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Validation of the flooding dose technique to determine fractional rates of protein synthesis in a model bivalve species, the blue mussel (*Mytilus edulis* L.)

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Abstract

For the first time, use of the flooding dose technique using ^{3}H-Phenylalanine is validated for measuring whole-animal and tissue-specific rates of protein synthesis in the blue mussel *Mytilus edulis* (61 mm shell length; 4.0 g fresh body mass). Following injection, the phenylalanine-specific radioactivities in the gill, mantle and whole-animal free pools were elevated within one hour and remained elevated and stable for up to 6 h following injection of ^{3}H-phenylalanine into the posterior adductor muscle. Incorporation of ^{3}H-phenylalanine into body protein was linear over time following injection and the non-significant intercepts for the regressions suggested incorporation into body protein occurred rapidly after injection. These results validate the technique for measuring rates of protein synthesis in mussels. There

were no differences in the calculated rates following 1-6 h incubation in gill, mantle or whole-animal and fractional rates of protein synthesis from the combined time course data were 9.5 ± 0.8 % d^{-1} for the gill, 2.5 ± 0.3 % d^{-1} for the mantle and 2.6 ± 0.3 % d^{-1} for the whole-animal, respectively (mean values ± SEM). The whole-animal absolute rate of protein synthesis was calculated as 18.9 ± 0.6 mg protein day^{-1}. The use of this technique in measuring one of the major components of maintenance metabolism and growth will provide a valuable and convenient tool in furthering our understanding of the protein metabolism and energetics of this keystone marine invertebrate and its ability to adjust and respond to fluctuations, such as that expected as a result of climate change.

Key words:

Bivalve, Flooding dose technique, Mussel, *Mytilus edulis*, Protein synthesis

1. Introduction

Proteins are central to life playing a key role in maintenance metabolism through the synthesis and breakdown of structural, catalytic and other metabolically-active proteins, with growth occurring when rates of protein synthesis exceed breakdown (Doherty and Whitfield, 2011). It has been estimated that protein synthesis accounts for 11 to 42% of the basal metabolism in ectothermic and endothermic animals with protein breakdown accounting for an additional energetic cost which, although currently unquantified, may be equivalent to the cost of synthesis (Houlihan et al., 1995a; Fraser and Rogers, 2007). Thus, the cycle of protein turnover (i.e. patterns of synthesis and breakdown) is a fundamental physiological process underlying not only changing patterns of tissue replacement and growth during ontogeny and ageing, but also providing the mechanisms allowing animals to adapt to changing environmental conditions and planes of nutrition. A number of techniques have been

developed to measure *in vivo* rates of protein synthesis (Waterlow, 1995; Houlihan et al., 1995a, 1995b; Wagenmakers, 1999). Essentially these techniques all adopt a similar approach in that a stable or radioactive isotope-labelled tracer is introduced to the animal and the rate of incorporation of the isotope into body protein, or its appearance in metabolic end-products over time, is measured in order to determine rates of protein synthesis. These methodologies, however, vary in their underlying methodological assumptions, choice of tracer (stable isotope or radioisotope tracer; use of labelled amino acids such as phenylalanine, leucine etc or uniformly-labelled protein), method of tracer administration (*e.g.* constant infusion, single high dose or multiple bolus injection), or choice of immediate precursor pool (*e.g.* free amino acids or amino-acyl-tRNAs) (Waterlow, 1995; Davis and Reeds, 2001).

The flooding dose technique of Garlick et al. (1980) has been one of the major tracer methods used to measure rates of protein synthesis in endotherms and ectotherms. It was originally developed to measure fractional rates of protein synthesis in rats but the methodology has been applied to other vertebrate and invertebrate taxa (see reviews by Houlihan et al., 1995a; Fraser and Rodgers, 2007; Carter and Mente, 2014). Using this technique, a labelled amino acid (usually L-[^{3}H]-phenylalanine in non-human studies) is injected as a single large dose to rapidly swamp all the body free amino acid pools with the label (hence the term 'flooding dose') and to ensure that the specific radioactivities of all the various body compartments are closely related. Validation work has shown that labelling in the free amino acid and the aminoacyl-transfer RNA are equilibrated following a flooding dose (Davis et al., 1999; Davis and Reeds, 2001; Caso et al., 2002). After a known incorporation period, samples can be obtained through biopsy (in humans, usually to measure rates of protein synthesis in muscle tissue) or through killing the animal and taking tissue samples for subsequent analysis of rates of protein synthesis in different tissues (e.g. liver, muscle), or homogenising the whole animal to obtain a whole-animal rate of protein

synthesis. The flooding dose technique of Garlick et al. (1980) has become one of the standard techniques for measuring *in vivo* rates of protein synthesis in most animals, including aquatic ectotherms, because of the ease of tracer administration through a single injection, the rapid flooding of the body free amino acid pools and the ability to selectively extract and measure free pool and protein-bound phenylalanine-specific radioactivities using standard biochemical assays. Validation of the flooding dose technique, however, is essential to ensure success and the following key criteria need to be fulfilled: (1) the phenylalanine-specific radioactivity of the intracellular free amino acid pools is rapidly elevated following injection; (2) the phenylalanine-specific radioactivity of the intracellular free amino acid pools either remain elevated and stable or show a slow linear decline over the incubation period (Garlick et al., 1980, 1983); (3) the uptake of radiolabel into body protein is linear over the incubation period; (4) the presence of a high dose of ^{3}H-L-phenylalanine does not stimulate or depress rates of protein synthesis following injection. When applying the flooding dose technique to a new species for the first time, validation studies that examine the changes in the free pool and protein-bound phenylalanine-specific radioactivities over time are essential (Fraser and Rodgers, 2007).

