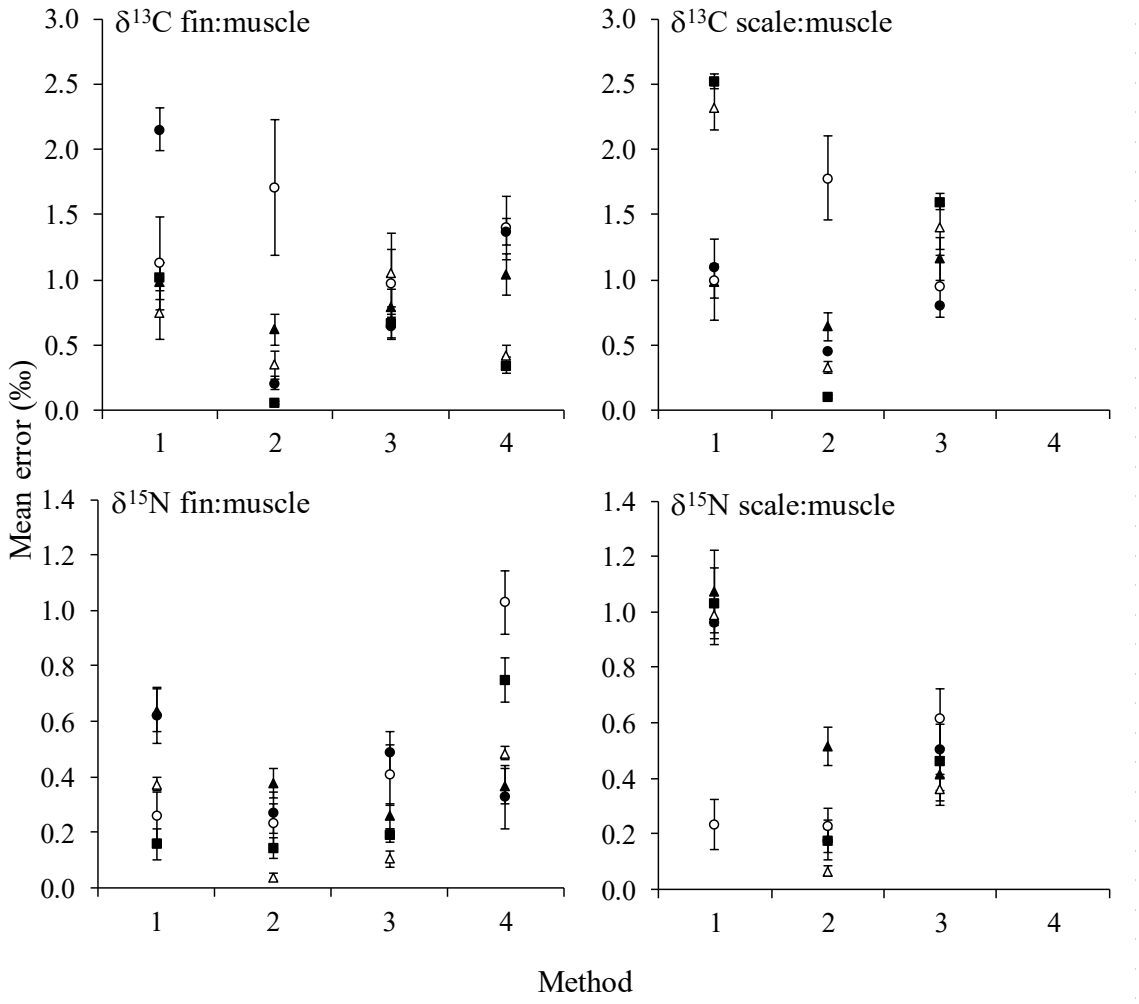

Figure 14. Mean error residuals per method for five cyprinid species sampled from wild populations used in determining stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values for dorsal muscle from pelvic fin tissue and scales (*cf.* Section 5.3.2 for details of methods); ▲ *P. parva*; ○ *T. tinca*; ● *R. rutilus*; Δ *S. cephalus*; ■ *L. leuciscus*. Error bars represent standard errors.

5.4.2. Relationships between $\delta^{13}C$ and $\delta^{15}N$ values in laboratory fish

There was a general pattern of ^{13}C enrichment and ^{15}N depletion from white dorsal muscle to pelvic fin tissue to scales in all species (Table 20). The differences between the stable isotope ratios of muscle and scales and muscle and fin for each species were significant for ^{13}C ($P < 0.01$) and other than for muscle and scales for *C. carpio* and muscle and fin for *P. promelas*, were significant for ^{15}N ($P < 0.05$). Using the mean differences in stable isotope ratio values between fin and muscle and scales and muscle as tissue conversion factors, for each species and the whole dataset, to estimate stable isotope values for muscle, revealed that errors were always significantly lower when using species-specific data ($P < 0.05$; Table 21) and were ≤ 0.25 ‰ for $\delta^{13}C$ and ≤ 0.30 ‰ for $\delta^{15}N$ (Fig. 15).

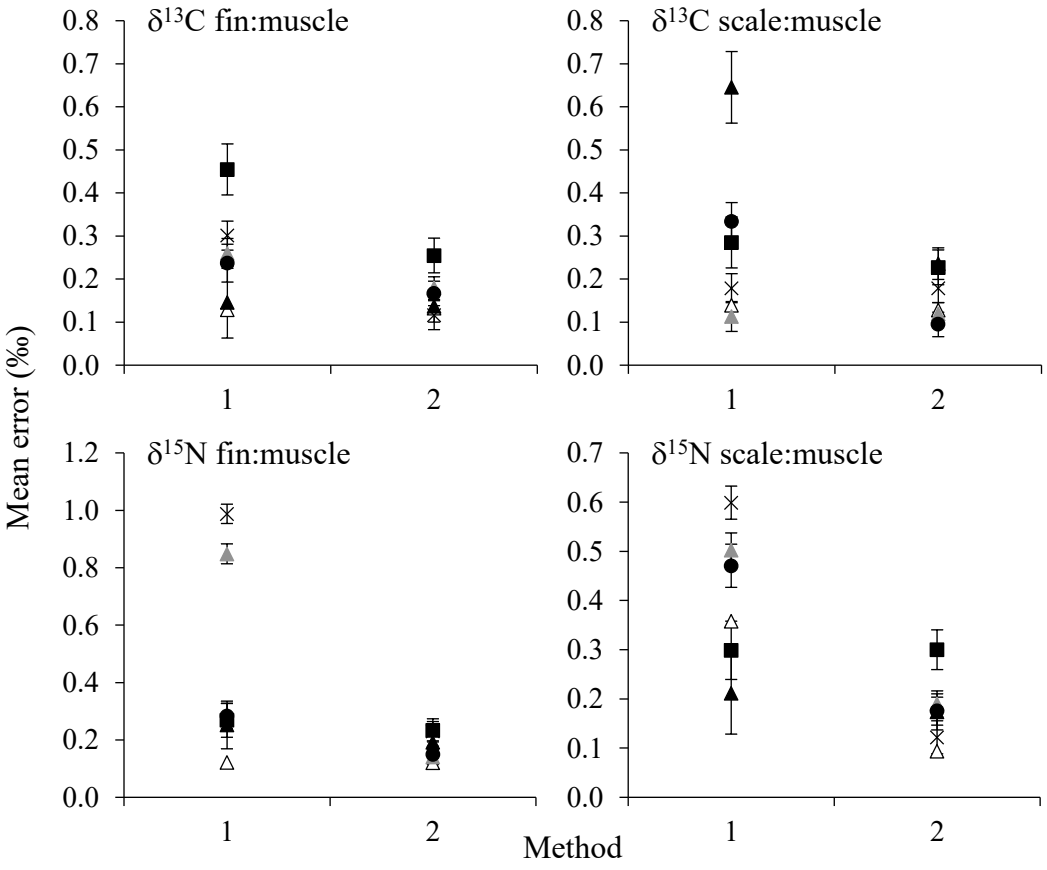

Figure 15. Mean error per method for six cyprinid species used in determining stable isotope ($\delta^{15}N$ and $\delta^{13}C$) values for dorsal muscle from pelvic fin tissue and scales, derived from a laboratory trial where the fish were fed a fixed diet for 120 days; method 1 is the general method and method 2 is the species-specific method; ▲ *B. barbus*; Δ *S. cephalus*; ● *C. auratus*; ■ *P. promelas*; × *C. carassius*; ▲ *C. carpio*. Error bars represent standard errors.

Table 20. Number, mean fork length and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values of pelletized fishmeal food source and white dorsal muscle, pelvic fin and scale tissues for laboratory fishes fed a fixed diet for 120 days. Errors around the means represent standard errors.

Species	Mean length (range) (mm)	Tissue	Mean $\delta^{13}C$ (‰)	95 % confidence interval	Mean $\delta^{15}N$ (‰)	95 % confidence interval
Fishmeal pellet *n* = 5			-22.15 ± 0.06	-22.32 - -21.99	8.51 ± 0.07	8.33 - 8.70
B. barbus	118	Muscle	-19.59 ± 0.04	-19.66 - -19.51	10.92 ± 0.04	10.84 - 10.99
n = 9	(105 - 127)	Fin	-18.31 ± 0.04	-18.38 - -18.24	10.60 ± 0.04	10.52 - 10.68
		Scales	-17.41 ± 0.04	-17.49 - -17.34	10.70 ± 0.04	10.63 - 10.78
C. auratus	62	Muscle	-20.58 ± 0.07	-20.71 - -20.45	12.28 ± 0.06	12.17 - 12.40
n = 9	(55 - 73)	Fin	-19.04 ± 0.07	-19.18 - -18.91	11.73 ± 0.06	11.62 - 11.85
		Scales	-18.65 ± 0.07	-18.78 - -18.52	11.24 ± 0.06	11.13 - 11.35
C. carassius	58	Muscle	-19.86 ± 0.05	-19.96 - -19.77	12.40 ± 0.09	12.22 - 12.58
n = 11	(50 - 66)	Fin	-18.25 ± 0.05	-18.35 - -18.16	11.13 ± 0.09	10.94 - 11.31
		Scales	-17.61 ± 0.05	-17.70 - -17.51	11.23 ± 0.09	11.05 - 11.41
C. carpio	78	Muscle	-20.46 ± 0.05	-20.56 - -20.36	11.55 ± 0.07	11.41 - 11.68
n = 8	(72 - 85)	Fin	-18.91 ± 0.05	-19.01 - -18.81	12.11 ± 0.07	11.97 - 12.25
		Scales	-18.25 ± 0.05	-18.35 - -18.15	11.48 ± 0.07	11.34 - 11.61
P. promelas	48	Muscle	-19.30 ± 0.06	-19.42 - -19.18	11.88 ± 0.07	11.74 - 12.03
n = 14	(40 - 53)	Fin	-18.41 ± 0.06	-18.53 - -18.29	11.70 ± 0.07	11.56 - 11.85
		Scales	-17.27 ± 0.06	-17.38 - -17.15	11.30 ± 0.07	11.15 - 11.44
S. cephalus	128	Muscle	-20.16 ± 0.05	-20.26 - -20.06	10.86 ± 0.05	10.78 - 10.95
n = 11	(94 - 183)	Fin	-18.91 ± 0.05	-19.01 - -18.81	10.79 ± 0.05	10.71 - 10.88
		Scales	-17.25 ± 0.05	-17.35 - -17.15	10.48 ± 0.05	10.39 - 10.56

Table 21. Tissue conversion factors between pelvic fin and dorsal muscle, and scales and dorsal muscle, for six cyprinid species held in laboratory conditions and fed a fixed diet for 120 days; 'General' represents combined data for the species; muscle values are derived by subtracting the values in the table from the fin and scale values (A) and outputs of generalised linear models testing differences in error residuals between the two muscle conversion methods for $\delta^{13}C$ and $\delta^{15}N$; *difference in error between method 1 (general method) and method 2 (species-specific method) is significant at $P < 0.05$ (B); overall model significant at $P < 0.01$ and the effect of species as a covariate was significant ($P < 0.05$). Errors around the means represent standard errors.

A)	General	*B. barbus*	*S. cephalus*	*C. carassius*	*C. carpio*	*C. auratus*	*P. promelas*
$\delta^{13}C$ fin: muscle	-1.31	-1.28	-1.25	-1.61	-1.56	-1.53	-0.88
$\delta^{13}C$ scale: muscle	-2.26	-2.17	-2.91	-2.25	-2.21	-1.93	-2.03
$\delta^{15}N$ fin: muscle	0.29	0.31	0.07	1.27	-0.56	0.55	0.18
$\delta^{15}N$ scale: muscle	0.57	0.21	0.39	1.17	0.07	1.04	0.59

B)		Method	
$n = 62$	Wald x^2	1	2
$\delta^{13}C$ fin: muscle	9.66	0.26 ± 0.02*	0.17 ± 0.02
$\delta^{13}C$ scale: muscle	12.72	0.30 ± 0.03*	0.17 ± 0.03
$\delta^{15}N$ fin: muscle	22.02	0.42 ± 0.04*	0.18 ± 0.04
$\delta^{15}N$ scale: muscle	28.59	0.39 ± 0.03*	0.19 ± 0.03

5.4.3. Diet-tissue discrimination factors of laboratory fish

The discrimination factors between the food resource and each tissue corresponded to the patterns of ^{13}C enrichment and ^{15}N depletion between the tissue types (Table 20), with muscle generally having lower discrimination factors than the other tissues for $\delta^{13}C$, and the converse for $\delta^{15}N$ (Table 22). For dorsal muscle, the $\Delta^{13}C$ for the six fishes ranged between 1.58 ± 0.11 ‰ for *C. auratus* and 2.86 ± 0.12 ‰ for *P. promelas* and, for $\Delta^{15}N$, ranged between 2.35 ± 0.08 ‰ for *S. cephalus* and 3.89 ± 0.15 ‰ for *C. carassius* (Table 22).

Application of the derived and standard discrimination factors to the mixing models resulted in differences in the predicted proportions of the putative food resources to *B. barbus* and *S. cephalus* diets (Fig. 16). Differences in outputs per resource were significant in all cases for *B. barbus*, with the standard discrimination factors resulting in significantly higher contributions of Fish and Molluscs than with the derived discrimination factors (Fish: $t_{(12)} = 3.26$, $P < 0.01$; Molluscs: $t_{(12)} = 4.45$, $P < 0.01$), and the opposite for Arthropods ($t_{(12)} = –5.70$, $P < 0.01$) (Fig. 16A). For *S. cephalus*, the use of standard discrimination factors also resulted in significantly higher proportions of Molluscs ($t_{(10)} = 3.56$, $P < 0.01$) and lower proportions of Arthropods ($t_{(10)} = –0.50$, $P < 0.01$) than with the derived values, although differences in estimates of the contributions of Fish were not significantly different between the models ($t_{(10)} = –0.22$, $P > 0.05$) (Fig. 16B).

Table 22. Mean isotopic discrimination (Δ) and 95 % confidence intervals (CI) between the pelletized fishmeal food source and white dorsal muscle (A), pelvic fin (B) and scale (C) tissues of the six laboratory cyprinid fishes. Errors around the means represent standard errors.

A)

Species	Mean $\Delta^{13}C$ (‰)	95 % CI	Mean $\Delta^{15}N$ (‰)	95 % CI
B. barbus	2.57 ± 0.06	2.73 - 2.40	2.40 ± 0.07	2.58 - 2.23
C. auratus	1.58 ± 0.11	1.87 - 1.28	3.77 ± 0.10	4.02 - 3.52
C. carassius	2.29 ± 0.08	2.50 - 2.08	3.89 ± 0.15	4.28 - 3.49
C. carpio	1.69 ± 0.08	1.91 - 1.47	3.03 ± 0.11	3.33 - 2.74
P. promelas	2.86 ± 0.12	3.16 - 2.55	3.37 ± 0.14	3.74 - 3.00
S. cephalus	1.99 ± 0.09	2.23 - 1.76	2.35 ± 0.08	2.56 - 2.14

Table 22 *continued*

B)

Species	Mean $\Delta^{13}C$ (‰)	95 % CI	Mean $\Delta^{15}N$ (‰)	95 % CI
B. barbus	3.84 ± 0.06	4.01 - 3.68	2.09 ± 0.07	2.26 - 1.91
C. auratus	3.11 ± 0.11	3.40 - 2.81	3.22 ± 0.10	3.47 - 2.97
C. carassius	3.90 ± 0.08	4.11 - 3.69	2.61 ± 0.15	3.01 - 2.22
C. carpio	3.24 ± 0.08	3.46 - 3.02	3.59 ± 0.11	3.89 - 3.30
P. promelas	3.74 ± 0.12	4.04 - 3.44	3.19 ± 0.14	3.55 - 2.82
S. cephalus	3.24 ± 0.09	3.48 - 3.01	2.28 ± 0.08	2.49 - 2.07

C)

Species	Mean $\Delta^{13}C$ (‰)	95 % CI	Mean $\Delta^{15}N$ (‰)	95 % CI
B. barbus	4.74 ± 0.06	4.90 - 4.57	2.19 ± 0.07	2.36 - 2.02
C. auratus	3.50 ± 0.11	3.80 - 3.21	2.73 ± 0.10	2.98 - 2.47
C. carassius	4.54 ± 0.08	4.75 - 4.34	2.72 ± 0.15	3.11 - 2.32
C. carpio	3.90 ± 0.08	4.12 - 3.68	2.96 ± 0.11	3.26 - 2.67
P. promelas	4.89 ± 0.12	5.19 - 4.58	2.78 ± 0.14	3.15 - 2.42
S. cephalus	4.90 ± 0.09	5.14 - 4.67	1.96 ± 0.08	2.17 - 1.75

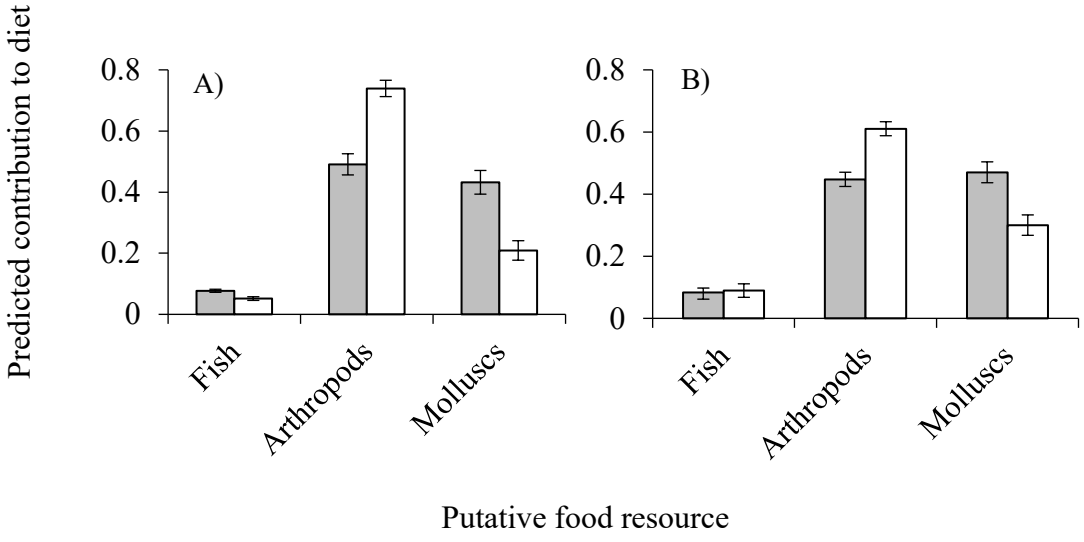

Figure 16. Predicted contributions to fish diet of putuative food resources for *B. barbus* (A) and *S. cephalus* (B) derived from two mixing models where model 1 (grey bars) utilised standard discrimination values (Post, 2002) and model 2 (white bars) used species-specifc values derived here for scales (Table 22). Error bars represent standard errors.

5.5. Discussion

5.5.1. Variations between isotopic signatures of tissues

Across the 10 cyprinid species, there was substantial variation in $\delta^{13}C$ and $\delta^{15}N$ values between their tissues, with a consistent pattern of $\delta^{13}C$ being most depleted in muscle and most enriched in scales. These results correspond with Sanderson *et al*. (2009) who reported that caudal fin tissues were enriched in $\delta^{13}C$ relative to muscle for Chinook salmon *Oncorhynchus tshawytscha* and rainbow trout *Oncorhynchus mykiss*. The opposite was apparent for $\delta^{15}N$, with muscle most enriched and scales most depleted. This is consistent with other fishes, with the fin tissue of Atlantic salmon *Salmo salar* smolts being depleted in $\delta^{15}N$ relative to muscle (Jardine *et al*., 2005). In addition, Pinnegar and Polunin (1999) found that muscle was significantly more enriched with $\delta^{15}N$ than other tissues in *O. mykiss*, which they attributed to factors such as the structural-protein amino acids in the muscle, rather than to lipid concentrations.

5.5.2. Utility of scales and fin clips as proxies for dorsal muscle tissue

The relationships between the $\delta^{13}C$ and $\delta^{15}N$ values in scales, pelvic fin tissue and white dorsal muscle were significant, with these relationships being consistent with those detected in other fish families. In salmonid species, significant relationships in stable isotope data between caudal fin and muscle tissue has revealed in *S. salar*, *O. tshawytscha*, *O. mykiss* and brook trout *Salvelinus fontinalis*, and between adipose fin and muscle tissue in *S. salar* and brown trout *Salmo trutta* (Jardine *et al*., 2005; Sanderson *et al*., 2009; Graham *et al*., 2013). In European freshwater fishes, Tronquart *et al*. (2012) quantified the relationships between muscle and fin stable isotope ratios, but not scales, for species including *R. rutilus*, *L. leuciscus* and *S. cephalus*, and revealed high correlations between fin and muscle stable isotope ratios.

As the variation between the tissues was generally predictable according to linear methods, this enabled conversion of fin and scale data to predict muscle values. Comparison of errors derived from the dataset indicated that the smallest errors generally resulted from application of species-specific equations and conversion factors, with the combined general equation from the dataset also being relatively accurate compared with using fin and scale isotopic data as muscle values directly without conversion.

Application of the general equations derived by Tronquart *et al*. (2012) for European freshwater fishes provided more accurate values than no conversion, but higher errors than the species-specific and general equations derived here. Consequently, whilst the outputs align with other studies suggesting that fin and scales can be used successfully within stable isotope studies as non-lethal surrogates of dorsal muscle (e.g., Jardine *et al*., 2005, 2011; Tronquart *et al*., 2012), there remains some conjecture as to how widely general conversion equations can be applied without incurring relatively high error values.

5.5.3. Discrimination factors for fish tissues

Across the species, the $\Delta^{15}N$ factors ranged from 2.35 to 3.89 ‰, with a mean of 3.13 ‰. Sweeting *et al*. (2007a) suggested that for European sea bass *Dicentrarchus labrax*, and other fishes generally, a similar value of 3.15 ‰ for $\Delta^{15}N$ was appropriate for fish muscle. By contrast, the results for $\delta^{13}C$ discrimination in muscle tissue were generally higher than those reported for other fishes (Post, 2002) with values ranging between 1.58 and 2.86 ‰, with a mean of 2.16 ‰. Discrimination factors for ^{13}C are generally more debated than those for ^{15}N, with ranges for $\delta^{13}C$ commonly cited between 0 ‰ (Peterson and Fry, 1987) and 1 ‰ (DeNiro and Epstein, 1978). In a review by Post (2002), a mean of 0.39 ± 1.3 ‰ was suggested for $\Delta^{13}C$ and this has been applied to mixing models in a recent study (Bašić *et al*., 2015). Conversely, Sweeting *et al*. (2007b) suggested that a value of 1.5 ‰ for $\Delta^{13}C$ was more appropriate for fishes, and that samples that had not undergone any treatment for lipid were likely to have even higher discrimination factors for ^{13}C, such as 2.27 ‰, which is much closer to the mean derived here (2.16 ‰). These arguments are important given the implications of applying $\Delta^{13}C$ values to interpretations of food web structure and the potential sensitivity of dietary mixing models to values of $\Delta^{13}C$ and $\Delta^{15}N$ (Gaye-Siessegger *et al*., 2004).

5.5.4. Mixing model performance according to discrimination factors

Previous studies have emphasised the importance of applying species-specific discrimination factors in isotopic mixing models in order to provide robust estimates of contributions to diet of putative food resources (Phillips and Gregg, 2001; Bond and Diamond, 2011; Phillips *et al*., 2014). Here, significant differences in model outputs were detected between the use of standard discrimination factors (Post, 2002) and the species-

specific factors derived within the Chapter. Not only were differences detected for each resource, the overall pattern of the relative importance of the putative food resources to fish diet also altered, with contributions to the diet of two macro-invertebrate groups having quite different outcomes between the models. Nevertheless, all outputs did indicate relatively low dietary contributions of fish compared with macro-invertebrates.

It can thus be argued that the specific discrimination factors calculated here are more appropriate for application in subsequent trophic studies on these and similar species than standard published values. However, some caution should be applied, as the discrimination factors were derived experimentally using a single food resource in the form of a pelletized formulated feed and general issues surrounding experimentally derived discrimination factors from formulated feeds have been strongly debated. For example, Caut *et al*. (2008) reported that discrimination factors for rats *Rattus rattus* altered markedly according to the isotopic ratio of their diet and they provided discrimination factors quite different from those from other studies. However, Perga and Grey (2010) suggested that these outputs might be less related to issues of discrimination and diet composition, and more to factors of experimental design and the effects of isotopic routing resulting from their formulated feeds. The feeds within Caut *et al*. (2008) contained a variety of dietary protein sources, with the possible preferential routing of essential amino acids to proteinaceous tissues, such as muscle, in the different protein sources potentially causing the differences observed between different diets (Perga and Grey, 2010). Thus, the discrimination factors recorded here might relate more to aspects of the composition of the pelletized feed, particularly its relatively high protein content (45 %), than to actual differences between the fishes and their more usual food resources of lower protein content. Notwithstanding, the extent of isotopic routing may vary considerably in prevalence and magnitude between ectotherms and endotherms (Kelly and Martínez del Rio, 2010) and so the explanations for the relationships provided by Perga and Grey (2010) considering the work on *R. rattus* may be less applicable to fish. Irrespective of this, there remains sufficient uncertainty in how representative these discrimination factors are in relation to cyprinid fish diets more generally to suggest that further work is necessary to substantiate and/or refine these discrimination factors, such as through using different feed formulae, particularly those with lower protein content and this issue is tackled within the proceeding chapter.

5.5.5. Summary and conclusions

This Chapter provides data that can be applied to studies on cyprinid fishes that incorporate stable isotope analyses, including evaluation of trophic positions and diet composition, and where non-lethal sampling is required or desired. In particular, data have also been provided on the use of scales for trophic studies that are otherwise unavailable (Tronquart *et al*., 2012), thereby increasing the utility of scales within stable isotope analysis. The application of the derived discrimination factors to Bayesian mixing models for *S. cephalus* and *B. barbus* emphasises the importance of these to obtaining robust dietary predictions and although it is not necessarily a recommendation that they should be applied unequivocally in future studies, as a minimum, they suggest sufficient uncertainty in the stable isotope discrimination factors of cyprinid fishes to warrant further investigation as the use of general values commonly cited and applied may be inappropriate.

Chapter 6. High variability in stable isotope diet-tissue discrimination factors of two omnivorous freshwater fishes in controlled *ex situ* conditions

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6.1. Summary

Diet-tissue discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) are influenced by variables including the tissues being analysed and the taxon of the consumer and its resources. Whilst differences in $\Delta^{13}C$ and $\Delta^{15}N$ are apparent between herbivorous and piscivorous fishes, there is less known for omnivorous fishes that consume both plant and animal material. Here, the omnivorous cyprinid fishes European barbel *Barbus barbus* and chub *Squalius cephalus* were held in tank aquaria and exposed to three diets that varied in their constituents (plant-based to fishmeal-based) and protein content (14 to 45 %). After 100 days and isotopic replacement in fish tissues to 98 %, samples of the food items, and dorsal muscle, fin tissue and scales were analysed for $\delta^{13}C$ and $\delta^{15}N$. For both species and all diets, muscle was always enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$ compared with fin tissue and scales. Across the different diets, $\Delta^{13}C$ ranged between 2.0 and 5.6 ‰ and $\Delta^{15}N$ ranged between 2.0 and 6.9 ‰. The diet based on plant material (20 % protein), always resulted in the highest discrimination factors for each tissue, whilst the diet based on fishmeal (45 % protein) consistently resulted in the lowest. The discrimination factors produced by non-fish diets were comparatively high compared with values in the literature, but were consistent with general patterns for some herbivorous fishes. These outputs suggest that the diet-tissue discrimination factors of omnivorous fishes will vary considerably between animal and plant resources and these specific differences need consideration in subsequent analyses, such as application to Bayesian mixing models for accurate predictions of their diet composition, and for calculation of trophic position.

6.2. Introduction

The application of stable isotope analysis (SIA) to ecological studies provides considerable insight into many aspects of species' interactions (Boecklen *et al*., 2011). Natural variations in stable isotope ratios of ^{13}C to ^{12}C ($\delta^{13}C$) and ^{15}N to ^{14}N ($\delta^{15}N$) have been applied widely to trophic and food web studies in aquatic environments (*cf.* Section 1.4.1) and have, for example, revealed the impacts of non-native fishes in native fish communities (Tran *et al.*, 2015), and the movements and ecology of endangered species (Seminoff *et al.*, 2012; Hamidan *et al.*, 2015). Critical to the interpretation of stable isotope ratios is the step-wise enrichment that occurs along trophic levels between consumer species and their prey resources (Boecklen *et al*., 2011; Section 1.4.1; Fig. 2), otherwise known as the isotopic discrimination (Martínez del Rio and Wolf, 2005).

An increasingly important use of stable isotope discrimination factors is within Bayesian mixing models that predict the proportional composition of consumer diets from data on their putative food resources (Jackson *et al.*, 2011). These models have been applied widely in recent years, including application to questions relating to the use of allochthonous and autochthonous food resources in freshwater consumers (Grey and Jackson, 2012) and the relative contributions of native and non-native taxa to consumer diet (Britton *et al.*, 2010d). A fundamental requirement of these models is robust estimates of the stable isotope discrimination factors between the resources and consumer tissue being analysed (Bond and Diamond, 2011; Phillips *et al.*, 2014). In general, they assume discrimination factors are constant across the size range of the consumer being studied and their dietary spectrum, overlooking potential differences that might occur through, for example, ontogenetic diet changes (Mill *et al.*, 2007; Phillips *et al.*, 2014).

This general application of constant discrimination factors across consumers within mixing models is potentially problematic, as studies increasingly suggest they can vary between species, ages, diet compositions, body sizes, sample preparations and tissue types (Locke *et al.*, 2013; Brush *et al.*, 2012; *cf.* Chapter 5). The commonly cited 'standard' values of 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.3 ‰ for $\delta^{13}C$ (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002) could thus be inappropriate for use in many models, resulting in dietary predictions whose variability within and between species could be due more to inappropriate discrimination factors than actual dietary variation (*cf.* Chapter 5). The specific food items that contribute to the diet of a consumer can

substantially influence the resulting stable isotope discrimination factors between the consumer and their overall diet (Caut *et al.*, 2008). For example, McCutchan *et al.* (2003) suggested that discrimination factors of $\delta^{15}N$ were lower in consumers with invertebrate-based diets (1.4 ± 0.21 ‰) than consumers with diets containing higher protein contents (3.3 ± 0.26 ‰) and mixed diets provided values between these (2.2 ± 0.30 ‰). In herbivorous fishes, discrimination factors for $\delta^{15}N$ have been recorded as high as 5.25 ‰ (Mill *et al.*, 2007), with Carassou *et al.* (2008) suggesting that herbivorous fishes have distinct stable isotope discrimination factors that distinguish them from piscivores.

Correspondingly, understanding the relationships between the long-term composition of the diet of consumer and their diet-tissue discrimination factors is a pre-requisite for obtaining robust dietary predictions from models (Parnell *et al.*, 2013; Phillips *et al.*, 2014). Whilst there are often clear discrimination differences apparent between herbivorous, insectivorous and piscivorous fishes, this potentially becomes more complex for omnivores, with their diets potentially comprising of a wide range of food resources with contrasting discrimination factors (Caut *et al.*, 2008; Florin *et al.*, 2011). This variability could be incorporated into models, and whilst this can be done via use of their weighted averages (Florin *et al.*, 2011), Robbins *et al.* (2010) suggested that erroneous estimates occurred in the dietary nitrogen of assimilated mixed diets when these were used in mixing models.

The aim of this Chapter was therefore to quantify and assess the extent to which stable isotope discrimination factors were significantly affected by diet composition in two omnivorous cyprinid fishes; European barbel *Barbus barbus* and chub *Squalius cephalus*, and across three tissue types; dorsal muscle, pelvic fin tissue ('fin-clip') and scales. The model species were selected to be representative of omnivorous freshwater fishes and although both tend to be rheophilic, they also tolerate lentic conditions (Britton and Pegg, 2011), and are relatively long-lived (> 15 years; Britton, 2007). They are also present across much of Eurasia and have socio-economic importance as angler-target species. Importantly, in the wild, both are highly omnivorous, with diets comprising a wide range of plant and animal taxa, including insect larvae, crustaceans, fish and macrophytes (Mann, 1976; Britton and Pegg, 2011). Thus, when estimations of their diet compositions are based on stable isotope data, these analyses may require awareness of differences in discrimination factors that might be present between common items in their diet. The objectives were thus to: (1) test the hypothesis that diet-tissue discrimination for the three tissues of each species will vary according to their exposure to three constant diets that

differ in their protein composition and content; and (2) assess how the diet-tissue discrimination factors relate to the difference in pre- and post-experimental stable isotope values of tissues as well as the change in body mass of individual fishes.

6.3. Materials and methods

6.3.1. Experimental design

The model fishes were sourced from pond aquaculture where their diets were a mix of natural foods (zooplankton and macro-invertebrates), supplemented with some formulated feeds. Their starting lengths were approximately 60 to 80 mm fork length (mean 69.4 ± 0.9 mm) and their body mass 2 to 7 g (mean 3.8 ± 0.2 g).

The experiment exposed the fish to three fixed diets which were fed *ad libitum* for 100 days. The duration of the experiment was balanced between feeding fish a single food source for an extended period and their tissues reaching an isotopic steady state with their new diet, i.e. turnover leading to equilibrium. Consumers are generally considered to have equilibrated to their food resources in four to five half-lives, i.e. 94 to 97 % isotopic replacement in their tissues (Hobson and Clark, 1992). Estimates of half-lives and isotopic replacement for the fishes over 100 days are provided by literature and calculated estimates (Thomas and Crowther, 2014; Vander Zanden *et al*., 2015). The estimated half-life for consumers of 1 g at 20 ºC is 23 days for $\delta^{13}C$ (100 days = 4.3 half-lives or 95 % replacement) and 25 days for $\delta^{15}N$ (100 days = 4.0 half-lives or 94 % replacement) (Thomas and Crowther, 2014). Estimates using the mean starting mass of fishes and equations from Vander Zanden *et al*. (2015) provided a half-life for both isotopes as low as 17 days (5.9 half-lives or 98.3 % replacement), whereas estimates from equations of Thomas and Crowther (2014) suggested half-life for $\delta^{13}C$ was 30 days (so 3.4 half-lives or 90 % replacement in 100 days) and for $\delta^{15}N$ was 32 days (3.1 half-lives or 88 % replacement).

