Assessing the reproducibility of fractional rates of protein synthesis in muscle tissue measured using the flooding dose technique.

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Abstract

The flooding dose technique of Garlick et al. (1980) has become the main method for measuring tissue and whole-animal rates of protein synthesis in ectotherms. However, single tissue samples are used to determine rates of protein synthesis and no studies have examined the pattern of flooding in large tissues such as the white muscle in fishes, which can comprise up to 55% of the wet body mass of a fish and which is poorly perfused. The present study has examined, for the first

time, the patterns of flooding and measured rates of protein synthesis in five different regions of the white muscle in the Arctic charr *Salvelinus alpinus* ranging in size from 25 g to 1.6 kg following a flooding dose injection of L-[^{3}H]-phenylalanine. The results indicate that the degree of flooding (*i.e.* free pool specific radioactivity relative to that of the injection solution) and elevation in free phenylalanine concentrations can vary between regions but the calculated fractional rates of protein synthesis were similar in four of the five regions studied. The variability in rates of protein synthesis increased with body size with greater variability observed between regions for fish > 1 kg in body mass. For consistency between studies, it is recommended that samples are taken from the epaxial muscle in the region below the dorsal fin when measuring fractional rates of white muscle synthesis in fishes.

Key words: Arctic charr; Flooding dose technique, Protein synthesis; Repeat measures; Reproducibility; *Salvelinus alpinus*; White muscle

1. Introduction

The synthesis of structural, catalytic and other metabolically-active proteins is one of the major metabolic costs in ectothermic and endothermic animals accounting for between 11 to 42% of basal metabolism (Houlihan et al., 1995a; Fraser and Rogers, 2007). As a result, a number of tracer techniques have been developed to measure *in vivo* rates of protein synthesis although the choice of tracer, method of administration and underlying methodological assumptions can vary (reviewed in McCarthy et al., 2016). The flooding dose technique of Garlick et al. (1980) is the major tracer method used to measure rates of protein synthesis

50 in aquatic ectotherms (Houlihan et al., 1995b; Fraser and Rodgers, 2007; Carter
51 and Mente, 2014). The aim of this technique is to inject a single high dose of amino
52 acid solution containing a labelled tracer (usually L-[^{3}H]-phenylalanine in aquatic
53 ectotherm studies) to rapidly swamp all the body free amino acid pools. After a
54 known incorporation period, the animal is killed and tissue samples can be taken
55 to determine tissue-specific rates of protein synthesis (*e.g.* liver, gill or muscle) or
56 the carcass can be homogenised and samples taken to obtain a whole-animal rate
57 of protein synthesis (Houlihan et al., 1995b; Fraser and Rodgers, 2007). The
58 flooding dose technique has been validated to measure rates of protein synthesis
59 in a range of aquatic ectotherm taxa including echinoderms, molluscs, crustaceans
60 and fishes (see Houlihan et al., 1995b; Fraser and Rodgers, 2007; McCarthy et al.,
61 2016) and has become a valuable technique in the study of metabolism and
62 ecophysiology of aquatic ectotherms .

63 In fishes, the flooding dose technique has been used to examine tissue-specific
64 and whole-animal rates of protein synthesis (Houlihan et al., 1995b) and
65 responses to the effects of nutritional state (Houlihan et al., 1995b; Carter and
66 Houlihan, 2001), contaminants (Houlihan et al., 1994; McCarthy and Fuiman,
67 2008) and abiotic factors such as temperature (McCarthy et al., 1999; Katersky
68 and Carter, 2007) and anoxia (Smith et al., 1996). Fractional rates of protein
69 synthesis are higher in metabolically-active organs with the general pattern being
70 liver > gill > gastrointestinal tract (intestine > stomach) >> white muscle (Carter
71 and Houlihan, 2001). However, white muscle tissue in fishes accounts for a larger
72 percentage of the whole animal (38-55% of wet body mass) compared to
73 metabolically-active organs (< 3.5% of wet body mass) (Foster et al., 1991; Martin
74 et al., 1993). Therefore, although the rate of synthesis in the white muscle on a

fractional basis (*i.e.* % of the protein mass synthesized day^{-1}) is low (usually < 1% day^{-1}: Table 1), the absolute rate of synthesis (*i.e.* g protein synthesized day^{-1}) in the white muscle can contribute between 33 and 45% of whole-animal rates of protein synthesis in fishes (Carter and Houlihan, 2001). As a result, whole-animal fractional rates of protein synthesis will track white muscle protein synthesis rates in fishes (usually 2–4 times higher; Katersky and Carter, 2010) and have been modelled using linear regression in order to predict whole-animal k_s values from white muscle rates (*e.g.* McCarthy et al., 1999; Carter and Houlihan, 2001; Katersky and Carter, 2010). Thus, the accurate measurement of rates of protein synthesis in the white muscle is important not only to understand protein dynamics in this tissue but for the whole-animal as well.

The axial white muscle in teleost fish comprises of a series of blocks or myotomes (Johnston and Altringham, 1991; Videler, 1993) that are poorly vascularised (Mosse, 1978; Johnston, 1981). Early work by Houlihan et al. (1986) on rainbow trout *Oncorhynchus mykiss* measured rates of protein synthesis in the 'anterior' (beneath the dorsal fin) and 'posterior' (beneath the adipose fin) regions of the epaxial white muscle following incorporation times of 20–60 minutes. The time course results indicated that flooding of the 'anterior' white muscle was not achieved after 60 minutes (attributed to poor vascularisation) and it was not possible to calculate synthesis rates for this region of the white muscle (Houlihan et al., 1986) although fractional rates of protein synthesis were calculated for the 'posterior' white muscle (Table 1). As a result, many subsequent studies measuring white muscle and/or whole-animal rates of protein synthesis have used longer incorporation times, usually 2-4 hours depending on body size and temperature, to ensure flooding occurs in the white muscle (see Table 1).

However, despite the work by Houlihan et al. (1986) indicating that distribution of radiolabel may not be uniform, there has been no examination of free-pool specific radioactivities in different regions of the white muscle after a longer incorporation period to determine whether uniform flooding has been achieved following injection and whether sampling location in the white muscle will affect the calculated rate of protein synthesis.

