Protein Synthesis, Degradation, and Retention: Mechanisms of Indeterminate Growth in Cephalopods

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ABSTRACT

This study is the first to examine the underlying process of growth in a cephalopod, the southern dumpling squid (*Euprymna tasmanica*), to ascertain the mechanism by which indeterminate growth is achieved in this live-fast, die-young group of animals. This is the first study to estimate rates of protein synthesis and growth of squid from 7 to 140 d of age, providing an understanding of both the pattern and the process of growth throughout the lifetime of a squid species. Younger and smaller individuals had greater rates of protein synthesis and protein synthesis retention efficiency, as well as more RNA, than did older and larger individuals. Variation in growth rates among older, larger individuals was a function of individuals with faster growth rates having greater protein synthesis retention efficiency and also greater concentrations of protein. Critically, growth did not cease in the adults and, with an average of 10% of protein synthesized being retained, the mechanism to support the nonasymptotic growth model of cephalopods is provided.

Introduction

Growth is the most basic of all biological functions, and estimates of growth rates are critical to our understanding of many facets of population biology and ecology, including the evolution of life-history characteristics, exploitation, conservation biology, and aquaculture. Understanding the basic mechanisms of growth allows us to predict and recognize how growth is altered by factors over which we may or may not have control, including diet and temperature. Most marine organisms have a lifetime growth pattern that is indeterminate and, when food is abundant, can be described by the von Bertalanffy growth curve (von Bertalanffy 1938). Critical elements of the von Bertalanffy growth curve are a slowing of growth and the plateau in size during the adult phase, with an increase in body size being postulated to be linked to slowing metabolic rates and limits to oxygen uptake associated with body size (von Bertalanffy 1957). Estimates of basic demographic information about cephalopods, particularly patterns of lifetime growth rates, have been highly controversial in the literature (Pauly 1998; Moltschaniwskyj 2004). The debate began when squid age estimates that were made using statoliths identified a range of growth curves fitted to size-at-age data, many of which did not have an asymptotic growth phase (Jackson and Choat 1992). In the oceanic squid species, in which a short asymptotic phase is evident, this occurs during the final phase of life. However, the absence of (or a much reduced) asymptotic growth phase (Jackson and Choat 1992) suggests distinct differences in the processes of growth and their relationship with size and age.

Given what we understand to date about cephalopods, and about squid in particular, it is unrealistic to expect this group of invertebrates to be physiologically similar to fish (Packard 1972; O'Dor and Webber 1986). Despite this, it has been suggested that it would be easier to generate models of growth that pretend that they are (Pauly 1998); however, the metabolic limitations of growth and the process of growth may not be the same for cephalopods and teleost fish. There is evidence that the relationship of metabolic rate with body size in cephalopods scales with a coefficient that is faster than that recorded in teleosts (e.g., Katesanevakis et al. 2005). Given that teleosts do show relationships between size and rates of protein synthesis and metabolic rates, it is suggested that central to the debate over the pattern of lifetime growth in cephalopods is a lack of understanding about the physiological processes responsible for growth.

Support for nonasymptotic growth by many cephalopod species is shown by continued production of muscle fibers in adults (Moltschaniwskyj 1994; Pecl and Moltschaniwskyj 1997; Martinez and Moltschaniwskyj 1999; Ho et al. 2004). Cutaneous respiration is possible in both the inner and the outer surfaces of the body that are continually exposed to seawater (Maden and Wells 1996), and this reduces the limitation of oxygen uptake associated with increasing mass to surface area that is observed in most aquatic organisms (Graham 2006). In addition, cephalopods appear to have adopted an extended-adolescent strategy (Rodhouse 1998), with a relatively short reproductive period and with reproduction fuelled directly

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from ingested food rather than from stored energy reserves (Moltschaniwskyj 2004). Evidence in *Euprymna tasmanica* of a response in protein metabolism due to mass (Carter et al. 2009) provides us with the means to examine the nature of the response across the lifetime of individuals. There is little doubt that fully understanding the observed growth patterns will be achieved by directly measuring the changes in the rates of protein synthesis and accretion throughout a squid's lifetime, as has been constructed for teleost fish (Houlihan et al. 1995*a*).