The three diets used in the experiment were all based on pelletized feeds; these were preferred to natural foods given that the experiment would expose the species to these diets over a set time period and thus variability in the stable isotope values of the feeds would vary little through use of single, homogenous batches, and arguably less than if natural food sources were utilised (*cf.* Chapter 5). They also enabled fish to be exposed

to set levels of the food over the course of the experiment. Correspondingly, the first diet was 'Red krill pellets', henceforth referred to as 'krill', comprising 13.7 % protein, 11.5 % fat, 6.5 % crude fibre and 3.8 % ash, and whose base ingredient was krill oil (order Euphausiacea). The second diet was 'Wheatgerm pellets', referred to as 'wheatgerm', comprising 20 % protein, 6 % fat (as oil), 2.5 % crude fibre and 2.5 % ash, and whose base ingredient was plant based. The third diet was crushed pelletized fishmeal, referred to as 'fishmeal', comprising 45 % protein, 10 % fat, 1.4 % crude fibre, 5.8 % ash, and whose base ingredient was marine fish. Note that data for the fishmeal diet were generated in the previous Chapter (5) and was not repeated here to avoid unnecessary use of live fishes in experiments.

All the fish were measured (fork length, nearest mm) and weighed (nearest 0.01g) prior to the commencement of the experiment. For the krill and wheatgerm diets, the fish were also anaesthetised (MS-222) and a small incision made to the abdomen to allow for the insertion of a passive integrated transponder tag (PIT tag) into the stomach cavity to enable individual identification, with a sample of pelvic fin tissue taken and immediately frozen for subsequent stable isotope analysis and to allow comparison with the other pelvic fin at the close of the experiment. Following their recovery in oxygenated water, they were randomly allocated into experimental tanks. The fish were released into four 90 l tanks at 20 °C on a 16: 8 light: dark cycle and water quality was maintained through a flow-through filtration system. A maximum of 11 fish were allocated per tank, with the species held separately. The krill and wheatgerm diets were assigned to each tank, and thus across the four tanks, each species was exposed to each diet with daily *ad libitum* feeding. At the end of the experimental period, the fish were removed from the tanks, identified according to their PIT tag, re-measured and weighed, euthanized (anaesthetic overdose, MS-222), and samples taken of dorsal muscle, pelvic fin tissue (the unclipped fin) and scales, which were removed from the anterior region above the lateral line and below the dorsal fin. As the fishes exposed to the fishmeal diet in Chapter 5 had not been PIT tagged, individual changes in their body masses could not be determined, but other than for the PIT tagging procedure, the fishes were subjected to the same experimental conditions as the fishes on the krill and wheatgerm diets, including the same tank sizes, filtration systems, water temperature and chemical parameters, light: dark cycle and feeding regime.

6.3.2. Stable isotope analysis

For the SIA, fin clips and dorsal muscle were rinsed with distilled water and dried. Scales were lightly cleaned with distilled water to remove mucus, with their outer portion removed and used as this represents the most recent growth and thus their stable isotope values represent the most recent diets of the fish (Grey *et al.*, 2009; Bašić *et al.*, 2015). All samples were oven dried at 60 ºC to constant mass prior to analysis. Lipids were not extracted from samples as C: N ratios were < 3.5 %, indicating low lipid content and thus extraction or normalisation would have little effect on $\delta^{13}C$ (Post *et al.*, 2007). The tissues were then analysed at the Cornell University Stable Isotope Laboratory, New York, USA (*cf*. Section 2.3.2.1 for details) and the $\delta^{13}C$ and $\delta^{15}N$ data were provided as ‰.

6.3.3. Relationships in $\delta^{13}C$ and $\delta^{15}N$ and diet-tissue discrimination factors

Following the SIA, the $\delta^{13}C$ and $\delta^{15}N$ data for each species were tested for the extent of their discrimination (Δ) between each tissue and diet using general linear models (GLMs). The dependent variable was $\delta^{13}C$ or $\delta^{15}N$ and the independent variables were diet (krill, wheatgerm or fishmeal) and tissue type (muscle, fin or scales). Mean differences in the $\delta^{13}C$ or $\delta^{15}N$ values between the diets and each tissue per species were tested via their estimated marginal means with pairwise comparisons with Bonferroni adjustments for multiple comparisons. The pairwise comparisons provided the species- and diet-specific discrimination factors through the mean difference between the adjusted values for $\delta^{13}C$ and $\delta^{15}N$ for each diet and each tissue type, as well as the significance of the difference in $\delta^{13}C$ and $\delta^{15}N$ between each tissue, per species and per diet.

6.3.4. Relationships in change of $\delta^{13}C$ and $\delta^{15}N$ with change in fish mass

To test the relationship between the extent of change in $\delta^{13}C$ and $\delta^{15}N$ in fin tissue and the growth of the fish, isotopic data were calculated by deducting the values of the fin clips taken at the end of the experiment from those gained from fin clip samples taken at the start. The individual fish were identified via their PIT tag code, enabling their increase in mass to be determined over the course of the experiment. Univariate linear regressions were then completed separately for each isotope ($\delta^{13}C$ and $\delta^{15}N$) and for each species and diet (krill and wheatgerm), where the independent variable was the increase in mass and the dependent variable was the difference in isotope value of the fin tissue between the

start and end of the experiment. All statistical analyses were completed using IBM SPSS Statistics (version 22.0).

6.4. Results

6.4.1. Stable isotope discrimination factors between diets and tissues

The stable isotope values of the fish fed on each diet indicated some considerable differences. Fish fed on fishmeal had the highest $\delta^{13}C$ values, wheatgerm had the highest $\delta^{15}N$, and those fed on krill had the lowest $\delta^{13}C$ and the lowest $\delta^{15}N$ (Table 23).

The muscle, fin and scale tissues were also distinct in their isotopic signatures, with the highest $\delta^{15}N$ in muscle, followed by fin tissue and then scales, with the converse relationship between the tissues for $\delta^{13}C$ (Table 23). These relationships were consistent in both species and across the three diets. The GLMs testing differences in stable isotope data per tissue and diet were significant ($\delta^{13}C$: $F_{17,155} = 164.76$, $P < 0.01$; $\delta^{15}N$: $F_{17,155} = 30.56$, $P < 0.01$) and revealed some significant differences across the tissues and diets (Tables 24, 25).

These differences in the isotopic values then translated into considerable variation in the discrimination factors (Δ) between the three diets. The largest differences in discrimination factors for each species were between the wheatgerm and fishmeal diets (Fig. 17). For wheatgerm, the highest $\Delta^{13}C$ recorded for *B. barbus* and *S. cephalus* was 5.6 and 5.0, respectively (both in scale tissue) and the highest $\Delta^{15}N$ recorded was 6.9 and 6.8, respectively (Fig. 17). In contrast, for fishmeal, the highest $\Delta^{13}C$ recorded for *B. barbus* and *S. cephalus* was 4.7 and 4.9, respectively (again, both in scale tissue) and the highest $\Delta^{15}N$ recorded for both species was only 2.4 (Fig. 17).

Table 23. Mean initial and final fork lengths and stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values per diet and species for muscle, fin and scale tissues. Errors around the means represent standard errors.

Diet	Species	Initial length (mm) Final length (mm)	Tissue	Mean $\delta^{13}C$ (‰)	95 % confidence intervals	Mean $\delta^{15}N$ (‰)	95 % confidence interval
1 =	Krill			-24.62 ± 0.11	-24.83 - -24.42	5.03 ± 0.18	4.67 - 5.39
2 =	Wheatgerm			-24.94 ± 0.26	-25.45 - -24.43	4.35 ± 0.15	4.04 - 4.65
3 =	Fishmeal			-22.15 ± 0.06	-22.32 - -21.99	8.51 ± 0.07	8.33 - 8.70
1	*B. barbus*	70 ± 2	Muscle	-21.79 ± 0.08	-21.96 - -21.63	11.03 ± 0.12	10.79 - 11.27
	n = 9	90 ± 2	Fin	-20.65 ± 0.08	-20.82 - -20.49	10.03 ± 0.12	9.79 - 10.27
			Scales	-20.23 ± 0.08	-20.40 - -20.07	9.25 ± 0.12	9.01 - 9.49
2	*B. barbus*	76 ± 2	Muscle	-20.98 ± 0.09	-21.16 - -20.80	11.22 ± 0.12	11.00 - 11.45
	n = 8	91 ± 1	Fin	-19.49 ± 0.09	-19.67 - -19.31	10.78 ± 0.12	10.56 - 11.01
			Scales	-19.16 ± 0.09	-19.33 - -18.98	9.89 ± 0.12	9.66 - 10.12
3	*B. barbus*	87 ± 2	Muscle	-19.59 ± 0.04	-19.66 - -19.51	10.92 ± 0.04	10.84 - 10.99
	n = 9	118 ± 2	Fin	-18.31 ± 0.04	-18.38 - -18.24	10.60 ± 0.04	10.52 - 10.68
			Scales	-17.41 ± 0.04	-17.49 - -17.34	10.70 ± 0.04	10.63 - 10.78
1	*S. cephalus*	69 ± 1	Muscle	-22.26 ± 0.07	-22.41 - -22.11	10.09 ± 0.13	9.83 - 10.34
	n = 10	90 ± 3	Fin	-21.88 ± 0.08	-22.04 - -21.73	9.62 ± 0.14	9.35 - 9.89
			Scales	-20.26 ± 0.07	-20.40 - -20.11	9.12 ± 0.13	8.86 - 9.37
2	*S. cephalus*	69 ± 2	Muscle	-21.48 ± 0.09	-21.66 - -21.30	11.02 ± 0.10	10.82 - 11.22
	n = 10	83 ± 2	Fin	-20.70 ± 0.09	-20.88 - -20.52	11.14 ± 0.10	10.94 - 11.33
			Scales	-19.93 ± 0.09	-20.11 - -19.75	10.04 ± 0.10	9.84 - 10.23
3	*S. cephalus*	97 ± 6	Muscle	-20.16 ± 0.05	-20.26 - -20.06	10.86 ± 0.05	10.78 - 10.95
	n = 11	128 ± 8	Fin	-18.91 ± 0.05	-19.01 - -18.81	10.79 ± 0.05	10.71 - 10.88
			Scales	-17.25 ± 0.05	-17.35 - -17.15	10.48 ± 0.05	10.39 - 10.56

Table 24. Differences in stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values between diets per fish tissue and species, according to pairwise comparisons from general linear models, where comparisons have undergone Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.05$. Errors around the means represent standard errors. Note the data for the fishmeal diet were obtained in Chapter 5.

Species	Tissue	Diet comparison	Difference in $\delta^{13}C$ (‰)	Difference in $\delta^{15}N$ (‰)
B. barbus	Muscle	Fishmeal vs. Wheatgerm	1.52 ± 0.17 *	- 0.31 ± 0.18
		Fishmeal vs. Krill	2.21 ± 0.17 *	- 0.11 ± 0.17
		Wheatgerm vs. Krill	0.69 ± 0.17 *	0.20 ± 0.18
	Fin	Fishmeal vs. Wheatgerm	1.33 ± 0.17 *	- 0.18 ± 0.18
		Fishmeal vs. Krill	2.34 ± 0.17 *	0.57 ± 0.17
		Wheatgerm vs. Krill	1.02 ± 0.17 *	0.75 ± 0.18 *
	Scales	Fishmeal vs. Wheatgerm	1.88 ± 0.17 *	0.81 ± 0.18 *
		Fishmeal vs. Krill	2.82 ± 0.17 *	1.45 ± 0.17 *
		Wheatgerm vs. Krill	0.94 ± 0.17 *	0.64 ± 0.18
S. cephalus	Muscle	Fishmeal vs. Wheatgerm	1.32 ± 0.10 *	- 0.16 ± 0.15
		Fishmeal vs. Krill	2.10 ± 0.16 *	0.18 ± 0.16 *
		Wheatgerm vs. Krill	0.78 ± 0.10 *	0.93 ± 0.16 *
	Fin	Fishmeal vs. Wheatgerm	1.79 ± 0.10 *	- 0.34 ± 0.15
		Fishmeal vs. Krill	2.97 ± 0.16 *	1.18 ± 0.16 *
		Wheatgerm vs. Krill	1.18 ± 0.11 *	1.52 ± 0.16 *
	Scales	Fishmeal vs. Wheatgerm	2.68 ± 0.10 *	0.44 ± 0.15
		Fishmeal vs. Krill	3.01 ± 0.16 *	1.36 ± 0.16 *
		Wheatgerm vs. Krill	0.33 ± 0.10 *	0.92 ± 0.16 *

Table 25. Differences in stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values between fish tissues per species for each diet according to pairwise comparisons from general linear models, where comparisons have undergone Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.05$. Errors around the means represent standard errors. Note the data for the fishmeal diet were obtained in Chapter 5.

Diet	Species	Tissue comparison	Difference in $\delta^{13}C$ (‰)	Difference in $\delta^{15}N$ (‰)
Fishmeal	*B. barbus*	Muscle vs. Fin	- 1.28 ± 0.17 *	0.32 ± 0.17
	$n = 9$	Muscle vs. Scales	- 2.17 ± 0.17 *	0.21 ± 0.17
		Fin vs. Scales	- 0.89 ± 0.17 *	- 0.10 ± 0.17
	S. cephalus	Muscle vs. Fin	- 1.25 ± 0.15 *	0.07 ± 0.15
	$n = 11$	Muscle vs. Scales	- 2.91 ± 0.15 *	0.39 ± 0.15
		Fin vs. Scales	- 1.66 ± 0.15	0.32 ± 0.15
Wheatgerm	*B. barbus*	Muscle vs. Fin	- 1.47 ± 0.18 *	0.44 ± 0.18
	$n = 8$	Muscle vs. Scales	- 1.81 ± 0.18 *	1.33 ± 0.18 *
		Fin vs. Scales	- 0.34 ± 0.18	0.89 ± 0.18 *
	S. cephalus	Muscle vs. Fin	- 0.77 ± 0.10 *	- 0.12 ± 0.16
	$n = 10$	Muscle vs. Scales	- 1.55 ± 0.10 *	0.98 ± 0.16 *
		Fin vs. Scales	- 0.78 ± 0.10 *	1.10 ± 0.16 *
Krill	*B. barbus*	Muscle vs. Fin	- 1.14 ± 0.17 *	1.00 ± 0.17 *
	$n = 9$	Muscle vs. Scales	- 1.56 ± 0.17 *	1.78 ± 0.17 *
		Fin vs. Scales	- 0.42 ± 0.17	0.78 ± 0.17 *
	S. cephalus	Muscle vs. Fin	- 0.38 ± 0.16	0.47 ± 0.17
	$n = 9$	Muscle vs. Scales	- 2.00 ± 0.16 *	0.97 ± 0.16 *
		Fin vs. Scales	- 1.62 ± 0.16 *	0.50 ± 0.17

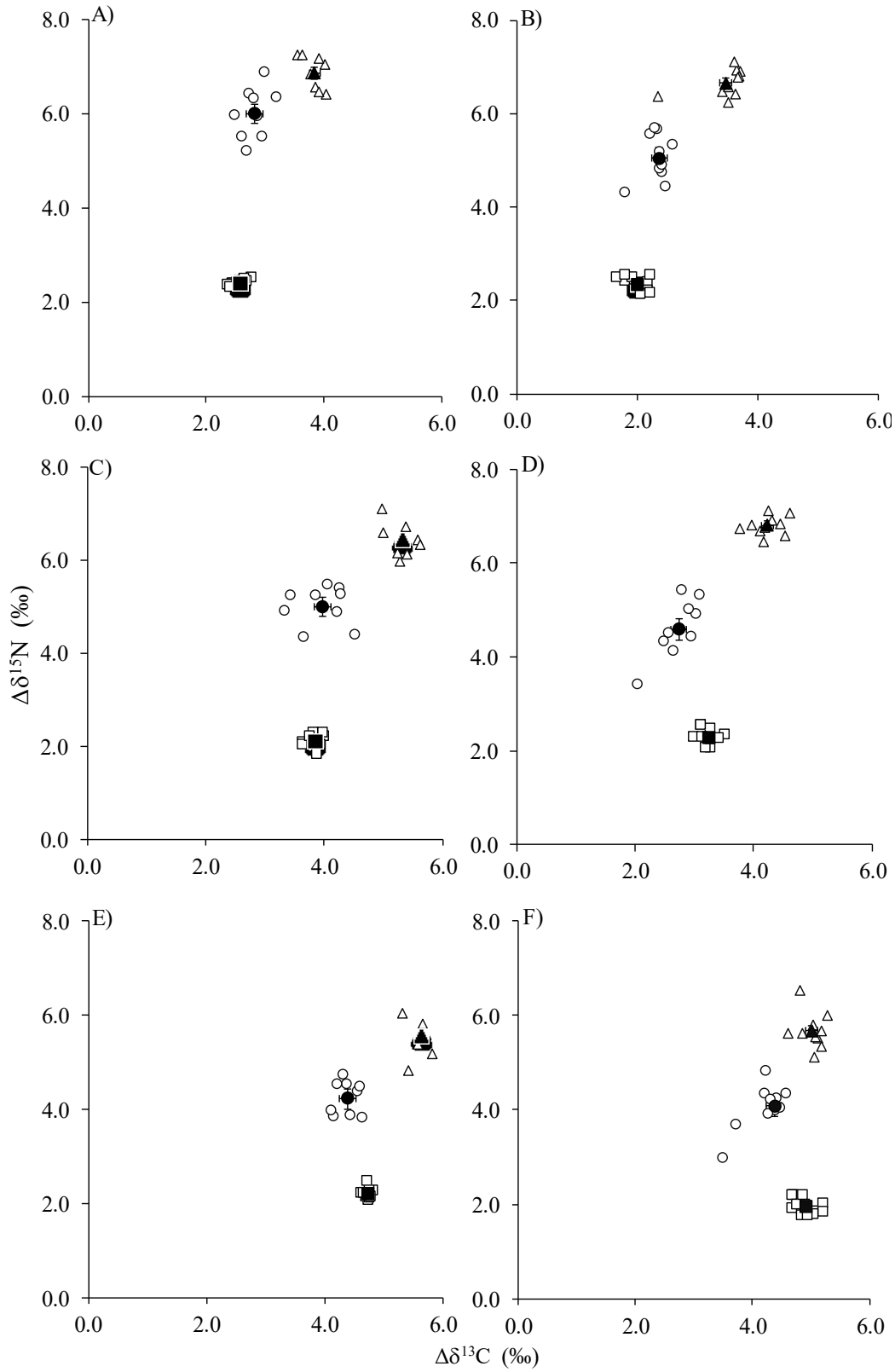

Figure 17. Stable isotope discrimination factors for *B. barbus* (A, C, E) and *S. cephalus* (B, D, F) for muscle (A, B), fin (C, D) and scales (E, F); Δ plant based 'wheatgerm' diet; □ fish based 'fishmeal' diet; and ○ invertebrate based 'krill' diet. Filled symbols are as per open symbols, except they represent mean values and standard errors.

6.4.2. Changes in stable isotope data in fin tissues

Across the experimental period, an increase in body mass was evident in all fishes fed on krill and wheatgerm (Table 26). Outputs of univariate linear regressions testing the extent of change in stable isotopes in the fin tissues of individual fish with their changes in body mass over the experiment revealed a lack of consistent pattern across the species and diets (Table 26). There were, however, some significant relationships between isotopic change and increase in mass for both species on krill ($P < 0.05$; Table 26).

The stable isotope data from the fin clips taken at the start and end of the experiment from the individual fish revealed the extent of isotopic change that occurred over the 100 day period (Table 27A). The GLMs testing the significance of the differences between these data were significant ($\delta^{13}C$: $F_{11,93} = 59.77$, $P < 0.01$; $\delta^{15}N$: $F_{11,93} = 122.46$, $P < 0.01$), with pairwise comparisons revealing significant decreases in the $\delta^{15}N$ of fin tissues between the start and end of the experiment for both krill and wheatgerm diets ($P < 0.01$), but for $\delta^{13}C$, differences were only significant for wheatgerm ($P < 0.01$) (Table 27B).

Table 26. Relationships between increase in mass of fishes over the experimental period and the shift in stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values determined by individual univariate linear regressions. Errors around the means represent standard errors.

Diet	Species	Mean increase in mass (g)	Stable isotope	R^2	F	P
Krill	*B. barbus*	4.2 ± 0.7	$\delta^{13}C$	0.05	0.29	0.61
	$n = 8$		$\delta^{15}N$	0.58	8.21	**0.03**
	S. cephalus	5.8 ± 0.8	$\delta^{13}C$	0.49	6.60	**0.04**
	$n = 9$		$\delta^{15}N$	0.64	12.47	**0.01**
Wheatgerm	*B. barbus*	4.2 ± 0.1	$\delta^{13}C$	0.05	0.27	0.62
	$n = 7$		$\delta^{15}N$	0.22	1.43	0.29
	S. cephalus	3.8 ± 0.2	$\delta^{13}C$	0.07	0.54	0.49
	$n = 9$		$\delta^{15}N$	0.24	2.24	0.18

Table 27. Mean stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values and diet discrimination factors of fin tissues between the start and end of the experiment (A) and differences according to pairwise comparisons from general linear models comparing stable isotope data of fin tissues between the start and end of the experiment where comparisons have undergone Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.01$ (B). Errors around the means represent standard errors.

A)

Diet	Species	Fin clip	Mean $\delta^{13}C$ (‰)	Mean $\Delta^{13}C$ (‰)	Mean $\delta^{15}N$ (‰)	Mean $\Delta^{15}N$ (‰)
Krill	*B. barbus*	Start	-20.96 ± 0.09		12.27 ± 0.13	
		End	-20.65 ± 0.08	3.97 ± 0.14	10.03 ± 0.12	5.00 ± 0.21
	S. cephalus	Start	-21.85 ± 0.07		12.53 ± 0.13	
		End	-21.88 ± 0.08	2.74 ± 0.13	9.62 ± 0.14	4.59 ± 0.23
Wheatgerm	*B. barbus*	Start	-20.82 ± 0.09		12.34 ± 0.12	
		End	-19.49 ± 0.09	5.31 ± 0.09	10.78 ± 0.12	6.43 ± 0.13
	S. cephalus	Start	-21.97 ± 0.09		12.53 ± 0.10	
		End	-20.70 ± 0.08	4.24 ± 0.10	11.14 ± 0.10	6.79 ± 0.10

B)

Diet	Species	Fin clip	Difference in $\delta^{13}C$ (‰)		Difference in $\delta^{15}N$ (‰)	
Krill	*B. barbus* (n = 9)	Start vs. End	0.30 ± 0.18		-2.24 ± 0.18	*
	S. cephalus (n = 10)	Start vs. End	-0.03 ± 0.17		-2.92 ± 0.17	*
Wheatgerm	*B. barbus* (n = 8)	Start vs. End	1.28 ± 0.19	*	-1.55 ± 0.19	*
	S. cephalus (n = 10)	Start vs. End	1.27 ± 0.12	*	-1.40 ± 0.14	*

6.5. Discussion

6.5.1. Diet-tissue discrimination factors

There was considerable variation in the stable isotope discrimination factors produced by the three diets in the tissues of the two fishes, as per the hypothesis. Across the diets, $\Delta^{13}C$ ranged between 2.0 and 5.6 ‰ and $\Delta^{15}N$ ranged between 2.0 and 6.9 ‰. For each tissue, the wheatgerm diet (20 % protein) always had the highest discrimination factors, whilst fishmeal (45 % protein) had the lowest. These diet-tissue discrimination factors contribute to a growing knowledge base on the general patterns of how the diet and feeding behaviour of fishes affects their stable isotope data. For example, whilst the discrimination factors for $\Delta^{15}N$ produced by krill and wheatgerm were high compared with most other fishes, the fishmeal values were relatively consistent with Sweeting *et al.* (2007a), as previously mentioned in Chapter 5, who suggested that white muscle of piscivorous European sea bass *Dicentrarchus labrax* had $\Delta^{15}N$ of 3.15 ‰. Their suggestion that this could be used for fishes generally was, however, not consistent with the means derived here for the krill and wheatgerm diets ($\Delta^{15}N$ of 5.1 to 6.9 ‰). Nevertheless, these latter elevated discrimination factors were largely in line with Mill *et al.* (2007), who recorded $\Delta^{15}N$ up to 5.25 ‰ in herbivorous fishes. They are also in general agreement with Carassou *et al.* (2008), who suggested that herbivorous fishes have distinct stable isotope discrimination factors that distinguish them from piscivorous fishes. Thus, an omnivorous fish with a strong proportion of plant material in their diet should be distinguishable from a conspecific with a fish-based diet through $\Delta^{15}N$.

For $\Delta^{13}C$, literature generally suggests values for dorsal white muscle of fishes of between 0 and 1 ‰ (e.g., Peterson and Fry, 1987; Phillips *et al.*, 2005; Cucherousset *et al.*, 2012b), with Post (2002) suggesting a mean of 0.39 ± 1.3 ‰, which has recently been applied to mixing models to estimate fish diet composition (e.g. Bašić *et al.*, 2015). However, Sweeting *et al.* (2007b) suggested that 1.5 ‰ was more appropriate for $\Delta^{13}C$ for fish muscle and samples that had not undergone lipid treatment could have higher values, such as 2.27 ‰. This latter inference is consistent with the outputs here for all diets, where the lowest $\Delta^{13}C$ for dorsal muscle was 2.0 and highest was 3.8 ‰, and where no sample had undergone lipid treatment due to the low C: N ratios.

Correspondingly, whilst there are some general patterns apparent in $\Delta^{13}C$ and $\Delta^{15}N$ across herbivorous and piscivorous species and diets, the basis of these differences

remains uncertain. The outputs suggest that the primary reason behind the differences between the three diets was their contrasting sources of dietary protein (McClelland *et al*., 2003). These differences could then relate to one of two opposing hypotheses. Firstly, the protein quality hypothesis suggests that discrimination in the stable isotopes of consumers and their prey will increase as protein quality decreases, and thus as carnivores tend to assimilate higher quality protein than herbivores, discrimination factors decrease with trophic level (Roth and Hobson, 2000). The results support this hypothesis, as fishmeal contained 45 % protein derived from marine fishes and had consistently low discrimination factors. Wheatgerm, although having the mid-range protein content of the three diets (20 %), was likely to have had the lowest quality protein as it was derived from plant material, and consistently produced the highest discrimination factors. This is generally consistent with Macko *et al.* (1986), who suggested that as dietary protein increases, the percentage of nitrogen in the diet increases and more amino acids are catabolised for energy, potentially reducing discrimination factors in protein rich diets. Secondly, the protein quantity hypothesis suggests that discrimination increases with dietary nitrogen concentration (i.e. decreasing C: N ratios), and thus as carnivores assimilate more protein than other fishes then discrimination increases with trophic level (Pearson *et al.*, 2003). This is contrary to the findings of the Chapter, as the diet with the highest protein content and trophic position (fishmeal) had the lowest discrimination factors.

When the two fish species were compared, then discrimination factors tended to be higher in *B. barbus* than *S. cephalus*, irrespective of diet and despite the species both being cyprinids. The reason for this difference between the species is not clear. Although somatic growth differences can result in differences in the isotopic enrichment of tissues (Thomas and Crowther, 2014; Vander Zanden *et al*., 2015) and have been used as an explanation for differences in discrimination factors between sexes (Kurle *et al*., 2014), the growth increments of each species per diet were similar here. However, the growth of the individual tissues within the species was not measured, and Reich *et al*. (2008) suggest that tissue growth may influence differences in isotopic incorporation among tissues. While the fishes grew at similar rates over the experimental period, differences in the growth of the tissues sampled may exist and could offer some explanation into the differences in discrimination values observed. Though this might relate to species-specific differences in metabolic activity and isotopic routing within tissues, as these can both influence the extent of discrimination (Caut *et al.*, 2008; Vander Zanden *et al*., 2015),

this is speculative and was not quantified. Notwithstanding, the differences demonstrate both the underlying complexity of understanding discrimination factors within and between species, and the importance of determining differences in species-specific discrimination factors across different prey items.

Resolution of the differences in discrimination factors between food items comprising of varying protein sources and content is required in studies that predict trophic level and diet composition using, for example, Bayesian mixing models (Parnell *et al.*, 2013; Phillips *et al.*, 2014). The outputs indicate the difficulty of estimating discrimination factors from consumers with mixed diets. Whilst a final diet comprising of a 50: 50 mix of the wheatgerm and fishmeal feed might have indicated a discrimination factor between their values when fed in isolation, that could have utility in subsequent analyses, this was not completed due to both ethical and logistical concerns. As the fishes both show natural shoaling behaviours, especially in smaller sizes (Britton and Pegg, 2011), then holding them individually for extended periods in order to tightly control their food intake can represent an unnatural and highly stressful environment. Whilst this can be overcome by holding numbers of fish together, it is then difficult to control the food intake of individual fish, with the potential for substantial deviations from the 50: 50 food ratio through selective feeding. Greer *et al*. (2015) suggested a mathematical method for calculating combined discrimination factors based on the known diet composition of a captive parrot, but this approach might have limited applicability in natural situations as it loses the isotopic variability that is often inherent in wild diets. For example, diet composition can vary considerably between individuals of the same species due to ontogenetic dietary shifts (Byström *et al*., 2012). Moreover, in omnivorous fishes, whilst stomach contents data can indicate high proportions of algae and plant based material in their diet, this does not mean that a high proportion will actually be assimilated into tissues, making it difficult to determine the relative importance of plant based materials as a food and energy source (e.g. Hamidan *et al*. 2015). In combination, these issues of varying diet-tissue discrimination factors between different food resources suggest that this variability should be captured within predictive models used for estimating the diet composition of omnivores, rather than relying on the use of a single discrimination factor covering all putative food items (Phillips *et al*., 2014).

The discrimination factors supplied here, produced in controlled conditions on fixed diets, thus provide strong evidence that tissue- and diet-specific discrimination factors require further consideration in fishes and, potentially, other taxa. Although general issues

surrounding experimentally-derived discrimination factors using formulated feeds have provoked debate in literature, for example, the study by Caut *et al.* (2008) and responses from Perga and Grey (2010), as previously mentioned in Chapter 5 (*cf.* Section 5.5.4), the extent of isotopic routing occurring within tissues may vary considerably in prevalence and magnitude between ectotherms and endotherms (Kelly and Martínez del Rio, 2010). Indeed, there is strong evidence to suggest that the rate in which stable isotopes turnover is significantly different between ecto- and endotherms, with the half-lives of the former being considerably longer (Thomas and Crowther, 2014; Vander Zanden *et al*., 2015). There also remains some uncertainty in how the discrimination between different food types will play out within omnivorous diets in the wild, as it is likely that they would consume foods that vary extensively in both protein quality and quantity, which may alter temporally. Where diet shifts occur regularly, the likelihood of tissues reaching isotopic equilibrium with each diet before a shift occurs is reduced. Furthermore, the consumption of food items that have different discrimination factors, may not be additive and linear and may vary with the degree of amino acid complementation determined by the entire diet (McClelland *et al*., 2003; MacNeil *et al*., 2006; Robbins *et al*., 2010), thus reducing the reliability of subsequent estimations of the assimilated diet where these interactions have not been factored into analyses within mixing models. Evidently further work is required in order to reconcile some of the differences identified here.

6.5.2. Stable isotope values across different tissues

It was also apparent in the outputs that there was variability in the stable isotope values between the tissue types, with a general pattern of muscle always being enriched in $\delta^{15}N$ and depleted in $\delta^{13}C$ compared with fin tissue and scales. This was consistent with the outcomes of Chapter 5, where the same relationships for wild and laboratory populations of the two model species used here were reported. These general patterns were also consistent with those between muscle and fin tissue for Chinook salmon *Oncorhynchus tshawytscha*, brook trout *Salvelinus fontinalis*, brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* and Atlantic salmon *Salmo salar* (Pinnegar and Polunin, 1999; Jardine *et al.*, 2005; Sanderson *et al.* 2009; Graham *et al*., 2013). Tronquart *et al.* (2012) also showed similar relationships between these tissues for 14 European freshwater fishes. These differences in isotopic enrichment between the tissues may relate to variation in amino acid profiles (Reich *et al*., 2008). The $\delta^{13}C$ and $\delta^{15}N$ of individual amino acids can vary significantly (McClelland and Montoya, 2002; Fogel and Tuross,

2003), and thus differences in amino acid composition of tissues can lead to differences in isotopic discrimination among them (Howland *et al*., 2003). The amino acid profiles of the tissue samples analysed here were not determined, but doing so may be worth consideration in future experiments where a range of tissues are sampled for use in stable isotope analysis. Additionally, the differences in tissues are likely to have been influenced by factors that determine isotopic turnover, including somatic growth and metabolic replacement (Martínez del Rio *et al*., 2009; Weidel *et al*., 2011; Thomas and Crowther, 2014; Vander Zanden *et al*., 2015). Due to the varying metabolic activity of tissues, the rate of isotopic turnover found in each often differs, with a strong relationship between turnover and metabolism, for example, in fishes, internal organs and blood plasma have shorter half-lives, and therefore faster turnover, than whole blood and muscle (Thomas and Crowther, 2014; Vander Zanden *et al*., 2015). Although, Weidel *et al*. (2011) state that many fish diet-switch studies conclude that growth is primarily responsible for $\delta^{13}C$ change, and found metabolic replacement had a negligible effect on turnover, this could relate to the use of predominately juvenile fish.

6.5.3. Summary and conclusions

The outputs reveal that for two omnivorous cyprinid fishes, there were differences across diet-tissue discrimination factors by diet and by species. Although further work is required to disentangle some of the processes involved, they nevertheless demonstrate that considerable attention must be made on the discrimination factors, according to the consumer and their putative food resources, in dietary studies of omnivorous fishes using SIA. Additionally, they also support the findings of Chapter 5, in that the destruction of fishes to take muscle samples is unnecessary and can be substituted by non-destructive sampling through use of scales or fin clips, thus facilitating a shift towards the use of non-lethal and more sustainable sampling methods for stable isotope tissue collection.