Despite comprising the largest tissue in the body of a fish, no published studies measuring white muscle fractional rates of protein synthesis in fish after Houlihan et al. (1986) have sampled from various locations in the white muscle, rather relying on a single sample from one location. Where sampling location is stated, tissue samples have been taken from the epaxial muscle below the dorsal fin or anterior to the dorsal fin, although many studies do not state sampling location (see Table 1). The implicit assumption has been that the distribution of radiolabel has been uniform throughout the tissue and that sampling from a single location will provide a representative measure of the phenylalanine-specific radioactivities in the free pool and protein and, therefore, a reliable estimate of the fractional rate of protein synthesis in the white muscle. However, this assumption has not been tested. The aims of the present study were to measure fractional rates of protein synthesis in the white muscle of the Arctic charr *Salvelinus alpinus* using a flooding dose injection of L-[^{3}H]-phenylalanine and to determine by sampling from 5 locations within the white muscle whether uniform flooding is achieved throughout the white muscle, whether fractional rates of protein synthesis are similar in each of these 5 regions (*i.e.* whether rates are reproducible), and to determine whether reproducibility is affected by body size

(which may affect perfusion rates into the white muscle) by measuring white muscle rates of protein synthesis in fish ranging in size from 25 g to 1.6 kg.

2. Materials and Methods

2.1 Fish Husbandry

Arctic charr *Salvelinus alpinus* from a commercial Scottish strain were obtained as eyed eggs from John Eccles Hatcheries, Orkney in February 2005 and reared in the freshwater aquarium facilities at the School of Ocean Sciences, Menai Bridge as outlined in Berrill and McCarthy (2008). From first feeding onwards, fish were reared in 1 x 1 x 0.75 m fibreglass rearing tanks, in a re-circulation system where water (average temperature 12.6^{o}C) was filtered and re-used, with *ca.* 10% of the re-circulated water replaced per day. Water flow rates were initially 0.1 litres sec^{-1} but were increased to 0.25 litres sec^{-1} as the fish increased in size. Fish were exposed to a natural photoperiod regime (53° N) and fed commercial salmon feed (EWOS Micro; EWOS Ltd, Bathgate, U.K.) by belt feeders throughout the light phase of the photoperiod, according to feed rates described by Johnston (2002). Food was not limiting as uneaten food was usually present on the tank floors. At monthly intervals from first feeding, a representative subsample of fish were anaesthetized (MS222, 0.1 g L^{-1}) and weighed (± 0.1g) in order to adjust the feed ration. The fish were reared for 28 months from first feeding on 4th April 2005 to 26th July 2007, during which time the fish increased in body mass from *ca.* 0.2 g to *ca.* 1.6 kg (Table 2).

2.2 Protein synthesis measurements

Fractional rates of protein synthesis in the white muscle of Arctic charr were

149 measured using the flooding dose technique of Garlick et al. (1980). Although no
150 time course validation trials were conducted in the present study, the use of the
151 flooding dose technique has been validated for use in salmonid fishes using both
152 intravenous (Houlihan et al., 1986; Carter et al., 1993; McCarthy et al., 1994) and
153 intraperitoneal injection (Owen et al., 1999; Lamarre et al., 2015) and validation
154 studies cover the same size range of animals as used in this present study (Martin
155 et al., 1993; Owen et al., 1999; Lamarre et al., 2015). See McCarthy et al. (2016) for
156 a discussion of the methodology and validation criteria for use of the flooding dose
157 technique to measure fractional rates of protein synthesis.
158 Measurements were made at regular intervals as the fish increased in size
159 (Table 2). Twenty four hours before measurement, food was removed from the
160 feeder and uneaten food was siphoned from the tank. Fish were injected into the
161 peritoneum (Houlihan et al., 1994; McCarthy et al., 1999; Lamarre et al., 2015)
162 without anaesthesia with a solution containing 135 mM L-phenylalanine and L-
163 [2,6-^{3}H]-phenylalanine (Amersham International, 37 × 106 Bq ml^{-1}). The specific
164 activity of the injection solution was measured in September 2005 as 1312 (± 87,
165 n = 4) disintegrations per minute per nanomole of phenylalanine (dpm nmole^{-1}
166 phe). Accounting for radioactive decay, the injection solution in July 2007 was
167 calculated to be 1178 dpm nmole^{-1} phe using the formula $A = A_0e^{-\lambda t}$ where A_0 is
168 the specific radioactivity of the injection solution in September 2005 (1312 dpm
169 nmole^{-1} phe), λ is the decay constant ($Ln2/t_{1/2}$ where $t_{1/2}$ is the half-life for tritium,
170 *i.e.* 12.33 years) and t is the time interval between September 2005 and July 2007
171 (1.92 years). However, due to the large size of the fish (>850 g) in July 2007, to
172 conserve radioactivity and reduce subsequent radiation waste, the injection
173 solution was diluted 50:50 with 135 mM L-phenylalanine prior to injection to give

174 a theoretical specific radioactivity of 589 dpm nmole^{-1} phe. The injection volume
175 administered to each group varied according to size (Table 2). The injection
176 volume usually used in protein synthesis studies in fish is 1 ml 100 g^{-1} body mass
177 (Table 1) but most studies have worked with fish < 250 g in body mass (Table 1).
178 In this study, to reduce the injection volume into the peritoneum and to conserve
179 radioactivity and reduce subsequent radiation waste, the injection volumes were
180 reduced (Table 2).
181 Following injection, the fish were returned to tanks containing aerated
182 freshwater and left for 2 to 4 hours incubation according to size (Table 2) to allow
183 uptake of radiolabel from the peritoneum into the body free amino acid pools and
184 incorporation into body protein. After the designated incubation time the fish
185 were killed using a Home Office Schedule 1 method, snap frozen in liquid nitrogen
186 and stored at -20°C until processing. In the laboratory, fish were part-thawed and
187 white muscle samples (*ca*. 150 mg) were obtained from 4 locations in the epaxial
188 white muscle and one location on the hypaxial white muscle (Fig. 1) whilst the
189 muscle tissue was still frozen. The locations were selected to be in the epaxial
190 muscle immediately behind the head (Region 1), below the dorsal fin (Region 2),
191 below the adipose fin (Region 3) and above the lateral line midway between dorsal
192 fin and the adipose fin (Region 4) and in the hypaxial muscle in front of the anal
193 fin (Region 5; Fig. 1). In most studies where sampling location is stated, tissue
194 samples have been taken from Region 2 (Table 1). Tissue samples (*ca.* 100 mg)
195 from each location in the white muscle were homogenised in 2 ml 0.2M perchloric
196 acid and centrifuged (6000 *g*, 4°C, 15 minutes) to separate the intracellular free
197 pool from the precipitated protein pellet. The subsequent treatment of the
198 samples to measure the white muscle free-pool (S_a) and protein-bound