In the aquatic environment, the majority of studies on protein synthesis using the flooding dose have focused on fish and crustaceans (Houlihan et al., 1995b; Fraser and Rodgers, 2007; Carter and Mente, 2014), although the technique has also been applied to other vertebrate and invertebrate taxa, albeit less often: amphibians (Fuery et al., 1998a, 1998b), reptiles (Fraser et al., 2001), holothurian echinoderms (Fraser et al., 2004) and gastropod (Fraser et al., 2002; Bowgen et al., 2007) and cephalopod (Houlihan et al., 1990b; Carter et al., 2009; Moltschaniwskyj and Carter, 2010; Moltschaniwskyj et al., 2013) molluscs. However, the flooding dose technique has not previously been used to determine rates of protein synthesis in bivalve molluscs, which is surprising given the key ecological

role, adaptive physiology for life in the intertidal and commercial importance of this taxa (e.g. Navarette and Menge, 1996; Ragnarsson and Raffaelli, 1999; Somero, 2002; Gosling, 2003; Gracey et al., 2008; Smith et al., 2014). *In vivo* rates of whole-animal protein synthesis have been measured in bivalve molluscs of the genus *Mytilus* using ^{15}N-labelled algae and a stochastic end-point model (*e.g.* Hawkins, 1985; Hawkins et al., 1989; Bayne and Hawkins, 1997). The results of these studies have provided some fundamental insights into the role that inter-individual variation in protein turnover plays in individual growth performance, in terms of both growth rates and growth efficiency (Hawkins et al., 1989), have shown links between genetic heterozygosity, protein turnover and growth performance (Hawkins et al., 1986; Bayne and Hawkins, 1997; Hawkins and Day, 1999) and examined the effects of salinity and acute temperature change in the laboratory (Hawkins et al., 1987; Hawkins and Hilbish, 1992) and seasonal changes in the field on protein metabolism in *M. edulis* (Hawkins, 1985).

The aim of the current study is to validate the flooding dose technique using L-[^{3}H]-phenylalanine to measure whole-animal and tissue-specific rates of protein synthesis in bivalve molluscs using the commercially and ecologically important species the blue mussel *Mytilus edulis* as a model. Validation will allow whole-animal and tissue-specific measures of protein synthesis in bivalves that are directly comparable with the range of other taxa that have been studied using the flooding dose technique of Garlick et al. (1980). In addition, whole-animal rates of protein synthesis can be compared with the data of Hawkins and co-workers (*op cit*) obtained using a stochastic endpoint model method.

2. Materials and Methods

2.1 Animal collection

Twenty five blue mussels (*Mytilus edulis* L.) were collected from the high intertidal zone at Tal y Foel, Menai Strait, North Wales in June 2010 and returned to the flow-through seawater

aquarium at the School of Ocean Sciences. On the shore, individual mussels were selected to be greater than 50mm shell length and free from any visible signs of damage such as chips or holes in the shell. The mussels [shell length, 61.0 mm ± 0.8 mm (mean ± SEM), range 54.2 to 68.8 mm] were left overnight in ambient environmental conditions for Menai Bridge (15.1 ºC, 33.1 PSU) and divided into five groups of five mussels the following morning for use in the validation experiment. Shell length was not different in the five time-course groups (ANOVA; $F_{3,24}$= 0.97, p = 0.45).

2.2 Radiolabel injection and incubation time course

Fractional rates of protein synthesis were measured using a modification of the flooding dose technique (Garlick et al., 1980; Houlihan et al., 1995a). Immediately prior to injection, the mussels were removed from seawater, blotted dry and the shell valves were notched in the vicinity of the posterior adductor muscle. Four groups of five mussels were injected, in turn, into the sinus of the posterior adductor muscle with a solution containing 135 mM L-phenylalanine and L-[2, 6-^{3}H]-phenylalanine (American Radiolabelled Chemicals (UK); 3.7 MBq ml^{-1}). The injection volume used was 10 µl g^{-1} wet body mass (Houlihan et al., 1995a; Fraser et al., 2002) with wet body mass (M_W) estimated from shell length (S_L) using the following calibration equation for mussels at Tal y Foel: $M_W = 0.00005S_L^{2.74}$ (n = 50, r^2 = 0.927, p < 0.001; Busbridge, unpublished results). The phenylalanine-specific radioactivity of the injection solution was 838 ± 33 disintegrations per minute per nanomole phenylalanine (dpm nmole^{-1} phe). Following injection, each group of five injected mussels was placed in a separate 5 l tank (20 x 15 x15cm) containing aerated seawater and left for either 1, 2, 4 or 6 h to allow incorporation of the injected radiolabel. The fifth group was notched but not injected and served as a control group (*i.e.* 0 h incorporation). After notching of the control group or following incubation of the injected groups, each mussel was dissected by cutting through the

posterior adductor muscle and the total fresh body tissue rinsed with distilled water, briefly blotted and weighed (fresh body mass 4.00 ± 0.22 g; range 2.22 to 6.58 g). Samples (*ca.* 200 mg) of tissue were removed from the gill and mantle and placed in microcentrifuge tubes and the remaining mussel tissue (henceforth referred to as the whole-animal) placed in a foil bag. All dissections were completed within 5 min and the samples were frozen in liquid nitrogen and stored at -80°C until further laboratory analysis.

2.3 Laboratory analyses

Duplicate gill and mantle tissue samples (ca. 100 mg) from each mussel were homogenised in 2 ml 0.2M perchloric acid (PCA) and centrifuged (6000 g, 4°C 15 min) to separate the intracellular free pool from the precipitated protein pellet and RNA. Whole-animal samples were homogenised in 40 ml 0.2M PCA, vortex mixed and duplicate 2 ml subsamples removed (Fraser et al., 2002) and treated in the same way as gill and mantle tissue samples. The treatment of gill, mantle and whole-animal samples to measure the free pool and protein-bound phenylalanine specific radioactivity followed the protocol outlined in Houlihan et al. (1995a) and McCarthy and Fuiman (2008). In brief, the protein pellet was solubilised in 0.3M NaOH and the protein content was measured using the Folin-phenol method of Lowry et al. (1951) as modified by Schacterle and Pollack (1973) using bovine serum albumin as a standard and RNA content measured using the Orcinol assay (Mejbaum, 1939) using Type IV calf liver RNA (SIGMA) as a standard. The protein-bound phenylalanine specific radioactivity (S_b, dpm $nmol^{-1}$ phe) was calculated from the radioactivity of the solubilised protein (dpm mg^{-1} protein), determined by scintillation counting (Perkin Elmer WinSpectralTM 1414 Liquid Scintillation counter using Optiphase 'HiSafe' scintillant at a counting efficiency of 37%), divided by the concentration of phenylalanine (nmol phe mg^{-1} protein) in that tissue or for the whole-animal (Houlihan et al., 1992; Wilson et al., 1996;

Morgan et al., 1998; McCarthy and Fuiman, 2008). The concentration of phenylalanine in blue mussel tissue and whole-animal protein was measured in replicate samples (n=10) by ion exchange analysis (PNAC, Department of Biochemistry, Cambridge University) for mussels collected from the same collection site providing values of 112.4, 134,3 and 192.2 nmol phe mg^{-1} protein for gill, mantle and whole-animal protein respectively. The phenylalanine-specific radioactivity of the gill, mantle and whole-animal free pools (S_a, dpm $nmol^{-1}$ phe) was calculated by converting phenylalanine in the free pool to β-phenylethylamine (PEA) and extracting through heptane into 0.01M sulphuric acid and measuring PEA content by fluorescence (Suzuki and Yagi, 1976) and PEA radioactivity by scintillation counting.