Chapter 7. *Ex situ* estimates of stable isotope turnover rates and discrimination factors in tissues of European barbel *Barbus barbus*

7.1. Summary

Interpretation of stable isotope data relies heavily upon knowledge of the turnover rate, which is the rate of isotopic change occurring within tissues after a consumer changes diet, and the discrimination factor between the consumer and its resources. Determining these values for consumers *in situ* can be problematic as they tend to assimilate a range of prey items that vary in isotopic content. The turnover rates and discrimination factors of carbon and nitrogen stable isotopes were estimated for the freshwater cyprinid European barbel *Barbus barbus* in *ex situ* controlled conditions. The fish were fed distinct formulated diets over two discrete time periods, with concomitant shifts in the stable isotope values of their tissues measured for estimating turnover rates, stable isotope values at equilibrium, and stable isotope discrimination factors. For ^{15}N turnover among tissues, the estimates from the best-fitting models ranked muscle as having the shortest half-life (84 days), followed by fin (91 days) and then scales (145 days). For ^{13}C, the half-life for muscle was 138 days and for scales, 91 days. Isotopic values at equilibrium were estimated as ranging between 6.75 and 9.63 ‰ for ^{15}N, and -0.27 and -17.64 ‰ for ^{13}C. The estimated diet-tissue discrimination factors were considerably higher than the standard values commonly cited in literature and ranged between 2.48 and 6.35 ‰ for $\delta^{15}N$ and 5.01 and 6.94 ‰ for $\delta^{13}C$. Thus, the estimation of these data in *ex situ* conditions provides considerable insight into the turnover rates and discrimination factors of *B. barbus* and emphasises the importance of estimating these parameters for consumers at the species level.

7.2. Introduction

Stable isotope analysis is an important tool in food web ecology that can be applied to reconstructing the trophic interactions and energy pathways within and between organisms (Fry, 2006). Natural variations of the ratios of ^{13}C to ^{12}C ($\delta^{13}C$) and ^{15}N to ^{14}N

($\delta^{15}N$) can be applied to Bayesian mixing models that predict the relative contributions of assimilated prey sources to consumer species (*cf.* Chapter 5), as well as identifying their trophic positions within the food web (*cf*. Chapter 2; Vander Zanden *et al*., 1997; Post, 2002). Recent applications have included identifying the feeding grounds of migratory species (Hertz *et al*., 2015; Madigan *et al*., 2016) and characterising trophic niche spaces and dietary overlaps between species (Shelton *et al.*, 2016; Wedchaparn *et al*., 2016).

The interpretation of stable isotope data is reliant upon two principal factors: the rate of isotopic change that occurs within tissues when a consumer undergoes a dietary shift, known as the turnover rate, and the discrimination factor between the consumer and its resources (*cf.* Chapters 5, 6; Boecklen *et al*., 2011). The turnover rate of stable isotopes in consumer tissues tends to be expressed as their half-life, defined as the time required for the stable isotope values in tissues to reach 50 % equilibration with the new diet (Vander Zanden *et al*., 2015). Identifying isotopic turnover rates is particularly important for assessing the trophic ecology of mobile and migratory species and species that undergo ontogenetic dietary shifts (e.g., MacAvoy *et al*., 2001; Buchheister and Latour, 2010; Hertz *et al*., 2015). This is because the isotopic characteristics of their diet are likely to be temporally variable and so if tissues are sampled prior to them reaching isotopic equilibrium with a new diet (generally considered to equate to four to five half-lives; Hobson and Clark, 1992) then erroneous data interpretations will result (O'Reilly *et al*., 2002). Estimates of isotopic half-lives are also important in the design of manipulative field studies and mesocosm experiments where, for example, the duration of the study could be confounded if it is of insufficient length for stable isotope equilibrium to be reached (Jackson *et al*., 2013; Tran *et al*., 2015). Importantly, variability has been shown in turnover rates between tissues of freshwater fishes and thus species-specific data are often required (e.g., McIntyre and Flecker, 2006; Church *et al.,* 2009; Carleton and Martínez del Rio, 2010). Similarly, stable isotope discrimination factors can vary between tissues, diet compositions, species, ages and sample preparations, and thus calculating precise values enables more accurate use of analytical techniques, such as statistical mixing models that are used to predict the diet composition of consumers (*cf*. Chapters 5, 6; Sweeting *et al*., 2007a, b; Caut *et al*., 2009).

The determination of tissue turnover rates of consumers in the wild can be problematic, as they tend to assimilate a range of prey items that vary in isotopic content, with single prey species also showing isotopic variation over time (Perga and Gerdeaux, 2005). This

results in increased uncertainty in the isotopic values of the prey items, and therefore the isotopic 'baselines' (*cf*. Section 1.4.1; Fig. 2), that contribute to the turnover rate. Additionally, consumers are unlikely to feed on the same proportions of prey items on a daily basis, and consumer isotopic turnover rates are also influenced by a number of other factors, including temperature fluctuations and life-history events, such as reproduction, during which nutrient allocation between somatic and gonadal growth may change (e.g., Bearhop *et al*., 2002; Bosley *et al*., 2002; Witting *et al*., 2004). Thus, an alternative approach for estimating turnover rates is the use of experimental diet-switch studies completed in controlled conditions (e.g., Herzka and Holt, 2000; Logan *et al*., 2006; Buchheister and Latour, 2010; Heady and Moore, 2012; Xia *et al*., 2013a, b). In these studies, the diet is fixed to provide prey with consistent stable isotope values that should then produce more reliable turnover estimates in the consumer tissues (Logan *et al*., 2006). Although these turnover estimates might have limited applicability in more wild scenarios, they at least enable greater understandings of the mechanisms involved in isotopic replacement (Buchheister and Latour, 2010; Heady and Moore, 2012). Furthermore, should reliable turnover rates be obtained from tissues then there is potential to utilise the natural variation in turnover rates between tissues to estimate the time since a resource shift has occurred, using the tissues as 'stable isotope clocks' (Phillips and Eldridge, 2006; Heady and Moore, 2012). Recent studies, such as Vander Zanden *et al*. (2015), have also attempted to provide general equations based from meta-analyses that provide half-life estimates using temperature and consumer starting mass, negating the use of diet-switch experiments to calculate turnover.

Isotopic turnover is driven by two general processes that occur concomitantly; the addition of new tissue from growth and from metabolic replacement (Xia *et al*., 2013a, b). Thus, the rate of turnover is influenced by both the growth rate, representing synthesis of new tissue from the new diet, and the metabolic rate, representing the balanced rate of breakdown of old tissue, synthesised during feeding on a previous diet, and the re-synthesis of tissue components made from the new diet (Hesslein *et al.,* 1993). With known growth rates, the proportional contributions of metabolism and growth to stable isotopic turnover can be estimated from non-linear regressions of the isotopic turnover trajectories (Buchheister and Latour, 2010). As the change in isotopic content within the tissues can be plotted on a temporal scale as well as a feature of increase in body mass, models can be based on either of two methodologies: time-based or growth-based. Time-based methods calculate turnover over a temporal scale and produce half-life estimates in

units of days and growth-based methods calculate turnover through an increase in body mass, producing half-life estimates in terms of weight gain in grams or an x-fold increase in body mass (Hobson and Clark, 1992; Hesslein *et al*., 1993; Fry and Arnold, 1982). The modelling method chosen is important as it can effect estimations of turnover rates as well as the diet-tissue discrimination factors (Martínez del Rio and Wolf, 2005; Kurle, 2009).

Correspondingly, the aim of this Chapter was to determine the turnover rates of ^{13}C and ^{15}N stable isotopes in the freshwater fish *B. barbus* under controlled conditions. The fish were fed distinct formulated diets over two discrete time periods, with concomitant shifts in stable isotope values of the fish tissues measured to determine their turnover rates, stable isotope values at equilibrium, and stable isotope discrimination factors. The specific objectives were thus to: (1) determine the discrimination factors and turnover rates of ^{13}C and ^{15}N in dorsal muscle, fin tissue and scales of *B. barbus* through the application of time- and growth-based models; (2) quantify the proportional contributions of metabolism and growth to the turnover rates of ^{13}C and ^{15}N in each tissue; (3) use an information-theoretic approach to determine the most appropriate model for estimating the turnover rates of ^{13}C and ^{15}N within each tissue; and (4) compare the turnover rate estimates from the final models with general estimates of turnover available from literature (e.g. Vander Zanden *et al*., 2015).

7.3. Materials and methods

7.3.1. Experimental design

The experiment utilised juvenile *B. barbus* that were sourced from pond aquaculture where they had been reared in outdoor ponds and fed on a mixture of natural food resources supplemented with formulated feeds. Their initial fork lengths and weights ranged between 75 and 85 mm and 4 and 7 g, respectively. Following their transfer to the laboratory, they were acclimated to conditions for 10 days before being measured and tagged with 12 mm passive integrated transponder (PIT) tags to enable their subsequent individual identification, following the PIT tagging procedure as described in Chapter 6 (*cf.* Section 6.3.1). The fish were then measured (fork length, nearest mm) and weighed (to the nearest 0.01g). The experimental design then used two feeding periods. The first

was on a set formulated diet, to provide all fish with similar isotopic values and lasted 125 days; the second immediately followed this and feeding was on an alternative formulated feed for a further 125 days during which the changes in the isotopic values of the fish were measured. These timescales were used as 125 days should have provided at least four isotopic half-lives and thus the fish would be close to their isotopic equilibrium at the end of each period (*cf*. Section 6.3.1; Hobson and Clark, 1992; Thomas and Crowther, 2014).

Correspondingly, following their tagging and measurement, the fish were transferred to 45 l tanks at 20 ºC where they were held in groups of 6 and were fed a 'control' diet *ad libitum* for 125 days. This feed consisted of crushed pelletized fishmeal (as used previously in Chapters 5 and 6) and was composed of 45 % protein, 10 % fat, 1.4 % crude fibre and 5.8% ash. The mean $\delta^{13}C$ and $\delta^{15}N$ values were -23.19 ± 0.11 ‰ and 9.34 ± 0.05 ‰ respectively (n = 5). At the end of the first 125 day feeding period, the fish were removed from their tanks, re-measured and weighed, and separated into three groups. The first two groups each comprised of 6 *B. barbus*. One of these groups was immediately euthanized with an overdose of anaesthetic (MS-222) and used to provide stable isotope data on the tissues of the fish at the start of the second feeding period. The second of these groups was then used as a 'control' group of fish that was kept in a separate 45 l tank and their diet maintained on the 'control' feed for the entirety of the subsequent 125 day feeding period. The third group of fish comprised of 24 fish that were used for the diet-switch experiment and were held in 45 l tanks in groups of 6. The new food source, hereafter referred to as the 'experimental' diet, was pelletized wheatgerm (as previously used in Chapter 6), a plant based feed that comprised 20 % protein, 6 % fat (as oil), 2.5 % crude fibre and 2.5 % ash and with stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$ of -25.35 ± 0.08 ‰ and 3.28 ± 0.02 ‰, respectively (n = 5). Thus, the control and experimental diets were isotopically distinct. For the diet-switch fish, on day 50, 75, 100 and 125, 6 fish were removed and euthanized (anaesthetic overdose, MS-222) with fish selected randomly from the tanks throughout the experimental period. Their feeding was at *ad libitum*, with husbandry conditions of 20 ºC on a 16: 8 light: dark cycle and with the tanks on a flow through, recirculating system. Environmental enrichment in the tanks was identical, comprising of artificial plants and plastic pipes of 65 mm diameter and 120 mm length for refugia.

Following euthanasia of the fish at each sampling time point, they were re-measured and weighed, with a sample of white dorsal muscle tissue excised from the anterior region

above the lateral line and below the dorsal fin, with scales (n = 5 to 10) removed from the area above the muscle sample and pelvic fin clips also taken. All samples were rinsed with distilled water, with scales also cleaned to ensure mucus and skin was removed and samples were then oven dried at 60 °C to constant mass. Following their drying, the fish tissues and diet samples were submitted to the Cornell University Stable Isotope Laboratory, New York, USA, for analysis (*cf*. Section 2.3.2.1 for details). The $\delta^{13}C$ and $\delta^{15}N$ data were provided per mille (‰) and were used subsequently in models to determine isotopic turnover rates.

7.3.2. Time-based modelling of stable isotope turnover rates

The time-based model estimated the stable isotope turnover rates in each of the different *B. barbus* tissues via modelling changes in $\delta^{13}C$ and $\delta^{15}N$ as an exponential function of time following the diet-switch, as described by Hobson and Clark (1992):

$$(1)\ \delta t = (\delta i - \delta f)\, e^{\,c\,t} + \delta f$$

where δt is the $\delta^{13}C$ or $\delta^{15}N$ value of fish at experimental time t, δf is the expected isotopic value for *B. barbus* in equilibrium with the new diet, δi is the initial $\delta^{13}C$ or $\delta^{15}N$ prior to the diet-switch, and c is the turnover constant. δf was estimated using non-linear regression. The mean $\delta^{13}C$ and $\delta^{15}N$ of the 6 fish collected before the diet-switch were used as the estimate of δi in the model; c was derived by fitting the exponential model in Eq. (1) to match the observed isotopic data, i.e. using the experimental time (t) as the independent variable and the corresponding $\delta^{13}C$ or $\delta^{15}N$ values of fish at time t (δt) as the dependent variable. The time period needed to achieve a 50 % turnover (half-life, $T_{0.5}$) of $\delta^{13}C$ or $\delta^{15}N$ was calculated as (Hobson and Clark, 1992):

$$(2)\ T_{0.5} = \ln (0.5) / c$$

To allow the relative contributions of growth and metabolism to stable isotope turnover to be separated, a second time-based model was also used, as described by Hesslein *et al*. (1993):

$$(3)\ \delta t = \delta f + (\delta i - \delta f)\, e^{\,-(k+m)\,t}$$

where δt, t, δf and δi are as previously defined in Eq. (1). m is the metabolic turnover constant derived by fitting the exponential model in Eq. (3) to match the observed isotopic data, i.e. using the experimental time (t) as the independent variable, and the

corresponding $\delta^{13}C$ or $\delta^{15}N$ values of fish at time t (δt) as the dependent variable. The growth rate constant k was represented by the specific growth rate; this was determined for each individual fish from (Sun *et al*., 2012):

(4) $k = \ln (Wf / Wi) / t$

where Wi is the initial weight of *B. barbus* on Day 0, and Wf is the final weight when sampled at time t. Any turnover of $\delta^{13}C$ and $\delta^{15}N$ in excess of growth was attributable to metabolic tissue replacement (m). Expected $\delta^{13}C$ and $\delta^{15}N$ changes due to growth alone was calculated using Eq. (3), where m was set to 0 (Hesslein *et al*., 1993). The relative contributions of growth (k) and metabolism (m) were calculated as the ratio of each parameter to the sum of the two parameters ($k + m$). This calculation yielded the contributing proportions of growth (P_g) and metabolism (P_m) to the turnover of $\delta^{13}C$ and $\delta^{15}N$. The half-life ($T_{0.5}$) of tissue turnover of $\delta^{13}C$ and $\delta^{15}N$ was calculated using (Tieszen *et al*., 1983):

(5) $T_{0.5} = - \ln (0.5) / (k + m)$

7.3.3. Growth-based modelling of stable isotope turnover rates

The changes $\delta^{13}C$ and $\delta^{15}N$ caused by the diet shift were then modelled as a function of increase in mass after the diet-switch. This was initially done by adjusting the time-based equation of Hobson and Clark (1992), Eq. (1), by substituting t for the increase in mass from Day 0 (m). The growth-based model was thus represented by:

(6) $\delta m = (\delta i - \delta f) e^{-c m} + \delta f$

where, t, δf, δi and c are as previously defined in Eq. (1) and δm is $\delta^{13}C$ or $\delta^{15}N$ at mass increase m. The increase in mass required to achieve a 50 % turnover (half-life, $G_{0.5}$) of $\delta^{13}C$ or $\delta^{15}N$ was calculated as (Hobson and Clark, 1992):

(7) $G_{0.5} = \ln (0.5) / m$

Similar to the time-based modelling, a second growth-based model was then also applied to enable the relative contributions of growth and metabolism to turnover to be separated. Changes in $\delta^{13}C$ and $\delta^{15}N$ were modelled as a function of relative growth after the diet-switch. This growth-based model was represented by (Fry and Arnold, 1982):

(8) $\delta W_R = \delta f + (\delta i - \delta f) W_R^{-c}$

where δi and δf are as previously defined in Eq. (1). The relative increase in weight of *B. barbus* (W_R) was calculated as the final wet weight divided by the initial wet weight, and the variable δW_R was the measured isotopic value for a fish given its increase in weight; c was the turnover rate constant and was derived by fitting the exponential model in Eq. (8) to match the observed isotopic data, i.e. using the relative mass increase W_R as the independent variable, and the $\delta^{13}C$ or $\delta^{15}N$ values corresponding to the W_R (δW_R) as the dependent variable. In the growth-based model, if $c = -1$, growth was entirely responsible for the isotopic turnover, whereas if $c < -1$, metabolism was contributing to turnover, with more negative values representing greater contributions by metabolism. The amount of relative growth needed to achieve a 50 % turnover (half-life, $G_{0.5}$) of $\delta^{13}C$ and $\delta^{15}N$ was calculated as (Buchheister and Latour, 2010):

(9) $G_{0.5} = e^{\ln(0.5)/c}$

where the growth-based half-life ($G_{0.5}$) represents the relative amount of growth needed for a 50 % conversion between the initial and final stable isotope values. Hence, the half-lifes estimated with the growth-based model are expressed as an x-fold mass increase. The fractions of new tissue derived from growth (D_g) and from metabolism (D_m) were calculated at the midpoint between the old and new isotopic values (Witting *et al.*, 2004):

(10) $D_g = 2 (G_{0.5} - 1) / G_{0.5}$

(11) $D_m = (2 - G_{0.5}) / G_{0.5}$

7.3.4. Model fitting and selection

To determine the best-fitting models for the stable isotope data across both the growth- and time-based methods, models were assessed using an information-theoretic approach to model selection. Models either estimated δf (the value of the stable isotope when in equilibrium with the diet) via non-linear regression or used the mean $\delta^{13}C$ and $\delta^{15}N$ data of the experimental fish on Day 125, therefore assuming that equilibrium had been reached. Additionally, models were parameterised to either include or exclude a metabolic contribution to turnover to examine the relative importance of metabolism to the turnover process in each tissue. Five models were generated (i; 1-5): model A, specific turnover parameter and δf estimated (time-based Eq. 1 and growth-based Eq. 6); model B, specific turnover parameter estimated, δf obtained from data (time-based Eq. 1 and growth-based Eq. 6); model C, specific turnover parameter and δf estimated (time-based

Eq. 3 and growth-based Eq. 8); model D, specific turnover parameter estimated, δf obtained from data (time-based Eq. 3 and growth-based Eq. 8) and lastly, model E no metabolic contribution to turnover and δf estimated (i.e., for time-based Eq. 3 and growth-based Eq. 8, m was set to 0 and c was set to -1, respectively). These model formulations were fitted to each isotope–tissue combination and were initially assessed separately for the growth- and time-based methods and then across all models and both methods to determine the overall best-fitting model for each isotope-tissue combination.

Evaluations of the best model parameterisation for each isotope-tissue combination were based on Akaike's information criterion corrected for small sample sizes (AICc). Model selection was performed using the *AICcmodavg* package in the R computing program (R Development Core Team, 2013). The model with the most empirical support generated $\Delta i = 0$ (from $\Delta i = \text{AICc}i - \text{AICc}_{min}$; *cf.* Section 4.3.2). Burnham and Anderson (2002) suggest that Δi values < 2 indicate substantial support for the model, whereas values from 4 to 7 suggest considerably less support, and Δi values > 10 indicate minimal support for that model.

7.3.5. Estimating stable isotope turnover rates

In addition to the time- and growth-based turnover models, estimates for the rates of stable isotope turnover among the tissues were also predicted from equations available from the literature. These equations used temperature, body mass and growth constants in order to obtain turnover estimates with the intention to remove the requirement for undertaking diet-switch experiments. Relevant equations were identified from three studies. Firstly, Buchheister and Latour (2010) developed a generalised model for predicting the time scale of isotopic turnover from growth-based turnover parameters; the turnover rate constant c, as determined in Eq. (8) and the specific growth rate k, as determined in Eq. (4). Their equation was developed to help evaluate isotopic equilibrium assumptions of fishes in the field. Secondly, Thomas and Crowther (2014) use literature-derived turnover estimates from animal species of differing body sizes to develop a predictive tool to estimate turnover rates in tissues of other taxa that only requires the input of the species' body mass and temperature. Lastly, Vander Zanden *et al.* (2015) also collected previously published half-life estimates and examined how half-life is related to body size, testing for tissue- and taxa-varying allometric relationships. They separated vertebrate ectotherms from invertebrates and combined half-life estimates for carbon, nitrogen and

sulphur stable isotopes, generating group specific intercepts for inclusion into their equation, along with body mass. All the estimated turnover times were produced as half-lives in terms of days and therefore the outputs allowed comparison with estimates generated from the time-based models used in the Chapter.

7.3.6. Final diet-tissue discrimination factors

The final step was estimating the discrimination factors (Δ) of $\delta^{13}C$ and $\delta^{15}N$ between the experimental diet and each tissue using (Minagawa and Wada, 1984):

(12) $\Delta = \delta f - \delta d$

where δf is the average isotopic ratio of the experimental fish collected on Day 125 and δd is the $\delta^{13}C$ or $\delta^{15}N$ of the experimental diet. Additionally, in case tissues had not reached equilibrium with the diet, discrimination factors were also calculated for each tissue type, using the δf value estimated in the best-fitting model for each isotope-tissue combination, as selected from the lowest AICc values.

7.3.7. Statistical analysis

Differences in the isotopic ratios between the sampling time points in the experiment and between the three types of tissue, i.e. muscle, fin and scales, were analysed using generalised linear models (GLM), as the data were not normally distributed, with either the sampling time points or tissue types as independent variables and $\delta^{13}C$ or $\delta^{15}N$ as dependent variables. Differences in the dependent variables according to the independent variables and their significance were determined from pairwise comparisons with Bonferroni adjustments for multiple comparisons. All statistical analyses were performed with IBM SPSS Statistics (version 22.0).

7.4. Results

7.4.1. Fish growth

All *B. barbus* individuals grew during the initial 125 day control feeding period prior to the diet-switch. At the start of this initial period, mean fork lengths and weights were 79.6

± 2.8 mm and 5.5 ± 0.6 g respectively, with these increasing to 89.3 ± 4.6 mm and 8.1 ± 1.3 g, by the end. During the subsequent 125 day diet-switch period, all *B. barbus* individuals in the control and experimental groups also increased in length and weight, with their increments varying according to the timing of the removal of the fish from the tanks (Table 28).

Specific growth rates (*k*) varied across individuals and sampling time points, and between the experimental and control groups (Table 28). Overall, mean growth rates increased with time and ranged from 0.004 to 0.006 at Day 50 and Day 125, respectively, in the experimental fish. No fish decreased in mass over the experimental period. The control fish, that were maintained on the pelletized fishmeal feed for the 125 day experimental period, had significantly higher *k* values than the experimental fish at the end of their feeding period (mean *k* control fish 0.010 vs. 0.006 of the experimental fish at Day 125; ANOVA: $F_{1,11} = 26.50$, $P < 0.001$).

Table 28. Number, fork length, weight, specific growth rate (*k*, as defined in Eq. 4), and relative growth (W_R; final wet weight divided by initial wet weight) of *B. barbus* fish in the experimental (A) and control group (B) at each sampling time point after the diet-switch. Errors around the means represent standard deviations.

A)

Time point	*n*	Fork length (mm)	Weight (g)	*k*	W_R
Day 0	6	90.17 ± 6.68	7.65 ± 1.44		
Day 50	6	102.50 ± 3.73	12.20 ± 0.89	0.004 ± 0.001	1.24 ± 0.08
Day 75	6	99.83 ± 4.02	11.89 ± 1.37	0.005 ± 0.001	1.44 ± 0.13
Day 100	5	103.20 ± 4.27	11.84 ± 1.43	0.005 ± 0.001	1.53 ± 0.22
Day 125	6	111.83 ± 4.40	16.58 ± 1.87	0.006 ± 0.001	2.11 ± 0.18

B)

Time point	*n*	Fork length (mm)	Weight (g)	*k*	W_R
Day 125	6	139.67 ± 8.96	30.28 ± 6.92	0.010 ± 0.002	4.28 ± 1.02

7.4.2. Stable isotope ratios of $\delta^{13}C$ and $\delta^{15}N$

Comparison of the $\delta^{13}C$ and $\delta^{15}N$ data for the 6 *B. barbus* euthanized on Day 0 of the diet-switch experiment with the control group of 6 *B. barbus* that were maintained on the pelletized fishmeal control diet over the second 125 day feeding period revealed that their $\delta^{13}C$ and $\delta^{15}N$ data were not significantly different and so had remained relatively constant over this second feeding period (GLM; $\delta^{13}C$: Wald $\chi^2 = 410.59$, *d.f.* = 17, $P > 0.05$; $\delta^{15}N$: Wald $\chi^2 = 801.02$, *d.f.* = 17, $P > 0.05$; Table 29). The only exception was $\delta^{13}C$ in fin tissue, where the difference was 1.24 ‰, suggesting the tissue may not have been in equilibrium with the control feed at the end of the initial 125 days (Table 29; Table 30A).

Following the diet-switch to the wheatgerm based experimental feed in the second feeding period, the euthanized fish at each subsequent sampling time point revealed significant shifts in $\delta^{15}N$ when these data were compared to the fish at Day 0 (Table 29). The only exception was fin tissue, where a significant shift was not apparent until Day 75 (Table 29; Table 30A; GLM: Wald $\chi^2 = 801.02$, *d.f.* = 17, $P < 0.05$). For $\delta^{13}C$, however, significant shifts in stable isotope ratios relative to the composition at Day 0 were only apparent on Day 125 for scales, whereas for dorsal muscle and fin tissue, no significant shifts were apparent (Table 29, Table 30A). After the diet-switch, the observed stable isotope values of the three fish tissues showed an overall increase in $\delta^{13}C$ and reduction in $\delta^{15}N$. There was a consistent hierarchy of muscle having the highest $\delta^{15}N$ values and scales the lowest; for $\delta^{13}C$, the opposite occurred, with scales having the highest $\delta^{15}N$ values and muscle the lowest (Table 29). The lack of significant changes in $\delta^{13}C$ of fin tissues throughout the experiment, coupled with the presence of a significant difference between stable isotope ratios on Day 0 and the control group on Day 125 indicate unreliable data, and thus $\delta^{13}C$ for fin tissue was not analysed further (Table 30A).

Table 29. Mean stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values of the control and experimental diets and tissues of *B. barbus* fish in the experimental and control groups at each sampling time point.; *significantly different from Day 0 at $P < 0.05$, according to pairwise comparisons from generalised linear models where comparisons have undergone Bonferroni adjustments for multiple comparisons. Errors around the means represent standard errors.

Tissue	Time point	$\delta^{13}C$	$\delta^{15}N$
Control diet		-23.19 ± 0.11	9.34 ± 0.05
Experimental diet		-25.35 ± 0.08	3.28 ± 0.02
Muscle	Day 0	-20.90 ± 0.13	12.28 ± 0.10
	Day 50	-20.75 ± 0.13	11.13 ± 0.10*
	Day 75	-21.08 ± 0.13	10.55 ± 0.10*
	Day 100	-20.99 ± 0.15	10.38 ± 0.10*
	Day 125	-20.34 ± 0.13	9.63 ± 0.10*
	Control Day 125	-21.16 ± 0.13	11.89 ± 0.10
Fin	Day 0	-19.14 ± 0.22	11.37 ± 0.16
	Day 50	-19.03 ± 0.22	10.87 ± 0.16
	Day 75	-19.04 ± 0.22	10.21 ± 0.16*
	Day 100	-19.58 ± 0.24	10.16 ± 0.18*
	Day 125	-19.40 ± 0.22	9.32 ± 0.16*
	Control Day 125	-20.38 ± 0.22*	11.75 ± 0.16
Scales	Day 0	-19.46 ± 0.18	11.21 ± 0.11
	Day 50	-19.13 ± 0.18	10.35 ± 0.11*
	Day 75	-19.05 ± 0.18	10.04 ± 0.11*
	Day 100	-18.79 ± 0.20	9.91 ± 0.12*
	Day 125	-18.41 ± 0.18*	9.33 ± 0.11*
	Control Day 125	-19.37 ± 0.18	11.36 ± 0.11

Table 30. Differences in stable isotope ($\delta^{13}C$ and $\delta^{15}N$) values between sampling time points per fish tissue (A) and between fish tissues per sampling time point (B) of *B. barbus* fish in the experimental and control groups. Differences were calculated according to pairwise comparisons from generalised linear models where comparisons have undergone Bonferroni adjustments for multiple comparisons; *difference is significant at $P < 0.05$. Errors around the means represent standard errors.

A)

Stable isotope	Time point comparison	Muscle	Fin	Scales
$\delta^{13}C$	Day 0 vs. Day 50	-0.15 ± 0.25	-0.11 ± 0.25	-0.33 ± 0.25
	Day 0 vs. Day 75	0.19 ± 0.25	-0.10 ± 0.25	-0.41 ± 0.25
	Day 0 vs. Day 100	0.09 ± 0.27	0.44 ± 0.27	-0.67 ± 0.27
	Day 0 vs. Day 125	-0.56 ± 0.25	0.26 ± 0.25	-1.04 ± 0.25*
	Day 0 vs. Control Day 125	0.26 ± 0.25	1.24 ± 0.25*	-0.09 ± 0.25
$\delta^{15}N$	Day 0 vs. Day 50	1.15 ± 0.18*	0.50 ± 0.18	0.86 ± 0.18*
	Day 0 vs. Day 75	1.73 ± 0.18*	1.16 ± 0.18*	1.17 ± 0.18*
	Day 0 vs. Day 100	1.90 ± 0.19*	1.21 ± 0.19*	1.30 ± 0.19*
	Day 0 vs. Day 125	2.65 ± 0.18*	2.05 ± 0.18*	1.88 ± 0.18*
	Day 0 vs. Control Day 125	0.39 ± 0.18	-0.39 ± 0.18	-0.15 ± 0.18

Table 30 *continued*

B)

Stable isotope	Tissue comparison	Day 0	Day 50	Day 75	Day 100	Day 125	Control Day 125
$\delta^{13}C$	Muscle vs. Fin	-1.76 ± 0.25*	-1.72 ± 0.25*	-2.04 ± 0.25*	-1.41 ± 0.28*	-0.94 ± 0.25*	-0.78 ± 0.25
	Fin vs. Scales	0.31 ± 0.25	0.10 ± 0.25	0.01 ± 0.25	-0.79 ± 0.28	-0.99 ± 0.25*	-1.01 ± 0.25
	Scales vs. Muscle	1.44 ± 0.25*	1.62 ± 0.25*	2.04 ± 0.25*	2.20 ± 0.28*	1.93 ± 0.25*	1.79 ± 0.25*
$\delta^{15}N$	Muscle vs. Fin	0.91 ± 0.18*	0.26 ± 0.18	0.34 ± 0.18	0.21 ± 0.20	0.31 ± 0.18	0.14 ± 0.18
	Fin vs. Scales	0.16 ± 0.18	0.52 ± 0.18	0.17 ± 0.18	0.25 ± 0.20	-0.01 ± 0.18	0.40 ± 0.18
	Scales vs. Muscle	-1.07 ± 0.18*	-0.78 ± 0.18*	-0.51 ± 0.18	-0.46 ± 0.20	-0.30 ± 0.18	-0.53 ± 0.18

7.4.3. Modelling stable isotope turnover

7.4.3.1. Time-based methods

For model A, estimates of parameter *c* and δ*f*, obtained through non-linear regression, explained more of the variation in the model for $\delta^{15}N$ than $\delta^{13}C$, with R^2 values ranging from 0.69 for scales to 0.92 for muscle versus 0.46 for scales and 0.58 for muscle (Table 31). The δ*f* estimates for $\delta^{15}N$ and $\delta^{13}C$ were not achieved within the experimental period, indicating that equilibrium had not been reached (Fig. 18). It should be noted that the δ*f* values for $\delta^{15}N$ and $\delta^{13}C$ used here were estimated in the growth-based version of model A due to model fitting difficulties when running the time-based version. In model B, where only parameter *c* was estimated through non-linear regression and δ*f* was the average of the stable isotope values of the experimental group at Day 125 (assuming that equilibrium had been reached by the tissues), the model explained similar levels of variation when compared to model A for $\delta^{13}C$, but slightly less for $\delta^{15}N$ (Table 31) and estimates for the turnover rate constant *c* were higher than in model A.

In model C, estimated contributions to turnover from growth and metabolism were separated, revealing differences between the tissues. Across both isotopes and all tissues, growth contributed more to turnover than metabolism (Table 31). Additionally, for $\delta^{15}N$ and $\delta^{13}C$, there was a clear reduction in the contribution to turnover from metabolism within the tissues, from muscle to fin to scales, but with $\delta^{15}N$ having relatively higher contributions from metabolism to turnover than $\delta^{13}C$ (Table 31; Fig. 19). For scale $\delta^{13}C$, metabolism was estimated to not contribute to turnover. Estimates of parameters *m* and δ*f* explained a high proportion of the variation within the model for $\delta^{15}N$, with R^2 ranging from 0.80 for scales to 0.94 for muscle, compared to 0.49 for scales and 0.58 for muscle for $\delta^{13}C$ (Table 31). The δ*f* values estimated for both $\delta^{13}C$ and $\delta^{15}N$ were not achieved, indicating that equilibrium had not been reached (Fig. 19).

For model D, where only parameter *m* was estimated through non-linear regression and δ*f* was the average of the stable isotope values of the experimental group at Day 125 (assuming that equilibrium had been reached by the tissues), differences between the relative contributions of growth and metabolism to turnover between the tissues were distinct. Across both isotopes and all tissues and in contrast to model C, metabolism contributed more to turnover than growth (Table 31). Patterns for $\delta^{15}N$ were similar to

model C, with a reduction in the contribution of metabolism to turnover from muscle to fin to scales (Table 31). However, the differences between the tissues were much reduced in this model, with contributions to turnover ranging from 72 % for muscle to 67 % for scales, versus 42 % for muscle to 0.6 % for scales in model C (Table 31). For $\delta^{13}C$, scales had a higher contribution from metabolism to turnover than muscle and across all tissues and isotopes estimates for the metabolic constant *m* were higher than model C.

Lastly, in model E, where there was no contribution of metabolism to turnover and δf was estimated through non-linear regression, more variation was again explained by the model for $\delta^{15}N$ than $\delta^{13}C$, with R^2 ranging from 0.80 for scales to 0.89 for muscle, versus 0.49 for scales and 0.58 for muscle. The δf values estimated for both $\delta^{13}C$ and $\delta^{15}N$ indicate that isotopic equilibrium had not been reached (Table 31).

Table 31. Parameter estimates and calculations from time-based methods of models A to E of stable isotope turnover in *B. barbus* tissues. *c*, turnover rate constant; *m*, metabolic constant; *k*, growth constant; *Pm*, relative contributions of metabolism to turnover; *Pg*, relative contributions of growth to turnover; $T_{0.5}$, half-life (days); δf, estimated equilibrium value; *estimate was obtained from the growth-based method of the corresponding model (Table 32). Errors around the means represent standard errors.