199 phenylalanine-specific radioactivity (S_b) (both dpm nmole^{-1} phe) was as described
200 in Houlihan et al. (1995a, 1995b). The free phenylalanine concentrations (nmol
201 phenylalanine g^{-1} wet body mass) in the 5 regions of the white muscle were
202 calculated for each fish and compared with a value of 55 nmol g^{-1} (Bystriansky et
203 al., 2007) in order to estimate the elevation in free phenylalanine concentrations
204 following injection.
205
206 **2.3 Calculations and statistical analysis**
207 Fractional rates of white muscle protein synthesis (k_s, expressed as a
208 percentage of the protein mass synthesized per day, % day^{-1}) were calculated as
209 $k_s = 100 \cdot ((S_b/S_a) \cdot (1440/t))$, where S_b and S_a are the protein-bound and free pool
210 phenylalanine specific radioactivities (dpm nmole^{-1} phe) and t is the incubation
211 time (between injection of the fish and freezing of the sample) in minutes for each
212 fish and 1440 is the number of minutes in a day (Garlick et al., 1983). Since
213 fractional rates of protein synthesis are affected by body size (Houlihan et al.,
214 1986; Houlihan et al., 1995c) and the fish sampled in this study ranged from *ca.*
215 25 g to *ca.* 1.5 kg body mass, the white muscle data were mass-corrected to a
216 standard body mass of 300 g prior to analysis using the equation $k_{s(std)} =$
217 $k_{s(obs)} \cdot (300/M_{obs})^{-0.26}$ (Duthie and Houlihan, 1982} where $k_{s(std)}$ is the mass-
218 corrected rate, $k_{s(obs)}$ and M_{obs} are the fractional rate of protein synthesis (% day^{-}
219 ^{1}) and body mass (g) for an individual fish and -0.26 is the mass-exponent for
220 protein synthesis (Houlihan et al., 1995c). Following scaling to a standard body
221 mass, measured fractional rates of protein synthesis in the five regions were
222 compared using a repeated-measures ANOVA followed by *post-hoc* pairwise
223 comparisons between all regions (n = 10 in total) using a paired t-test applying a

Bonferroni correction (i.e. significance level was $p < 0.005$). The degree of flooding in the white muscle free pool following injection in the 8 groups was compared using a one-way ANOVA followed by multiple pairwise *post-hoc* comparisons using Scheffe's test. All data are presented as mean values ± one standard deviation. All data were tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test) and met the assumptions for parametric statistics. Percentage and proportional data were arcsine transformed prior to statistical analysis. Statistical analyses were conducted using SPSS v22.

3. Results and Discussion

3.1 Mean white muscle free pool phenylalanine-specific radioactivities

The mean free pool phenylalanine-specific radioactivities (S_a) in the white muscle for each sampling group after 2-4 hours incubation (depending on size) are presented in Table 2. To standardize and allow the degree of flooding to be compared across groups, the S_a values for each fish (*i.e.* the mean of the 5 regions for that fish) were expressed as a percentage of the specific radioactivity (SR) of the injection solution used. The mean S_a values for each group of fish ranged from 40.6% to 81.3% with an overall average value of 59.7% (Table 2). A one way ANOVA indicated a significant difference between the degree of flooding between groups ($F_{7,30} = 6.54$, $p < 0.001$) and *post-hoc* comparisons indicted that the level of flooding was significantly lower in Group 5 compared to Groups 1 and 8 ($p = 0.002$ and $p = 0.01$ respectively), tended to be lower in group 7 compared to group 1 ($p = 0.064$) but were similar between all other groups (all $p > 0.11$).

Previous studies that have measured rates of protein synthesis in the white muscle in fishes have introduced radiolabel either through intravenous injection

249 (IV) into the caudal vein or by injection into the peritoneum (IP) (Table 1).
250 Introduction directly into the bloodstream results in white muscle S_a values that
251 are closer to the SR of the injection solution (average value is 79% for the data
252 presented in Table 1) compared to IP injection (average value is 64% for the data
253 presented in Table 1). However, IV injection is technically more challenging than
254 IP injection, takes longer to perform and can be more stressful for the fish (IDM,
255 pers. obs.). In the present study, the average S_a value for the white muscle free
256 pool expressed as percentage of the injection solution was 59% (Table 2) which is
257 close to the average for the white muscle following IP injection for previous
258 studies (64%, Table 1). It is likely that S_a values will never attain parity with the
259 injection solution as the injected dose will be diluted by free phenylalanine
260 present in the body free amino acid pools and, especially with IP injection, it is
261 likely that some radiolabel will be lost from the injection site following withdrawal
262 of the needle. Fraser et al. (2004) report that these two factors combined resulted
263 in a 27% reduction in the body wall S_a values in the Antarctic holothurian
264 *Heterocucumis steini* following a flooding dose injection with dilution accounting
265 for a 12.5% reduction and the remainder assumed lost by leakage. However, the
266 important consideration is that there is sufficient radioactivity introduced into the
267 free pool and incorporated into body protein to enable accurate measurement of
268 synthesis rates. The white muscle free pool S_a values measured in the present
269 study (Table 2) fall within the range of S_a values reported in validation studies for
270 measuring fractional rates of white muscle protein synthesis in fishes (see Table
271 1).
272
273 **3.2. Mean white muscle free phenylalanine concentrations**

In addition to comparing tissue or whole-animal S_a values to the injection solution, many studies also calculate the elevation in free pool phenylalanine concentrations above control values in order to determine the degree of flooding (see Table 1). Measurement of the phenylalanine concentrations in the present study indicated that the elevation of free phenylalanine in the white muscle was on average 7.8 times higher than the estimated background level of 55 nmol phe g^{-1} with a 5.9 fold to 11.2 fold elevation observed among the sampling groups (Table 2). A one way ANOVA indicated that the elevation in free phenylalanine concentrations varied between groups ($F_{7,30}$ = 4.68, p = 0.001) and *post-hoc* comparisons indicted that the elevation in free phenylalanine was significantly higher in Group 1 compared to Groups 2 and 4 (p = 0.02 and p = 0.03 respectively) and tended to be lower in group 5 compared to group 1 (p = 0.06) but were similar between all other groups (all p > 0.21).