Fractional rates of protein synthesis (k_s, expressed as a percentage of the protein mass synthesised per day, % d^{-1}) were calculated for gill, mantle and whole-animal samples as $k_s = 100.((S_b/S_a).(1440/t))$, where S_b and S_a are the protein-bound and free pool phenylalanine specific radioactivities (dpm $nmole^{-1}$ phe) and t is the incubation time (between injection and freezing) in minutes and 1440 is the number of minutes in a day (Garlick et al., 1983). Whole-animal absolute rates of protein synthesis (A_s, mg protein synthesised per day, mg d^{-1}) were calculated as $A_s = (k_s/100).(M_{prot})$ where M_{prot} is the whole-animal protein mass (mg). RNA concentrations were expressed as the capacity for protein synthesis (C_s, μg RNA mg^{-1} protein) and as RNA activity (k_{RNA}, mg protein synthesised per mg RNA per day, mg mg^{-1} d^{-1}) calculated as $(10.k_s)/C_s$ (Sugden and Fuller, 1991).

2.4 Statistical analyses

All data are presented as mean values ± SEM. All data were tested for normality (Kolmogorov-Smirnov test) and equal variance (Levene's test) prior to statistical analysis and data were log_{10}-transformed if necessary to meet assumption for parametric statistical tests.

Least-squares linear regression analysis was used to describe the incorporation of radiolabel into gill, mantle and whole-animal protein over time. Free pool S_a values and fractional rates of protein synthesis after 1, 2, 4 and 6 h incubation were compared using a one-way ANOVA. Similarly, fractional rates of protein synthesis, C_s and k_{RNA} values in gill, mantle and the whole mussel were compared using a one-way ANOVA. Where the ANOVA was significant, post-hoc pairwise comparisons were conducted using Tukey's HSD test. All statistical analyses were conducted using SPSS for Windows v20 using a significance value of $\alpha = 0.05$.

3. Results

3.1. Time course validation

The intracellular free pool specific radioactivities (S_a) in the gill, mantle and whole-animal were elevated within one hour of injection (Fig. 1a-c) and remained elevated over the 6 h time course with average S_a values of 642 ± 23 dpm $nmol^{-1}$ phe for the gill, 562 ± 14 dpm $nmol^{-1}$ phe for the mantle and 501 ± 36 dpm $nmol^{-1}$ phe for the whole-animal, respectively, which were equivalent to 77 ± 3 %, 67 ± 2 % and 60 ± 4 % of the specific radioactivity of the injection solution. In the mantle and whole-animal, S_a values were similar over the six hour time course (mantle, $F_{3,19}$= 0.36, p = 0.78; whole-animal, $F_{3,19}$= 1.21, p = 0.34). In the gill, S_a was significantly higher after 2 h incubation compared with 4 h ($F_{3,19}$= 5.94, p = 0.006; Tukey *post-hoc* pairwise comparison, p = 0.003) but the other free pool S_a values were similar between 1 and 6 h incubation (Tukey *post-hoc* pairwise comparisons, p = 0.14 to p = 0.99) (Fig. 1a). Thus, phenylalanine-specific radioactivities in the gill, mantle and whole-animal free pools remained elevated and stable over the 6 h time-course experiment.

The time course incorporation of ^{3}H phenylalanine into gill, mantle and whole-animal protein pools are shown in Fig. 2a-c. The incorporation rates of radiolabel into all three

protein pools over the 6 h time course all exhibited significant linear relations and were described by the following equations:

Gill $S_b = 0.408 + 2.292t$ ($r^2 = 0.989$, n = 5, $p < 0.001$)

Mantle $S_b = 0.255 + 0.427t$ ($r^2 = 0.968$, n = 5, $p = 0.002$)

Whole-animal $S_b = 0.119 + 0.445t$ ($r^2 = 0.967$, n = 5, $p = 0.002$)

where S_b is the protein-bound phenylalanine specific radioactivity (dpm $nmol^{-1}$ phe) and t is incubation time (h). The intercept values in each regression line were not significantly different from zero ($p = 0.15$ to 0.45).

3.2 Rates of protein synthesis

Fractional rates of protein synthesis for gill and mantle tissue and the whole-animal calculated from the time-course data are presented in Fig. 3. Fractional rates of protein synthesis were highest in gill tissue at *ca.* 8-10 % d^{-1} compared to mantle tissue and whole-animal rates where rates of protein synthesis were *ca.* 2–3.5 % d^{-1}. There were no differences in the calculated rates following 1-6 h incubation in the gill ($F_{3,19}= 0.64$, $p = 0.60$), mantle ($F_{3,19}= 1.27$, $p = 0.32$) and whole-animal ($F_{3,19}= 0.93$, $p = 0.45$) and the average fractional rates of protein synthesis from the combined time course data were 9.5 ± 0.8 % d^{-1} for the gill, 2.5 ± 0.3 % d^{-1} for the mantle and 2.6 ± 0.3 % d^{-1} for the whole-animal, respectively (Table 1). The whole body absolute rate of protein synthesis was calculated as 18.9 ± 0.6 mg protein day^{-1}.

Data on the capacity for protein synthesis, RNA activity and fractional rates of protein synthesis for gill, mantle and whole-animal tissue are presented in Table 1. The capacity for protein synthesis (C_s, Table 1) was significantly different between gill, mantle and whole animal tissue ($F_{2,57}= 20.03$, $p < 0.001$; Tukey's HSD pairwise comparisons, gill *vs* mantle or whole animal $p < 0.01$, mantle *vs* whole animal $p < 0.05$). However, RNA activity was

significantly higher in gill tissue compared with the mantle and whole-animal ($F_{2,57}$= 37.34, p < 0.001; Tukey's HSD pairwise comparisons, gill *vs.* mantle or whole animal both p < 0.01; mantle *vs* whole animal p > 0.05). This increased RNA activity resulted in significantly higher fractional rates of protein synthesis in gill tissue compared to the mantle and whole-animal (ANOVA on $\log_{10}$-transformed data; $F_{2,57}$= 68.93, p < 0.001; Tukey's HSD pairwise comparisons, Gill vs. mantle or whole animal both p < 0.01, mantle *vs* whole animal p > 0.05) (Table1).