Model	Stable isotope	Tissue	Parameter estimate				R^2	δf (‰)			$T_{0.5}$
A	^{13}C	Muscle	*c*	-0.005	±	0.001	0.58	-19.74	*		138.29
		Scales	*c*	-0.008	±	0.002	0.46	-18.06	*		90.73
	^{15}N	Muscle	*c*	-0.012	±	0.001	0.92	9.25	*		56.80
		Fin	*c*	-0.011	±	0.001	0.83	8.89	*		61.75
		Scales	*c*	-0.009	±	0.001	0.69	8.78	*		80.49
B	^{13}C	Muscle	*c*	-0.011	±	0.002	0.55	-20.27	±	0.04	63.81
		Scales	*c*	-0.012	±	0.004	0.45	-18.41	±	0.13	56.09
	^{15}N	Muscle	*c*	-0.015	±	0.001	0.86	9.63	±	0.13	45.05
		Fin	*c*	-0.015	±	0.001	0.76	9.32	±	0.10	47.55
		Scales	*c*	-0.013	±	0.002	0.65	9.33	±	0.09	51.64

Table 31 *continued*

Model	Stable isotope	Tissue	Parameter estimate				R^2	$k + m$			P_m			P_g			δf (‰)			$T_{0.5}$		
C	^{13}C	Muscle	*m*	0.001	±	0.003	0.58	0.005	±	0.0005	0.15	±	0.01	0.85	±	0.01	-19.93	±	0.39	122.41	±	6.55
		Scales	*m*	0.000	±	0.004	0.49	0.004	±	0.0005	0.00	±	0.00	1.00	±	0.00	-17.64	±	0.87	145.38	±	9.72
	^{15}N	Muscle	*m*	0.003	±	0.001	0.94	0.008	±	0.0004	0.42	±	0.01	0.58	±	0.01	8.44	±	0.34	84.25	±	2.84
		Fin	*m*	0.003	±	0.002	0.84	0.007	±	0.0004	0.37	±	0.01	0.63	±	0.01	8.07	±	0.57	91.89	±	3.42
		Scales	*m*	0.000	±	0.001	0.80	0.005	±	0.0004	0.06	±	0.00	0.94	±	0.00	7.87	±	0.53	139.61	±	8.51
D	^{13}C	Muscle	*m*	0.006	±	0.002	0.56	0.010	±	0.0005	0.62	±	0.04	0.38	±	0.04	-20.27	±	0.04	78.19	±	5.06
		Scales	*m*	0.007	±	0.003	0.47	0.011	±	0.0005	0.67	±	0.04	0.33	±	0.04	-18.41	±	0.13	65.65	±	3.46
	^{15}N	Muscle	*m*	0.010	±	0.001	0.88	0.015	±	0.0004	0.72	±	0.02	0.28	±	0.02	9.63	±	0.13	48.18	±	1.59
		Fin	*m*	0.009	±	0.001	0.79	0.014	±	0.0004	0.70	±	0.02	0.30	±	0.02	9.32	±	0.10	51.20	±	1.82
		Scales	*m*	0.008	±	0.002	0.70	0.013	±	0.0004	0.67	±	0.03	0.33	±	0.03	9.33	±	0.09	57.04	±	2.31
E	^{13}C	Muscle	*m*	0.000			0.58	0.005	±	0.0003	0.00			1.00			-19.82	±	0.12	145.38	±	9.72
		Scales	*m*	0.000			0.49	0.004	±	0.0005	0.00			1.00			-17.64	±	0.24	145.38	±	9.72
	^{15}N	Muscle	*m*	0.000			0.89	0.004	±	0.0004	0.00			1.00			6.86	±	0.17	145.38	±	9.72
		Fin	*m*	0.000			0.81	0.004	±	0.0004	0.00			1.00			6.75	±	0.23	145.38	±	9.72
		Scales	*m*	0.000			0.80	0.004	±	0.0004	0.00			1.00			7.74	±	0.16	145.38	±	9.72

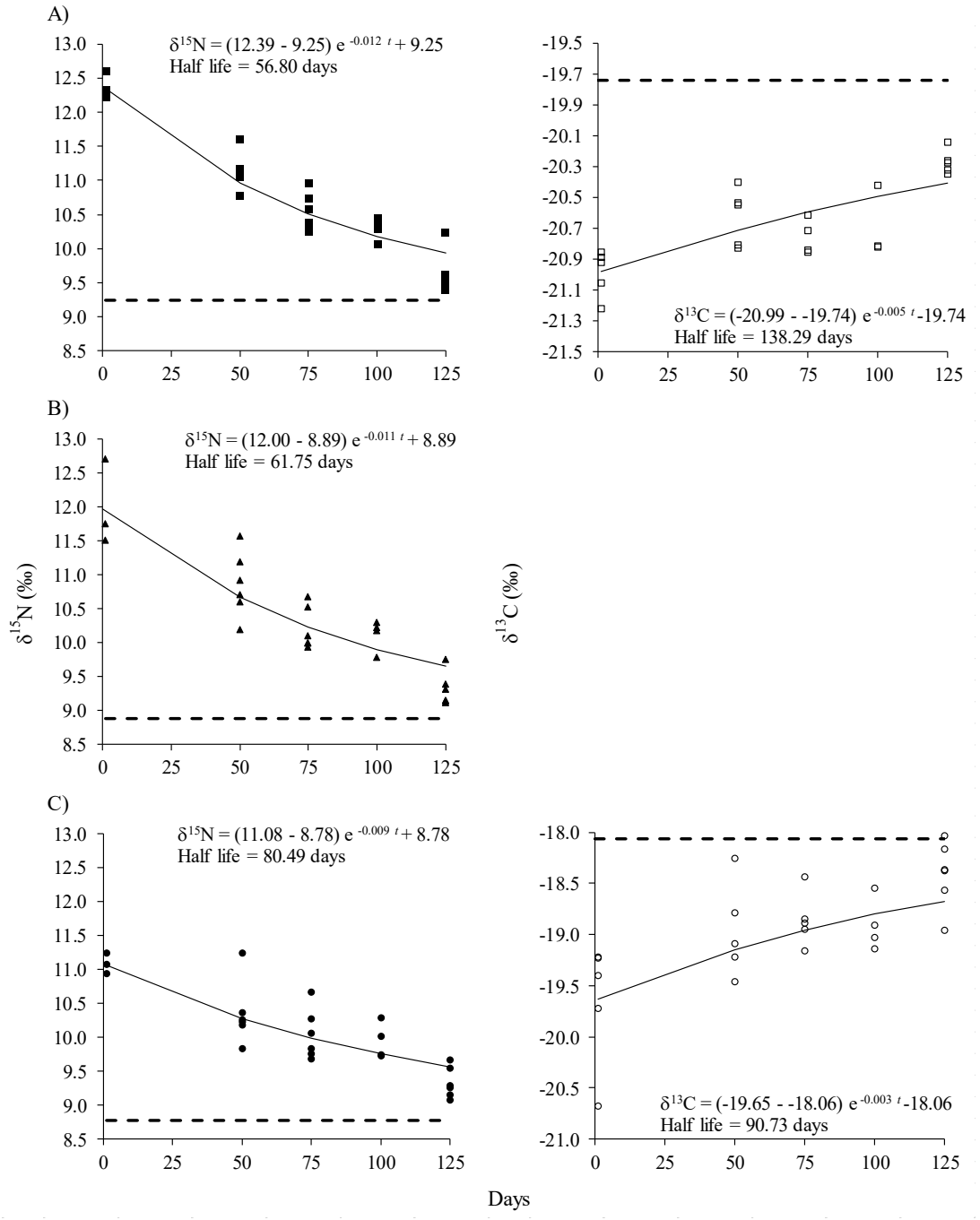

Figure 18. Changes in *B. barbus* stable isotope ($\delta^{15}N$: filled symbols and $\delta^{13}C$: open symbols) values estimated from the time-based method of model A for muscle (A), fin (B), and scales (C). The horizontal dashed lines represent the expected final isotopic values of the tissues in equilibrium with experimental feed (δf).

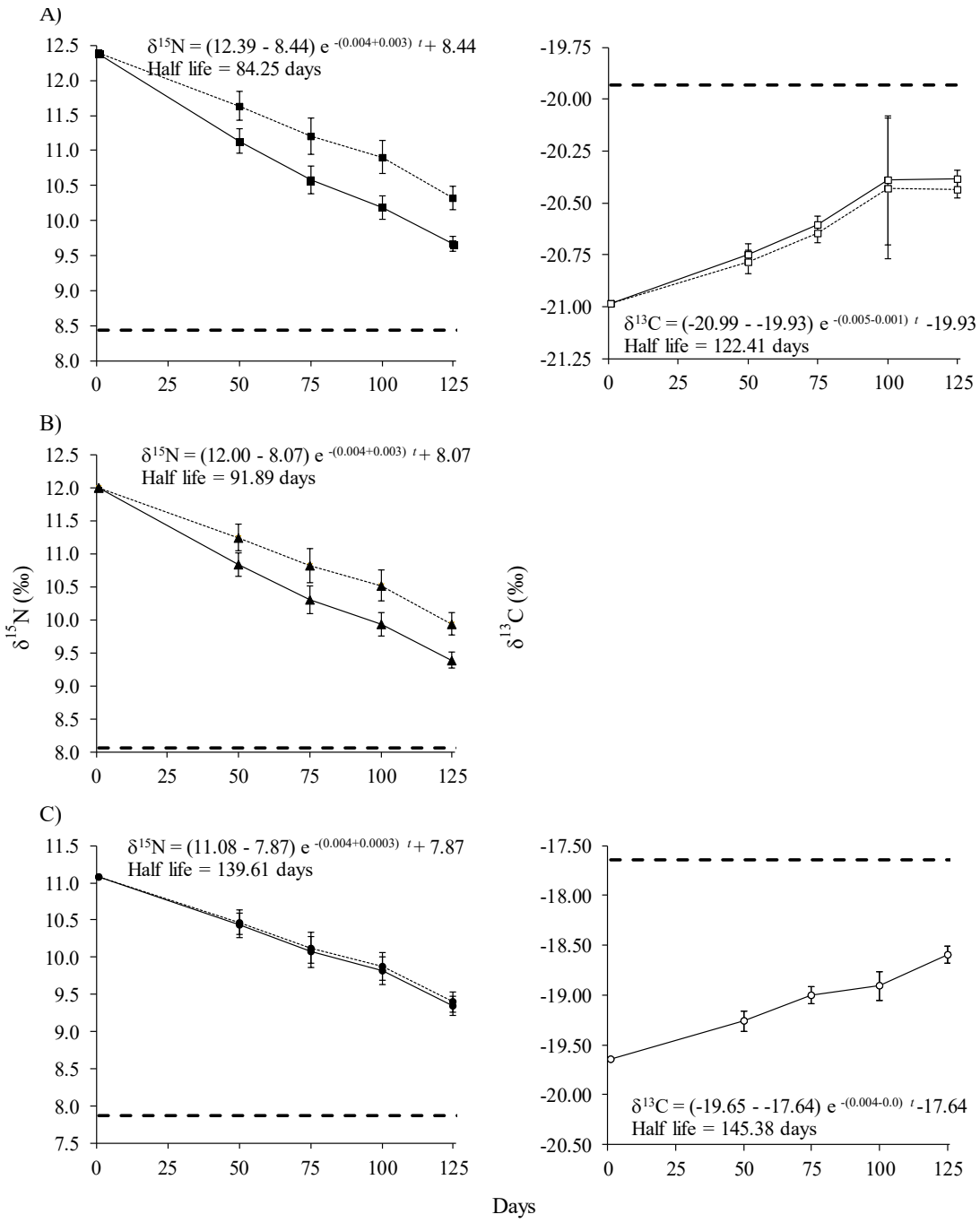

Figure 19. Changes in *B. barbus* stable isotope ($\delta^{15}N$: filled symbols and $\delta^{13}C$: open symbols) values estimated from the time-based method of model C for muscle (A), fin (B), and scales (C). Solid lines represent the isotopic values with the contribution of growth and metabolism, and dotted lines are the isotopic values with the contribution of growth alone ($m = 0$). The horizontal dashed lines represent the expected final isotopic values of the tissues in equilibrium with experimental feed (δf). Error bars represent standard deviations.

7.4.3.2. Growth-based methods

Similar to the time-based version of the model, estimates of parameter *c* and δ*f* in model A explained more of the variation in $\delta^{15}N$ than $\delta^{13}C$ with R^2 ranging from 0.79 for scales to 0.91 for muscle, compared to 0.58 for scales and 0.62 for muscle (Table 32). The δ*f* values for $\delta^{15}N$ and $\delta^{13}C$ were not achieved by Day 125, indicating that isotopic equilibrium had not been reached (Fig. 20). In model B, where only parameter *m* was estimated through non-linear regression and δ*f* was the average of the stable isotope values of the experimental group at Day 125 (assuming that equilibrium had been reached by the tissues), similar levels of variation were explained when compared to model A for $\delta^{13}C$, but were slightly less for $\delta^{15}N$ (Table 32). Estimates for the turnover rate constant *m* were also higher than in model A.

In model C, where estimated contributions from growth and metabolism were separated, a similar pattern to the time-based version of the model was revealed. For $\delta^{15}N$, there was again a reduction in the contribution to turnover from metabolism from muscle to fin to scales, but for $\delta^{13}C$ contributions from growth and metabolism for muscle and scales were very similar, with both showing minimal contributions of metabolism to turnover (Table 32). Estimates of turnover rate constant *c* and δ*f* explained a high proportion of the variation for $\delta^{15}N$, with R^2 ranging from 0.79 for scales to 0.91 for muscle, versus 0.49 and 0.59 for $\delta^{13}C$ (Table 32). The δ*f* values estimated for $\delta^{15}N$ were similar to those generated in time-based version of model C and revealed isotopic equilibrium had not been reached (Fig. 21). It should be noted that the δ*f* values for $\delta^{13}C$ used here were estimated in the time-based version of model C due to model fitting difficulties when running this growth-based version of the model.

In model D, where only parameter *c* was estimated through non-linear regression and δ*f* was the average of the stable isotope values of the experimental group at Day 125 (assuming that equilibrium had been reached), there were differences between the relative contributions of growth and metabolism to turnover between the tissues. In contrast to model C, metabolism contributed more to turnover than growth for $\delta^{15}N$ and there was an almost even contribution from growth and metabolism to turnover for $\delta^{13}C$ in both muscle and scales (Table 32). Patterns for $\delta^{15}N$ were similar to model C, with a reduction in the contribution of metabolism to turnover from muscle to fin to scales, but with differences between the tissues much reduced, with values ranging from 61 % for muscle to 55 % for scales, compared to 44 % for muscle to 0.8 % for scales in model C. Across

all tissues and isotopes, estimates for the turnover rate constant *c* were lower than model C (Table 32).

Finally, in model E, where there was no contribution of metabolism to turnover and δ*f* was estimated through non-linear regression, similarly to all proceeding models, more variation was explained in the model for $\delta^{15}N$ than $\delta^{13}C$ with R^2 values ranging from 0.88 for muscle to 0.79 for scales, versus 0.57 for muscle to 0.49 for scales for $\delta^{13}C$. The δ*f* values estimated for both $\delta^{13}C$ and $\delta^{15}N$ were not achieved by Day 125, indicating that equilibrium had not been reached (Table 32).

Table 32. Parameter estimates and calculations from growth-based methods of models A to E of stable isotope turnover in B. *barbus* tissues. *m* and *c*, turnover rate constants; *Dm*, relative contribution of metabolism to turnover; *Dg*, relative contribution of growth to turnover; $G_{0.5}$, half-life; δ*f*, estimated equilibrium value. Growth-based half-lives of models B, D and E are expressed as an x-fold increase in body mass (x BM); *estimate was obtained from the time-based method of the corresponding model (Table 31). Errors around the means represent standard errors.

Model	Stable isotope	Tissue	Parameter estimate				R^2	δ*f* (‰)			$G_{0.5}$	
A	^{13}C	Muscle	*m*	-0.08	±	0.09	0.62	-19.74	±	0.96	8.24	g
		Scales	*m*	-0.19	±	0.11	0.58	-18.06	±	0.52	3.66	g
	^{15}N	Muscle	*m*	-0.24	±	0.04	0.91	9.25	±	0.24	2.89	g
		Fin	*m*	-0.22	±	0.06	0.81	8.89	±	0.41	3.19	g
		Scales	*m*	-0.16	±	0.05	0.79	8.78	±	0.45	4.25	g
B	^{13}C	Muscle	*m*	-0.19	±	0.04	0.60	-20.27	±	0.04	3.60	g
		Scales	*m*	-0.24	±	0.07	0.50	-18.41	±	0.13	2.93	g
	^{15}N	Muscle	*m*	-0.31	±	0.02	0.78	9.63	±	0.13	2.25	g
		Fin	*m*	-0.29	±	0.03	0.67	9.32	±	0.10	2.40	g
		Scales	*m*	-0.26	±	0.03	0.62	9.33	±	0.09	2.64	g

Table 32 *continued*

Model	Stable isotope	Tissue	Parameter estimate			R^2	D_m	D_g	δf (‰)			$G_{0.5}$	
C	^{13}C	Muscle	*c*	-1.13	± 0.17	0.59	0.08	0.92	-19.93	*		1.84	x BM
		Scales	*c*	-1.00	± 0.19	0.49	0.00	1.00	-17.64	*		2.00	x BM
	^{15}N	Muscle	*c*	-2.13	± 0.43	0.91	0.44	0.56	8.96	±	0.38	1.39	x BM
		Fin	*c*	-1.81	± 0.62	0.82	0.36	0.64	8.45	±	0.72	1.47	x BM
		Scales	*c*	-1.13	± 0.64	0.79	0.08	0.92	8.02	±	1.23	1.85	x BM
D	^{13}C	Muscle	*c*	-2.02	± 0.40	0.54	0.42	0.58	-20.27	±	0.04	1.41	x BM
		Scales	*c*	-2.47	± 0.74	0.48	0.51	0.49	-18.41	±	0.13	1.32	x BM
	^{15}N	Muscle	*c*	-3.20	± 0.23	0.89	0.61	0.39	9.63	±	0.13	1.24	x BM
		Fin	*c*	-3.00	± 0.31	0.80	0.59	0.41	9.32	±	0.10	1.26	x BM
		Scales	*c*	-2.73	± 0.32	0.74	0.55	0.45	9.33	±	0.09	1.29	x BM
E	^{13}C	Muscle	*c*	-1.00		0.57	0.00	1.00	-19.82	±	0.12	2.00	x BM
		Scales	*c*	-1.00		0.49	0.00	1.00	-17.64	±	0.24	2.00	x BM
	^{15}N	Muscle	*c*	-1.00		0.88	0.00	1.00	6.87	±	0.17	2.00	x BM
		Fin	*c*	-1.00		0.81	0.00	1.00	6.76	±	0.23	2.00	x BM
		Scales	*c*	-1.00		0.79	0.00	1.00	7.75	±	0.16	2.00	x BM

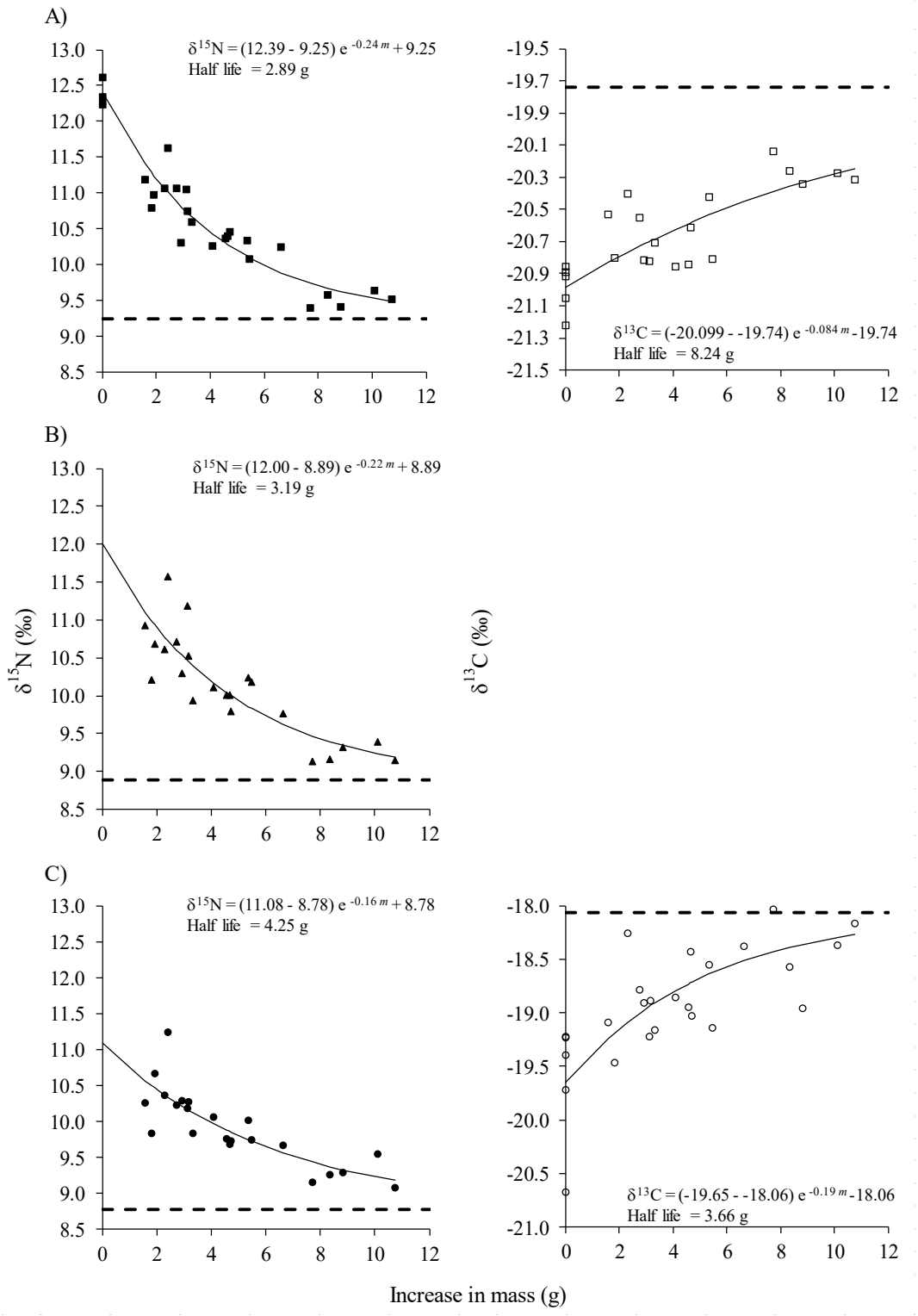

Figure 20. Changes in *B. barbus* stable isotope ($\delta^{15}N$: filled symbols and $\delta^{13}C$: open symbols) values estimated from the growth-based method of model A for muscle (A), fin (B), and scales (C). The horizontal dashed lines represent the expected final isotopic values of the tissues in equilibrium with experimental feed (δf).

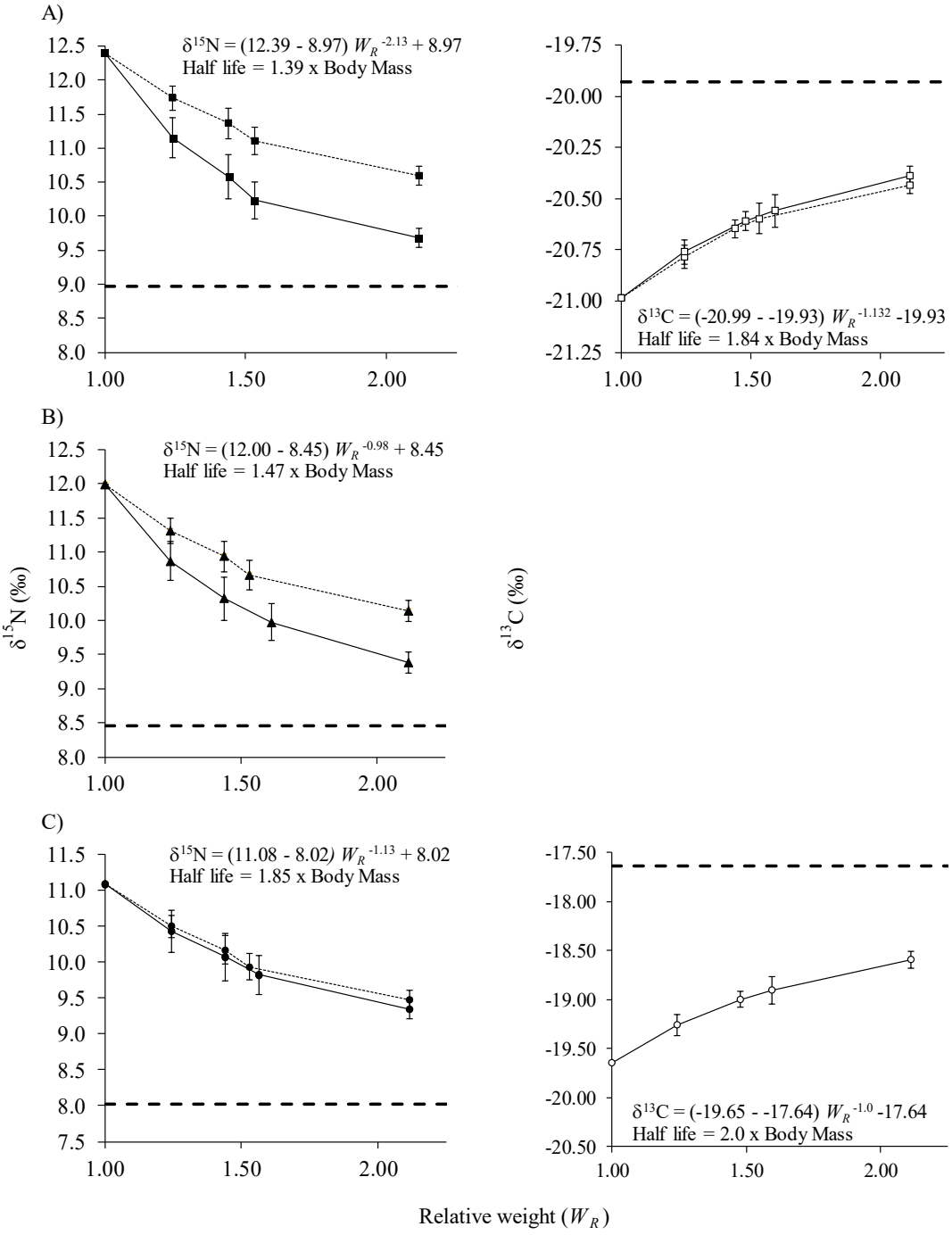

Figure 21. Changes in *B. barbus* stable isotope ($\delta^{15}N$: filled symbols and $\delta^{13}C$: open symbols) values estimated from the growth-based method of model C for muscle (A), fin (B), and scales (C). Solid lines represent the isotopic values with the contribution of growth and metabolism, and dotted lines are the isotopic values with the contribution of growth alone (c = -1). The horizontal dashed lines represent the expected final isotopic values of the tissues in equilibrium with experimental feed (δf). Error bars represent standard deviations.

7.4.4. Fitting the stable isotope turnover models

The best-fitting models, as indicated by having the lowest AICc values, varied within and between each method (growth- or time-based), isotope and tissue. For time-based modelling, the best-fitting models varied between the isotope–tissue combinations. For $\delta^{13}C$, muscle and scales were best described by model A, for $\delta^{15}N$, muscle and fin were best described by model C and scale $\delta^{15}N$ turnover was best described by model E (Table 33A). Within the growth-based modelling of $\delta^{13}C$, muscle and scales were best described by model B, fin and scales by model E, and muscle $\delta^{15}N$ turnover by model B (Table 33A). Only muscle and scales for $\delta^{15}N$ were best described by the same model when analysed separately for time- and growth-based methods (Table 33B). When model fitting was compared across all models and both methods, $\delta^{13}C$ was always best described by growth-based models and $\delta^{15}N$ by time-based models. In no cases was model D selected as the best-fit. Additionally, there was variability within the model Δi values for both carbon and nitrogen stable isotopes, with there being substantial support ($\Delta i < 2$) for almost all models for $\delta^{13}C$ across both time- and growth-based methods, in contrast to $\delta^{15}N$, where most models had considerably less or minimal support when compared to the best-fitting model (Table 33).

Table 33. Comparisons of Δi among models A to E, where; the equilibrium value (δf) was either estimated (model A, B and E); or obtained from the experimental Day 125 data (model C and D); separation of the relative contributions of metabolism and growth to turnover was allowed (model B and D); or had no contribution of metabolism to turnover (model E). Models were fitted using the time- and growth-based methods for each isotope and tissue and are compared across time and growth-based methods separately (A) and combined (B). The best-fitting models are identified in bold face, models with $\Delta i < 2$ have considerable support and are underlined (*cf*. Section 7.3.4 for full model descriptions).

A)		ΔAICc									
Stable		Time-based method					Growth-based method				
isotope	Tissue	Model A	Model B	Model C	Model D	Model E	Model A	Model B	Model C	Model D	Model E
$\delta^{13}C$	Muscle	**0.00**	1.53	2.68	1.13	0.05	1.40	**0.00**	1.23	2.60	1.06
	Scales	**0.00**	0.43	0.65	0.03	0.65	0.18	**0.00**	1.97	1.53	1.97
$\delta^{15}N$	Muscle	12.63	17.82	**0.00**	12.44	12.09	0.80	1.52	**0.00**	2.77	4.93
	Fin	4.28	7.36	**0.00**	4.43	2.11	1.98	1.04	0.67	1.22	**0.00**
	Scales	10.12	12.90	2.53	9.19	**0.00**	2.57	3.04	2.55	4.90	**0.00**
B)		ΔAICc									
Stable		Time-based method					Growth-based method				
isotope	Tissue	Model A	Model B	Model C	Model D	Model E	Model A	Model B	Model C	Model D	Model E
$\delta^{13}C$	Muscle	1.05	2.58	3.73	2.18	1.10	1.40	**0.00**	1.23	2.60	1.06
	Scales	1.43	1.86	2.08	1.46	2.08	0.18	**0.00**	1.97	1.53	1.97
$\delta^{15}N$	Muscle	12.63	17.82	**0.00**	12.44	12.09	7.96	8.68	7.16	9.94	12.09
	Fin	4.28	7.36	**0.00**	4.43	2.11	4.09	3.15	2.78	3.33	2.11
	Scales	10.12	12.90	2.53	9.19	**0.00**	2.57	3.04	2.55	4.90	**0.00**

7.4.5. Estimated stable isotope turnover rates

Half-life estimates varied considerably and were slightly more wide-ranging across ^{13}C than ^{15}N, from 37 to 159 days for ^{13}C, compared to 40 to 141 days for ^{15}N (Table 34). In comparison to the turnover estimates produced from the best-fitting models across the time-based methods, for ^{13}C, Buchheister and Latour's (2010) equation was the closest estimate for the half-life of muscle tissue; 141 days compared to 138 in model A and for scales Vander Zanden *et al.*'s (2015) equation was closest; 61 days compared to 91 in model A. For half-life estimates of ^{15}N, Buchheister and Latour's (2010) equation was closest for all three tissues, estimating half-lives within 10 days of those estimated from the best-fitting models (Table 34).

7.4.6. Diet-tissue discrimination factors

The diet-tissue discrimination factors calculated for ^{13}C, where δf was the average stable isotope value of the experimental fish at Day 125 (assuming equilibrium had been reached), produced discrimination factors ranging from 5.01 ‰ for muscle to 6.94 ‰ for scales (Table 35B). For ^{15}N, isotopic discriminations were higher than ^{13}C for muscle and fin, but lower for scales and the differentiation between the tissue types was also narrower for ^{15}N, with discrimination factors ranging from 6.04 ‰ for fin to 6.35 ‰ for muscle. The isotopic discrimination for ^{15}N calculated using δf values from the best-fitting models, as identified by having the lowest Δi (Table 33), were lower than those produced from the experimental data, however, the differentiation between the tissues was increased; 2.48 ‰ for scales, 4.79 ‰ for fin and 5.16 ‰ for muscle (Table 35B). As the best-fitting model for ^{13}C for both muscle and scales was model B, where the δf values were taken from the experimental fish on Day 125, the isotopic discriminations are the same as for the experimental data.

Table 34. Estimates of carbon and nitrogen stable isotope turnover rates, as half-lives ($T_{0.5}$), generated from equations. Time-based methods produce half lifes in days (d^{-1}) and growth-based methods as a gain in mass (g) or as an x-fold increase in body mass (x BM). Body temperature (BT), body mass (BM) and growth constants are used within the equations. Values in bold are from the best-fitting models ($\Delta i = 0$; Table 33); *mass value converted from x BM to grams (g) from the initial weights of the fish that underwent the diet-switch. Errors around the means represent standard errors.

Method	Reference	Equation	Tissue	^{13}C $T_{0.5}$				^{15}N $T_{0.5}$			
Time-based	Hobson & Clark (1992)	$\delta t = (\delta i - \delta f)\ e^{c\,t} + \delta f$	Muscle	**138.29**			d^{-1}	56.80			d^{-1}
			Fin					61.75			d^{-1}
			Scales	**90.73**			d^{-1}	80.49			d^{-1}
	Hesslein *et al.* (1993)	$\delta t = (\delta i - \delta f)\ e^{-(k+m)t} + \delta f$	Muscle	122.41	±	6.55	d^{-1}	**84.25**	±	**2.84**	d^{-1}
			Fin					**91.89**	±	**3.42**	d^{-1}
			Scales	145.38	±	9.72	d^{-1}	**145.38**	±	**9.72**	d^{-1}
	Thomas & Crowther (2014)	$\log_{10}(^{13}C\ T_{0.5}) = 1.6668 + 0.1935 * \log_{10} BM + -0.0153 * BT$	Muscle	37.03	±	0.20	d^{-1}	39.55	±	0.22	d^{-1}
		$\log_{10}(^{15}N\ T_{0.5}) = 1.6884 + 0.1933 * \log_{10} BM + -0.0149 * BT$									
	Vander Zanden *et al.* (2015)	Vertebrate ectotherm muscle: $\ln(T_{0.5}) = 0.22 * \ln(BM) + 3.28$	Muscle	42.26	±	0.27	d^{-1}	42.26	±	0.27	d^{-1}
		Vertebrate ectotherm whole body: $\ln(T_{0.5}) = 0.22 * \ln(BM) + 3.65$	Muscle	61.18	±	0.38	d^{-1}	61.18	±	0.38	d^{-1}
	Buchheister & Latour (2010)	$T_{0.5} = \ln(50 / 100) / k\,c$	Muscle	140.82	±	12.16	d^{-1}	75.02	±	6.48	d^{-1}
			Fin					88.22	±	7.62	d^{-1}
			Scales	159.44	±	13.76	d^{-1}	141.38	±	12.21	d^{-1}

Table 34 *continued*

Method	Reference	Equation	Tissue	^{13}C $G_{0.5}$			^{15}N $G_{0.5}$			
Growth-based	Hobson & Clark (1992)	$\delta t = (\delta i - \delta f)\ e^{c\ m} + \delta f$	Muscle	**3.60**		g	2.89			g
			Fin				3.19			g
			Scales	**2.93**		g	4.25			g
	Fry & Arnold (1982)	$\delta W_R = (\delta i - \delta f)\ W_R^{c} + \delta f$	Muscle	1.84		x BM	1.39			x BM
			Fin				1.47			x BM
			Scales	2.00		x BM	**2.00***			x BM
			Muscle	6.83	± 0.18	g	3.17	±	0.08	g
			Fin				3.82	±	0.10	g
			Scales	8.13	± 0.22	g	**8.13***	**±**	**0.22**	g

Table 35. Stable isotope equilibrium values (δ*f*) of *B. barbus* fish tissues, either obtained from experimental data or estimated from best-fitting models (Δ*i* = 0; Table 33) (A) and the corresponding isotopic discrimination factors between the experimental diet and tissues (B). Errors around the means represent standard errors along with 95 % confidence intervals (CI).