Both the average and range of values observed for the elevation in free phenylalanine concentrations in the present study are within the range of values observed for the white muscle in previous studies following IV (average 8.6 fold increase; range 2-fold to 17-fold; Table 1) or IP injection (average 8.6 fold increase; range 8.1-fold to 11.6-fold; Table 1). Taken together, the elevation in free phenylalanine concentrations and the phenylalanine specific radioactivity in the white muscle relative to that of the injection solution indicate that flooding has been achieved in the white muscle tissue in the present study. It is interesting to note that flooding has been achieved despite the reduction in the injection dose compared to the 1 ml 100 g^{-1} standard (Table 1). Houlihan et al. (1986) found that 60 minutes incubation was not sufficient to achieve flooding in the anterior region of the epaxial white muscle following a dose of 0.35 ml 100 g^{-1} and recommended

299 that future work use a larger dose (1 ml 100 g^{-1}). Subsequent studies have
300 followed this recommendation to measure fractional rates of protein synthesis in
301 the white muscle (Table 1). However, the results of the present study indicate that
302 the dose can be substantially reduced and flooding of the white muscle free amino
303 acid pools can still be achieved (Table 2). To the authors' knowledge, only one
304 previous study, Martin et al. (1993) working on 1.3-1.6 kg Atlantic salmon *Salmo*
305 *salar*, has reduced the specific radioactivity of the injection by dilution with 150
306 mM phenylalanine. In their study, Martin et al. (1993) report white muscle S_a
307 values of *ca.* 200 dpm $nmol^{-1}$ phe attaining 60-70% of the SR of the injection
308 solution following a 5-fold dilution and IV injection. However, their time course
309 trial validated the flooding dose technique for the white muscle, even with this
310 reduced level of radioactivity in body tissue. Reducing the radiolabel dose in
311 future studies is to be encouraged as this will reduce experimental costs in terms
312 of the amount of radiolabel used and the amount of radioactivity required for
313 disposal.
314
315 **3.3 Variability in flooding between different regions of the white muscle**
316 The analyses presented thus far have examined flooding in the white muscle as
317 a whole by examining mean tissue values, however, following on from Houlihan
318 et al. (1986), it is possible that flooding within the tissue is not uniform. Therefore,
319 the degree of flooding (*i.e.* expressing S_a relative to the SR of the injection solution)
320 in the 5 regions of the white muscle for each group of fish is presented in Table 3.
321 There was considerable variation in the degree of flooding between regions within
322 groups with values ranging from 35.5% (Group 5, Region 4) to 94.1 (Group 1,
323 Region 5). However, the general pattern across the 8 groups was for the regions

to rank in the following order: Region 5 (66.1%) > Region 3 (60.3%) > Region 1 (59.0%) > Region 2 (54.5%) > Region 4 (52.2%). The elevation in free phenylalanine concentrations in the 5 regions of the white muscle is also presented in Table 3. Again, there was considerable variation in elevation values between regions and between groups but the highest elevation values above estimated baseline free phenylalanine were observed in Region 2 (ranked 1 in 5/8 groups and ranked 2 in 3/8 groups) and the lowest in Region 1 (ranked 4 in 6/8 groups and ranked 5 in 1/8 groups) respectively.

To the authors' knowledge, this is the first study that has examined S_a values and free phenylalanine concentrations in multiple locations in a tissue of an ectotherm following a flooding dose injection to determine whether flooding is uniform throughout the tissue. Houlihan et al. (1986) examined flooding in the 'anterior' (beneath the dorsal fin, *i.e.* equivalent to Region 2 in the present study) and 'posterior' (beneath the adipose fin, *i.e.* equivalent to Region 3 in the present study) regions of the white muscle following incorporation times of 20–60 minutes. The results indicated little evidence of flooding in the anterior white muscle after 60 minutes incubation with free phenylalanine concentrations increasing 1.5-fold above baseline compared to the expected 5.6 fold increase expected with uniform distribution throughout the body (Houlihan et al., 1986, their Fig. 1) with a very low S_a value. In contrast, the posterior white muscle showed a 4-fold increase in free phenylalanine and the free pool S_a value was 69% of the SR of the injection solution (Table 1). This difference in radiolabel distribution in the white muscle was attributed to the relatively poor perfusion of the white muscle fibres (Stevens, 1968; Johnston, 1981) and Houlihan et al. (1986) recommended that a higher dose be utilised in future studies in order to ensure

flooding of the white muscle tissue. Although subsequent studies did adopt this recommendation, together with a longer incorporation time (*e.g.* Carter et al., 1993; McCarthy et al., 1994; Owen et al., 1999; see Table 1), no studies have employed multiple sampling to verify that flooding has been achieved within the muscle tissue. The results of the present study show that flooding throughout the white muscle can be achieved (and with a reduced injection dose) following a longer incorporation time of 2-4 hours (dependent on body size). However, it would appear that distribution of radiolabel within the white muscle is not uniform as the degree of flooding varied between regions (Table 3). The pattern of perfusion and rate of distribution to different regions of the white muscle in salmonid fishes are not known and so the results of the present study cannot be related to any known distribution patterns. It is interesting to note that the elevation in free phenylalanine concentrations measured in the different regions of the white muscle varied and did not show the same rankings as seen for the S_a values. This may suggest variation in baseline free phenylalanine levels in different regions of the white muscle that would warrant further investigation.

3.4 White muscle fractional rates of protein synthesis

Following size correction to standard body mass of 300 g, the fractional rates of protein synthesis in the white muscle ranged between and 0.46 and 0.76% day^{-1} with a mean synthesis rate of 0.61 ± 0.07% day^{-1}. These synthesis rates fall within the range of white muscle fractional rates of protein synthesis observed in fishes (Table 1) and are comparable to the synthesis rates in Arctic charr recently measured by Lamarre et al. (2015) following size-correction (*i.e.* 0.74 and 0.77% d^{-1} for charr weighing 108 and 257 g following size-correction to 300 g body