4. Discussion

The results of the present study validate the use of the 'flooding dose' method of Garlick et al. (1980) using ^{3}H-phenylalanine to determine, for the first time, whole animal and tissue-specific fractional rates of protein synthesis in bivalve molluscs. The blue mussel, *Mytilus edulis*, was chosen as a direct comparison for previous determinations of protein synthesis carried out using the stochastic end-point model and the stable isotope ^{15}N (*e.g.* Hawkins, 1985; Hawkins et al., 1986; Hawkins et al., 1989). The time-course data presented in Figs 1 and 2 fulfil the first three validation criteria for the technique (see Introduction) as following injection into the posterior adductor muscle, free pool phenylalanine-specific radioactivities in the gill, mantle and whole-animal were elevated within one hour of injection (criteria 1) and remained elevated and stable (criteria 2) for a further 5 h (Fig. 1). Subsequent incorporation of radiolabel into body protein was linear (Fig. 2; criteria 3) and the non-significant intercepts for the regression lines describing the time-course incorporation rates of radiolabel into gill, mantle and whole-animal protein suggest rapid equilibration within the body free amino acid pools and incorporation into body protein occurred rapidly after injection. The fourth validation criteria, that the swamping of the body free amino acid pools with phenylalanine does not affect rates of protein synthesis, has been examined in humans

and shown to be the case (e.g. McNurlan et al., 1979; Garlick et al., 1980; McNurlan et al., 1991; Garlick et al., 1994; but see Rennie et al., 1994). However, this validation criteria has been little studied in non-mammalian animals (Loughna and Goldspink, 1985) and it has been assumed that there is no stimulatory effect of the flooding dose on rates of protein synthesis (Houlihan et al., 1995a; Fraser and Rodgers, 2007). Clearly, further validation of this criteria is required for aquatic ectotherms but may be problematic due to differing time scales of tracer administration and incorporation using different methodologies.

The free pool phenylalanine-specific radioactivities in the gill, mantle and whole-animal were lower than the specific radioactivity of the injection solution (with mean values of 77%, 67% and 60%, respectively). However, these values are within the range of phenylalanine-specific radioactivities attained within tissue and whole-animal free pools for a range of taxa with the percentage similarity to the specific radioactivity of the injection dependent on method of administration. Injection directly into the circulating body fluid via a blood vessel or sinus has resulted in free pool phenylalanine-specific radioactivities that are usually > 80 % the value of the injection solution (e.g. Houlihan et al., 1990a, 1990b; Foster et al., 1991; McCarthy et al., 1994) compared to injection into a body cavity such as the coelom (73%, Fraser et al., 2004) or peritoneum (60%, McCarthy et al., 1999) or the uptake from bathing in radiolabel (74%, Houlihan et al., 1995c; 42%, McCarthy and Fuiman, 2008). It is unlikely that the lower specific radioactivities observed in the gill and mantle are the result of the injection volume remaining as a bolus in the adductor muscle and not distributing throughout the body within the 6 h incubation period because the whole-animal free pool phenylalanine-specific radioactivity also differs from that of the injection solution and therefore does not include any residual undistributed injection solution within the posterior adductor muscle. In addition, the non-significant intercepts for the time-course incorporation of radiolabel into body protein suggest rapid distribution throughout the body.

The lower tissue/whole-animal free pools reported here are most likely due to the combined effect of dilution of the injection solution by baseline phenylalanine in the free amino acid pools in the mussel and by leakage of radiolabel from the injection site following withdrawal of the needle. Fraser et al. (2004) report that these two factors combined resulted in a 27% reduction in the body wall free-pool phenylalanine-specific radioactivity in the Antarctic holothurian *Heterocucumis steini* following a flooding dose injection with dilution accounting for a 12.5% reduction and the remainder assumed lost by leakage.

The values obtained here for *M. edulis* at 15°C compare favourably with those obtained by Hawkins and co-workers using ^{15}N-labelled algae measuring the tracer flux through the animal by the appearance of ^{15}N in excreted ammonia and using a stochastic end-point model to determine absolute protein synthesis rates (Hawkins, 1985; Hawkins et al., 1986, 1989; Hawkins and Hilbish, 1992) (Fig. 4). Although the data set is limited, absolute rates of protein synthesis reported in the present study are of a magnitude expected for mussels of this size at 15°C (rate predicted from regression, 20.9 mg d^{-1}; observed rate 18.9 mg d^{-1}) providing confidence in the measurements obtained. Synthesis rate data are compared to shell length as body mass data are not comparable (wet mass data are presented in the present study and dry mass data presented by Hawkins and co-workers) Similar concordance between measurements using the flooding dose technique and the stochastic endpoint method has also been reported for fish (Carter et al., 1994; Houlihan et al., 1995b). However, further cross-calibration studies between methodologies in aquatic ectotherms are recommended.

In most studies of protein metabolism in aquatic ectotherms, rates of protein synthesis have been reported on a fractional basis, i.e. as a percentage of the protein mass synthesised per day. Tables 2 and 3 summarise the whole-animal and tissue fractional rates of protein synthesis published for molluscs. The molluscan dataset set is limited to 10 studies in total compared to the larger data sets available for crustaceans [15 studies cited in review by