A)

Data	Tissue	$\delta f^{13}C$	95 % CI	$\delta f^{15}N$	95 % CI
Experimental	Muscle	-20.34 ± 0.10	-20.52 - -20.15	9.63 ± 0.09	9.45 - 9.81
(Day 125)	Fin	-19.40 ± 0.10	-19.59 - -19.21	9.32 ± 0.09	9.14 - 9.50
	Scale	-18.41 ± 0.10	-18.60 - -18.22	9.33 ± 0.09	9.15 - 9.51
Best-fitting model	Muscle	-20.34 ± 0.10	-20.52 - -20.15	8.44 ± 0.34	7.75 - 9.14
(Δ*i* = 0)	Fin			8.07 ± 0.57	6.90 - 9.24
	Scale	-18.41 ± 0.10	-18.60 - -18.22	7.74 ± 0.16	7.41 - 8.07

B)

Data	Tissue	$\Delta^{13}C$	95 % CI	$\Delta^{15}N$	95 % CI
Experimental	Muscle	5.01 ± 0.17	4.58 - 5.45	6.35 ± 0.16	5.93 - 6.77
(Day 125)	Fin	5.95 ± 0.17	5.51 - 6.38	6.04 ± 0.16	5.62 - 6.46
	Scale	6.94 ± 0.17	6.50 - 7.37	6.05 ± 0.16	5.63 - 6.47
Best-fitting model	Muscle	5.01 ± 0.17	4.58 - 5.45	5.16 ± 0.02	5.11 - 5.21
(Δ*i* = 0)	Fin			4.79 ± 0.02	4.74 - 4.84
	Scale	6.94 ± 0.17	6.50 - 7.37	2.48 ± 0.02	2.44 - 2.53

7.5. Discussion

This *ex situ* diet-switch experiment using juvenile *B. barbus* enabled estimations of carbon and nitrogen stable isotope turnover rates, expressed as half-lives, as well as separation of the relative contributions from metabolism and growth to turnover, along with determinations of tissue-specific discrimination factors. Equilibrium between the experimental diet and the tissues was not reached within the time-frame of the experiment, with this reflected in the half-life estimates. These outputs are discussed in turn in the following sub-sections.

7.5.1. Estimated stable isotope turnover rates

The results indicated that the nitrogen stable isotope signatures of the *B. barbus* tissues reflected and approached those of their new diet, with the turnover processes being mathematically predictable using turnover models (Buchheister and Latour, 2010; Xia *et al*., 2013b). For ^{15}N turnover among the fish tissues, in the best-fitting models, there was a consistent ranking of muscle having the shortest turnover time (84.3 days), followed by fin (91.2 days) and scales having the longest (145.4 days). This hierarchy is supported by other studies that show dramatically different turnover rates between fish tissues (Buchheister and Latour, 2010; Xia *et al*., 2013b), although some other studies have detected only minor inter-tissue differences in turnover rates (e.g., Hesslein *et al.,* 1993; Sweeting *et al*., 2005; McIntyre and Flecker, 2006). This suggests that tissue turnover differentiation, as well as the relative ordering of turnover rates among tissues, may be species-specific. For example, Heady and Moore (2012) calculated ^{15}N half-lives for muscle, fin and scales in rainbow trout *Oncorhynchus mykiss* and found fin had the fastest rate of turnover (12.9 days), followed by muscle (39.0 days) and scales (40.0 days). These turnover estimates are shorter than those produced here, however the authors recognised that their turnover rates were fast, approximately 71 % faster for muscle than estimates from a previous *O. mykiss* isotope diet-switch study (Church *et al*., 2009) and this was attributed to experimental conditions (Heady and Moore, 2012). Buchheister and Latour (2010) studied summer flounder *Paralichthys dentatus* liver, blood and muscle tissue and found muscle to have the slowest turnover (84.9 days) which is comparable to the muscle half-life estimated here (84.3 days). Xia *et al*. (2013b) measured turnover in grass carp *Ctenopharyngodon idellus*, and

estimated ^{15}N turnover in muscle tissue to be 68.3 days. Where differences between tissue turnover rates are observed, they generally follow the pattern that more metabolically active tissues show higher stable isotope turnover rates than less active tissues (Tieszen *et al*., 1983).

The results of the carbon isotopic signatures of *B. barbus* showed that changes within the tissues did occur, but unlike nitrogen, they did not approach those of the experimental feed, which was more negative in $\delta^{13}C$ compared to the control feed, with the tissues becoming less negative in $\delta^{13}C$ over the course of the experimental period. The carbon isotopic turnover processes were mathematically predictable following the turnover models, but the variation explained within the models was reduced compared to ^{15}N. The best-fitting models for muscle and scales produced half-life estimates of 138.3 and 90.7 days, respectively, suggesting that muscle ^{13}C turnover is slower than scales and for both tissues ^{13}C turnover is slower than ^{15}N. There are fewer comparisons available in the literature for ^{13}C turnover in fish tissue, as most studies concentrate on nitrogen stable isotopes (e.g., Logan *et al*., 2006; MacNeil *et al*., 2006; Heady and Moore, 2012; Xia *et al*., 2013a) and this is the first known attempt to calculate carbon turnover in fish scales. Though, Buchheister and Latour (2010) estimated that muscle tissue had a carbon stable isotope half-life of 68.9 days in *P. dentatus,* compared to 84.9 days for nitrogen.

A possible explanation of the difficulty encountered with determining the turnover of ^{13}C in *B. barbus* tissues is the issue of the differences between the diet-switch feeds not being sufficiently large enough. In a study by MacNeil *et al*. (2006), control and treatment diets maintained relative $\delta^{15}N$ differences of more than 200 ‰ during their experiment. They highlight that a considerable challenge in studying stable isotope dynamics has been in selecting suitable control and treatment diets that differ substantially in stable isotope values as differences of 3 ‰ are in fact at the 5th to 6th decimal place when converted to actual concentrations (Pinnegar and Polunin, 1999). Most studies of isotopic turnover have not achieved differences of more than 3 ‰ between diets (MacNeil *et al*., 2006). Here, the difference between control and experimental feeds for $\delta^{15}N$ was 6.06 ‰, although differences between feeds for $\delta^{13}C$ were small, only 2.16 ‰, which may account for the minimal changes in $\delta^{13}C$, which might limit conventional analyses of turnover (Sweeting *et al*., 2005). Consequently, the data obtained for ^{15}N turnover derived here is likely to be more reliable than ^{13}C.

A potential confounding factor of diet-switch experiments in general is that tissues are not fully equilibrated to the control diet before the diet-switch occurs, leaving tissues either closer to, or further from, their equilibrium level with the new diet. However, here, control fish tissues showed little change in isotopic signatures, with relatively small variations between tissues sampled before the diet-switch and those maintained on the control feed and sampled on Day 125, suggesting that the fish were initially near isotopic equilibrium with the control diet. Given that the pre- and post-diet-switch periods were equal in their duration this indicates that the turnover rate of the control feed was faster than that of the experimental feed. A possible explanation for this is the differing protein source and content of the different feeds. Few studies have controlled for the potential effects of protein composition on stable isotope turnover, but MacNeil *et al*. (2006) observed a difference in the rate of $\delta^{15}N$ uptake and elimination within tissues when shifts occurred between high and low (elimination) and low and high (uptake) $\delta^{15}N$ concentration. The diet-switch here was from a high (9.34 ‰) to a low (3.28 ‰) $\delta^{15}N$ concentration, which may have influenced the rate of turnover occurring within the tissues, creating a difference between the two diets.

7.5.2. Proportional contributions of metabolism and growth to turnover

The proportional contributions of growth and metabolism to the turnover rates of ^{13}C and ^{15}N could only be calculated from models C, D and E and so the contributions discussed are those generated from those models with the lowest AICc value for each tissue and isotope. For muscle, fin and scale tissue, growth was the predominant contributor to ^{15}N turnover, accounting for 58, 63 and 100 % of isotopic change, respectively. For ^{13}C, growth accounted for 100 % of turnover in muscle, but in scales, metabolism was slightly dominant, accounting for 51 % of the change in isotopes in the best-fitting model. However, other models with substantial support ($\Delta i < 2$; Burnham and Anderson, 2002) suggest that growth accounted for up to 100 % of turnover for ^{13}C in scales, which is a more realistic estimate as scale collagen is deposited only during seasonal growth and is generally thought not to turnover metabolically (Hutchinson and Trueman, 2006). Hesslein *et al.* (1993) utilised juvenile broad whitefish *Coregonus nasus* to examine $\delta^{13}C$ and $\delta^{15}N$ in response to a dietary shift and attributed 90 % of the observed isotopic changes in the fish to growth. The dominant influence of growth on turnover is supported here and elsewhere for ectotherms (e.g., Herzka and Holt, 2000; MacAvoy *et al.,* 2001; Bosley *et*

al., 2002; Perga and Gerdeaux, 2005; Heady and Moore, 2012) and is attributed to ectothermic animals having lower metabolic activities than endotherms (Bosley *et al*., 2002; Tominaga *et al*., 2003).

Perga and Gerdeaux (2005) suggested that as fish, and other ectotherms, have a discontinuous pattern of growth over the year, the $\delta^{13}C$ and $\delta^{15}N$ of muscle may only reflect food consumed during periods of growth. They found that muscle exhibits a slow and discontinuous turnover and that food consumed during nearly half of the year cannot be detected within the tissue (Perga and Gerdeaux, 2005). Herzka and Holt (2000) also found that growth explained over 90 % of the observed variability in turnover of both carbon and nitrogen isotopes. However, one caveat to these experiments is that the majority focused solely on muscle tissue, and for most fishes, the roles of growth versus metabolism on tissue-specific turnover rates have not been investigated. Where multiple tissues have been compared, contrasting results have been found, showing that the isotopic turnover rate substantially varies depending on the relative metabolic activity of various tissues (e.g., Herzka *et al*., 2001; Logan *et al*., 2006; McIntyre and Flecker, 2006; Carleton and Martínez del Rio, 2010) and several authors have found that high metabolic rates do appear capable of elevating muscular $\delta^{15}N$ signatures (Herzka and Holt, 2000; Gaye-Siessegger *et al*., 2004). Additionally, some studies have noted that results may not be consistent across all fish life stages as the majority of *ex situ* diet-switch experiments have utilised juveniles (Hesslein *et al*., 1993; Herzka and Holt, 2000; Perga and Gerdeaux, 2005). Indeed, experiments on older or larger fishes and those with lower specific growth rates found replacement to be a major proportion of total turnover, in some cases accounting for 80 % of isotopic change in dorsal muscle tissue (Suzuki *et al*., 2005; Logan *et al*., 2006; Tarboush *et al*., 2006). Heady and Moore (2012) found that metabolism contributed more to ^{15}N turnover for faster turnover tissues contributing 68 % for fin, 6.1 % for muscle, and 0.7 % for scales. The estimated contributions derived here are similar for scales, but for muscle, the influence of metabolism was much stronger, accounting for 42 and 44 % of turnover in the time- and growth-based models, respectively.

Size, growth rate, tissue type and turnover rate may thus influence the relative contributions of growth and metabolism to turnover. Moreover, experimental design, and in particular temperature, may also have a significant effect, as recognised by Heady and Moore (2012) for their fast turnover rates. Furthermore, Frazer *et al*. (1997) examined $\delta^{13}C$ and $\delta^{15}N$ of larval Antarctic krill *Euphausia superba Dana* and found an effect of

metabolism on turnover only at the higher of two rearing temperatures; and in studies that utilised similar sized fishes, higher water temperatures reduced the half-lives of carbon in muscle tissue (Bosley *et al*., 2002; Witting *et al*., 2004). Caution must be applied where the contributions of metabolism to turnover are estimated and represented by a metabolic constant, as in reality, it is the turnover observed that is not attributable to growth (MacNeil *et al*., 2006). It should be recognised, therefore, that this constant includes all other processes that contribute to turnover, such as inter-tissue recycling of nutrients, preferential isotopic routing and amino-acid effects, and these processes may operate differently during isotopic uptake and elimination (MacNeil *et al*., 2006). There is a considerable lack of analysis of the relative fates of ^{13}C and ^{15}N in turnover in fishes, and without such specific research providing a mechanistic basis for predicting stable isotope dynamics, the biological processes that contribute to the metabolic constant, specifically, and that underpin isotopic turnover more generally, cannot be fully understood. Nonetheless, the majority of studies do support the speculations by Tieszen *et al.* (1983) that metabolic activity is positively correlated with turnover.

7.5.3. Using AICc to determine best-fitting models

An information-theoretic approach to model selection highlighted differences between the best-fitting models for carbon and nitrogen stable isotope turnover, with time-based methods providing better fits for ^{15}N turnover and growth-based methods for ^{13}C turnover. All of the time-based models that were selected for ^{15}N turnover included specific-growth rates and metabolic constants. Weidel *et al*. (2011) also found that models including a tissue replacement parameter were generally better supported than models predicting turnover based solely on growth, but with regard to ^{13}C turnover. The growth-based models that were best-fitting for ^{13}C were generated from Hobson and Clark's (1992) equation, which was developed from a stable isotope study in birds, which are endothermic animals and that have a higher basal metabolic rate than ectotherms, such as fishes (Tieszen *et al*., 1983; Hobson and Clark, 1992; Herzka and Holt, 2000). Thus, whilst model B provided the best-fit for ^{13}C turnover of muscle and scales according to Δi, it is questionable that equilibrium was reached during the experimental time-frame when other substantially supported models of ^{13}C turnover of muscle and scales suggest otherwise. These data were also potentially unreliable due to the limited change of isotopic values observed within the

tissues, and therefore, the use of the turnover estimates for ^{13}C from model B is not recommended.

7.5.4. Comparing turnover estimates from models with general equations

In addition to the assessed models, general equations available from the literature were applied to the data to generate estimates of stable isotope turnover. This allowed comparison of half-lives between the equations and the best-fitting models for each isotope-tissue combination. Buchheister and Latour's (2010) equation was closest for ^{15}N turnover for all tissues and ^{13}C for muscle. Although the best-fitting ^{13}C time-based model for scales produced a half-life close to Vander Zanden *et al*.'s (2015) equation, as previously mentioned, the data for ^{13}C may be questionable and therefore Buchheister and Latour's (2010) equation could provide a more realistic estimate. Buchheister and Latour (2010) generated their equation from a fish diet-switch experiment, which, when compared to Thomas and Crowther (2014) and Vander Zanden *et al*. (2015), who calculated their equations from broad data sets encompassing many different species and types of animal under varied experimental conditions, could explain the proximity of their half-life estimates to those observed here. Although a limitation to Buchheister and Latour's (2010) equation is that it requires accurate estimates of the specific growth rate (k) and the turnover constant (c), whereas the others only require body mass and temperature.

The range of half-lives produced from the experimental data reveal how model selection can influence turnover and equilibrium estimates, and the associated discrimination factors which are critical when applying stable isotope data to food web ecology. Consequently, the results of previous studies that have used such general equations to justify their experimental time-frame or for determination of the extent of isotopic turnover observed (e.g. Milardi *et al*., 2015) may be confounded as outputs here suggest that equilibrium may not have been reached. It is therefore suggested that where possible, researchers should use half-life estimates derived for the species in question, or if not available, from a closely related taxa, or of similar biology (e.g. ecto- or endo-thermic) and generated from comparable temperature conditions to those experienced by the species.

7.5.5. Diet-tissue discrimination factors

The diet-tissue discrimination factors observed here are considerably higher than the standard values that are commonly cited (i.e., 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.3 ‰ for $\delta^{13}C$; DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002) and that have been recently applied to a stable isotope mixing model (Bašić *et al*., 2015). However, these values are increasingly recognised as inappropriate for application across a wide range of species, tissues and diets. In Chapter 6, where the same wheatgerm diet was fed to *B. barbus* continuously for 100 days, discrimination factors for $\delta^{15}N$ were 5.31 ‰ and 6.43 ‰ for $\delta^{13}C$ in fin tissue. These values are comparable to the estimates here that were calculated using δf as the stable isotope values from the experimental fish sampled on Day 125; 5.95 ‰ for $\delta^{13}C$ and 6.04 ‰ for $\delta^{15}N$, which demonstrates consistency in discrimination factors for this wheatgerm diet in *B. barbus*. However, the outputs of this *ex situ* experiment suggest that the experimental fish used in Chapter 6 would not have reached equilibrium with their diet within the time-frame of 100 days. Heady and Moore's (2012) best-supported $\delta^{15}N$ diet-tissue discrimination factors for *O. mykiss* were 3.4 ‰ for muscle, 1.6 ‰ for fin, and 2.2 ‰ for scales. Results here for $\delta^{15}N$ are alike for scales using δf from the best-fitting model (2.44 ‰), but discrimination factors calculated for muscle and fin are much higher, 5.16 and 4.79 ‰, respectively. A potential reason for this is that Heady and Moore (2012) found fin tissue to have a faster turnover rate than muscle.

As highlighted previously (*cf.* Chapter 6), differences between the discrimination factors derived here and those elsewhere may be attributed to the protein compositions of the diets which can influence the diet-tissue discrimination, as authors have suggested a strong association between amino acid composition and tissue $\delta^{15}N$ enrichment (e.g., Pinnegar and Polunin, 1999; Schmidt *et al*., 2004). This creates difficulties when comparisons are made between discrimination factors that have been estimated from diets that have different protein compositions. Here, the protein sources, and hence, amino acid profiles, of the control and experimental diets were different (fishmeal based to plant based) and this might have impacted on the discrimination factors measured.

7.5.6. Summary and conclusions

The outputs of this Chapter reveal that *B. barbus* tissues exhibit variation in their turnover rates and discrimination factors. The results add to a growing body of evidence that suggest

stable isotope turnover is faster in tissues with higher metabolic activity and within muscle, fin and scale tissue, growth is the dominant contributor to isotopic change. Nevertheless, contribution from metabolism should not be disregarded or underestimated, particularly in muscle tissue. This is the first known instance where carbon and nitrogen stable isotope turnover in scales has been estimated, providing novel values for ^{13}C turnover in all fishes and the first for ^{15}N in *B. barbus*. In addition, the application of general equations can be a valuable tool where specific turnover estimates are unavailable and/or an experimental diet-switch study cannot be undertaken, however, it is recommended that where possible, the equations are derived from the species in question or from a species with a similar biology in order to derive the best half-life estimates to therefore allow allocation of a sufficient time-frame to ensure stable isotope turnover has fully occurred and the tissues have reached equilibrium with the diet.

Chapter 8. General discussion

8.1. Introduction

The use of fish scales within studies on fish and fisheries ecology is long established, particularly with regard to their use within investigations on population demographics, such as age structure and growth rates (e.g., Jackson *et al*., 2007; Vilizzi *et al*., 2013; Yates *et al*., 2016). More recently, they are increasingly applied within research investigating the long-term trophic ecology of fishes, primarily due to the expansion and popularisation of contemporary stable isotope techniques, coupled with a desire to utilise material collected in a non-destructive manner (Gerdeaux and Perga, 2006; Trueman and Moore, 2007; Yao *et al*., 2016). In addition, the successful extraction of DNA from scales in sufficient quantities for completing modern genetic analyses is also possible and has been used, for example, to investigate the phylogeography of European barbel *Barbus barbus* in Great Britain (Antognazza *et al*., 2016). Notably, all of these applications are possible from removing a small number of scales from a fish in a non-lethal and marginally invasive manner, with their subsequent long-term storage causing minimal degradation in their quality (Al-Absy and Carlander, 1988). Despite these advantages, there remain some significant concerns regarding the use of scales within ecological studies due to a series of substantial knowledge gaps regarding tackling the inherent issues associated with their use. These include the difficulties of scale interpretation for ageing and a lack of information on stable isotope turnover rates as well as the isotopic discrimination between a fish's scales and its food resources. Thus, the overarching aim of thesis was to overcome some of these paucities in knowledge via the completion of a range of *in*- and *ex-situ* studies completed on freshwater fishes in temperate systems. These outputs, whilst focussed on fishes of the Cyprinidae family, should have applicability more widely. In the subsequent sub-sections, the results of the research are discussed in wider detail and in response to the issues outlined regarding their application to population demographics and trophic studies, followed with recommendations for future research directions.

8.2. Investigating the ecological interactions of non-native fish

The investigation into how the impacts of non-native fish can be studied, through utilising a range of spatial scales, was successful in providing a series of new insights into the interactions of a model native and two non-native fishes. Importantly, it also emphasised a series of inherent issues that were apparent when applying data from fish scales to the ecological study. These included difficulties of estimating the accuracy and precision of ages derived from scales, and understanding the discrimination factors and isotopic turnover rates when using scales within stable isotope analyses. Nevertheless, through use of three cyprinid species, two considered non-native (goldfish *Carassius auratus* and common carp *Cyprinus carpio*) and one considered native (crucian carp *Carassius carassius*) and across three approaches of differing size and complexity, Chapter 2 was still successful in revealing a range of ecological consequences for the native fish from the non-native fishes, although these impacts were a function of spatial scale.

The initial approach utilised the fishes in co-habitation aquaria and revealed *C. carassius* growth rates were significantly suppressed when they were present in sympatry with *C. auratus* or *C. carpio.* These results from this controlled feeding experiment were interesting due to their contrast with the field study of Tarkan *et al*. (2009), who detected no detrimental consequences for the somatic growth rates of *C. carassius* populations when they were present in ponds with *C. auratus*. Consequently, the following approach then developed this further through the completion of an experiment in semi-wild conditions, using pond enclosures, which enabled the testing of the interactions of the fishes in a less artificial system where the fishes would exploit natural food resources. The results indicated that when *C. auratus* and *C. carassius* were in sympatry, there were no negative impacts on the growth rates of either species, with them occupying similar trophic positions and niche sizes. Despite some resource sharing, the growth rates suggested there was no inter-specific competition apparent between the fishes, most likely due to the food resources not being limiting, a distinction from the controlled feeding experiment completed in aquaria. In contrast, *C. carpio* had a strong influence on both *Carassius* species in sympatry, with their presence resulting in significant increases in *Carassius* trophic position and niche size, but suppression in their growth rates. The final approach was completed within non-replicated ponds using only *C. carassius* and *C. carpio*. The results were similar to the pond enclosures, with *C. carassius* increasing in trophic niche size in the presence of *C. carpio*.

In respect to ecological theory, the results from the pond experiments were contrary to the niche variation hypothesis that predicts that the increased competitive interactions that result from an introduction of a non-native species would result in diet constriction, leading to increased diet specialisation in the post-introduction period and translating into a reduced trophic niche size (Van Valen, 1965; Thomson, 2004; Olsson *et al.,* 2009). Instead, the results were consistent with Svanbäck and Bolnick (2007) who suggested that larger trophic niches can result from increased resource competition, as the competing species exploit a wider dietary base to maintain their energetic requirements, although it should be noted that despite increasing their niche size in the presence of *C. carpio*, the *Carassius* fishes were still unable to maintain their growth rates at levels observed in allopatry. Thus, these results suggest that the interaction of native *C. carassius* and non-native *C. auratus* had minimal ecological implications for *C. carassius*, but when in the presence of the globally invasive *C. carpio* (*cf.* Section 1.5), the ecological impacts were significant. Accordingly, for native fishes such as *C. carassius*, negative trophic and demographic consequences would be predicted from introduced *C. carpio*, whereas impacts of invasive *C. auratus* appear to be primarily related to a reduced genetic integrity through hybridisation (Hänfling *et al*., 2005).

The range of results detected in Chapter 2 between the three approaches highlights the importance of considering spatial scale and complexity across ecological experiments, especially where co-habitation experiments in controlled conditions are to be extrapolated to wild situations. Moreover, inherent issues were encountered regarding the current methods and techniques that were applied. Specifically, these related to: (i) whether the destructive sampling of fishes to obtain muscle tissue for stable isotope analysis is necessary when there are potential non-lethal alternatives, such as scales; (ii) whether the standard diet-tissue discrimination factors commonly cited are appropriate for wide-spread application, as studies indicate they may be species- and tissue-specific; (iii) what time-frame should be allowed in order for tissues to reach equilibrium with a new diet, i.e., what is the rate of turnover of stable isotopes within the tissues of these fishes?; and (iv) how sample sizes, and the error and subjectivity that surrounds obtaining fish age estimates, may affect the accuracy and precision of subsequent analyses, as numbers were low and no age validation was completed in Tarkan *et al*. (2009). The focus of the following research was therefore to attempt to resolve these specific procedural problems in order to increase the efficacy of future experimental studies completed in field contexts.

8.3. Issues with scale age estimates: dealing with precision and uncertainty

It was outlined in Section 8.2 that despite their wide use in ecological research, there remain some fundamental issues with the use of fish scales in studies on age and growth. In investigations such as Tarkan *et al*. (2009), where sample sizes are limited, there is considerable uncertainty in how precise the subsequent growth rate data are. In addition, the difficulties associated with ageing fish from their scales, especially where validation methods are inapplicable, means that there is potential for the accuracy of ageing to be variable across the data-set, leading to considerable confounds in subsequent analyses.

The use of fish scales to age fish is also a fundamental component in determining the ecological status of fish fauna under the Water Framework Directive (WFD, 2000; *cf*. Section 1.2). When large numbers of fish are sampled within monitoring programmes to assess ecological status, then scale sub-sampling strategies are usually employed. It was demonstrated within Chapter 3 that the use of sub-sampling regimes can significantly influence the precision of the growth data subsequently produced. Across the three cyprinid species studied (roach *Rutilus rutilus*, dace *Leuciscus leuciscus* and chub *Squalius cephalus*) and between age groups, there was consistency in the number of scales that needed to be aged in order to achieve a desired level of precision. The results indicated that to achieve a 10 % precision, between 7 and 12 scales per age group would require ageing. However, given that the ages of fish are rarely, if ever, known in advance of scale ageing, then the effect of sub-sampling scales on precision was also tested, with ageing 10 fish per 5 mm length category (e.g. 51 to 55 mm, 56 to 60 mm etc.) never significantly reducing precision.

Knowledge of the impacts of sub-sampling, and the number of scales required in order to achieve an acceptable level of precision, is important in order to improve the application of scale ageing to relevant studies. Furthermore, it ensures that researchers have the information required to formulate the most appropriate sampling strategies in the design of new fish population sampling programmes and protocols according to the species being studied and their objectives, and ensures that interpretations and evaluations of the data are reliable. Whilst precision is an important aspect of age determination, as it represents the reproducibility of individual age estimates from a given structure (Kalish *et al*., 1995), the focus for researchers should primarily be improving ageing accuracy, which is the proximity of an age estimate to the true age (Kalish *et al*., 1995), as precise age estimates can be obtained that are not accurate and although *vice versa* can also occur, the

implications for inaccurate age estimates are likely to have more negative consequences for subsequent calculations, such as growth rate analyses, than imprecise estimates. Thus, Chapter 4 investigated how the inherent errors in age estimates, that can arise from features of fish scales that lead to uncertainty, can be utilised within analytical methods to provide more robust estimates of age structure and growth rate analyses.

Errors in estimating the ages of fish from hard structures are difficult to eliminate completely given the subjective nature of the process (*cf.* Section 1.3; Musk *et al*., 2006). Hence, if data subjectively generated from ageing, is to be used in research and monitoring programmes, then it can be argued strongly that rather than disregarding this, it should be integral to subsequent analyses. Consequently, a statistical model was developed to incorporate uncertainty levels into growth rate calculations via their application to age estimates within a bootstrapping methodology. This then produced adjusted von Bertalanffy growth function (VBGF) parameters where the ageing uncertainty had been incorporated. The model results and evaluations revealed that across *R. rutilus, L. Leuciscus* and *S. cephalus*, the extent of ageing uncertainty increased with fish age, with significant non-linear relationships. Comparison of the original versus the adjusted VBGF parameters revealed some significant differences, with general patterns of higher $L\infty_{\text{-adjusted}}$ and lower $k_{\text{-adjusted}}$ than the original estimates, indicating that these were produced from fish that were under-aged, a common characteristic of scale ageing identified in the literature review (*cf*. Appendix 2). These adjusted VBGF parameters also impacted length-at-age estimates, with shifts toward slower growth rates.

The development of this simple method based on bootstrapping procedures provides a highly useful ecological tool that works with uncertainty in scale age estimates. It has potential for application beyond scales, to other hard structures used for ageing, such as otoliths, and could be used for a broad range of freshwater and marine species. One issue, however, remains within the method proposed, as there exists some subjectivity around translating each age estimate and uncertainty level per species into a distribution of probable ages (*cf*. Section 4.3.3). Whilst the distributions were based on literature review wherever possible, they also required some additional input and therefore some inherent error might remain. Notwithstanding, here its application to populations of riverine fishes revealed that it produced adjusted VBGF parameters that better reflect the uncertainty in the original data.

The uncertainty incorporated, adjusted VBGF parameters, can then be used, for example, within fish stock assessment models that utilise the von Bertalanffy growth function to determine the growth of fish, such as 'stock synthesis', which is a statistical age-structured population modelling framework (Methot and Wetzel, 2013). These stock assessment models have far reaching implications; by 2012, 5 stocks in the United States of America, 10 tuna/ billfish stocks in three oceans, 4 European stocks, and 12 Australian stocks had been assessed using this approach (Methot and Wetzel, 2013), demonstrating the importance of ensuring the parameters used within them are as accurate as possible to ensure sustainable fishing rates can be correctly estimated. In incorporating the inherent error into age estimates, the proposed method should thus enable improved management decision-making in fish and fisheries ecology.

8.4. Obtaining stable isotope data from fish scales

The use of stable isotope analysis (SIA) has increased dramatically within freshwater ecological studies over the past 20 years, due to a reduction in costs of analysis and increased awareness of the ability of SIA to provide insights into trophic interactions within ecosystems (Grey, 2006). This flourish in the application of SIA has been cautioned by some researchers (e.g., Gannes *et al*., 1997; Martínez del Rio *et al*., 2009; Phillips *et al*., 2014), resulting from the lack of understanding of the fundamental processes that drive the isotopic changes within tissues, including diet-tissue discrimination factors (Δ), the rate of stable isotope turnover, and the aspects that influence these. Variability in isotopic discrimination has been attributed to a range of factors such as diet, temperature, species, tissue, sample preparation, protein source, content and concentration, as well as amino acid composition (Gannes *et al*., 1997; McClelland *et al*., 2003; MacNeil *et al*., 2006; Barnes *et al.,* 2007; Robbins *et al*., 2010; Kurle *et al*., 2014). Additional concerns have also been raised with regard to the ethics of destructive sampling to obtain muscle tissue, which is principally collected for SIA in fishes, and the problem that this raises in relation to the study of endangered or protected species (Sanderson *et al*., 2009; Huang *et al*., 2013; Hamidan *et al*., 2015). Consequently, a recent onus has been placed on investigating the use of tissues that can be collected non-lethally. This present change in approach requires information regarding how non-destructively sampled tissues, such as scales and fin tissue, compare to those that tend to be collected through lethal sampling, such as dorsal muscle. The advantage of using scales over fin tissue is that many research institutes have

collections of scales kept in archives covering extensive temporal scales, opening the possibility of these being utilised to identify major long-term changes in the trophic niches of these fishes. In addition, scales are collected routinely within fish stock assessment exercises and thus developing knowledge on the use of scales in stable isotope ecology could increase access to a large volume of material to help answer relevant questions. Thus, Chapters 5, 6 and 7 investigated some of these issues, as highlighted in Chapter 2, with the results evaluated below, along with the contributions they have made towards the progression of the utilisation of scales within SIA.

8.4.1. Scales as a non-lethal surrogate for dorsal muscle tissue

The application of scales for stable isotope analyses within food web and trophic investigations is growing, given the recent ethical shift towards increasing the use of non-lethally sampled tissues. However, compared to fin tissues, there remains very little information on the stable isotopes of scales. The results of Chapter 5 revealed that scales are appropriate to use as a proxy for muscle tissue in a range of cyprinid fishes and their stable isotope values can be successfully converted to muscle values through the application of simple linear regression equations with relatively low error, especially when species-specific methods are used.

The results also suggested that the production and use of species-specific diet-tissue discrimination factors are more suitable and informative than the use of standard values that are commonly cited (i.e., 3.4 ± 0.98 ‰ for $\delta^{15}N$ and 0.39 ± 1.3 ‰ for $\delta^{13}C$; DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002), as the difference between the results can be marked. Consequently, the use of standard discrimination values in order to quantitatively determine the relative contributions of putative food resources to the diets of fishes, as well as their use within calculations of trophic position, as was done in Chapter 2, should be avoided wherever possible, and specific discrimination factors that relate to the focal species should be used to obtain the most accurate predictions, where possible. In a recent study, Yao *et al.* (2016) utilised a historical scale archive of two omnivorous cyprinid fishes, bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*, which are important aquaculture species that are also highly invasive in North America. The study revealed trends in resource use in response to resource availability and was successful in detecting dynamic trophic interactions between

the species, regarding their trophic niche positions and widths, over an 11 year period. However, their methodology assumed that the discrimination factor per trophic level was 3.4 ‰ for $\delta^{15}N$. The results of Chapter 5 indicate that this might not be appropriate, with lower values, such as between 1.96 and 2.96 ‰, found for scale $\Delta^{15}N$, more likely to be representative. Thus, the provision of specific diet-scale discrimination data for a range of cyprinids, especially those with high ecological and socio-economic importance, will contribute to promoting the application of scales within stable isotope studies and will increase knowledge on the variability that exists between species in their diet-scale discriminations. It also further promotes the application of scale archives to answering research questions based on long-term ecological and/or environmental changes, as currently there remains an absence of exploitation of these invaluable sources of data.

8.4.2. Effect of diet composition on tissue-specific isotopic discrimination

Along with their application to food web and trophic investigations, an increasingly important use of stable isotope discrimination factors is within statistical mixing models that predict the proportional composition of consumer diets from data on their putative food resources (Jackson *et al.*, 2011). A fundamental requirement of these models is robust estimates of the stable isotope discrimination factors between the prey resources and the consumer tissue being analysed (Bond and Diamond, 2011; Phillips *et al.*, 2014). However, diet-tissue discrimination factors are influenced by numerous variables, including the tissues being analysed and the taxon of the consumer and its food resources. Whilst differences in $\Delta^{13}C$ and $\Delta^{15}N$ are apparent between herbivorous and piscivorous fishes, there is less known for omnivores that consume both plant and animal material, hence Chapter 6 aimed to quantify and assess the extent to which stable isotope discrimination factors were significantly affected by diet source and protein content in two omnivorous cyprinids, *B. barbus* and *S. cephalus,* and across scale, fin and muscle tissues. The results revealed that the diet based on plant material (20 % protein), always resulted in the highest discrimination factors for each tissue, whilst the diet based on marine fishes (45 % protein) consistently resulted in the lowest, suggesting that the diet-tissue discrimination factors of omnivorous fishes will vary considerably between animal and plant food items. Additionally, there were species-specific differences, with discrimination factors tending to be higher in *B. barbus* than *S. cephalus*, irrespective of diet and despite the species being closely related.