374 mass). The mass-corrected rates of protein synthesis measured in the 5 regions of
375 the white muscle are presented in Table 4. For each region, among individuals
376 there was a 1.8 to 2-fold difference between the minimum and maximum
377 fractional rates of protein synthesis rates measured. This observed individual
378 variability in rates of protein synthesis in fishes is not uncommon with previous
379 studies reporting up to 4-fold variation in white muscle rates between individuals
380 (Houlihan et al., 1994; Katersky and Carter, 2007). Fractional rates of protein
381 synthesis were significantly different between region (Repeated measures
382 ANOVA, $F_{4,148} = 49.92$, $p < 0.001$) and pairwise *post-hoc* comparisons (with
383 Bonferroni correction) indicated that that rate of protein synthesis was
384 significantly higher in Region 4 compared to the other four regions (all $p < 0.001$)
385 with similar rates of protein synthesis being recorded in Regions, 1, 2, 3 and 5 (all
386 $p > 0.007$).
387 Given the variability in mass-corrected rates of protein synthesis, to clearly
388 visualize any differences in the measured rates of protein synthesis between the
389 5 regions for each fish, the mass-corrected fractional rates of protein synthesis in
390 each region were expressed as a proportion of the global average of the 5 values
391 for that fish to scale all values so they were proportional to 1. Rates of protein
392 synthesis in Region 4 were, on average, 1.15 times larger than the global average
393 for each fish whilst rates in the other four regions were more similar to the overall
394 average (0.94-0.99 times the global average) (Table 4; Fig. 2a).
395 To the authors' knowledge, this is the first time repeat measures of protein
396 synthesis have been made in multiple regions of a tissue/organ in an ectotherm,
397 although Houlihan et al. (1986) attempted to sample two locations in the white
398 muscle in rainbow trout. However, concurrent multiple measures of protein

399 synthesis have been made in one medical study. Heys et al. (1991) sampled either
400 2 or 3 biopsies from 9 patients with breast cancer (n = 6 and n = 3 respectively)
401 following an IV flooding injection of L-[1-^{13}C]leucine to measure fractional rates
402 of protein synthesis in the tumour. Following removal of the tumour by surgery, 2
403 or 3 biopsies were taken at random from the periphery of the tumour for analysis
404 to determine rates of protein synthesis. The aim of the study by Heys and co-
405 workers was to quantify inter-tumour variability in rates of protein synthesis
406 (which may be as a result of regional differences in tumour structure) to
407 determine whether this may explained differences in rates of protein synthesis
408 reported by earlier studies. Although Heys et al. (1991) present the actual
409 synthesis data, so it is possible to determine that the rates are significantly
410 repeatable [Intraclass Correlation Coefficient = 0.88 calculated from the data
411 presented in Table 3 of Heys et al. (1991); see Lessells and Boag (1987)], no tissue
412 free leucine data are provided for the biopsy samples to assess whether flooding
413 was uniform. Thus, to the authors' knowledge, the present study is the first to test
414 for differences in flooding and measured rates of protein synthesis in different
415 regions of a tissue/organ following a flooding dose injection.

416 To determine whether the observed variability in rates of protein synthesis
417 between regions was related to body size, the proportional rates of protein
418 synthesis in the 5 regions for each fish were plotted against body mass (Fig. 2a).
419 Figure 2a shows that, with the exception of two fish, the variability in synthesis
420 rates between regions is relatively low, *i.e.* in the range 0.9 to 1.1, for fish < 1 kg
421 body mass whilst variability between regions appears to increase in fish larger
422 than *ca.* 1 kg body mass. The coefficient of variation for the five measures of
423 protein synthesis for each fish is plotted against its body mass in Figure 2b. This

plot confirms the pattern suggested in Figure 2a with CV values of < 10% generally observed for fish < 1 kg in body mass (Table 5; 21/33 fish < 1 kg body mass have a CV of < 10%). It is possible that the increased variability in rates of protein synthesis between different regions of the white muscle with increasing size may be due to the effect of size on the perfusion rate to different regions of the white muscle. However, in the absence of any studies looking at the pattern and rates of perfusion in the muscle of salmonid fishes, this hypothesis cannot be confirmed.

3.5 Conclusions

The present study has examined the patterns of flooding and measured rates of protein synthesis in different regions of the white muscle in the Arctic charr following a flooding dose injection of L-[^{3}H]-Phenylalanine. The results have shown that the degree of flooding (*i.e.* free pool specific radioactivity relative to that of the injection solution) and elevation in free phenylalanine concentrations can vary between regions but the calculated fractional rates of protein synthesis were similar in 4 of the 5 regions studied. The variability in rates of protein synthesis increased with body size with greater variability observed between regions for fish > 1 kg in body mass. However, for consistency between studies, it is recommended that samples are taken from the epaxial muscle in the region below the dorsal fin when measuring fractional rates of white muscle synthesis in fishes.

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452

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569 **Figure legends**

570

571 **Figure 1.** Diagram of Arctic charr *Salvelinus alpinus* showing the 5 regions in the
572 epaxial (Regions 1 to 4) and hypaxial (Region 5) white muscle where tissue
573 samples were removed to measure rates of protein synthesis following a flooding
574 dose injection of L-[^{3}H]-phenylalanine.

575

576 **Figure 2.** Variability in rates of protein synthesis in the white muscle of Arctic
577 charr *Salvelinus alpinus* (n = 38) ranging in size from 25 g to 1.6 kg body mass
578 (Note: log scale on the abcissas). (a) Rates of protein synthesis in 5 regions of the
579 white muscle expressed as a proportion of the overall mean value for the 5
580 measurements for each fish. See Figure 1 for the location of each region in the
581 white muscle. (b) The coefficient of variation (%) for the five measures of protein
582 synthesis for each fish.