326 Carter and Mente (2014) plus Rastrick and Whiteley (2013)] and for fishes [*ca.* 70 published
327 studies; Web of Science using Garlick et al. (1980) as a cited reference, search conducted
328 22/7/15]. The molluscan data focuses primarily on whole-animal rates in the Antarctic limpet
329 *Nacella concinnus*, on whole-animal and tissue rates in the dumpling squid *Euprymna*
330 *tasmanicus* together with the whole-animal data for *Mytilus edulis* by Hawkins and co-
331 workers (Tables 2 and 3). The whole-animal data presented in Table 2 can be described by
332 the following equation: $Log_{10}k_s = 0.044T - 0.306$ ($r^2 = 0.821$, n = 16, $p < 0.0001$), where k_s is
333 the fractional rate of protein synthesis measured using the flooding dose technique and T is
334 water temperature (°C). This predicts a fractional rate of protein synthesis of 2.26 % d^{-1} at
335 15°C which is close to the measured value (2.56 % d^{-1}), indicating that the rate measured in
336 the present study is of the magnitude expected (although factors such as size and feeding
337 history have not been accounted for in this estimate). One of the advantages of the flooding
338 dose technique is that it enables rates of protein synthesis to be measured in tissues and
339 organs as well as in the whole-animal. Tissue rates of protein synthesis are important because
340 they can indicate specific responses to environmental change that may be masked in the
341 whole-animal (Fraser and Rogers, 2007). The available data on tissue-specific fractional rates
342 of protein synthesis in molluscs are limited to 5 studies (Table 3) but in comparison to the
343 rates obtained in other aquatic ectotherms, the data show the same patterns in the ranking of
344 the relative rates obtained for different tissues. Fractional rates of protein synthesis are higher
345 in metabolically-active tissues such as the liver/hepatopancreas, gonads, gill and gut organs
346 compared to muscle tissue [Table 3; crustaceans (*Carcinus maenas*, Houlihan et al., 1990a;
347 *Nephrops norvegicus*, Mente et al., 2011); fish (*Oncorhynchus mykiss*, Houlihan et al., 1986,
348 Foster et al., 1991; *Salmo salar* Martin et al., 1993)], usually associated with high tissue
349 capacities for protein synthesis and RNA activity (Table 1; Houlihan et al., 1990a, 1990b;
350 Foster et al., 1991; Carter et al., 2009). However, the exception appears to be mantle tissue in

young, fast-growing cephalopods (Table 3) where as part of their r-selected life histories ('living life in the fast lane', Carter et al., 2009), growth is rapid in order to reach maturity and spawn within their short lifespans (usually < 1 year). Metabolically-active tissues such as the gills are likely to have higher rates of protein synthesis to allow for protein remodelling and flexibility in enzyme production in keeping with the function of the gill as a specialised site for both gas and ion exchange (Lyndon and Houlihan, 1998).

In summary, the results of the present study validate the use of the flooding dose technique to measure whole-animal and tissue-specific rates of protein synthesis in the blue mussel *Mytilus edulis*. The use of this technique in measuring one of the major components of maintenance metabolism and growth will provide a valuable tool in furthering our understanding of the intriguing relationship between protein synthesis rates, growth rates and energy expenditure previously suggested by Hawkins and Day (1999) for this species. In addition, the comparative study of rates of protein turnover in a range of aquatic ectotherm species within the 'fast-slow pace of life' continuum (Ricklefs and Wikelski, 2002) will provide valuable insight into the physiological costs and energetic trade-offs associated with fast growth/early maturation versus slow growth/delayed maturation (e.g. cephalopods versus long-lived fish species). Indeed, validation of the flooding dose technique for bivalve molluscs will allow protein turnover rates to be studied in a range of bivalve molluscs where lifespan can vary from less than 5 years (e.g. *Cerastoderma edule*, Malham et al., 2012) to decades (e.g. *Glycymeris glycymeris*, Brocas et al., 2013) or hundreds of years (e.g. *Arctica islandica*, Butler et al., 2013) to determine the contribution of changing rates of protein turnover to the ageing process and senescence. Finally, the study of tissue-specific and whole-animal rates of protein synthesis will also provide understanding on the ability of this keystone marine invertebrate to adjust and respond to environmental fluctuations, such as that expected as a result of climate change.

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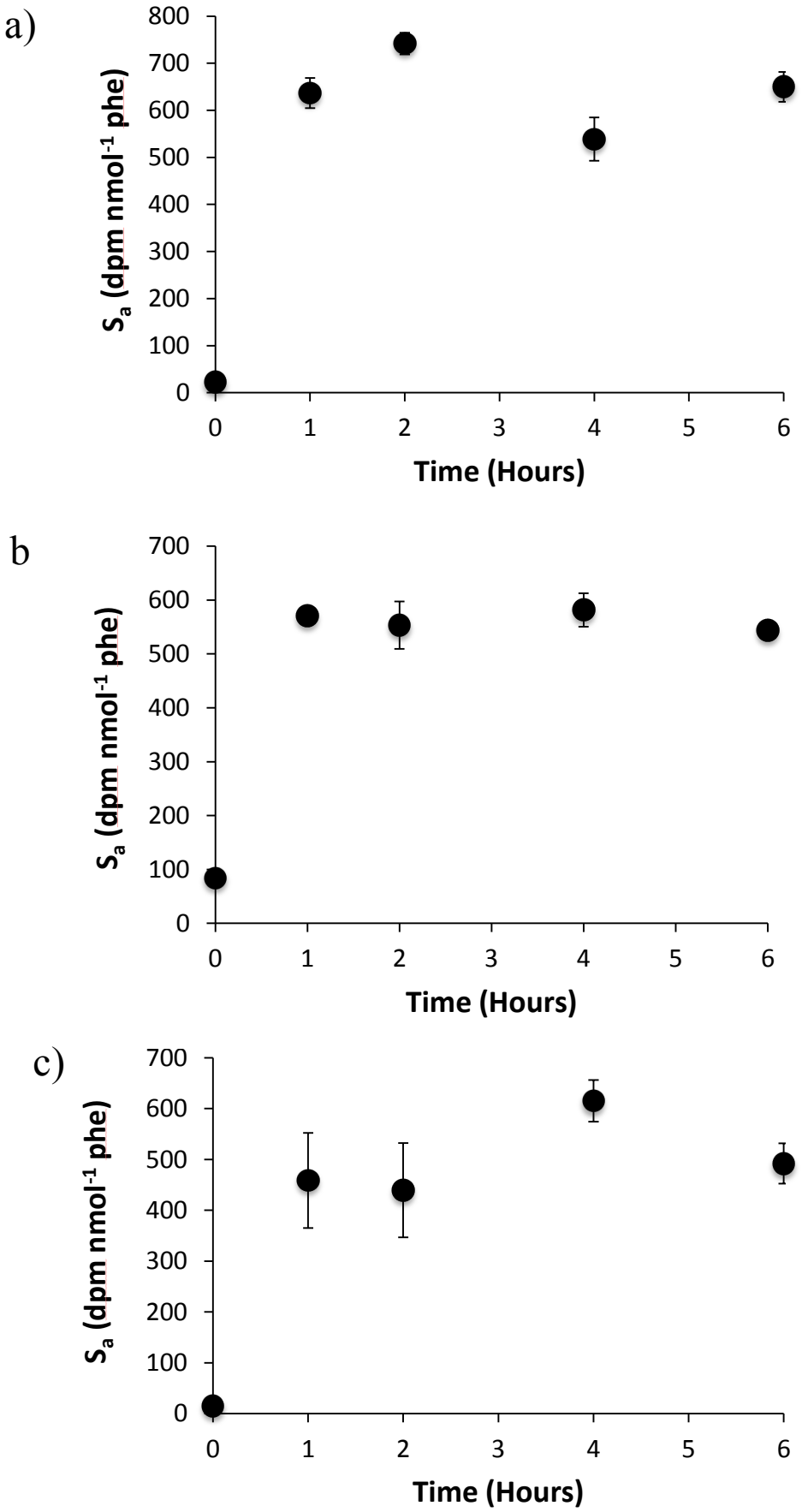
Figure Legends