Awareness of the requirement for using species-specific discrimination factors within the analyses of stable isotope data is increasing and consequently, these are being progressively factored into studies. However, issues regarding the influence of different food resources on the resulting diet-tissue discrimination factor still require resolution. For example, in a recent investigation, Garcia *et al*. (2016) explored the temporal variability in basal food resources in an omnivorous fish, the onesided livebearer *Jenynsia multidentata*, over a five year period. In the absence of species-specific discrimination factors, the authors used experimentally derived values from a similar species, which outputs from Chapter 5 suggest should be more representative than the use of standard values. However, the outcomes of Chapter 6 indicate that the putative food resources that they analysed, which were either pelagic or benthic, could discriminate differently among the tissues and this was not recognised by the authors. Additionally, the species-specific or tissue-specific isotopic turnover rates were unknown. Thus, the results of the study potentially have several important confounds, but these remain unquantifiable.

The issues of varying diet-tissue discrimination factors between different food resources, as revealed in Chapter 6, suggest that rather than relying on the use of a single discrimination factor covering all putative food items (Phillips *et al*., 2014), as per Garcia *et al*. (2016), this variability must be captured within the predictive model used for estimating the diet composition of a consumer and particularly of an omnivore. Therefore, resolution of the differences in discrimination factors between food items comprising of varying protein content and source is required in order to obtain the maximum value from studies that predict trophic level and diet composition, such as those using Bayesian mixing models (Parnell *et al.*, 2013; Phillips *et al.*, 2014). Hence, although the outputs from Chapter 6 contribute to achieving this goal, questions on how and why these specific differences in diet-tissue discrimination occur need further investigation in order to facilitate the consideration of these variables in subsequent analyses and allow for more accurate predictions of diet composition and trophic position to be made.

8.4.3. The rate of carbon and nitrogen stable isotope turnover

Identifying isotopic turnover rates is particularly important for assessing the trophic ecology of mobile and migratory species, and species that undergo ontogenetic dietary shifts (MacAvoy *et al*., 2001; Buchheister and Latour, 2010; Hertz *et al*., 2015). Estimates

of turnover, as isotopic half-lives, are also important in the design of manipulative field studies and mesocosm experiments where, for example, erroneous outcomes could result if the time-frame is of insufficient length for stable isotope equilibrium to be reached within the tissues analysed (Jackson *et al*., 2013; Tran *et al*., 2015). The aim of Chapter 7 was thus to determine turnover rates for carbon and nitrogen stable isotopes from a range of *B. barbus* tissues, using a variety of approaches including time- and growth-based models and general equations. For ^{15}N turnover among tissues, the estimates from the best-fitting models ranked muscle as having the shortest half-life at 84 days, followed by fin at 91 days and then scales at 145 days. For ^{13}C, the half-life for muscle was 138 days and for scales, 91 days. Comparison of the derived turnover rates with those estimated from general equations revealed some similarities, with the equation provided by Buchheister and Latour (2010) producing estimates within 10 days of those from the best-fitting models for ^{15}N turnover. The process of determining half-life estimates from a range of models was beneficial in highlighting how model selection can influence turnover and equilibrium estimates, and the associated discrimination factors, which are critical when applying stable isotope data to food web ecology.

It is evident that scales can be, and are being, used successfully within SIA (*cf.* Sections 8.4.1 and 8.4.2) and over recent years there has been an increase in their specific application to trophic studies, which is beneficial for the non-destructive sampling of fishes and will encourage researchers to exploit historical scale archives. However, a major problem exists that is likely to confound the outputs of these studies as there is scarce information available regarding the rate of stable isotope turnover occurring within them, not only for cyprinids, but fishes in general. This is a potentially serious issue, as without this knowledge, inferences made regarding trophic interactions and positioning as well as dietary analyses are highly likely to be inaccurate and misguided, due to the scale tissue not reaching equilibrium with the diet, jeopardising the robustness and validity of any outputs. It is therefore surprising that Chapter 7 is one of only a few attempts to estimate stable isotope turnover within scales, providing the first known values for ^{13}C turnover in all fishes and the first for ^{15}N turnover in *B. barbus.* Although it is thought that metabolic turnover in scales is negligible (Hutchinson and Trueman, 2006), significant isotopic addition within scales, through growth, has been demonstrated here and thus the use of the outer portions of scales that have been formed on a recent diet can be used. Therefore the half-life estimates provided should have utility in future trophic investigations, providing

researchers with a better estimate of the time-frames and growth required for stable isotope turnover in scales.

In hindsight, the initial investigation into the application of scales in SIA should have been the determination of their turnover rate, as this would have provided a better guideline for the time-frames necessary for equilibrium to be reached. As this is a prerequisite of accurate calculations of diet-tissue discrimination factors, the issues regarding the experimental time-frames for the SIA completed in Chapters 5 and 6 would have been resolved. Thus, the calculation of these novel data in *ex situ* conditions provides considerable insight into the turnover rates and discrimination factors of *B. barbus*, emphasising the importance of estimating these parameters for consumers at the species level, but also indicating that the utilisation of general equations can be a valuable tool where specific turnover estimates are unavailable.

8.5. Future directions

The outputs of this thesis have been successful in providing valuable information with regard to overcoming many of the existing knowledge gaps that are apparent in the use of scales in ecological studies, as highlighted in Chapter 2. Nevertheless, some unresolved problems remain, especially regarding their use in SIA, as there is such limited information currently available for fishes in general and especially for cyprinids. It is thus argued that the main focus for future work should be to continue to resolve these issues caused by this lack of data.

Much of the variation in diet-tissue discrimination factors occurs between fish in different trophic groups, with this highlighted in Chapters 5 and 6 where there were distinct differences between the closely related species. The reasons for this variability are often poorly understood (Vander Zanden *et al.*, 1997) and it remains unclear whether the cause of this variation is random or due to specific, predictable influences (Gannes *et al*., 1997). If explanations can be found for some of this variation, then it will greatly contribute to the development of this area of ecology and will improve the interpretation of stable isotope data and ensure that stable isotope techniques can be applied with greater confidence and lead to more robust and reliable understandings.

There remains some uncertainty in how the different discrimination factors between types of food item will result within omnivorous diets in the wild, as it is likely that omnivorous fishes would consume foods that vary extensively in both protein quality and quantity. Additionally, as diet shifts of omnivores may occur regularly in response to food availability, this reduces the likelihood of tissues reaching equilibrium with each diet before a shift occurs. So where these interactions have not been factored into analyses within mixing models, the reliability of subsequent estimations of the assimilated diet could be questionable. Chapter 6 highlighted that further work was required in order to reconcile some of the issues identified, and consequently, a next step could be to perform a similar experiment but use a diet that contained a 50: 50 mix of two distinct types of food, rather than feeding the fish one type or another, and to house the fish in tanks individually to ensure that each type of food was consumed in the same proportion. This would more closely resemble the mixed consumption of food items that omnivores experience in wild situations. As mentioned in Chapter 6, housing the fishes individually was inappropriate for *B. barbus* and *S. cephalus*, due to ethical concerns. However, this design could be more suitable for another species of cyprinid, such as *C. auratus*, that is used as a model experimental species across various disciplines including behavioural, genetic and developmental biology and is tolerant to solitary conditions (e.g., Sánchez-Vázquez *et al*., 1996; Thompson and Walton, 2004).

Equilibrium between the experimental diet and the fish tissues analysed was not reached within the time-frame of the stable isotope turnover experiment conducted in Chapter 7. However, the results suggested that the fish were initially near isotopic equilibrium with the control diet. Thus, given that the pre- and post-diet-switch periods were equal in their duration and experimental conditions, this indicates that the turnover rate of the control feed was faster than that of the experimental feed, suggesting that the rate of isotopic change occurring within tissues is affected by diet. As Chapter 6 indicated that isotopic discrimination is also affected by diet, then in combination with the results of Chapter 7, this suggests that the interactions between turnover rate, diet-tissue discrimination and diet should be explored further. Consequently, conducting a comparable study over a longer time-frame, to allow equilibrium within the tissues to be reached, would facilitate a more accurate determination of the stable isotope turnover rates of carbon and nitrogen for these fishes and tissues, as this could be measured directly, rather than relying upon predicted values extrapolated from fitting exponential models to the data. It would be beneficial to

increase the frequency of sampling during the early phases post diet-switch as this would provide more data points for the exponential models to be fitted to, which would be advantageous as this is the period where most rapid isotopic change occurs and may solve some of the model fitting difficulties encountered when fitting some of the time- and growth-based models within the Chapter. Additionally, performing a diet-switch between two diets that were more separated in their stable isotope values would be advantageous as this was indicated as a potential confounding factor, particularly with regard to the turnover of ^{13}C.

As growth rates have been found to influence turnover rates (e.g. Weidel *et al*., 2011), and temperature significantly affects growth in ectothermic fishes (Jobling, 1997; Pörtner *et al*., 2001), than it seems probable that temperature will also affect the rate of turnover and diet-tissue discrimination factors (e.g. Bosley *et al*., 2002). Indeed, Barnes *et al*. (2007) found that temperature affected the discrimination of ^{13}C and ^{15}N when rearing European sea bass *Dicentrarchus labrax* on identical diets at 11 and 16 °C. They therefore concluded that temperature would confuse the interpretation of $\delta^{15}N$ as an indicator of trophic level, or $\delta^{13}C$ as an indicator of trophic source, when comparing populations exposed to different temperatures. Thus, single discrimination values are not necessarily applicable in all environments. Additionally, Bloomfield *et al*. (2011) studied the effects of temperature on ^{13}C and ^{15}N turnover and discrimination in omnivorous black bream *Acanthopagrus butcheri* reared at 16°C or 23°C and fed either a fishmeal or vegetable feed. For $\delta^{15}N$ they found increased turnover and smaller discrimination factors at warmer temperatures and temperature and tissue $\delta^{13}C$ values were also affected by diet (Bloomfield *et al*., 2011). Moreover, the fish reared on the vegetable feed showed greater $\delta^{15}N$ changes and larger discrimination than those reared on a fishmeal feed, concurring with the outputs from Chapter 6. Consequently, further experimentation is required in order to determine the effects of temperature on the turnover rate, and the resulting diet-tissue discrimination factors, in order to reveal whether these patterns are applicable more widely. Experimental conditions should look to replicate the range of temperatures that the focal species would experience in the wild in order to be most beneficial for researchers and enable extrapolation of findings to observational studies.

In combination, these points demonstrate that the trophic discrimination of $\delta^{13}C$ and $\delta^{15}N$ can be considerably different to values typically used in food-web analyses, and the effects of diet composition and temperature are also potentially significant. These findings

are particularly relevant for ecological studies that are reconstructing food webs where organisms from different trophic levels may experience different environmental conditions. They are also relevant for investigations carried out within temperate regions, where seasonal shifts in temperature can be significant, as well as long-term studies or those that require repeated measurements over extended time-frames, where the temperature may have changed substantially. These should all consider whether the influence of temperature is impacting the rate of stable isotope turnover occurring within tissues and the discrimination between the consumer and their diet. In order to better interpret stable isotope data, the effects of environmental variability and dietary composition on isotopic discrimination factors and tissue turnover rates must be further explored, clarified and then validated.

8.6. Final conclusions

The vast array of information that is available for extraction from fish scales is potentially invaluable to ecologists and fisheries scientists alike. The contribution that the data obtained from them is able to make towards the study of fish regarding population dynamics, food web structure and trophic interactions is unsurpassed for a tissue that can be non-lethally collected. Whilst many of the issues that this research has examined still require further work to be resolved fully, the investigations completed have made a considerable contribution to the enhancement of the practical application of scales within ecological studies, enabling researchers to reduce the error surrounding data collection when applying sub-sampling strategies, proposing a simple methodology to incorporate the inherent uncertainty in fish ages estimates into growth analyses, and providing vital lacking information regarding tissue conversions, diet-tissue discriminations and turnover rates that is required to improve scale use within stable isotope analysis.

References

Al-Absy, A.H. and Carlander, K.D., 1988. Notes: Criteria for Selection of Scale-Sampling Sites in Growth Studies of Yellow Perch. *Transactions of the American Fisheries Society*, *117*(2), pp.209-212.

Allen, M.S. and Hightower, J.E., 2010. Fish population dynamics: mortality, growth, and recruitment. In: W.A. Hubert and M.C. Quist (eds.), *Inland Fisheries Management in North America*, 3rd edition. American Fisheries Society, Bethesda, Maryland, pp.43-79.

Amarasekare, P., 2003. Competitive coexistence in spatially structured environments: a synthesis. *Ecology Letters*, *6*(12), pp.1109-1122.

Antognazza, C.M., Andreou, D., Zaccara, S. and Britton, R.J., 2016. Loss of genetic integrity and biological invasions result from stocking and introductions of *Barbus barbus*: insights from rivers in England. *Ecology and Evolution*, *6*(5), pp.1280-1292.

Artero, C., Murie, D.J., Koenig, C.C., Berzins, R., Bouchon, C. and Lampert, L., 2015. Age, growth, and mortality of the Atlantic goliath grouper *Epinephelus itajara* in French Guiana. *Endangered Species Research*, *28*(3), pp.275-287.

Bagenal, T.B. and Tesch, F.W., 1978. Age and growth. In: T.B. Bagenal (ed.), *Methods for the assessment of Fish production in Fresh Waters*. IBP Handbook No 3 (3rd edition). Blackwell Scientific Publication, Oxford, U.K., 101–136.

Baiser, B., Russell, G.J. and Lockwood, J.L., 2010. Connectance determines invasion success via trophic interactions in model food webs. *Oikos*, *119*(12), pp.1970-1976.

Baker, T.T. and Timmons, L.S., 1991. Precision of ages estimated from five bony structures of Arctic char (*Salvelinus alpinus*) from the Wood River System, Alaska. *Canadian Journal of Fisheries and Aquatic Sciences*, *48*(6), pp.1007-1014.

Barnes, C., Sweeting, C.J., Jennings, S., Barry, J.T. and Polunin, N.V., 2007. Effect of temperature and ration size on carbon and nitrogen stable isotope trophic fractionation. *Functional Ecology*, *21*(2), pp.356-362.

Bašić, T. and Britton, J.R., 2015. Utility of fish scales from stock assessment surveys in stable isotope analysis for initial assessments of trophic relationships in riverine fish communities. *Journal of Applied Ichthyology*, *31*(2), pp.296-300.

Bašić, T. and Britton, J.R., 2016. Characterising the trophic niches of stocked and resident cyprinid fishes: consistency in partitioning over time, space and body sizes. *Ecology and Evolution*.

Bašić, T., Britton, J.R., Jackson, M.C., Reading, P. and Grey, J., 2015. Angling baits and invasive crayfish as important trophic subsidies for a large cyprinid fish. *Aquatic Sciences*, *77*(1), pp.153-160.

Beamish, R.J. and McFarlane, G.A., 1983. The forgotten requirement for age validation in fisheries biology. *Transactions of the American Fisheries Society*, *112*(6), pp.735-743.

Beamish, R.J. and McFarlane, G.A., 1995. A discussion of the importance of aging errors, and an application to walleye pollock: the world's largest fishery. In: D.H. Secor, J.M. Dean and S.E. Campana (eds.), *Recent Developments in Fish Otolith Research.* University of South Carolina Press, Columbia, pp.545-565.

Beardsley, H. and Britton, J.R., 2012. Contribution of temperature and nutrient loading to growth rate variation of three cyprinid fishes in a lowland river. *Aquatic Ecology*, *46*(1), pp.143-152.

Bearhop, S., Waldron, S., Votier, S.C. and Furness, R.W., 2002. Factors that influence assimilation rates and fractionation of nitrogen and carbon stable isotopes in avian blood and feathers. *Physiological and Biochemical Zoology*, *75*(5), pp.451-458.

Ben-David, M. and Schell, D.M., 2001. Mixing models in analyses of diet using multiple stable isotopes: a response. *Oecologia*, *127*(2), pp.180-184.

Benejam, L., Alcaraz, C., Sasal, P., Simon-Levert, G. and García-Berthou, E., 2009. Life history and parasites of the invasive mosquitofish (*Gambusia holbrooki*) along a latitudinal gradient. *Biological Invasions*, *11*(10), pp.2265-2277.

Bereiter-Hahn, J. and Zylberberg, L., 1993. Regeneration of teleost fish scale. *Comparative Biochemistry and Physiology Part A: Physiology*, *105*(4), pp.625-641.

Beverton, R.J.H. and Holt, S.J. 1957. *On the dynamics of exploited fish populations*. Chapman and Hall, London, UK.

Bloomfield, A.L., Elsdon, T.S., Walther, B.D., Gier, E.J. and Gillanders, B.M., 2011. Temperature and diet affect carbon and nitrogen isotopes of fish muscle: can amino acid nitrogen isotopes explain effects?. *Journal of Experimental Marine Biology and Ecology*, *399*(1), pp.48-59.

Boecklen, W.J., Yarnes, C.T., Cook, B.A. and James, A.C., 2011. On the use of stable isotopes in trophic ecology. *Annual Review of Ecology, Evolution, and Systematics*, *42*, pp.411-440.

Böhm, M., Schultz, S., Koussoroplis, A.M. and Kainz, M.J., 2014. Tissue-specific fatty acids response to different diets in common carp (*Cyprinus carpio* L.). *PloS one*, *9*(4), e94759.

Bolland, J.D., Britton, J.R. and Cowx, I.G., 2007. Lifetime consequences of variable 0 year group length in riverine populations of chub *Leuciscus cephalus* (L.). *Journal of Fish Biology*, *71*(6), pp.1810-1819.

Bolnick, D.I., Ingram, T., Stutz, W.E., Snowberg, L.K., Lau, O.L. and Paull, J.S., 2010. Ecological release from interspecific competition leads to decoupled changes in population and individual niche width. *Proceedings of the Royal Society of London B: Biological Sciences*, *277*(1689), pp.1789-1797.

Bond, A.L. and Diamond, A.W., 2011. Recent Bayesian stable-isotope mixing models are highly sensitive to variation in discrimination factors. *Ecological Applications*, *21*(4), pp.1017-1023.

Bosley, K.L., Witting, D.A., Chambers, R.C. and Wainright, S.C., 2002. Estimating turnover rates of carbon and nitrogen in recently metamorphosed winter flounder

Pseudopleuronectes americanus with stable isotopes. *Marine Ecology Progress Series*, *236*, pp.233-240.

Britton, J.R., 2007. Reference data for evaluating the growth of common riverine fishes in the UK. *Journal of Applied Ichthyology*, *23*(5), pp.555-560.

Britton, J.R., 2010. Scale circuli patterns differentiate between hatchery-reared and wild *Rutilus rutilus* during evaluation of fish stocking. *Journal of fish biology*, *77*(10), pp.2454-2459.

Britton, J.R. and Pegg, J., 2011. Ecology of European barbel *Barbus barbus*: implications for river, fishery, and conservation management. *Reviews in Fisheries Science*, *19*(4), pp.321-330

Britton, J.R., Boar, R.R., Grey, J., Foster, J., Lugonzo, J. and Harper, D.M., 2007. From introduction to fishery dominance: the initial impacts of the invasive carp *Cyprinus carpio* in Lake Naivasha, Kenya, 1999 to 2006. *Journal of Fish Biology*, *71*(sd), pp.239-257.

Britton, J.R., Cowx, I.G. and Peirson, G., 2004a. Sources of error in the ageing of stocked cyprinids. *Fisheries Management and Ecology*, *11*(6), pp.415-417.

Britton, J.R., Cowx, I.G., Axford, S.N., Frear, P.A., Eggleton, M.A. and Gido, K.B., 2004b. An overview of recruitment patterns of roach *Rutilus rutilus* (L.) between 1969 and 2001 in the rivers of England and their influence on population abundance. *Ecohydrology and Hydrobiology*, *4*, pp.91-102.

Britton, J.R., Cucherousset, J., Davies, G.D., Godard, M.J. and Copp, G.H., 2010a. Non-native fishes and climate change: predicting species responses to warming temperatures in a temperate region. *Freshwater Biology*, *55*(5), pp.1130-1141.

Britton, J.R., Cucherousset, J., Grey, J. and Gozlan, R.E., 2011a. Determining the strength of exploitative competition from an introduced fish: roles of density, biomass and body size. *Ecology of Freshwater Fish*, *20*(1), pp.74-79.

Britton, J.R., Davies, G.D. and Harrod, C., 2010b. Trophic interactions and consequent impacts of the invasive fish *Pseudorasbora parva* in a native aquatic foodweb: a field investigation in the UK. *Biological Invasions*, *12*(6), pp.1533-1542.

Britton, J.R., Davies, G.D. and Pegg, J., 2013. Spatial variation in the somatic growth rates of European barbel *Barbus barbus*: a UK perspective. *Ecology of Freshwater Fish*, *22*(1), pp.21-29.

Britton, J.R., Gozlan, R.E. and Copp, G.H., 2011b. Managing non-native fish in the environment. *Fish and fisheries*, *12*(3), pp.256-274.

Britton, J.R., Harper, D.M. and Oyugi, D.O., 2010c. Is the fast growth of an equatorial *Micropterus salmoides* population explained by high water temperature?. *Ecology of Freshwater Fish*, *19*(2), pp.228-238.

Britton, J.R., Harper, D.M., Oyugi, D.O. and Grey, J., 2010d. The introduced *Micropterus salmoides* in an equatorial lake: a paradoxical loser in an invasion meltdown scenario?. *Biological Invasions*, *12*(10), pp.3439-3448.

Broussonet, M., 1786. Observations sur la ŕeǵeneration de quelques parties du corps des poissons. *Histoire de l'Academie Royale des Sciences*, pp.684–688.

Broussonet, M., 1789. Memoir on the regeneration of certain parts of the bodies of fishes. *The Literary Magazine and British Review for 1789*, *3*, pp.111–113.

Brown, C.A. and Gruber, S.H., 1988. Age assessment of the lemon shark, *Negaprion brevirostris*, using tetracycline validated vertebral centra. *Copeia*, pp.747-753.

Brown, P., Green, C., Sivakumaran, K.P., Stoessel, D. and Giles, A., 2004. Validating otolith annuli for annual age determination of common carp. *Transactions of the American Fisheries Society*, *133*(1), pp.190-196.

Brush, J.M., Fisk, A.T., Hussey, N.E. and Johnson, T.B., 2012. Spatial and seasonal variability in the diet of round goby (*Neogobius melanostomus*): stable isotopes indicate that stomach contents overestimate the importance of dreissenids. *Canadian Journal of Fisheries and Aquatic Sciences*, *69*(3), pp.573-586.

Brusher, J.H. and Schull, J., 2009. Non-lethal age determination for juvenile goliath grouper Epinephelus itajara from southwest Florida. *Endangered Species Research*, *7*(3), pp.205-212.

Buchheister, A. and Latour, R.J., 2010. Turnover and fractionation of carbon and nitrogen stable isotopes in tissues of a migratory coastal predator, summer flounder (*Paralichthys dentatus*). *Canadian Journal of Fisheries and Aquatic Sciences*, *67*(3), pp.445-461.

Burnham, K.P. and Anderson, D.R., 2002. *Model selection and multimodel inference: a practical information-theoretic approach*, (2nd edition). Springer-Verlag, New York, USA.

Byström, P., Huss, M. and Persson, L., 2012. Ontogenetic constraints and diet shifts in Perch (*Perca fluviatilis*): mechanisms and consequences for intra-cohort cannibalism. *Freshwater Biology*, *57*(4), pp.847-857.

Campana, S.E., 2001. Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *Journal of Fish Biology*, *59*(2), pp.197-242.

Campana, S.E. and Neilson, J.D., 1985. Microstructure of fish otoliths. *Canadian Journal of Fisheries and Aquatic Sciences*, *42*(5), pp.1014-1032.

Campana, S.E., Annand, M.C. and McMillan, J.I., 1995. Graphical and statistical methods for determining the consistency of age determinations. *Transactions of the American Fisheries Society*, *124*(1), pp.131-138.

Candy, S.G., Nowara, G.B., Welsford, D.C. and McKinlay, J.P., 2012. Estimating an ageing error matrix for Patagonian toothfish (*Dissostichus eleginoides*) otoliths using between-reader integer errors, readability scores, and continuation ratio models. *Fisheries Research*, *115*, pp.14-23.

Carassou, L., Kulbicki, M., Nicola, T.J. and Polunin, N.V., 2008. Assessment of fish trophic status and relationships by stable isotope data in the coral reef lagoon of New Caledonia, southwest Pacific. *Aquatic Living Resources*, *21*(1), pp.1-12.

Carlander, K.D., 1974. Difficulties in ageing fish in relation to inland fishery management. In: T.B. Bagenal (ed.), *Ageing of Fish, Proceedings of an International Symposium*. Unwin Brothers Limited, Surrey, U.K.

Carleton, S.A. and Martínez del Rio, C., 2010. Growth and catabolism in isotopic incorporation: a new formulation and experimental data. *Functional Ecology*, *24*(4), pp.805-812.

Cass, A.J. and Beamish, R.J., 1983. First evidence of validity of the fin-ray method of age determination for marine fishes. *North American Journal of Fisheries Management*, *3*(2), pp.182-188.

Casselman, J.M., 1990. Growth and relative size of calcified structures of fish. *Transactions of the American Fisheries Society*, *119*(4), pp.673-688.

Caut, S., Angulo, E. and Courchamp, F., 2008. Caution on isotopic model use for analyses of consumer diet. *Canadian Journal of Zoology*, *86*(5), pp.438-445.

Caut, S., Angulo, E. and Courchamp, F., 2009. Variation in discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$): the effect of diet isotopic values and applications for diet reconstruction. *Journal of Applied Ecology*, *46*(2), pp.443-453.

Chang, W.Y., 1982. A statistical method for evaluating the reproducibility of age determination. *Canadian Journal of Fisheries and Aquatic Sciences*, *39*(8), pp.1208-1210.

Charnov, E.L. and Berrigan, D., 1991. Evolution of life history parameters in animals with indeterminate growth, particularly fish. *Evolutionary Ecology*, *5*(1), pp.63-68.

Chen, Y., Jackson, D.A. and Harvey, H.H., 1992. A comparison of von Bertalanffy and polynomial functions in modelling fish growth data. *Canadian Journal of Fisheries and Aquatic Sciences*, 49(6), pp.1228-1235.

Church, M.R., Ebersole, J.L., Rensmeyer, K.M., Couture, R.B., Barrows, F.T. and Noakes, D.L., 2009. Mucus: a new tissue fraction for rapid determination of fish diet switching using stable isotope analysis. *Canadian Journal of Fisheries and Aquatic Sciences*, *66*(1), pp.1-5.

Coggins, L.G. and Pine, W.E., 2010. Development of a Temperature-Dependent Growth Model for the Endangered Humpback Chub Using Capture-recapture Data. *Open Fish Journal,* 3, pp.122-131.

Connell, J.H., 1983. On the prevalence and relative importance of interspecific competition: evidence from field experiments. *American Naturalist*, pp.661-696.

Cope, J.M. and Punt, A.E., 2007. Admitting ageing error when fitting growth curves: an example using the von Bertalanffy growth function with random effects. *Canadian Journal of Fisheries and Aquatic Sciences*, *64*(2), pp.205-218.

Copp, G.H. and Sayer, C.D., 2010. *Norfolk Biodiversity Action Plan–Local Species Action Plan for Crucian Carp (*Carassius carassius*)*. Norfolk Biodiversity Partnership Reference: LS⁄ 3. Centre for Environment, Fisheries and Aquaculture Science, Lowestoft, Suffolk.

Copp, G.H., Bianco, P.G., Bogutskaya, N.G., Erős, T., Falka, I., Ferreira, M.T., Fox, M.G., Freyhof, J., Gozlan, R.E., Grabowska, J. and Kováč, V., 2005. To be, or not to be, a non-native freshwater fish?. *Journal of Applied Ichthyology*, *21*(4), pp.242-262.

Copp, G.H., Tarkan, A.S., Godard, M.J., Edmonds, N.J. and Wesley, K.J., 2010. Preliminary assessment of feral goldfish impacts on ponds, with particular reference to native crucian carp. *Aquatic invasions*, *5*(4), pp.413-422.

Copp, G.H., Warrington, S. and Wesley, K.J., 2008. Management of an ornamental pond as a conservation site for a threatened native fish species, crucian carp *Carassius carassius*. *Hydrobiologia*, *597*(1), pp.149-155.

Cornell University Stable Isotope Laboratory, 2016. *Preparation Guidelines.* Available at: www.cobsil.com/iso_main_preparation_guidlines.php (last accessed 24/09/2016).

Cowx, I.G. and Broughton, N.M., 1986. Changes in the species composition of anglers' catches in the River Trent (England) between 1969 and 1984. *Journal of fish biology*, *28*(5), pp.625-636.

Cowx, I.G. and Gerdeaux, D., 2004. The effects of fisheries management practises on freshwater ecosystems. *Fisheries Management and Ecology*, *11*(3-4), pp.145-151.

Cragg-Hine, D. and Jones, J.W., 1969. The growth of dace *Leuciscus leuciscus* (L.), roach *Rutilus rutilus* (L.) and chub *Squalius cephalus* (L.) in Willow Brook, Northamptonshire. *Journal of Fish Biology*, *1*(1), pp.59-82.

Craig, M.T., 2011. *Epinephelus itajara*. The IUCN Red List of Threatened Species 2011: e.T195409A8961414. Available at: www.iucnredlist.org (last accessed 26/09/2016).

Crawley, M.J., 2005. Statistics: an Introduction using R. Wiley, Hoboken, New Jersey.

Crichton M. I., 1935. Scale-absorption in salmon and sea trout. *Fishery Board for Scotland, Salmon Fisheries*, *4*, pp.1-8.

Crowl, T.A., Townsend, C.R. and McIntosh, A.R., 1992. The impact of introduced brown and rainbow trout on native fish: the case of Australasia. *Reviews in fish biology and fisheries*, *2*(3), pp.217-241.

Cucherousset, J. and Olden, J.D., 2011. Ecological impacts of nonnative freshwater fishes. *Fisheries*, *36*(5), pp.215-230.

Cucherousset, J., Boulêtreau, S., Azémar, F., Compin, A., Guillaume, M. and Santoul, F., 2012a. "Freshwater Killer Whales": Beaching Behavior of an Alien Fish to Hunt Land Birds. *PloS one*, *7*(12), e50840.

Cucherousset, J., Boulêtreau, S., Martino, A., Roussel, J.M. and Santoul, F., 2012b. Using stable isotope analyses to determine the ecological effects of non-native fishes. *Fisheries Management and Ecology*, *19*(2), pp.111-119.

Cucherousset, J., Copp, G.H., Fox, M.G., Sterud, E., van Kleef, H.H., Verreycken, H. and Záhorská, E., 2009. Life-history traits and potential invasiveness of introduced pumpkinseed *Lepomis gibbosus* populations in northwestern Europe. *Biological Invasions*, *11*(9), pp.2171-2180.

Cyr, H., Downing, J.A., Lalonde, S., Baines, S.B. and Pace, M.L., 1992. Sampling larval fish populations: choice of sample number and size. *Transactions of the American Fisheries Society*, *121*(3), pp.356-368.

Dahl, K., 1907. The scales of the herring as a means of determining age, growth and migration. *Reports of the Directorate of Fisheries, Technological Investigations Series*, *2*(6), pp.1–39.

Davis, A.M., Blanchette, M.L., Pusey, B.J., Jardine, T.D. and Pearson, R.G., 2012. Gut content and stable isotope analyses provide complementary understanding of ontogenetic dietary shifts and trophic relationships among fishes in a tropical river. *Freshwater Biology*, 57(10), pp.2156-2172.

Declerck, S., Louette, G., Bie, T.D. and Meester, L.D., 2002. Patterns of diet overlap between populations of non-indigenous and native fishes in shallow ponds. *Journal of Fish Biology*, *61*(5), pp.1182-1197.

DeNiro, M.J. and Epstein, S., 1978. Influence of diet on the distribution of carbon isotopes in animals. *Geochimica et cosmochimica acta*, *42*(5), pp.495-506.

DeNiro, M.J. and Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et cosmochimica acta,* 45(3), pp.341–351.

Doering-Arjes, P., Cardinale, M. and Mosegaard, H., 2008. Estimating population age structure using otolith morphometrics: a test with known-age Atlantic cod (*Gadus morhua*) individuals. *Canadian Journal of Fisheries and Aquatic Sciences*, *65*(11), pp.2342-2350.

Dortel, E., Massiot-Granier, F., Rivot, E., Million, J., Hallier, J.P., Morize, E., Munaron, J.M., Bousquet, N. and Chassot, E., 2013. Accounting for age uncertainty in growth modeling, the case study of yellowfin tuna (*Thunnus albacares*) of the Indian Ocean. *PloS one*, *8*(4), e60886.

Englund, V., Niemela, E., Lansman, M. and Heino, M., 1999. Variations in Atlantic salmon, *Salmo salar* L., smolt age in tributaries of the River Teno, Finland. *Fisheries Management and Ecology, 6*, pp.83-86.

Environment Agency, 2016. *Annual fisheries report 2014 to 2015*. Environment Agency, Bristol, UK. Available at: www.gov.uk (last accessed on 28/09/2016).

Fincel, M.J., Vandehey, J.A. and Chipps, S.R., 2012. Non-lethal sampling of walleye for stable isotope analysis: a comparison of three tissues. *Fisheries Management and Ecology*, *19*(4), pp.283-292.

Finlay, J.C., Khandwala, S. and Power, M.E., 2002. Spatial scales of carbon flow in a river food web. *Ecology*, *83*(7), pp.1845-1859.

Florin, S.T., Felicetti, L.A. and Robbins, C.T., 2011. The biological basis for understanding and predicting dietary-induced variation in nitrogen and sulphur isotope ratio discrimination. *Functional Ecology*, *25*(3), pp.519-526.

Fogel, M.L. and Tuross, N., 2003. Extending the limits of paleodietary studies of humans with compound specific carbon isotope analysis of amino acids. *Journal of Archaeological Science*, *30*(5), pp.535-545.

Fontagne, S., Geurden, I., Escaffre, A.M. and Bergot, P., 1998. Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. *Aquaculture*, *161*(1), pp.213-223.

Francis, R.I.C.C., 1990. Back-calculation of fish length: a critical review. *Journal of Fish Biology*, *36*(6), pp.883-902.

Francis, R.I.C.C., Campana, S.E. and Neil, H.L., 2010. Validation of fish ageing methods should involve bias estimation rather than hypothesis testing: a proposed approach for bomb radiocarbon validations. *Canadian Journal of Fisheries and Aquatic Sciences*, *67*(9), pp.1398-1408.