583

584

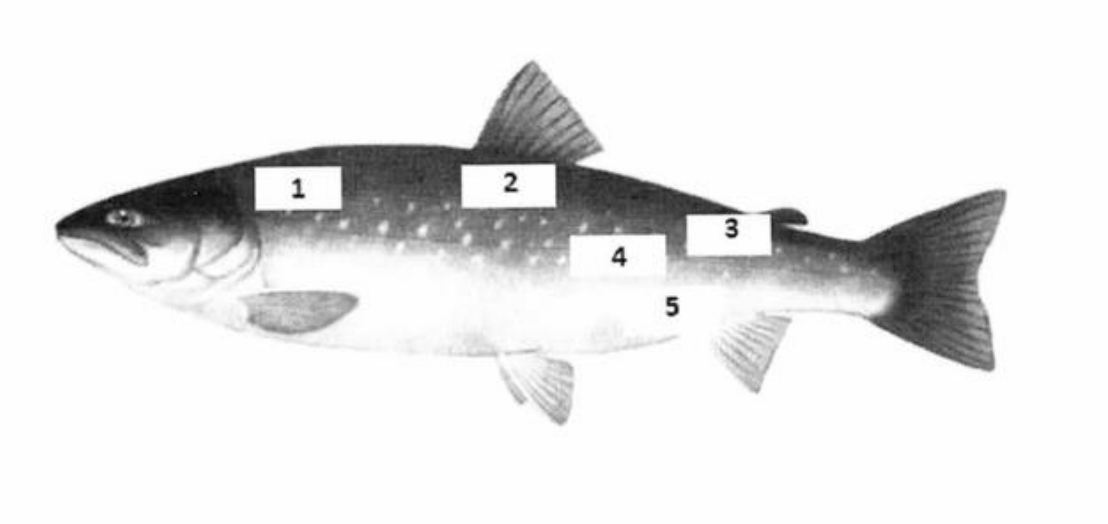
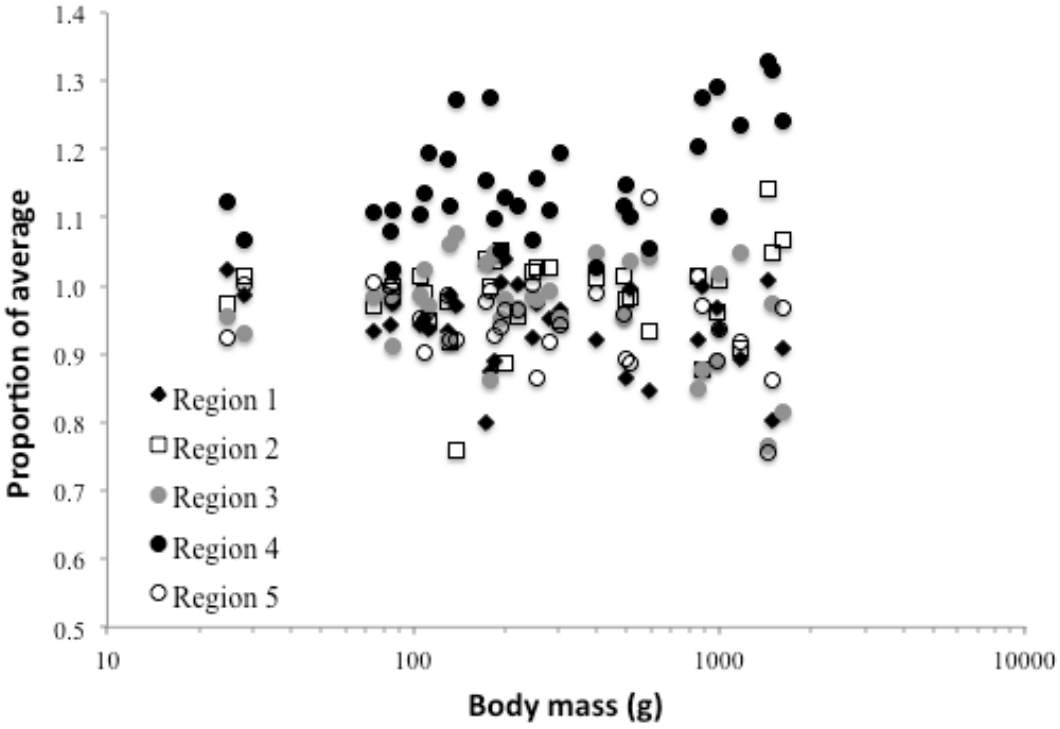


Figure 1

a)


1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
Proportion of average
Region 1
Region 2
Region 3
Region 4
Region 5
10
100
1000
10000
Body mass (g)

b)

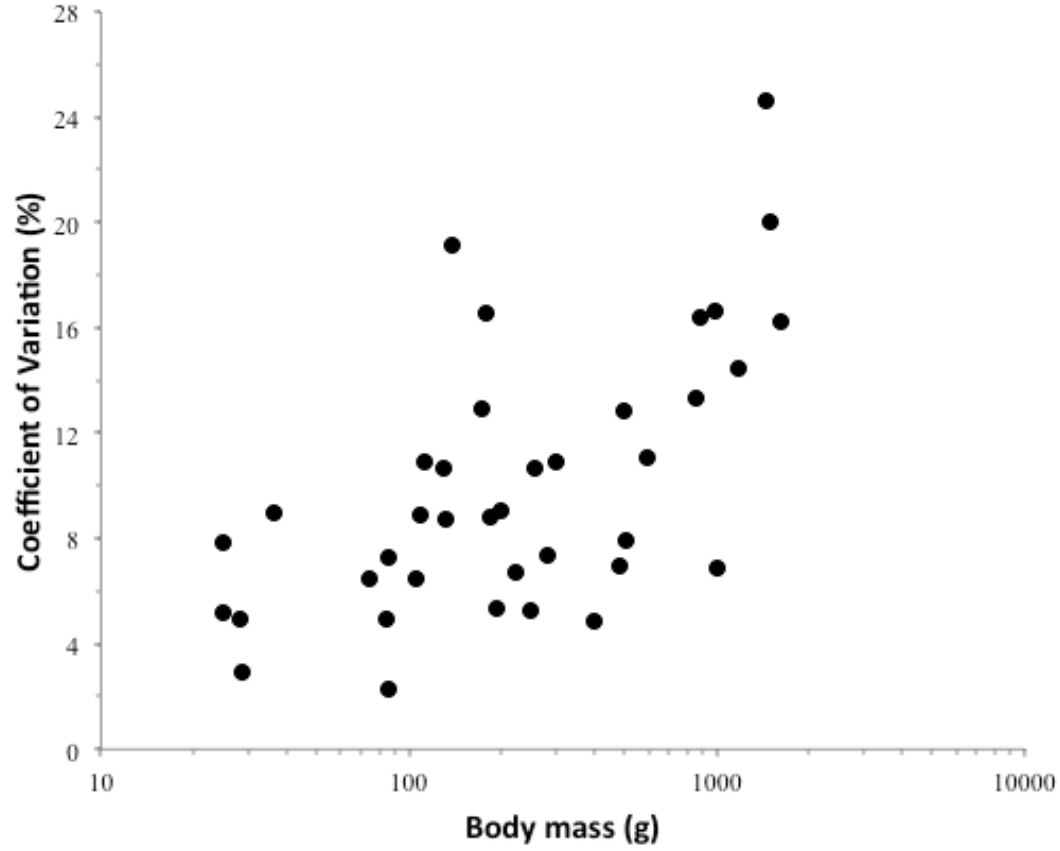

28
24
20
16
12
8
4
0
Coefficient of Variation (%)
10
100
1000
10000
Body mass (g)