Fig. 1. Intracellular free pool phenylalanine-specific radioactivity (S_a, dpm $nmol^{-1}$ phe) in (a) gill, (b) mantle and (c) whole-animal tissue of blue mussel *Mytilus edulis* (shell length 61.0 mm ± 0.8 mm; fresh body mass 4.00 ± 0.22 g) after 1–6 h incubation following a single flooding dose injection of a solution containing 135 mM phenylalanine/L-[2,6-^{3}H]-phenylalanine (3.6 MBq ml^{-1}) into the posterior adductor muscle. S_a values for uninjected control mussels are plotted at time zero. Data are presented as mean values ± SEM (n = 5).

Fig. 2. Protein-bound phenylalanine-specific radioactivity (S_b, dpm $nmol^{-1}$ phe) in (a) gill, (b) mantle and (c) whole-animal tissue of blue mussel *Mytilus edulis* (shell length, 61.0 mm ± 0.8 mm; fresh body mass 4.00 ± 0.22 g) after 1–6 h incubation following a single flooding dose injection of a solution containing 135 mM phenylalanine/L-[2,6-^{3}H]-phenylalanine (3.6 MBq ml^{-1}) into the posterior adductor muscle. S_b values for uninjected control mussels are plotted at time zero. Data are presented as mean values ± SEM (n = 5).

Fig. 3. Fractional rates of protein synthesis (k_s, % d^{-1}) in the blue mussel *Mytilus edulis* (shell length, 61.0 mm ± 0.8 mm; fresh body mass 4.00 ± 0.22 g) after 1–6 h incubation following a single flooding dose injection of a solution containing 135 mM phenylalanine/L-[2,6-^{3}H]-phenylalanine (3.6 MBq ml^{-1}). Data (mean ± SEM, n = 5) are presented for (a) gill, (b) mantle and (c) whole-animal.

Fig. 4. Absolute rates of protein synthesis (A_s, mg Protein day^{-1}) for *Mytilus edulis* at 15°C. Data are taken from the present study (open circle) and from Hawkins et al. (1986), Hawkins et al. (1989) and Hawkins and Hilbish (1992). Data from Hawkins and co-workers have been corrected back to their original rates from the mass-corrected data reported based on dry mass data presented in these papers.


a)
800
700
600
500
400
300
200
100
0
S_a (dpm nmol^{-1} phe)
0 1 2 3 4 5 6
Time (Hours)
b
700
600
500
400
300
200
100
0
S_a (dpm nmol^{-1} phe)
0 1 2 3 4 5 6
Time (Hours)
c)
700
600
500
400
300
200
100
0
S_a (dpm nmol^{-1} phe)
0 1 2 3 4 5 6
Time (Hours)