Fraser, C.M., 1916. Growth of the spring salmon. *Transaction of Pacific Society Seattle, Second Annual Meeting* 1915, pp.29–39.

Frazer, T.K., Ross, R.M., Quetin, L.B. and Montoya, J.P., 1997. Turnover of carbon and nitrogen during growth of larval krill, *Euphausia superba Dana*: a stable isotope approach. *Journal of Experimental Marine Biology and Ecology*, *212*(2), pp.259-275.

Frear, P.A. and Cowx, I.G., 2003. Factors affecting coarse fish recruitment. R&D Technical Report W2-048/TR. Available at: www.gov.uk (last accessed on 28/09/2016).

Frie, R.V., 1982. Measurement of fish scales and back calculation of body lengths using a digitizing pad and microcomputer. *Fisheries*, *7*(5), pp.5-8.

Frisk, M.G., Miller, T.J. and Fogarty, M.J., 2001. Estimation and analysis of biological parameters in elasmobranch fishes: a comparative life history study. *Canadian Journal of Fisheries and Aquatic Sciences*, *58*(5), pp.969-981.

Fry, B. 2006. *Stable isotope ecology*. Springer Science & Business Media, New York.

Fry, B. and Arnold, C., 1982. Rapid $^{13}C/^{12}C$ turnover during growth of brown shrimp (*Penaeus aztecus*). *Oecologia*, *54*(2), pp.200-204.

Fry, B., Mumford, P.L., Tam, F., Fox, D.D., Warren, G.L., Havens, K.E. and Steinman, A.D., 1999. Trophic position and individual feeding histories of fish from Lake Okeechobee, Florida. *Canadian Journal of Fisheries and Aquatic Sciences*, *56*(4), pp.590-600.

Galster, B.J., Wuellner, M.R. and Graeb, B.D.S., 2012. Walleye *Sander vitreus* and smallmouth bass *Micropterus dolomieu* interactions: an historic stable-isotope analysis approach. *Journal of Fish Biology*, *81*(1), pp.135-147.

Gannes, L.Z., O'Brien, D.M. and Martínez del Rio, C., 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. *Ecology*, *78*(4), pp.1271-1276.

Garcia, A.M., Claudino, M.C., Mont'Alverne, R., Pereyra, P.E.R., Copertino, M. and Vieira, J.P., 2016. Temporal variability in assimilation of basal food sources by an omnivorous fish at Patos Lagoon Estuary revealed by stable isotopes (2010–2014). *Marine Biology Research*, pp.1-10.

García-Berthou, E., 2001. Size-and depth-dependent variation in habitat and diet of the common carp (*Cyprinus carpio*). *Aquatic Sciences*, *63*(4), pp.466-476.

Garner, P., 1997. Sample sizes for length and density estimation of 0+ fish when using point sampling by electrofishing. *Journal of Fish Biology*, *50*(1), pp.95-106.

Gaye-Siessegger, J., Focken, U., Muetzel, S., Abel, H. and Becker, K., 2004. Feeding level and individual metabolic rate affect $\delta^{13}C$ and $\delta^{15}N$ values in carp: implications for food web studies. *Oecologia*, *138*(2), pp.175-183.

Gerdeaux, D. and Perga, M.E., 2006. Changes in whitefish scales ^{13}C during eutrophication and reoligotrophication of subalpine lakes. *Limnology and Oceanography*, *51*(1 part 2), pp.772-780.

Gido, K.B. and Brown, J.H., 1999. Invasion of North American drainages by alien fish species. *Freshwater Biology*, *42*(2), pp.387-399.

Gomez, S.E., Ferrè, H., Cassarà, H. and Bordone, S., 1997. Cultivo de peces ornamentales (*Carassius auratus* y *Cyprinus carpio*) en sistemas semiintensivos en la Argentina. *AQUATEC Bol Téc*, *4*, pp.1-13.

Goodyear, C.P., 1995. Mean size at age: an evaluation of sampling strategies with simulated red grouper data. *Transactions of the American Fisheries Society*, *124*, pp.746–755.

Gotthard, K., 2001. Growth strategies of ectothermic animals in temperate environments. In: D. Atkinson and M. Thorndyke (eds.), *Environment and animal development.* BIOS Scientific, Oxford, pp.287-304.

Gozlan, R.E., 2008. Introduction of non-native freshwater fish: is it all bad?. *Fish and Fisheries*, *9*(1), pp.106-115.

Gozlan, R.E., Britton, J.R., Cowx, I. and Copp, G.H., 2010. Current knowledge on non-native freshwater fish introductions. *Journal of Fish Biology*, *76*(4), pp.751-786.

Graham, C.T., Harrison, S.S. and Harrod, C., 2013. Development of non-lethal sampling of carbon and nitrogen stable isotope ratios in salmonids: effects of lipid and inorganic components of fins. *Isotopes in Environmental and Health Studies*, *49*(4), pp.555-566.

Greer, A.L., Horton, T.W. and Nelson, X.J., 2015. Simple ways to calculate stable isotope discrimination factors and convert between tissue types. *Methods in Ecology and Evolution*, *6*(11), pp.1341-1348.

Grey, J. and Jackson, M.C., 2012. 'Leaves and eats shoots': Direct terrestrial feeding can supplement invasive red swamp crayfish in times of need. *PloS one*, *7*, e42575.

Grey, J., 2006. The use of stable isotope analyses in freshwater ecology: current awareness. *Polish Journal of Ecology, 54*(4), pp.563-584.

Grey, J., Graham, C.T., Britton, J.R. and Harrod, C., 2009. Stable isotope analysis of archived roach (*Rutilus rutilus*) scales for retrospective study of shallow lake responses to nutrient reduction. *Freshwater Biology*, *54*(8), pp.1663-1670.

Guinan Jr., M.E., Kapuscinski, K.L. and Teece, M.A., 2015. Seasonal diet shifts and trophic position of an invasive cyprinid, the rudd *Scardinius erythrophthalmus* (Linnaeus, 1758), in the upper Niagara River. *Aquatic Invasions*, *10*(2), pp.217-225.

Guo, Z., Liu, J., Lek, S., Li, Z., Ye, S., Zhu, F., Tang, J. and Cucherousset, J., 2012. Habitat segregation between two congeneric and introduced goby species. *Fundamental and Applied Limnology/Archiv für Hydrobiologie*, *181*(3), pp.241-251.

Guo, Z., Liu, J., Lek, S., Li, Z., Zhu, F., Tang, J. and Cucherousset, J., 2014. Trophic niche differences between two congeneric goby species: evidence for ontogenetic diet shift and habitat use. *Aquatic Biology, 20*(1)*,* pp.23-33.

Hagen, P.T., Oxman, D.S. and Agler, B.A., 2001. Developing and deploying a high resolution imaging approach for scale analysis. *North Pacific Anadromous Fish Commission.* Doc. 567. Department of Fish and Game, Mark, Tag, and Age Lab, Alaska.

Hamidan, N., Jackson, M.C. and Britton, J.R., 2015. Diet and trophic niche of the endangered fish *Garra ghorensis* in three Jordanian populations. *Ecology of Freshwater Fish*, *25*(3), pp.455-464.

Hänfling, B., Bolton, P., Harley, M. and Carvalho, G.R., 2005. A molecular approach to detect hybridisation between crucian carp (*Carassius carassius*) and non-indigenous carp species (*Carassius* spp. and *Cyprinus carpio*). *Freshwater Biology*, *50*(3), pp.403-417.

Hanisch, J.R., Tonn, W.M., Paszkowski, C.A. and Scrimgeour, G.J., 2010. $\delta^{13}C$ and $\delta^{15}N$ signatures in muscle and fin tissues: nonlethal sampling methods for stable isotope analysis of salmonids. *North American Journal of Fisheries Management*, *30*(1), pp.1-11.

Heady, W.N. and Moore, J.W., 2012. Tissue turnover and stable isotope clocks to quantify resource shifts in anadromous rainbow trout. *Oecologia*, *172*(1), pp.21-34.

Heidarsson, T., Antonsson, T. and Snorrason, S.S., 2006. The relationship between body and scale growth proportions and validation of two back-calculation methods using individually tagged and recaptured wild Atlantic salmon. *Transactions of the American Fisheries Society*, *135*(5), pp.1156-1164.

Hertz, E., Trudel, M., Brodeur, R.D., Daly, E.A., Eisner, L., Farley Jr, E.V., Harding, J.A., MacFarlane, R.B., Mazumder, S., Moss, J.H. and Murphy, J.M., 2015. Continental-scale variability in the feeding ecology of juvenile Chinook salmon along the coastal Northeast Pacific Ocean. *Marine Ecology Progress Series*, *537*, pp.247-263.

Herzka, S.Z. and Holt, G.J., 2000. Changes in isotopic composition of red drum (*Sciaenops ocellatus*) larvae in response to dietary shifts: potential applications to settlement studies. *Canadian Journal of Fisheries and Aquatic Sciences*, *57*(1), pp.137-147.

Herzka, S.Z., Holt, S.A. and Holt, G.J., 2001. Documenting the settlement history of individual fish larvae using stable isotope ratios: model development and validation. *Journal of Experimental Marine Biology and Ecology*, *265*(1), pp.49-74.

Hesslein, R.H., Hallard, K.A. and Ramlal, P., 1993. Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by $\delta^{34}S$, $\delta^{13}C$, and $\delta^{15}N$. *Canadian Journal of Fisheries and Aquatic Sciences*, *50*(10), pp.2071-2076.

Hickley, P. and Chare, S., 2004. Fisheries for non-native species in England and Wales, Angling or the environment? *Fisheries Management and Ecology, 11*(3-4), pp.203–212.

Hickley, P. and Dexter, K.F., 1979. A comparative index for quantifying growth in length of fish. *Aquaculture Research*, *10*(4), pp.147-151.

Hobson, K.A. and Clark, R.G., 1992. Assessing avian diets using stable isotopes I: turnover of ^{13}C in tissues. *Condor*, *94*, pp.181-188.

Holopainen, I.J., Tonn, W.M. and Paszkowski, C.A., 1992. Effects of fish density on planktonic communities and water quality in a manipulated forest pond. *Hydrobiologia*, *243*(1), pp.311-321.

Holopainen, I.J., Tonn, W.M. and Paszkowski, C.A., 1997. Tales of two fish: the dichotomous biology of crucian carp (*Carassius carassius* (L.)) in northern Europe. *Annales Zoologici Fennici*, *34*, pp.1-22.

Horká, P., Ibbotson, A., Jones, J.I., Cove, R.J. and Scott, L.J., 2010. Validation of scale-age determination in European grayling *Thymallus thymallus* using tag-recapture analysis. *Journal of Fish Biology*, *77*(1), pp.153-161.

Howland, M.R., Corr, L.T., Young, S.M., Jones, V., Jim, S., Van Der Merwe, N.J., Mitchell, A.D. and Evershed, R.P., 2003. Expression of the dietary isotope signal in the compound-specific δ13C values of pig bone lipids and amino acids. *International Journal of Osteoarchaeology*, *13*(1-2), pp.54-65.

Huang, W., Yano, S., Zhang, J.M. and Wang, Y.R., 2013. Spatial and temporal variation in stable isotopes signatures of periphyton and an endangered fish in a flow-reduced river reach. *International Journal of Environmental Research*, *7*(3), pp.533-538.

Hulme, M., Jenkins, G.J., Lu, X., Turnpenny, J.R., Mitchell, T.D., Jones, R.G., Lowe, J., Murphy, J.M., Hassell, D., Boorman, P., McDonald, R. and Hill, S., 2002. *Climate Change Scenarios for the United Kingdom: The UKCIP02 Scientific Report*, Tyndall Centre for Climate Change Research, School of Environmental Sciences, University of East Anglia, Norwich, UK.

Human, K.G. and Gordon, D.M., 1996. Exploitation and interference competition between the invasive Argentine ant, *Linepithema humile*, and native ant species. *Oecologia*, *105*(3), pp.405-412.

Hutchinson, J.J. and Trueman, C.N., 2006. Stable isotope analyses of collagen in fish scales: limitations set by scale architecture. *Journal of Fish Biology*, *69*(6), pp.1874-1880.

Hyslop, E.J., 1980. Stomach contents analysis—a review of methods and their application. *Journal of fish biology*, *17*(4), pp.411-429.

Ibáñez, A.L., Britton, J.R. and Cowx, I.G., 2008. Relationship between scale growth checks, circuli formation rate and somatic growth in *Rutilus rutilus* (L.) a fish farm-reared cyprinid. *Journal of Fish Biology*, *72*(4), pp.1023-1034.

Jackson, A.L., Inger, R., Parnell, A.C. and Bearhop, S., 2011. Comparing isotopic niche widths among and within communities: SIBER–Stable Isotope Bayesian Ellipses in R. *Journal of Animal Ecology*, *80*(3), pp.595-602.

Jackson, J.R., 2007. Earliest references to age determination of fishes and their early application to the study of fisheries. *Fisheries*, *32*(7), pp.321-328.

Jackson, M.C. and Britton, J.R., 2014. Divergence in the trophic niche of sympatric freshwater invaders. *Biological invasions*, *16*(5), pp.1095-1103.

Jackson, M.C., Allen, R., Pegg, J. and Britton, J.R., 2013. Do trophic subsidies affect the outcome of introductions of a non-native freshwater fish? *Freshwater Biology*, *58*(10), pp.2144-2153.

Jackson, M.C., Britton, J.R., Cucherousset, J., Guo, Z., Stakėnas, S., Gozlan, R.E., Godard, M.G., Roussel, J.M. and Copp, G.H., 2016a. Do non-native pumpkinseed Lepomis gibbosus affect the growth, diet and trophic niche breadth of native brown trout Salmo trutta?. *Hydrobiologia*, *772*(1), pp.63-75.

Jackson, M.C., Donohue, I., Jackson, A.L., Britton, J.R., Harper, D.M. and Grey, J., 2012. Population-level metrics of trophic structure based on stable isotopes and their application to invasion ecology. *PloS one*, *7*(2), e31757.

Jackson, M.C., Grey, J., Miller, K., Britton, J.R. and Donohue, I., 2016b. Dietary niche constriction when invaders meet natives: evidence from freshwater decapods. *Journal of Animal Ecology*, *85*(4), pp.1098-1107.

Jackson, Z.J., Quist, M.C., Larscheid, J.G., Thelen, E.C. and Hawkins, M.J., 2007. Precision of scales and dorsal spines for estimating age of common carp. *Journal of Freshwater Ecology*, *22*(2), pp.231-239.

Jacob, U., Mintenbeck, K., Brey, T., Knust, R. and Beyer, K., 2005. Stable isotope food web studies: a case for standardized sample treatment. *Marine Ecology Progress Series*, *287*, pp.251-253.

Jardine, T.D., Gray, M.A., McWilliam, S.M. and Cunjak, R.A., 2005. Stable isotope variability in tissues of temperate stream fishes. *Transactions of the American Fisheries Society*, *134*(5), pp.1103-1110.

Jardine, T.D., Hunt, R.J., Pusey, B.J. and Bunn, S.E., 2011. A non-lethal sampling method for stable carbon and nitrogen isotope studies of tropical fishes. *Marine and Freshwater Research*, *62*(1), pp.83-90.

Jeffries, D.L., Copp, G.H., Lawson Handley, L., Olsén, K.H., Sayer, C.D. and Hänfling, B., 2016. Comparing RADseq and microsatellites to infer complex phylogeographic patterns, an empirical perspective in the Crucian carp, *Carassius carassius*, L. *Molecular ecology*, *25*(13), pp. 2997-3018.

Jobling, M., 1997. Temperature and growth: modulation of growth rate via temperature change. In: C.M. Wood and D.G. McDonald (eds.), *Society for Experimental Biology Seminar Series 61: Global Warming: Implications for freshwater and marine fish*, Cambridge University Press, UK, pp.225-253.

Jones, J.I. and Waldron, S., 2003. Combined stable isotope and gut contents analysis of food webs in plant-dominated, shallow lakes. *Freshwater Biology*, *48*(8), pp.1396-1407.

Jones, M., Mandelik, Y. and Dayan, T., 2001. Coexistence of temporally partitioned spiny mice: roles of habitat structure and foraging behavior. *Ecology*, *82*(8), pp.2164-2176.

Kakareko, T., Kobak, J., Grabowska, J., Jermacz, Ł., Przybylski, M., Poznańska, M., Pietraszewski, D. and Copp, G.H., 2013. Competitive interactions for food resources between invasive racer goby *Babka gymnotrachelus* and native European bullhead *Cottus gobio*. *Biological Invasions*, *15*(11), pp.2519-2530.

Kalish, J.M., Beamish, R.J., Brothers, E.B., Casselman, J.M., Francis, R.I.C.C., Mosegaard, H., Panfili, J., Prince, E.D., Thresher, R.E., Wilson, C.A. and Wright,

P.J, 1995. Glossary for otolith studies. In: D.H. Secor, J.M. Dean and S.E. Campana (eds.), *Recent Developments in Fish Otolith Research*, University of South Carolina Press, Columbia, pp.723–729.

Kamler, E., Kamiński, R., Wolnicki, J., Sikorska, J. and Wałowski, J., 2012. Effects of diet and temperature on condition, proximate composition and three major macro elements, Ca, P and Mg, in barbel *Barbus barbus* juveniles. *Reviews in Fish Biology and Fisheries*, *22*(3), pp.767-777.

Kelly, L.J. and Martínez del Rio, C., 2010. The fate of carbon in growing fish: an experimental study of isotopic routing. *Physiological and Biochemical Zoology*, *83*(3), pp.473-480.

Kelly, M.H., Hagar, W.G., Jardine, T.D. and Cunjak, R.A., 2006. Nonlethal sampling of sunfish and slimy sculpin for stable isotope analysis: how scale and fin tissue compare with muscle tissue. *North American Journal of Fisheries Management*, *26*(4), pp.921-925.

Kimura, D.K. and Anderl, D.M., 2005. Quality control of age data at the Alaska Fisheries Science Center. *Marine and Freshwater Research*, *56*(5), pp.783-789.

Kimura, D.K. and Lyons, J.J., 1991. Between-reader bias and variability in the age-determination process. *Fishery Bulletin*, *89*(1), pp.53-60.

King, A.J., 2005. Ontogenetic dietary shifts of fishes in an Australian floodplain river. *Marine and Freshwater Research*, *56*(2), pp.215-225.

Klumb, R.A., Bozek, M.A. and Frie, R.V., 1999. Validation of the Dahl–Lea and Fraser–Lee back-calculation models by using oxytetracycline-marked bluegills and bluegill× green sunfish hybrids. *North American Journal of Fisheries Management*, *19*(2), pp.504-514.

Koch, J.D., Quist, M.C. and Hansen, K.A., 2009. Precision of hard structures used to estimate age of bowfin in the upper Mississippi River. *North American Journal of Fisheries Management*, *29*(2), pp.506-511.

Koch, J.D., Schreck, W.J. and Quist, M.C., 2008. Standardised removal and sectioning locations for shovelnose sturgeon fin rays. *Fisheries Management and Ecology*, *15*(2), pp.139-145.

Koehn, J.D., 2004. Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater biology*, *49*(7), pp.882-894.

Korsu, K., Huusko, A. and Muotka, T., 2009. Does the introduced brook trout (*Salvelinus fontinalis*) affect the growth of the native brown trout (*Salmo trutta*)? *Naturwissenschaften, 96*(3), pp.347–353.

Kritzer, J.P., Davies, C.R. and Mapstone, B.D., 2001. Characterizing fish populations: effects of sample size and population structure on the precision of demographic parameter estimates. *Canadian Journal of Fisheries and Aquatic Sciences*, *58*(8), pp.1557-1568.

Kurle, C.M., 2009. Interpreting temporal variation in omnivore foraging ecology via stable isotope modelling. *Functional Ecology*, *23*(4), pp.733-744.

Kurle, C.M., Koch, P.L., Tershy, B.R. and Croll, D.A., 2014. The effects of sex, tissue type, and dietary components on stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) in mammalian omnivores. *Isotopes in Environmental and Health Studies*, *50*(3), pp.307-321.

Kwak, T.J., Waters, D.S. and Pine III, W.E., 2006. Age, growth, and mortality of introduced flathead catfish in Atlantic rivers and a review of other populations. *North American Journal of Fisheries Management*, *26*(1), pp.73-87.

Layman, C.A., Araujo, M.S., Boucek, R., Hammerschlag-Peyer, C.M., Harrison, E., Jud, Z.R., Matich, P., Rosenblatt, A.E., Vaudo, J.J., Yeager, L.A. and Post, D.M., 2011. Applying stable isotopes to examine food-web structure: an overview of analytical tools. *Biological Reviews*, *87*(3), pp.545-562.

Layman, C.A., Winemiller, K.O., Arrington, D.A. and Jepsen, D.B., 2005. Body size and trophic position in a diverse tropical food web. *Ecology*, *86*(9), pp.2530-2535.

Lea, E., 1910. On the methods used in the herring investigations. *Publications de Circonstance, International Council for the Exploration of the Sea*, *53*, pp.7–25.

Lee, R. M., 1920. A review of the methods of age and growth determination in fishes by means of scales. *Fisheries Investigations, Series II, Marine Fisheries, Great Britain Ministry of Agriculture Fisheries and Food, 4*(2), pp.1–35.

Lee, R.M., 1912. An investigation into the methods of growth determination in fishes by means of scales. *Journal du Conseil*, *1*(63), pp.3-34.

Leim, A.H., 1924. The life history of the shad (*Alosa sapidissima* (Wilson)) with special reference to the factors limiting its abundance. *Contributions to Canadian Biology and Fisheries*, *2*(1), pp.161-284.

Lester, N.P., Shuter, B.J. and Abrams, P.A., 2004. Interpreting the von Bertalanffy model of somatic growth in fishes: the cost of reproduction. *Proceedings of the Royal Society of London B: Biological Sciences*, *271*, pp.1625-1631.

Liao, H., Sharov, A.F., Jones, C.M. and Nelson, G.A., 2013. Quantifying the effects of aging bias in Atlantic striped bass stock assessment. *Transactions of the American Fisheries Society*, *142*(1), pp.193-207.

Linfield, R.S.J., 1974. The errors likely in ageing roach *Rutilus rutilus* (L.), with special reference to stunted populations. In: T.B. Bagenal (ed.), *Ageing of Fish, Proceedings of an International Symposium*. Unwin Brothers Limited, Surrey, U.K.

Lintermans, M., 2004. Human-assisted dispersal of alien freshwater fish in Australia. *New Zealand Journal of Marine and Freshwater Research*, *38*(3), pp.481-501.

Locke, S.A., Bulté, G., Forbes, M.R. and Marcogliese, D.J., 2013. Estimating diet in individual pumpkinseed sunfish *Lepomis gibbosus* using stomach contents, stable isotopes and parasites. *Journal of Fish Biology*, *82*(2), pp.522-537.

Logan, J., Haas, H., Deegan, L. and Gaines, E., 2006. Turnover rates of nitrogen stable isotopes in the salt marsh mummichog, *Fundulus heteroclitus*, following a laboratory diet switch. *Oecologia*, *147*(3), pp.391-395.

Lorenzoni, M., Corboli, M., Ghetti, L., Pedicillo, G. and Carosi, A., 2007. Growth and reproduction of the goldfish *Carassius auratus*: a case study from Italy. In: F.

Gherardi (ed.), *Biological invaders in inland waters: Profiles, distribution, and threats* (pp. 259-273). Springer, Netherlands, pp.259–273.

Lorenzoni, M., Ghetti, L., Pedicillo, G. and Carosi, A., 2010. Analysis of the biological features of the goldfish Carassius auratus auratus in Lake Trasimeno (Umbria, Italy) with a view to drawing up plans for population control. *Folia Zoologica*, *59*(2), p.142.

Lougheed, V.L., Crosbie, B. and Chow-Fraser, P., 1998. Predictions on the effect of common carp (*Cyprinus carpio*) exclusion on water quality, zooplankton, and submergent macrophytes in a Great Lakes wetland. *Canadian Journal of Fisheries and Aquatic Sciences*, *55*(5), pp.1189-1197.

Lowe, S., Browne, M., Boudjelas, S. and De Poorter, M., 2000. 100 of the world's worst invasive alien species: a selection from the global invasive species database. Species Survival Commission, World Conservation Union (IUCN), New Zealand.

MacAvoy, S.E., Macko, S.A. and Garman, G.C., 2001. Isotopic turnover in aquatic predators: quantifying the exploitation of migratory prey. *Canadian Journal of Fisheries and Aquatic Sciences*, *58*(5), pp.923-932.

Macko, S.A., Estep, M.L.F., Engel, M.H. and Hare, P.E., 1986. Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta*, *50*(10), pp.2143-2146.

MacNeil, M.A., Drouillard, K.G. and Fisk, A.T., 2006. Variable uptake and elimination of stable nitrogen isotopes between tissues in fish. *Canadian Journal of Fisheries and Aquatic Sciences*, *63*(2), pp.345-353.

Madigan, D.J., Chiang, W.C., Wallsgrove, N.J., Popp, B.N., Kitagawa, T., Choy, C.A., Tallmon, J., Ahmed, N., Fisher, N.S. and Sun, C.L., 2016. Intrinsic tracers reveal recent foraging ecology of giant Pacific bluefin tuna at their primary spawning grounds. *Marine Ecology Progress Series*, *553*, pp.253-266.

Mahe, K., Rabhi, K., Bellamy, E., Elleboode, R., Aumond, Y., Huet, J. and Roos, D., 2016. Comparison between the opercular bones, the scales and the otoliths to investigate the growth of the brilliant pomfret (*Eumegistus illustris*) off the coast of Reunion

Island (SW Indian Ocean). *International Journal of Fisheries and Aquatic Studies*, *4*(1 Part C), pp.176-179.

Mann, R.H.K. and Steinmetz, B., 1985. On the accuracy of age-determination using scales from rudd, *Scardinius erythrophthalmus* (L.), of known age. *Journal of Fish Biology*, 26(5), pp.621-628.

Mann, R.H.K., 1973. Observations on the age, growth, reproduction and food of the roach *Rutilus rutilus* (L.) in two rivers in southern England. *Journal of Fish Biology*, *5*(6), pp.707-736.

Mann, R.H.K., 1974. Observations on the age, growth, reproduction and food of the dace, *Leuciscus leuciscus* (L.), in two rivers in southern England. *Journal of Fish Biology*, *6*(3), pp.237-253.

Mann, R.H.K., 1976. Observations on the age, growth, reproduction and food of the chub *Squalius cephalus* (L.) in the River Stour, Dorset. *Journal of Fish Biology*, *8*(3), pp.265-288.

Martin, C.W., Valentine, M.M. and Valentine, J.F., 2010. Competitive interactions between invasive Nile tilapia and native fish: the potential for altered trophic exchange and modification of food webs. *PLoS One*, *5*, e14395.

Martínez del Rio, C. and Wolf, B.O., 2005. Mass balance models for animal isotopic ecology. In: J.M. Starck and T. Wang (eds.), *Physiological and Ecological Adaptations to Feeding in Vertebrates*, Science Publishers, New Hampshire, pp.141–174.

Martínez del Rio, C., Wolf, N., Carleton, S.A. and Gannes, L.Z., 2009. Isotopic ecology ten years after a call for more laboratory experiments. *Biological Reviews*, *84*(1), pp.91-111.

McClelland, J.W. and Montoya, J.P., 2002. Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology*, *83*(8), pp.2173-2180.

McClelland, J.W., Holl, C.M. and Montoya, J.P., 2003. Relating low $\delta^{15}N$ values of zooplankton to N_2-fixation in the tropical North Atlantic: insights provided by

stable isotope ratios of amino acids. *Deep Sea Research Part I: Oceanographic Research Papers*, *50*(7), pp.849-861.

McCutchan, J.H., Lewis, W.M., Kendall, C. and McGrath, C.C., 2003. Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos*, *102*(2), pp.378-390.

McIntyre, P.B. and Flecker, A.S., 2006. Rapid turnover of tissue nitrogen of primary consumers in tropical freshwaters. *Oecologia*, *148*(1), pp.12-21.

Methot, R.D. and Wetzel, C.R., 2013. Stock synthesis: a biological and statistical framework for fish stock assessment and fishery management. *Fisheries Research*, *142*, pp.86-99.

Milardi, M., Käkelä, R., Weckström, J. and Kahilainen, K.K., 2015. Terrestrial prey fuels the fish population of a small, high-latitude lake. *Aquatic Sciences*, pp.1-12.

Mill, A.C., Pinnegar, J.K. and Polunin, N.V.C., 2007. Explaining isotope trophic-step fractionation: why herbivorous fish are different. *Functional Ecology*, *21*(6), pp.1137-1145.

Minagawa, M. and Wada, E., 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. *Geochimica et cosmochimica acta*, *48*(5), pp.1135-1140.

Morgan, D.L., Gill, H.S., Maddern, M.G. and Beatty, S.J., 2004. Distribution and impacts of introduced freshwater fishes in Western Australia. *New Zealand Journal of Marine and Freshwater Research*, *38*(3), pp.511-523.

Musk, R.S., Britton, J.R. and Axford, S.N., 2006. The effect of subjective fish scale ageing on growth and recruitment analyses: a case study from the UK. *Acta ichthyologica et Piscatoria*, *36*(1), pp.81-84.

Myers, R.A. and Drinkwater, K., 1989. The influence of Gulf Stream warm core rings on recruitment of fish in the northwest Atlantic. *Journal of Marine Research*, *47*(3), pp.635-656.

Nagiec, M., Czerkies, P., Goryczko, K., Witkowski, A. and Murawska, E., 1995. Mass-marking of grayling, *Thymallus thymallus* (L.), larvae by fluorochrome tagging of otoliths. *Fisheries Management and Ecology*, *2*(3), pp.185-195.

Nelson, J.S., Grande, T.C. and Wilson, M.V., 2016. *Fishes of the World* (5th edition). John Wiley and Sons, Inc., Hoboken, New Jersey.

Neuheimer, A.B. and Taggart, C.T., 2007. The growing degree-day and fish size-at-age: the overlooked metric. *Canadian Journal of Fisheries and Aquatic Sciences*, *64*(2), pp.375-385.

NMFS (National Marine Fisheries Service), 2006. *Status report on the continental United States distinct population segment of the goliath grouper (*Epinephelus itajara*), Florida*. National Marine Fisheries Service, St. Petersburg, Florida.

Nuevo, M., Sheehan, R.J. and Heidinger, R.C., 2004. Accuracy and precision of age determination techniques for Mississippi River bighead carp *Hypophthalmichthys nobilis* (Richardson 1845) using pectoral spines and scales. *Archiv für Hydrobiologie*, *160*(1), pp.45-56.

Nunn, A.D., Cowx, I.G. and Harvey, J.P., 2002. Recruitment patterns of six species of cyprinid fishes in the lower River Trent, England. *Ecology of Freshwater Fish*, *11*(2), pp.74-84.

Nunn, A.D., Harvey, J.P. and Cowx, I.G., 2007. The food and feeding relationships of larval and 0+ year juvenile fishes in lowland rivers and connected waterbodies. I. Ontogenetic shifts and interspecific diet similarity. *Journal of Fish Biology*, *70*(3), pp.726-742.

Olsson, K., Stenroth, P., Nyström, P. and Graneli, W., 2009. Invasions and niche width: does niche width of an introduced crayfish differ from a native crayfish?. *Freshwater Biology*, *54*(8), pp.1731-1740.

O'Reilly, C.M., Hecky, R.E., Cohen, A.S. and Plisnier, P.D., 2002. Interpreting stable isotopes in food webs: recognizing the role of time averaging at different trophic levels. *Limnology and oceanography*, *47*(1), pp.306-309.

Oyugi, D.O., Cucherousset, J. and Britton, J.R., 2012a. Temperature-dependent feeding interactions between two invasive fishes competing through interference and exploitation. *Reviews in Fish Biology and Fisheries*, *22*(2), pp.499-508.

Oyugi, D.O., Cucherousset, J., Baker, D.J. and Britton, J.R., 2012b. Effects of temperature on the foraging and growth rate of juvenile common carp, *Cyprinus carpio*. *Journal of Thermal Biology*, *37*(1), pp.89-94.

Oyugi, D.O., Cucherousset, J., Ntiba, M.J., Kisia, S.M., Harper, D.M. and Britton, J.R., 2011. Life history traits of an equatorial common carp *Cyprinus carpio* population in relation to thermal influences on invasive populations. *Fisheries Research*, *110*(1), pp.92-97.

Pardo, S.A., Cooper, A.B. and Dulvy, N.K., 2013. Avoiding fishy growth curves. *Methods in Ecology and Evolution*, *4*(4), pp.353-360.

Parkos III, J.J., Santucci, Jr, V.J. and Wahl, D.H., 2003. Effects of adult common carp (*Cyprinus carpio*) on multiple trophic levels in shallow mesocosms. *Canadian Journal of Fisheries and Aquatic Sciences*, *60*(2), pp.182-192.

Parnell, A.C., Inger, R., Bearhop, S. and Jackson, A.L., 2010. Source partitioning using stable isotopes: coping with too much variation. *PloS one*, *5*, e9672.

Parnell, A.C., Phillips, D.L., Bearhop, S., Semmens, B.X., Ward, E.J., Moore, J.W., Jackson, A.L., Grey, J., Kelly, D.J. and Inger, R., 2013. Bayesian stable isotope mixing models. *Environmetrics*, *24*(6), pp.387-399.

Paszkowski, C.A., Tonn, W.M., Piironen, J. and Holopainen, I.J., 1990. Behavioral and population-level aspects of intraspecific competition in crucian carp. *Annales Zoologici Fennici,* 27, pp. 77-85.

Pearson, S.F., Levey, D.J., Greenberg, C.H. and Martínez Del Rio, C., 2003. Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. *Oecologia*, *135*(4), pp.516-523.

Penttinen, O.P. and Holopainen, I.J., 1992. Seasonal feeding activity and ontogenetic dietary shifts in crucian carp, *Carassius carassius*. *Environmental Biology of Fishes*, *33*(1-2), pp.215-221.

Perga, M.E. and Gerdeaux, D., 2005. 'Are fish what they eat' all year round?. *Oecologia*, *144*(4), pp.598-606.

Perga, M.E. and Grey, J., 2010. Laboratory measures of isotope discrimination factors: comments on Caut, Angulo & Courchamp (2008, 2009). *Journal of Applied Ecology*, *47*(4), pp.942-947.

Peterson, B.J. and Fry, B., 1987. Stable isotopes in ecosystem studies. *Annual Review of Ecology and Systematics*, 18, pp.293-320.

Phelps, Q.E., Edwards, K.R. and Willis, D.W., 2007. Precision of five structures for estimating age of common carp. *North American Journal of Fisheries Management*, *27*(1), pp.103-105.

Phillips, D.L. and Eldridge, P.M., 2006. Estimating the timing of diet shifts using stable isotopes. *Oecologia*, *147*(2), pp.195-203.