Figure 2

Table 1. A summary of the published data for white muscle rates of protein synthesis (k_s, %/d) in fishes using the flooding dose technique. Data are presented for fish size (body mass, g), water temperature (°C) and method of tracer administration [intravenous (IV) or intraperitoneal (IP) injection], injection dose, incorporation time (minutes) and location in the epaxial white muscle from where samples were collected. The phenylalanine-specific radioactivities (SR) of the injection solution (IJ) and the white muscle free amino acid pool (WM FP) are presented (both dpm nmole^{-1} phe) and the latter also presented as a percentage of the former (% IJ). In addition the elevation in white muscle free phenylalanine concentrations (Free Phe, nmol phe g^{-1}) above basal control (*i.e.* uninjected) levels is presented.

Species	Size (g)	T°C	Dose (/100g)	IJ SR	Time (mins)	Sample location	WM FP	% IJ	Free Phe	k_s (%/d)	Reference
Lates calcarifer	3	21 - 33	1 ml IV	1123	60 130	Not stated	1255 929	112 83	–	0.5–1.9	Katersky and Carter (2007)
Salmo salar	200-300	14	1 ml IV	1600	81, 120	Below dorsal fin	1323	82	8x	–	Carter et al. (1993)[d]
	1300-1600	9	1 ml IV	300[a]	20-60	Not stated	180-210	60-70	–	2.5	Martin et al. (1993)
Sparus aurata	90-100	_	1 ml IV	1473	30-180	Not stated	936	64	2x	0.5-2.6	Carter et al. (2012)
Oncorhynchus mykiss	84	12	0.35 ml IV	2450	20-60	Anterior epaxial Posterior epaxial	– 1700	– 69	– 4x	– 0.2–0.5	Houlihan et al. (1986)
	60-70	15	1 ml IV	1400	20-40	Not stated	1064	70	–	0.2–0.7	McMillan and Houlihan (1988)
	50	-	1 ml IV	1210	40	Below dorsal fin	1057	87	17x	0.2–0.3	Foster et al. (1991)
	65	10	1 ml IV	1250	278	Below dorsal fin	1091	87	12x	–	McCarthy et al. (1994)[d]
Ctenopharnygodon idella	10-30	22	1 ml IV	1836	60-206	Below dorsal fin	–	–	–	0.8-2.5	Carter et al. (1992)

Gadus morhua	247 174	5 15	1 ml IV	1563	30-90	Not stated	1350 1225	86 78	–	< 0.1 < 0.1	Foster et al. (1992)
Limanda limanda	250	7	1 ml IP	1600	120-240	Not stated	1300	81	–	0.15	Houlihan et al. (1994)
Anarhichas lupus	40-65	5-14	1 ml IP	2682	169-208	Not stated	1648	62	–	0.5-1.0	McCarthy et al. (1999)
Salmo salar	37	12	1 ml IP	32.0 APE[b]	120-360	Anterior to dorsal fin	20.5 APE[b]	64	–	–	Owen et al. (1999)[d]
Dicentrarchus labrax	8	18	1 ml IP	2250	160-100	Not stated	1616	72	11x	0.31	Houlihan et al. (1995a)
Tautogolabrus adspersus	174	0 8	1 ml IP	–	240-1440	Not stated	800 900	– –	8.1x	– –	Lewis and Driedzic (2007)
Salvelinus alpinus	108 257	12	1 ml IP	–[c]	60- 480 240	Not stated	–[c]	40	11.6x	0.96 0.80	Lamarre et al. (2015)

a = 5-fold dilution of injection solution prior to measurement;
b = protein synthesis measured using ^{15}N-phenylalanine. APE = Atom Percent Excess;
c = protein synthesis measured using D_5-phenylalanine. No data provided on APE of IJ or WM FP. WM FP expressed as a % of injected dose;
d = White muscle FP SR used as an estimate of the whole-animal FP SR to measure whole-animal fractional rates of protein synthesis;
– = data not presented in original paper

Table 2. A summary of the injection protocol (injection volume, ml; incubation time, minutes) for the 8 groups of Arctic charr *Salvelinus alpinus* used to measure fractional rates of white muscle synthesis. The mean (± SD) white muscle free pool phenylalanine specific radioactivity (S_a, disintegrations $minute^{-1}$ $nanomole^{-1}$ phenylalanine) for each group is presented and expressed as a percentage (mean ± SD) of the specific radioactivity of the injection solution. The specific radioactivity of injection solution was 1312 (± 87, n = 4) dpm $nmole^{-1}$ phe in September 2005 and calculated as 1178 dpm $nmole^{-1}$ phe in July 2007 but diluted 50:50 with 135 mM L-Phenylalanine prior to injection*. Values with the same letter (down columns) are significantly different from each other ($p < 0.05$).

	Days post 1st feeding	Body Mass (g)	Volume (ml)	Dose (ml 100 g^{-1})	Time (min)	S_a (dpm $nmole^{-1}$)	S_a (% Injection)	Free Phe elevation
Group 1, n = 5 14 Sept 2005	163	28.6 (25.0–36.5)	0.2	0.7	118	1066 ± 145	81.3 ± 11.1[a]	11.2 ± 2.0
Group 2, n = 5 24 Nov 2005	234	88.4 (74.9–109.6)	0.7	0.8	165	755 ± 45	57.5 ± 3.4	5.9 ± 2.1
Group 3, n = 5 19 Jan 2006	290	133.2 (105.6–184.3)	0.5	0.4	168	726 ± 154	54.7 ± 11.7	7.5 ± 1.4
Group 4, n = 5 17 March 2006	347	177.7 (138.6–200.5)	0.5	0.3	193	772 ± 93	58.8 ± 7.1	6.1 ± 1.6
Group 5, n = 5 22 March 2006	352	261 (221–301)	0.5	0.2	224	533 ± 173	40.6 ± 13.2[a,b]	6.4 ± 1.6
Group 6, n = 5 30 June 2006	452	497 (397–593)	0.5	0.1	210	717 ± 144	54.6 ± 11.0	8.6 ± 3.2
Group 7, n = 4 26 July 2007	843	933 (850–1010)	1.0*	0.1	243	364 ± 132*	50.9 ± 9.0	7.3 ± 1.3
Group 8, n = 4 26 July 2007	843	1437 (1185–1616)	1.0*	0.07	240	425 ± 123*	76.7 ± 20.9[b]	9.6 ± 1.6