593

Fig. 1

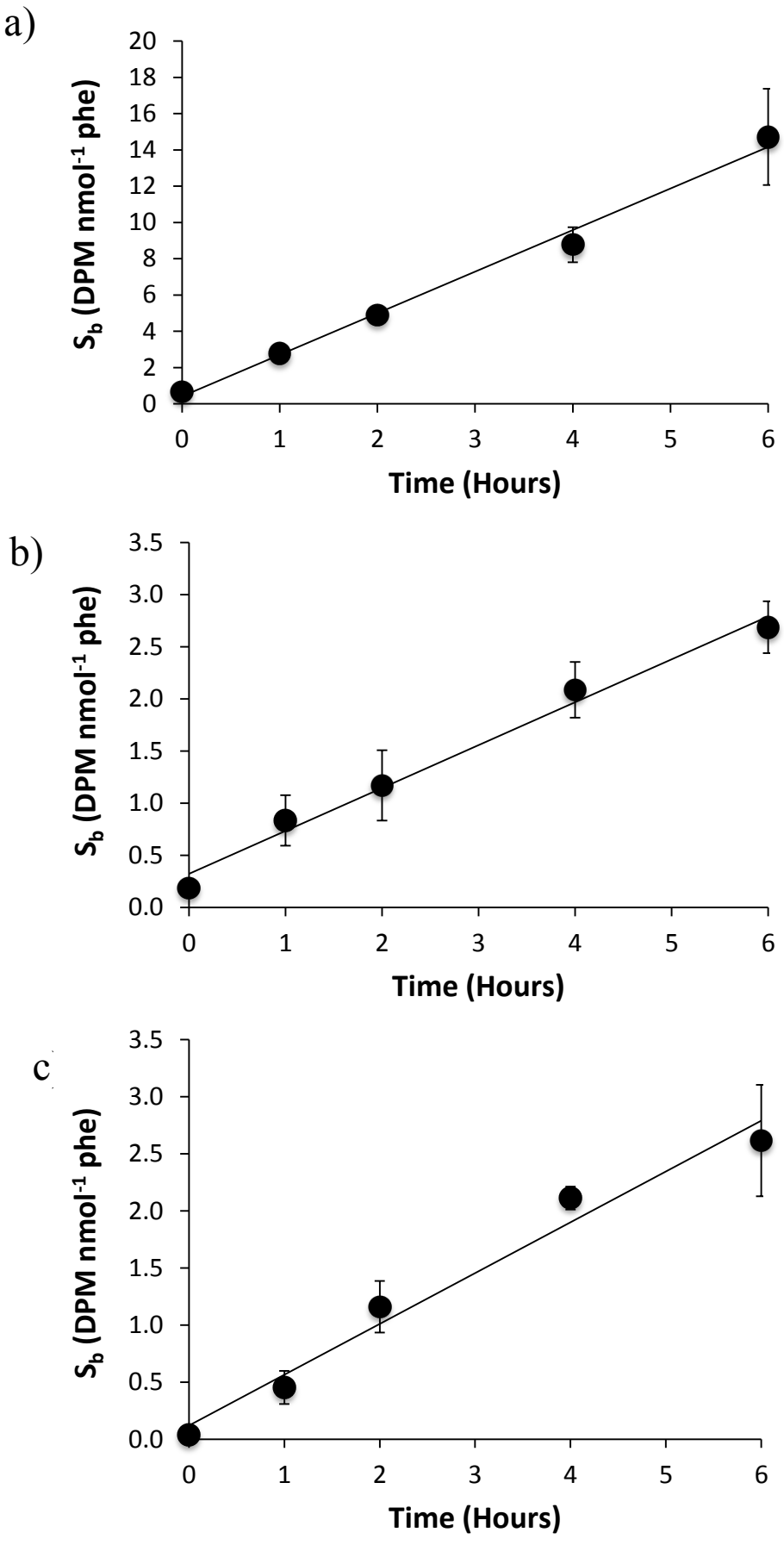
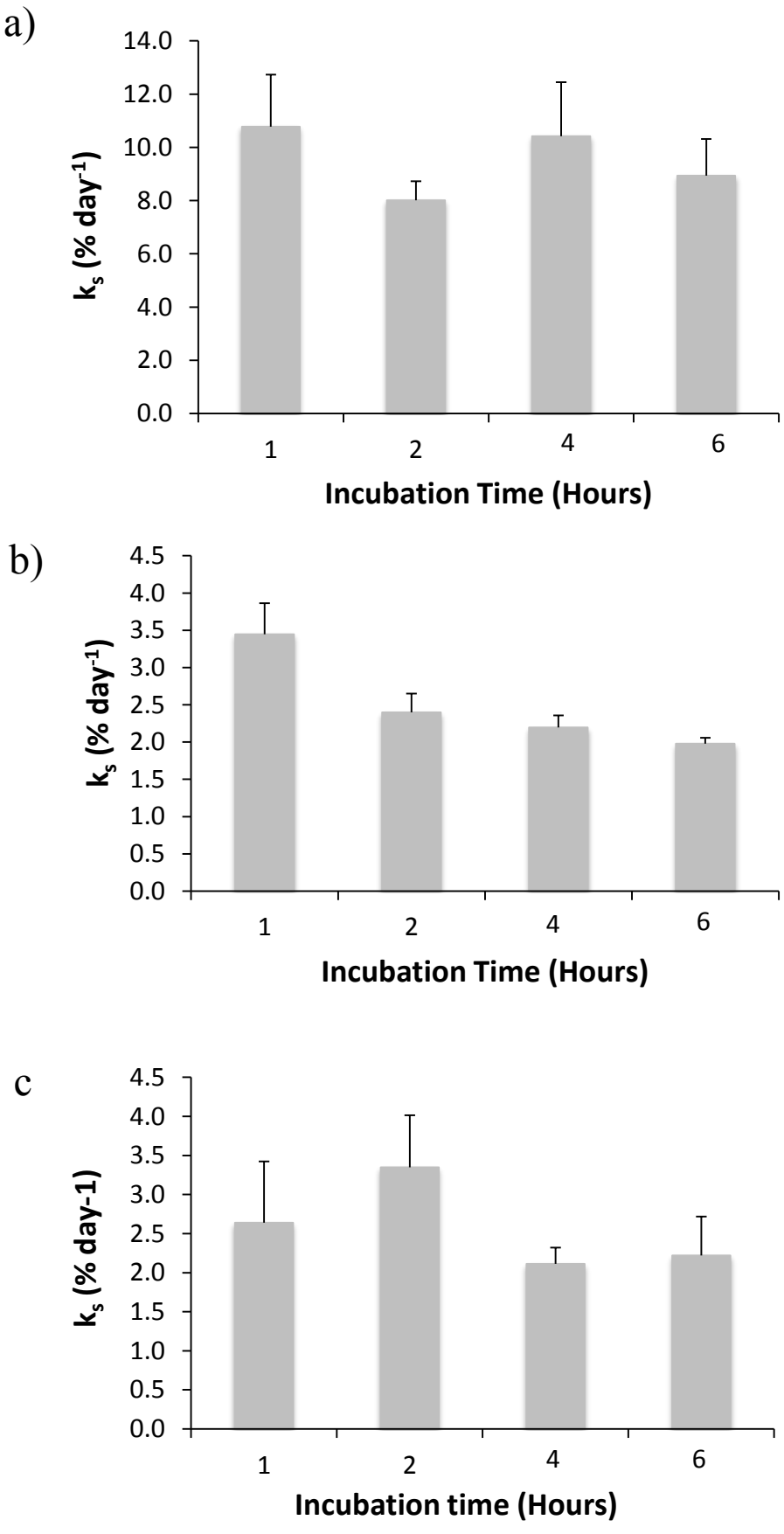

a)
S_b (DPM nmol^{-1} phe)
Time (Hours)
b)
S_b (DPM nmol^{-1} phe)
Time (Hours)
c
S_b (DPM nmol^{-1} phe)
Time (Hours)

Fig. 2


a)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
k_s (% day^{-1})
1
2
4
6
Incubation Time (Hours)
b)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
k_s (% day^{-1})
1
2
4
6
Incubation Time (Hours)
c
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
k_s (% day-1)
1
2
4
6
Incubation time (Hours)