Phillips, D.L. and Gregg, J.W., 2001. Uncertainty in source partitioning using stable isotopes. *Oecologia*, *127*(2), pp.171-179.

Phillips, D.L., Inger, R., Bearhop, S., Jackson, A.L., Moore, J.W., Parnell, A.C., Semmens, B.X. and Ward, E.J., 2014. Best practices for use of stable isotope mixing models in food-web studies. *Canadian Journal of Zoology*, *92*(10), pp.823-835.

Phillips, D.L., Newsome, S.D. and Gregg, J.W., 2005. Combining sources in stable isotope mixing models: alternative methods. *Oecologia*, *144*(4), pp.520-527.

Pierce, C.L., Rasmussen, J.B. and Leggett, W.C., 1996. Back-calculation of fish length from scales: empirical comparison of proportional methods. *Transactions of the American Fisheries Society*, *125*(6), pp.889-898.

Pilling, G.M., Kirkwood, G.P. and Walker, S.G., 2002. An improved method for estimating individual growth variability in fish, and the correlation between von Bertalanffy growth parameters. *Canadian Journal of Fisheries and Aquatic Sciences*, *59*(3), pp.424-432.

Pinnegar, J.K. and Polunin, N.V.C., 1999. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: implications for the study of trophic interactions. *Functional Ecology*, *13*(2), pp.225-231.

Pörtner, H.O., Berdal, B., Blust, R., Brix, O., Colosimo, A., De Wachter, B., Giuliani, A., Johansen, T., Fischer, T., Knust, R. and Lannig, G., 2001. Climate induced temperature effects on growth performance, fecundity and recruitment in marine fish: developing a hypothesis for cause and effect relationships in Atlantic cod (*Gadus morhua*) and common eelpout (*Zoarces viviparus*). *Continental Shelf Research*, *21*(18), pp.1975-1997.

Post, D.M., 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. *Ecology*, *83*(3), pp.703-718.

Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J. and Montana, C.G., 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. *Oecologia*, *152*(1), pp.179-189.

Prajneshu and Venugopalan, R., 1999. von Bertalanffy growth model in a random environment. *Canadian Journal of Fisheries and Aquatic Sciences*, *56*(6), pp.1026-1030.

Przybylski, M. and García-Berthou, E., 2004. Age and growth of European bitterling (*Rhodeus sericeus*) in the Wieprz-Krzna Canal, Poland. *Ecohydrology and Hydrobiology*, *4*, pp.207-213.

Quist, M.C., Jackson, Z.J., Bower, M.R. and Hubert, W.A., 2007. Precision of hard structures used to estimate age of riverine catostomids and cyprinids in the upper Colorado River basin. *North American Journal of Fisheries Management*, *27*(2), pp.643-649.

Quist, M.C., Pegg, M.A. and DeVries, D.R., 2012. Age and growth. In: A.V. Alexander, D.L. Parrish and T.M. Sutton (eds.), *Fisheries techniques*, 3rd edition. American Fisheries Society, Bethesda, Maryland, pp.677-731.

R Development Core Team, 2013. *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria.

R Development Core Team, 2014. *R: A language and environment for statistical computing.* R Foundation for Statistical Computing, Vienna, Austria.

Radford, A., Riddington, G. and Gibson, H., 2007. Economic evaluation of inland fisheries: the economic impact of freshwater angling in England and Wales. Environment Agency. Science Report SC050026/SR2, Bristol, UK.

Rahel, F.J. and Olden, J.D., 2008. Assessing the effects of climate change on aquatic invasive species. *Conservation Biology*, *22*(3), pp.521-533.

Reich, K.J., Bjorndal, K.A. and Martínez del Rio, C., 2008. Effects of growth and tissue type on the kinetics of ^{13}C and ^{15}N incorporation in a rapidly growing ectotherm. *Oecologia*, *155*(4), pp.651-663.

Remon, J., Bower, D.S., Gaston, T.F., Clulow, J. and Mahony, M.J., 2016. Stable isotope analyses reveal predation on amphibians by a globally invasive fish (*Gambusia holbrooki*). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *26*(4), pp.724-735.

Richards, L.J., Schnute, J.T., Kronlund, A.R. and Beamish, R.J., 1992. Statistical models for the analysis of ageing error. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(9), pp.1801-1815.

Ricker, W.E., 1975. Computation and interpretation of biological statistics of fish populations. Dept. of Fisheries and Oceans. *Bulletin of the Fisheries Research Board of Canada,* 191, pp.1-382.

Ricker, W.E., 1992. Back-calculation of fish lengths based on proportionality between scale and length increments. *Canadian Journal of Fisheries and Aquatic Sciences*, *49*(5), pp.1018-1026.

Robbins, C.T., Felicetti, L.A. and Florin, S.T., 2010. The impact of protein quality on stable nitrogen isotope ratio discrimination and assimilated diet estimation. *Oecologia,* 162, pp.571-579.

Robillard, S.R. and Marsden, J.E., 1996. Comparison of otolith and scale ages for yellow perch from Lake Michigan. *Journal of Great Lakes Research*, *22*(2), pp.429-435.

Rogers-Bennett, L. and Rogers, D.W., 2016. A Two-Step Growth Curve: Approach to the von Bertalanffy and Gompertz Equations. *Advances in Pure Mathematics*, *6*, pp.321-330.

Roth, J.D. and Hobson, K.A., 2000. Stable carbon and nitrogen isotopic fractionation between diet and tissue of captive red fox: implications for dietary reconstruction. *Canadian Journal of Zoology*, *78*(5), pp.848-852.

Roussel, J.M., Perrier, C., Erkinaro, J., Niemelä, E., Cunjak, R.A., Huteau, D. and Riera, P., 2014. Stable isotope analyses on archived fish scales reveal the long-term effect of nitrogen loads on carbon cycling in rivers. *Global Change Biology*, *20*(2), pp.523-530.

Rowe, D.K., 2007. Exotic fish introductions and the decline of water clarity in small North Island, New Zealand lakes: a multi-species problem. *Hydrobiologia*, *583*(1), pp.345-358.

Ruesink, J.L., 2005. Global analysis of factors affecting the outcome of freshwater fish introductions. *Conservation Biology*, *19*(6), pp.1883-1893.

Ruetz III, C.R., Hurford, A.L. and Vondracek, B., 2003. Interspecific interactions between brown trout and slimy sculpin in stream enclosures. *Transactions of the American Fisheries Society*, *132*(3), pp.611-618.

Ruiz-Navarro, A., Gillingham, P.K. and Britton, J.R., 2016a. Predicting shifts in the climate space of freshwater fishes in Great Britain due to climate change. *Biological Conservation*, *203*, pp.33-42.

Ruiz-Navarro, A., Gillingham, P.K. and Britton, J.R., 2016b. Shifts in the climate space of temperate cyprinid fishes due to climate change are coupled with altered body sizes and growth rates. *Global Change Biology*, *22*, pp.3221-3232.

Rybczynski, S.M., Walters, D.M., Fritz, K.M. and Johnson, B.R., 2008. Comparing trophic position of stream fishes using stable isotope and gut contents analyses. *Ecology of Freshwater Fish*, *17*(2), pp.199-206.

Sánchez-Vázquez, F.J., Madrid, J.A., Zamora, S., Iigo, M. and Tabata, M., 1996. Demand feeding and locomotor circadian rhythms in the goldfish, *Carassius auratus*: dual and independent phasing. *Physiology and Behavior*, *60*(2), pp.665-674.

Sanderson, B.L., Tran, C.D., Coe, H.J., Pelekis, V., Steel, E.A. and Reichert, W.L., 2009. Nonlethal sampling of fish caudal fins yields valuable stable isotope data for threatened and endangered fishes. *Transactions of the American Fisheries Society*, *138*(5), pp.1166-1177.

Sayer, C.D., Copp, G.H., Emson, D., Godard, M.J., Zięba, G. and Wesley, K.J., 2011. Towards the conservation of crucian carp *Carassius carassius*: understanding the extent and causes of decline within part of its native English range. *Journal of Fish Biology*, *79*(6), pp.1608-1624.

Schmidt, K., McClelland, J.W., Mente, E., Montoya, J.P., Atkinson, A. and Voss, M., 2004. Trophic-level interpretation based on $\delta^{15}N$ values: implications of tissue-specific fractionation and amino acid composition. *Marine Ecology Progress Series*, *266*, pp.43-58.

Schneider, J.C., Laarman, P.W. and Gowing, H., 2000. Age and growth methods and state averages. In: J. C. Schneider (ed.), *Manual of fisheries survey methods II: with periodic updates.* Fisheries Special Report, vol. 25. Michigan Department of Natural Resources, Ann Arbor, Michigan.

Secor, D.H., Trice, T.M. and Hornick, H.T., 1995. Validation of otolith-based ageing and a comparison of otolith and scale-based ageing in mark-recaptured Chesapeake Bay striped bass, *Morone saxatilis*. *Fishery Bulletin*, *93*(1), pp.186-190.

Seminoff, J.A., Benson, S.R., Arthur, K.E., Eguchi, T., Dutton, P. H., Tapilatu, R.F. and Popp, B.N., 2012. Stable isotope tracking of endangered sea turtles: validation with satellite telemetry and $\delta^{15}N$ analysis of amino acids. *PLoS One* 7, e37403.

Sharp, D. and Bernard, D.R., 1988. Precision of estimated ages of lake trout from five calcified structures. *North American Journal of Fisheries Management*, *8*(3), pp.367-372.

Sheath, D.J., Williams, C.F., Reading, A.J. and Britton, J.R., 2015. Parasites of non-native freshwater fishes introduced into England and Wales suggest enemy release and parasite acquisition. *Biological Invasions*, *17*(8), pp.2235-2246.

Shelton, J.M., Bird, M.S., Samways, M.J. and Day, J.A., 2016. Non-native rainbow trout (*Oncorhynchus mykiss*) occupy a different trophic niche to native Breede River redfin (*Pseudobarbus burchelli*) which they replace in South African headwater streams. *Ecology of Freshwater Fish*. In press.

Shirvell, C.S., 1981. Validity of fin-ray ageing for brown trout. *Journal of Fish Biology*, *18*(4), pp.377-383.

Shuter, B.J., Lester, N.P., LaRose, J., Purchase, C.F., Vascotto, K., Morgan, G., Collins, N.C. and Abrams, P.A., 2005. Optimal life histories and food web position: linkages among somatic growth, reproductive investment, and mortality. *Canadian Journal of Fisheries and Aquatic Sciences*, *62*(4), pp.738-746.

Sikstrom, C.B., 1983. Otolith, pectoral fin ray, and scale age determinations for Arctic grayling. *The Progressive Fish-Culturist*, *45*(4), pp.220-223.

Skinner, M.M., Martin, A.A. and Moore, B.C., 2016. Is lipid correction necessary in the stable isotope analysis of fish tissues?. *Rapid Communications in Mass Spectrometry*, *30*(7), pp.881-889.

Smith, D.C., Robertson, S.G., Fenton, G.E. and Short, S.A., 1995. Age determination and growth of orange roughy (*Hoplostethus atlanticus*): a comparison of annulus counts with radiometric ageing. *Canadian Journal of Fisheries and Aquatic Sciences*, *52*(2), pp.391-401.

Smith, S.H., 1954. Method of producing plastic impressions of fish scales without using heat. *The Progressive Fish-Culturist*, *16*(2), pp.75-78.

Spiegel, J.R., Quist, M.C. and Morris, J.E., 2010. Precision of scales and pectoral fin rays for estimating age of highfin carpsucker, quillback carpsucker, and river carpsucker. *Journal of Freshwater Ecology*, *25*(2), pp.271-278.

Sterner, R.W. and George, N.B., 2000. Carbon, nitrogen, and phosphorus stoichiometry of cyprinid fishes. *Ecology*, *81*(1), pp.127-140.

Sun, Z.L., Gao, Q.F., Dong, S.L., Shin, P.K. and Wang, F., 2012. Estimates of carbon turnover rates in the sea cucumber *Apostichopus japonicus* (Selenka) using stable isotope analysis: the role of metabolism and growth. *Marine Ecology Progress Series*, *457*, pp.101-112.

Suzuki, K.W., Kasai, A., Nakayama, K. and Tanaka, M., 2005. Differential isotopic enrichment and half-life among tissues in Japanese temperate bass (*Lateolabrax japonicus)* juveniles: implications for analyzing migration. *Canadian Journal of Fisheries and Aquatic Sciences*, *62*(3), pp.671-678.

Svanbäck, R. and Bolnick, D.I., 2007. Intraspecific competition drives increased resource use diversity within a natural population. *Proceedings of the Royal Society of London B: Biological Sciences*, *274*(1611), pp.839-844.

Svanbäck, R., Eklöv, P., Fransson, R. and Holmgren, K., 2008. Intraspecific competition drives multiple species resource polymorphism in fish communities. *Oikos*, *117*(1), pp.114-124.

Sweeting, C.J., Barry, J., Barnes, C., Polunin, N.V.C. and Jennings, S., 2007a. Effects of body size and environment on diet-tissue $\delta^{15}N$ fractionation in fishes. *Journal of Experimental Marine Biology and Ecology*, *340*(1), pp.1-10.

Sweeting, C.J., Barry, J.T., Polunin, N.V.C. and Jennings, S., 2007b. Effects of body size and environment on diet-tissue $\delta^{13}C$ fractionation in fishes. *Journal of Experimental Marine Biology and Ecology*, *352*(1), pp.165-176.

Sweeting, C.J., Jennings, S. and Polunin, N.V.C., 2005. Variance in isotopic signatures as a descriptor of tissue turnover and degree of omnivory. *Functional Ecology*, *19*(5), pp.777-784.

Syväranta, J., Vesala, S., Rask, M., Ruuhijärvi, J. and Jones, R.I., 2008. Evaluating the utility of stable isotope analyses of archived freshwater sample materials. *Hydrobiologia*, *600*(1), pp.121-130.

Tarboush, R.A., MacAvoy, S.E., Macko, S.A. and Connaughton, V., 2006. Contribution of catabolic tissue replacement to the turnover of stable isotopes in *Danio rerio*. *Canadian Journal of Zoology*, *84*(10), pp.1453-1460.

Tarkan, A.S., Almeida, D., Godard, M.J., Gaygusuz, Ö., Rylands, M., Sayer, C.D., Zięba, G. and Copp, G.H., 2016. A review and meta-analysis of growth and life-history traits of a declining European freshwater fish, crucian carp *Carassius carassius*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *26*(1), pp.212-224.

Tarkan, A.S., Copp, G.H., Zięba, G., Godard, M.J. and Cucherousset, J., 2009. Growth and reproduction of threatened native crucian carp *Carassius carassius* in small ponds of Epping Forest, south-east England. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *19*(7), pp.797-805.

Tarkan, A.S., Gaygusuz, Ö., Godard, M.J. and Copp, G.H., 2011. Long-term growth patterns in a pond-dwelling population of crucian carp, *Carassius carassius*: environmental and density-related factors. *Fisheries Management and Ecology*, *18*(5), pp.375-383.

Thomas, S.M. and Crowther, T.W., 2014. Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. *Journal of Animal Ecology*, *84*(3), pp.861-870.

Thompson, R.R. and Walton, J.C., 2004. Peptide effects on social behavior: effects of vasotocin and isotocin on social approach behavior in male goldfish (*Carassius auratus*). *Behavioral Neuroscience*, *118*(3), p.620.

Thomson, D., 2004. Competitive interactions between the invasive European honey bee and native bumble bees. *Ecology*, *85*(2), pp.458-470.

Tieszen, L.L., Boutton, T.W., Tesdahl, K.G. and Slade, N.A., 1983. Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}C$ analysis of diet. *Oecologia*, *57*(1-2), pp.32-37.

Tilman, D., 2004. Niche tradeoffs, neutrality, and community structure: a stochastic theory of resource competition, invasion, and community assembly. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(30), pp.10854-10861.

Tominaga, O., Uno, N. and Seikai, T., 2003. Influence of diet shift from formulated feed to live mysids on the carbon and nitrogen stable isotope ratio ($\delta^{13}C$ and $\delta^{15}N$) in

dorsal muscles of juvenile Japanese flounders, *Paralichthys olivaceus*. *Aquaculture*, *218*(1), pp.265-276.

Tracey, D.M. and Horn, P.L., 1999. Background and review of ageing orange roughy (*Hoplostethus atlanticus*, Trachichthyidae) from New Zealand and elsewhere. *New Zealand Journal of Marine and Freshwater Research*, *33*(1), pp.67-86.

Tran, T.N.Q., Jackson, M.C., Sheath, D., Verreycken, H. and Britton, J.R., 2015. Patterns of trophic niche divergence between invasive and native fishes in wild communities are predictable from mesocosm studies. *Journal of Animal Ecology*, *84*(4), pp.1071-1080.

Tronquart, N.H., Mazeas, L., Reuilly-Manenti, L., Zahm, A. and Belliard, J., 2012. Fish fins as non-lethal surrogates for muscle tissues in freshwater food web studies using stable isotopes. *Rapid Communications in Mass Spectrometry*, *26*(14), pp.1603-1608.

Trueman, C.N. and Moore, A., 2007. Use of the stable isotope composition of fish scales for monitoring aquatic ecosystems. *Terrestrial Ecology*, *1*, pp.145-161.

Van Oosten, J., 1929. Life history of the lake herring (*Leucichthys artedi* Le Sueur) of Lake Huron as revealed by its scales, with a critique of the scale method. *Bulletin of the Bureau of Fisheries*, *44*(1053), pp.265-428.

Van Valen, L., 1965. Morphological variation and width of ecological niche. *American Naturalist*, pp.377-390.

Vander Zanden, M.J., Cabana, G. and Rasmussen, J.B., 1997. Comparing trophic position of freshwater fish calculated using stable nitrogen isotope ratios ($\delta^{15}N$) and literature dietary data. *Canadian Journal of Fisheries and Aquatic Sciences*, *54*(5), pp.1142-1158.

Vander Zanden, M.J., Casselman, J.M. and Rasmussen, J.B., 1999. Stable isotope evidence for the food web consequences of species invasions in lakes. *Nature*, *401*, pp.464-467.

Vander Zanden, M.J., Clayton, M.K., Moody, E.K., Solomon, C.T. and Weidel, B.C., 2015. Stable isotope turnover and half-life in animal tissues: a literature synthesis. *PloS one*, *10*, e0116182.

Veer, G. and Nentwig, W., 2015. Environmental and economic impact assessment of alien and invasive fish species in Europe using the generic impact scoring system. *Ecology of Freshwater Fish*, *24*(4), pp.646-656.

Victor, B.C. and Brothers, E.B., 1982. Age and growth of the fallfish *Semotilus corporalis* with daily otolith increments as a method of annulus verification. *Canadian Journal of Zoology*, *60*(11), pp.2543-2550.

Vilizzi, L. and Walker, K.F., 1999. Age and growth of the common carp, *Cyprinus carpio*, in the River Murray, Australia: validation, consistency of age interpretation, and growth models. *Environmental Biology of Fishes*, *54*(1), pp.77-106.

Vilizzi, L., 1998. Observation on ontogenetic shifts in the diet of 0+ carp, *Cyprinus carpio*, from the River Murray, Australia. *Folia Zoologica*, *47*(3), pp.225-229.

Vilizzi, L., 2012. The common carp, *Cyprinus carpio*, in the Mediterranean region: origin, distribution, economic benefits, impacts and management. *Fisheries Management and Ecology, 19*(2), pp.93-110.

Vilizzi, L., Copp, G.H. and Britton, J.R., 2013. Age and growth of European barbel *Barbus barbus* (Cyprinidae) in the small, mesotrophic River Lee and relative to other populations in England. *Knowledge and Management of Aquatic Ecosystems, 409*(9), pp.1-18.

Vilizzi, L., Tarkan, A.S. and Copp, G.H., 2015. Experimental evidence from causal criteria analysis for the effects of common carp *Cyprinus carpio* on freshwater ecosystems: a global perspective. *Reviews in Fisheries Science and Aquaculture*, *23*(3), pp.253-290.

von Bertalanffy, L., 1938. A quantitative theory of organic growth (inquiries on growth laws. II). *Human Biology*, *10*(2), pp.181-213.

Wainright, S.C., Fogarty, M.J., Greenfield, R.C. and Fry, B., 1993. Long-term changes in the Georges Bank food web: trends in stable isotopic compositions of fish scales. *Marine Biology*, *115*(3), pp.481-493.

Watkins, C.J., Klein, Z.B., Terrazas, M.M. and Quist, M.C., 2015a. Influence of sectioning location on age estimates from common carp dorsal spines. *North American Journal of Fisheries Management*, *35*(4), pp.690-697.

Watkins, C.J., Ross, T.J., Hardy, R.S. and Quist, M.C., 2015b. Precision of hard structures used to estimate age of mountain whitefish (*Prosopium williamsoni*). *Western North American Naturalist*, *75*(1), pp.1-7.

Webber, D.N. and Thorson, J.T., 2016. Variation in growth among individuals and over time: A case study and simulation experiment involving tagged Antarctic toothfish. *Fisheries Research*, *180*, pp.67-76.

Weber, M.J. and Brown, M.L., 2011a. Comparison of common carp (*Cyprinus carpio*) age estimates derived from dorsal fin spines and pectoral fin rays. *Journal of Freshwater Ecology*, *26*(2), pp.195-202.

Weber, M.J. and Brown, M.L., 2011b. Relationships among invasive common carp, native fishes and physicochemical characteristics in upper Midwest (USA) lakes. *Ecology of Freshwater Fish*, *20*(2), pp.270-278.

Wedchaparn, O., Zhao, L., Fan, Y., He, D., Zhang, H., Ivan, L.N., Liu, Q. and Ayisi, C.L., 2016. Comparison of the trophic niches between two planktivorous fishes in two large lakes using stable isotope analysis. *Biochemical Systematics and Ecology*, *68*, pp.148-155.

Weidel, B.C., Carpenter, S.R., Kitchell, J.F. and Vander Zanden, M.J., 2011. Rates and components of carbon turnover in fish muscle: insights from bioenergetics models and a whole-lake ^{13}C addition. *Canadian Journal of Fisheries and Aquatic Sciences*, *68*(3), pp.387-399.

WFD (Water Framework Directive), 2000. Directive 2000/60/EC of the European Parliament and of the Council establishing a framework for the Community action in the field of water policy. Available at:

http://ec.europa.eu/environment/water/water-framework/index_en.html (last accessed on 28/09/2016).

Wheeler, A., 2000. Status of the crucian carp, *Carassius carassius* (L.), in the UK. *Fisheries Management and Ecology, 7*(4), pp.315-322.

Winker, H., Weyl, O.L.F., Booth, A.J. and Ellender, B.R., 2010. Validating and corroborating the deposition of two annual growth zones in asteriscus otoliths of common carp *Cyprinus carpio* from South Africa's largest impoundment. *Journal of Fish Biology*, *77*(10), pp.2210-2228.

Witting, D.A., Chambers, R.C., Bosley, K.L. and Wainright, S.C., 2004. Experimental evaluation of ontogenetic diet transitions in summer flounder (*Paralichthys dentatus*), using stable isotopes as diet tracers. *Canadian Journal of Fisheries and Aquatic Sciences*, *61*(11), pp.2069-2084.

Woodford, D.J., Impson, N.D., Day, J.A. and Bills, I.R., 2005. The predatory impact of invasive alien smallmouth bass, *Micropterus dolomieu* (Teleostei: Centrarchidae), on indigenous fishes in a Cape Floristic Region mountain stream. *African Journal of Aquatic Science*, *30*(2), pp.167-173.

Xia, B., Gao, Q.F., Dong, S.L. and Wang, F., 2013a. Carbon stable isotope turnover and fractionation in grass carp *Ctenopharyngodon idella* tissues. *Aquatic Biology*, *19*(3), pp.207-216.

Xia, B., Gao, Q.F., Li, H., Dong, S.L. and Wang, F., 2013b. Turnover and fractionation of nitrogen stable isotope in tissues of grass carp *Ctenopharyngodon idellus*. *Aquaculture Environment Interactions*, *3*(2), pp.177-186.

Yao, X., Huang, G., Xie, P. and Xu, J., 2016. Trophic niche differences between coexisting omnivores silver carp and bighead carp in a pelagic food web. *Ecological Research*, *31*(6), pp.831-839.

Yates, J.R., Watkins, C.J. and Quist, M.C., 2016. Evaluation of Hard Structures Used to Estimate Age of Common Carp. *Northwest Science*, *90*(2), pp.195-205.

Yule, D.L., Stockwell, J.D., Black, J.A., Cullis, K.I., Cholwek, G.A. and Myers, J.T., 2008. How systematic age underestimation can impede understanding of fish population

dynamics: lessons learned from a Lake Superior cisco stock. *Transactions of the American Fisheries Society*, *137*(2), pp.481-495.

Živkov, M.T., Trichkova, T.A. and Raikova-Petrova, G.N., 1999. Biological reasons for the unsuitability of growth parameters and indices for comparing fish growth. *Environmental Biology of Fishes*, *54*(1), pp.67-76.

Appendix 1: R code for fitting the three-parameter von Bertalanffy growth function (VBGF) using the nls() function.

For a more in depth review of model fitting, see:

http://derekogle.com/fishR/examples/oldFishRVignettes/VonBertalanffy.pdf

The VBGF equation is in the form of:

Length = Linf - ((Linf - t0 * exp (-k*Estimated age))

```
#Read in libraries required:
    library(FSA)
    library(nlstools)
#Read in data file containing fish lengths (Length), age estimates (Age) and uncertainty
#levels (Conf.):
    dta <- read.table("Roach.river.colne.csv", sep = ",", header = TRUE)
    attach(dta)
```

Step 1: Generate VBGF parameters for original data

```
#Calculate estimates for VBGF parameters for original data, without uncertainty levels:
#Starting parameters are estimated for optimisation:
    svTypical1 <- vbStarts(Length~Age, data=dta, type="typical")
#von Bertalanffy growth equation is defined:
    vbTypical1 <-Length~Linf*(1-exp(-K*(Age-t0)))
#nls() function applied to data:
    fitTypical1 <- nls(vbTypical1, data=dta, start=svTypical1)
```

Step 2: Apply bootstrapping to obtain VBGF parameters for adjusted data

```
#Read in BEAs and SD's generated from the pre-set distributions of probable ages:
    BEAs<-read.table("BEAs.roach.csv", sep = ",", header = FALSE)
    sds <-read.table("sd.roach.csv", sep = ",", header = FALSE)
#Set up bootstrap:
    boot.size <- 1000
    store.data<-array(NA,c(boot.size,3))
    xx<-dim(dta)
    xx<-xx[1]
    intial.store<-array(NA,c(boot.size))
#Generate VBGF parameters for adjusted data using uncertainty levels:
    for(boot in 1:boot.size){
      new.age <- array(NA,xx)
      for(x in 1:xx){
         a <- AGE[x]
         b <- Conf.[x]
         c<- BEAs[a,b]
         d<- sds[a,b]
         new.age[x]<-(rnorm(1,c,d))}
      svTypical2 <- vbStarts(Length~new.age, data=dta)
      vbTypical2 <-Length~Linf*(1-exp(-K*(new.age-t0)))
      fitTypical2 <- nls(vbTypical2, data=dta, start=svTypical2)
      store.data[boot,1]<-coef(fitTypical2)[1]
      store.data[boot,2]<-coef(fitTypical2)[2]
      store.data[boot,3]<-coef(fitTypical2)[3]}
```

Appendix 2: Key outputs of the literature review into the uncertainty of age estimates gained from hard structures.

Species	Habitat	Structure(s)	Output	Reference
Carpiodes velifer, Carpiodes cyprinus, Carpiodes carpio	Freshwater	Scales, Pectoral fin rays	➢ Precision between readers low for scales. ➢ Precision of ageing structures decreased as age increased. ➢ Estimates for *C. velifer* scales were generally 1-3 years less than fin rays. ➢ Agreement between readers increased with increased confidence.	Spiegel *et al.* (2010)
Cyprinus carpio	Freshwater	Otoliths	➢ Annulus counts are reliable indicators of ages 3 - 14 years.	Brown *et al.* (2004)
Cyprinus carpio	Freshwater	Scales, Vertebrae, Opercula bones, Otoliths, Pectoral fin rays	➢ Most ages overestimated by scales, vertebrae, and opercula up to age 6 but underestimated over 10. ➢ Ages from pectoral fin rays nearly as precise as otoliths up to age 13. ➢ Fin rays underestimated ages of fish older than 13. ➢ For fish age 14+ discrepancies were as high as 6 years. ➢ Maximum ages from scales, vertebrae, and opercula were underestimated by as much as 12 years. ➢ Scales aged to a maximum of 15, versus otolith age of 24.	Phelps *et al.* (2007)
Cyprinus carpio	Freshwater	Scales, Dorsal spines	➢ Ages from scales as much as 8 years less and 7 years greater than dorsal spines. ➢ Discrepancies occurred in young as well as old fish. ➢ Scale ages and age ranges were lower than dorsal spines. ➢ Scales estimated to be age 6 by 1 reader were assigned ages 2 - 12 by other reader. ➢ Neither reader felt confident in ageing scales beyond age 1.	Jackson *et al.* (2007)

Species	Habitat	Structure(s)	Output	Reference
Cyprinus carpio	Freshwater	Otoliths	➢ Due to decrease in width of translucent zones, interpretations of otoliths with >8 growth zones became difficult. ➢ The maximum number of growth zones counted was 14.	Winker *et al.* (2010)
Gadus morhua	Marine	Otoliths	➢ Age misclassification rates increased considerably when older individuals were considered. ➢ Pen cod showed an error rate of ~5% for ages 2–4 years and 11% for ages 2–6, wild fish showed an error rate of 14% for ages 2–4 years.	Doering-Arjes *et al.* (2008)
Gila robusta, Semotilus atromaculatus, Catostomus discobolus, C. commersonii, C. latipinnis	Freshwater	Scales, Pectoral fin rays, Cleithra, Opercula bones	➢ Scale ages agreed with less than 20% of otolith ages, many disagreements differed by more than 5 years. ➢ Disagreements were due to a lower scale age relative to otolith age and most discrepancies occurred for age 5 and older fish. ➢ There was a high agreement between scale age and otolith ages for all fish with an otolith age less than 5. ➢ Age estimates from scales were up to 9 years (frequently 5 years) less than otolith ages.	Quist *et al.* (2007)
Hypophthal-michthys nobilis	Freshwater	Scales, Pectoral spines	➢ Agreement for age 1 fish using scales was 100% and 20% for age 2. ➢ Accuracy of scale readings was 100% for age 1 and 60% for age 2. ➢ Fish scales were over-aged by up to 3 years, 100% of scales read were aged within 3 years of known age. ➢ Accuracy of interpreting the scales of known-age fish was 78%. ➢ Scales yielded lower age estimates than spine sections for older fish. ➢ Scales are harder to read as fish get older. ➢ Suggested using pectoral spine sections to age fish of 2 years and older because they showed more and sharper annuli than scales.	Nuevo *et al.* (2004)

Species	Habitat	Structure(s)	Output	Reference
Rhodeus sericeus	Freshwater	Scales	➢ Annuli clearly visible in all parts of scales. ➢ The number of annuli corresponded to the number of bands in operculum bone. ➢ Shape of scales changed with fish size. Due to change the radius does not occupy constant position, shifts towards the caudal part. ➢ In the anterior part annuli were crowded near the margin. ➢ In the lateral part annuli had no fixed position producing some difficulties in radial measurement of the annuli.	Przybylski & García-Berthou (2004)
Rutilus rutilus	Freshwater	Scales, Opercula	➢ For fish up to 10, the ages from scales and opercula's had few discrepancies. ➢ Scales had clearer inner but opercula's clearer outer annuli in fish >9 years. ➢ Roach over 10 years were determined from opercula bones as distinguishing annuli near scale edge either difficult or impossible.	Mann (1973)
Rutilus rutilus	Freshwater	Scales	➢ Re-ageing exercise revealed only 69% agreement with the original ages, with a significant decrease in agreement with age. ➢ Agreement above 80% at ages 1, 2, and 4 and reduced to 6% at age 9.	Musk *et al*. (2006)
Salvelinus namaycush	Freshwater	Scales, Cleithra, Opercula bones, Otoliths, Vertebrae	➢ Counts of annuli on all structures from immature fish were similar. ➢ Fewer annuli on scales than on other structures from larger fish. ➢ For scales, otoliths, opercula bones, and vertebrae, sampling SEs were higher for older larger fish than for younger smaller fish. ➢ Differences in mean counts were greater for larger, older fish. ➢ Counts from scales were skewed toward younger ages. ➢ Scales are useful in determining the age of immature fish.	Sharp & Bernard (1998)

Species	Habitat	Structure(s)	Output	Reference
Scavdinius evythvophthalmus	Freshwater	Scales	➢ 1 of 6 correctly aged all material, others <50% success. ➢ 15% of incorrect ages resulted from misinterpretation of false or true annuli from age 2 onwards, 85% from overlooking the first annulus. ➢ In faster-growing fish where the first annulus was further from the centre there were no such errors. ➢ Only two fish were incorrectly aged by more than one year. ➢ Age 1 aged 100% accurately, 2: 64%, 3: 42%, 4: 44% and 5: 83%. ➢ Only 6.9% were incorrectly aged, this may have increased had samples contained scales from fish > 10 years.	Mann & Steinmetz (1985)
Semotilus corporalis	Freshwater	Otoliths	➢ Majority of otolith annuli were relatively easy to identify at low magnification, in contrast to scales which are often hard to read.	Victor & Brothers (1982)
Squalius cephalus	Freshwater	Scales, Opercula	➢ Annuli on scales up to age 10 not difficult to see, but care necessary to avoid overlooking first annulus near scale centre. ➢ Annuli formed on scale edge of older fish could not be separated. ➢ Ageing using only scale examination did result in some under-ageing compared with ages obtained from opercula bones.	Mann (1976)
Thymallus arcticus	Freshwater	Scales, Otoliths, Fin rays	➢ Reader agreement twice as high for otoliths and fin rays than scales. ➢ Scale ages differed by up to 5 but generally within 3 years. ➢ Scale age never exceed otolith age, differed by as much as 6 years. ➢ Maximum age determined from scales was 7, versus 12 from otoliths. ➢ Discrepancies in age assessment started at scale ages 2 and 3. ➢ Scales developed a dense edge in which annuli could not be distinguished.	Sikstrom (1983)

Species	Habitat	Structure(s)	Output	Reference
Thymallus thymallus	Freshwater	Scales	➢ Accuracy of scales high in 1-2 years (error 4.15%) decreased in older. ➢ In >4 year fish underestimation occurred and error in reading scales rose to 51.9% in 5 year-old fish. ➢ Scale ages underestimated tag-recapture age by as much as 3 years. ➢ The validation of 1+ year aged fish demonstrated that scale reading was correct in 97% of cases, with this decreasing with age. ➢ Annuli formed on scales of young fish easier to read than older fish, due to poor visibility of annuli at edge of scales. ➢ Significant and increasing deviation between scale-read age and tag age ≥5, age of older and maximum age cannot be accurately determined from scales.	Horka *et al.* (2010)