Table 3. Variability in level of flooding in the free pool in 5 regions of the white muscle in 8 groups of Arctic charr *Salvelinus alpinus*. (n = 38) following a flooding dose injection of 135 mM L-Phenylalanine/L- [2, 6-^{3}H]phenylalanine. For each region sampled in the white muscle, the phenylalanine specific radioactivity (S_a, dpm nmole^{-1} phe) values are expressed as a proportion of the specific radioactivity of the injection solution (Inj. Sol., dpm nmole^{-1} phe) and the elevation in the free phenylalanine concentrations (nmole^{-1} phe g^{-1} wet body mass) above an estimated basal value (55 nmol^{-1} g^{-1}; Bystriansky et al., 2007) is calculated (Elevation, *i.e.* an X-fold increase above estimate basal values). Data are presented as mean values ± Standard Deviation. See Figure 1 for the location of each region in the white muscle.

	Body Mass (g)	Inj. Sol.	Flooding	Region 1	Region 2	Region 3	Region 4	Region 5
Group 1	28.6	1312	Proportion	0.84 ± 0.10	0.80 ± 0.25	0.71 ± 0.16	0.77 ± 0.31	0.94 ± 0.10
N = 5	(25.0 – 36.5)		Elevation	10.0 ± 1.1	13.8 ± 4.3	10.5 ± 4.4	11.1 ± 3.0	10.6 ± 3.4
Group 2	88.4	1312	Proportion	0.59 ± 0.12	0.52 ± 0.14	0.67 ± 0.19	0.51 ± 0.12	0.59 ± 0.10
N = 5	(74.9 – 109.6)		Elevation	5.3 ± 2.5	6.5 ± 3.3	6.6 ± 3.9	5.1 ± 1.4	5.8 ± 2.0
Group 3	133.2	1312	Proportion	0.58 ± 0.18	0.48 ± 0.19	0.57 ± 0.21	0.46 ± 0.12	0.65 ± 0.21
N = 5	(105.6 – 184.3)		Elevation	6.9 ± 1.8	8.8 ± 3.5	7.1 ± 1.8	8.1 ± 2.0	6.5 ± 1.8
Group 4	177.7	1312	Proportion	0.48 ± 0.14	0.55 ± 0.09	0.59 ± 0.25	0.59 ± 0.20	0.63 ± 0.24
N = 5	(138.6 – 200.5)		Elevation	5.8 ± 1.4	6.2 ± 3.1	6.2 ± 1.9	5.4 ± 2.3	6.7 ± 3.4
Group 5	261	1312	Proportion	0.44 ± 0.10	0.38 ± 0.11	0.46 ± 0.20	0.36 ± 0.08	0.44 ± 0.15
N =5	(221 – 301)		Elevation	6.0 ± 2.5	6.9 ± 3.1	5.7 ± 0.9	7.3 ± 3.0	6.4 ± 1.8
Group 6	497	1312	Proportion	0.57 ± 0.17	0.57 ± 0.17	0.59 ± 0.17	0.49 ± 0.21	0.57 ± 0.15
N = 5	(397 – 593)		Elevation	7.4 ± 3.3	11.0 ± 5.1	6.6 ± 2.7	7.1 ± 5.9	11.0 ± 4.8
Group 7	933	589	Proportion	0.45 ± 0.05	0.46 ± 0.08	0.47 ± 0.06	0.44 ± 0.17	0.73 ± 0.26
N = 4	(850 – 1010)		Elevation	7.0 ± 1.8	7.8 ± 1.2	7.6 ± 1.5	6.9 ± 1.8	7.4 ± 2.0
Group 8	1437	589	Proportion	0.79 ± 0.28	0.65 ± 0.28	0.76 ± 0.18	0.56 ± 0.07	0.78 ± 0.22
N = 4	(1185 – 1616)		Elevation	9.6 ± 3.1	11.8 ± 2.3	10.2 ± 2.7	5.6 ± 3.0	10.9 ± 4.0

Table 4. Variability in rates of protein synthesis in 5 regions of the white muscle in Arctic charr *Salvelinus alpinus*. (n = 38). For each region sampled in the white muscle, rates of protein synthesis are presented as the fractional rate (k_s, % day^{-1}) and expressed as a proportion of the overall average synthesis rate for each fish (Proportion). Data are mass-corrected to a standard body mass of 300 g and are presented as mean values ± standard deviation for each region together with the minimum and maximum values. Muscle samples were collected from the epaxial muscle immediately behind the head (Region 1), below the dorsal fin (Region 2), below the adipose fin (Region 3) and above the lateral line midway between dorsal fin and the adipose fin (Region 4) and in the hypaxial muscle in front of the anal fin (Region 5). See Figure 1 for the location of each region in the white muscle.

	k_s			Proportion		
	Mean ± SD	Min	Max	Mean ± SD	Min	Max
Region 1	0.57 ± 0.08[a]	0.39	0.72	0.94 ± 0.04	0.80	1.04
Region 2	0.60 ± 0.07[a]	0.40	0.77	0.99 ± 0.06	0.76	1.14
Region 3	0.59 ± 0.08[a]	0.36	0.71	0.97 ± 0.07	0.77	1.11
Region 4	0.70 ± 0.09[b]	0.49	0.96	1.15 ± 0.08	1.02	1.33
Region 5	0.58 ± 0.08[a]	0.35	0.70	0.95 ± 0.06	0.76	1.13

Mean values with the same letter are not significantly different from each other

Table 5. The effect of body size on the variability in fractional rates of protein synthesis measured in 5 regions of the white muscle in Arctic charr *Salvelinus alpinus*. (n = 38). The coefficient of variation (CV = 100·SD/mean) of the 5 measures was calculated for each fish and the mean ± standard deviation CV for each size group is presented together with the minimum and maximum CV values for each size group.

Body Mass	n	CV	Min	Max
< 100 g	9	5.6 ± 2.2	2.3	8.9
100 – 250 g	13	10.0 ± 4.2	5.3	19.1
250 – 500 g	6	8.9 ± 3.0	4.9	12.8
500 – 1000 g	5	13.1 ± 3.7	7.9	16.7
> 1000 g	5	16.4 ± 6.6	6.9	24.6