595 Fig. 3

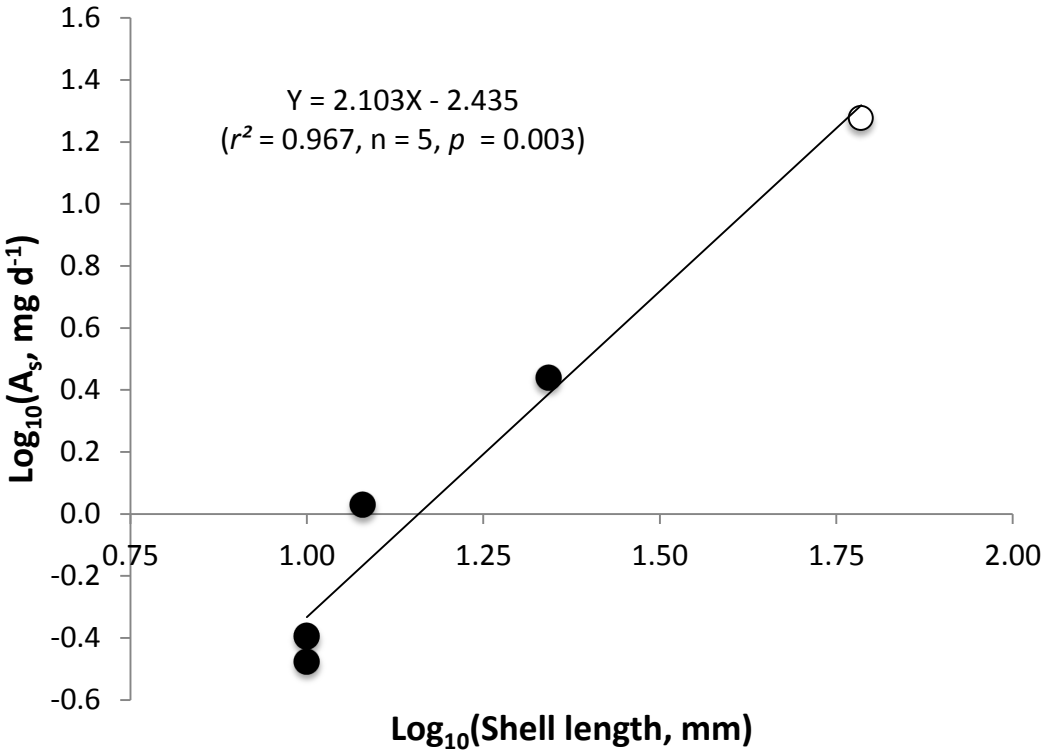

Y = 2.103X - 2.435
(r^2 = 0.967, n = 5, p = 0.003)
$Log_{10}(A_s, mg\ d^{-1})$
Log_{10}(Shell length, mm)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
0.75
1.00
1.25
1.50
1.75
2.00

Fig. 4

Table 1.

Protein metabolism in the gill, mantle and whole-animal tissue of the blue mussel *Mytilus edulis* (shell length, 61.0 mm ± 0.8 mm; fresh mass 4.00 ± 0.22 g, 15°C).

	Gill	Mantle	Whole-animal
k_s (% d^{-1})	9.54 ± 0.78	2.51 ± 0.30[a]	2.58 ± 0.29[a]
C_s (µg RNA mg^{-1} protein)	50.81 ± 1.88	40.44 ± 2.62	32.69 ± 1.39
k_{RNA} (mg protein mg^{-1} RNA d^{-1})	1.98 ± 0.15	0.62 ± 0.07[a]	0.83 ± 0.11[a]

Mean ± SEM (n=20).

k_s fractional rate of protein synthesis, C_s capacity for protein synthesis; k_{RNA} RNA activity.

Same letter, along a row, indicate no significant difference following one-way ANOVA and Tukey HSD pairwise comparisons.

624 **Table 2.** Summary of whole-animal fractional rates of protein synthesis (k_s, % d^{-1}) for
625 Phylum Mollusca.

Species	Tracer	Size	T°C	k_s (% d^{-1})	Reference
Bivalvia					
Mytilus edulis	^{3}H	61 mm S_L 4.00 g M_W	15°C	2.58	This study
	^{15}N	10 mm S_L 10 mg M_D	15°C	5.4	Hawkins et al. (1989)
	^{15}N	45-57 mm S_L 523 mg M_D	9°C 13°C 13°C	0.29 0.58 0.53	Hawkins (1985)
Gastropoda					
Nacella concinna	^{3}H	25.2 mm S_L 2.05 g M_W	0.58°C -0.36°C -1.05°C -1.35°C	0.40 0.56 0.35 0.27	Fraser et al. (2002)
	^{3}H	25.7 mm S_L 1.98 g M_W	-0.47°C	0.80	Fraser et al. (2007)
		27.7 mm S_L 2.24 g M_W	-1.62°C	0.55	
	^{3}H	23.9 mm S_L 1.50 g M_W	-1.5°C	0.50	Fraser et al. (2007)
		24.6 mm S_L 1.59 g M_W	1.0°C	0.85	
		24.3 mm S_L 1.55 g M_W	3.5°C	0.50	
		23.5 mm S_L 1.21 g M_W	6.0°C	0.55	
Cephalopoda					
Octopus vulgaris	^{3}H	199 g M_W	22.0°C	3.8	Houlihan et al. (1990b)
Euprymna tasmanica	^{3}H	2.8 g M_W	18.0°C	9.45	Carter et al. (2009)
		14.8 g M_W		1.49	
	^{3}H	60 d old 0.92 g M_W	20.0°C	4.78	Moltschaniwskyj and Carter (2010)
		100 d old 3.72 g M_W		3.77	
		140 d old 6.54 g M_W		3.10	

626 T°C - Temperature; S_L – shell length M_W – wet mass; M_D – dry mass

627 **Table 3.** Summary of fractional rates of protein synthesis (k_s, % d^{-1}) in various body tissues
628 for Phylum Mollusca.

Species	Tracer	Size	Tissue	k_s (% d^{-1})	T°C & Reference
Bivalvia					
Mytilus edulis	^{3}H	61 mm S_L	Gill	9.54	15°C
		4.00 g M_W	Mantle	2.51	This study
Gastropoda					
Helix aspersa	^{3}H	Awake	Hepato[1]	3.3	22 - 25°C
		4.2 g M_W	Foot[2]	2.6	Pakay et al. (2002)
		Aestivate	Hepato[1]	0.8	
		3.8 g M_W	Foot[2]	1.4	
Octopus vulgaris	^{3}H	14.8 g M_W	Arm	2.92	22°C
			Arm Tip	3.10	Houlihan et al.
			Brain	3.64	(1990b)
			Branchial heart	3.19	
			Gill	2.96	
			Mantle	2.33	
			Renal Appendage	2.36	
			Stomach	2.94	
Euprymna tasmanica	^{3}H	2.8 g M_W	Arms	5.37	18°C
			Digestive Gland	11.31	Carter et al. (2009)
			Anterior Mantle	6.13	
			Posterior Mantle	6.86	
		14.8 g M_W	Arms	1.43	20°C
			Digestive Gland	9.24	Carter et al. (2009)
			Anterior Mantle	0.56	
			Posterior Mantle	0.36	
	^{3}H	Immature	Gonad*	9.67	18°C
		3.33 g M_W	Mantle	4.56	Moltschaniwskyj and Carter (2013)
		Mature	Gonad	12.23	18°C
		5.58 g M_W	Mantle	1.67	Moltschaniwskyj and Carter (2013)

629 S_L – shell length; M_W – wet mass; M_D – dry mass.
630 [1] = Hepatopancreas; [2] = Foot muscle; * estimated from Figure 2a in Moltschaniwskyj and
631 Carter (2013)