Protein, which is the major component of most animals, is expensive to produce, accounting for between 20% and 40% of total energy expenditure (Houlihan 1991). Basal metabolism is thought to be a reflection of rates of protein synthesis (Houlihan et al. 1986). Protein growth, the increase in mass with time, is a function of two underlying processes: the rate at which protein is synthesized and the rate at which protein is degraded. Synthesized protein that is not degraded is accreted as growth; the proportion of synthesized protein retained for growth is a measure of the protein synthesis retention efficiency. Patterns in the of protein synthesis retention efficiency (i.e., synthesized protein used for growth) during the lifetime of an individual will relate to their pattern of growth. For most marine species, growth is asymptotic (e.g., described by the von Bertalanffy [1938] and Gompertz [1825] models), and we would expect the protein synthesis retention efficiency to decline during the lifetime of an individual and approach zero due to slowing rates of protein synthesis relative to rates of protein degradation. There is evidence that protein turnover in fish is related to body size, with rates of both protein synthesis and degradation slowing with increasing body size (Houlihan 1991). We hypothesize that cephalopods with nonasymptotic growth (exponential, linear, power, or logarithmic growth patterns) should be characterized by protein synthesis retention efficiencies that do not approach zero. Furthermore, rates of protein synthesis are such that they allow for protein degradation but there is sufficient protein synthesized to allow significant protein accretion to occur throughout an individual's lifetime, including in the older and larger individuals in a population. Finally given the metabolic cost of protein synthesis, we expect to see the rate of protein synthesis decrease with body size at a slower rate than has been recorded in teleost fish (Houlihan et al. 1986) and mammals (Makrides 1983).

Most biological conversion processes scale allometrically with body mass at rates similar or identical to mass-specific metabolic rates. The mass exponent for protein synthesis is similar to that for the mass exponent of metabolism for rainbow trout (Houlihan et al. 1986), and it is assumed to also occur in marine ectotherms; however, this has not been explored in detail. In teleosts, RNA : protein has been used as a correlate of both protein synthesis (Houlihan et al. 1989; Carter et al. 1993*b*) and growth (Houlihan et al. 1995*b*; see review in Carter and Houlihan 2001). In seeking predictors of past and current growth rates, a number of cephalopod studies have explored the use of biochemical measures, for example, protein concentration, RNA, and DNA. However, in cephalopods these are not always correlated with growth (Moltschaniwskyj 2004), and when they are it is often related to only recent growth (Clarke et al. 1989). Highly variable protein concentrations among individuals has led to the suggestion that protein concentration may be a better predictor of recent somatic growth rates (Pierce et al. 1999). By directly assessing the relationships of RNA and protein content with protein synthesis, we will obtain a better understanding of the use of biochemical correlates of growth in cephalopods and the capacity to assess the mass-specific rates of biological conversions in ectotherms.

The aim of this study was to quantify, for the first time, the fractional rates of protein synthesis and accretion throughout the lifetime of a species with indeterminate nonasymptotic growth, so as to determine the underlying processes of growth. This has rarely been done for any species, with perhaps the rat as the only other example (Waterlow 2006). The temperate dumpling squid *E. tasmanica* (Pfeffer, 1884) was chosen for this study for several critical reasons. It is a small (<10-g), solitary, benthic species. As such, individuals can be held individually in captivity without experiencing stress associated with the removal of social structure and with confinement. It is easy to handle, allowing individuals to be used in experiments, and like many small cephalopods, it has a life span of 4–5 mo (Jackson 1989; Tracey et al. 2003), allowing laboratory-reared individuals to be held for most of their lifetime in the duration of an experiment. Finally, in vivo quantification of rates of protein synthesis requires the use of a validated and reliable method. This is possible through the application of a technique developed by Garlick et al. (1980), which uses a single large-dose injection of radioactive phenylalanine. This technique has been applied and successfully validated for a range of species, including many marine fish (e.g., Houlihan et al. 1988; Carter et al. 1993*a*, 1993*b*, 1998; Katersky and Carter 2007) and, more critically, three cephalopod species, including *E. tasmanica* (Houlihan et al. 1998, 1990; Carter et al. 2009). Injection is possible only in individuals that are large and robust enough to be handled; therefore, an alternative method of introducing the radioactive phenylalanine into the tissues of very small marine animals is through bathing them in a solution of the material (Houlihan et al. 1995*a*). Bathing has not been attempted for newly hatched cephalopods; as such, this study represents the first application (and subsequent validation) of the use of bathing to introduce radioactive phenylalanine into the tissues of very small cephalopods. This is also the first study to estimate rates of protein synthesis and growth of an ectotherm throughout most of its lifetime, providing an understanding of both the lifetime patterns and the processes of growth.

Material and Methods

Experimental Design

Egg masses were obtained from three female *Euprymna tasmanica* individuals caught off the northern coast of Tasmania. The wild females, males, egg masses, and all individuals grown during the experiment were held in individual tanks within a 1,000-L recirculating seawater system maintained at 18°C. Females were fed mysid shrimps (*Tasmanomysis oculata*, *Paramesopodopsis rufa*, *Tenagomysis* spp.) ad lib. and mated with a

number of males until egg masses were deposited in the tank. As juveniles hatched, they were removed from the tank with the egg mass and transferred to individual containers, where they were provided with on-grown *Artemia* (boosted with SuperSelco) until they were 5 d old, at which point mysid shrimps were provided (all prey items were provided ad lib.). The 45 individuals used in the experiment were kept in isolation for their entire lifetimes. This ensured that when the larger, older animals became reproductively mature and produced gametes, these gametes were not released during the experiment.

To measure the rate of change in protein synthesis and accretion across the range of ages of dumpling squid, estimates of protein synthesis, protein accretion, and proximal analysis were obtained by terminally sampling individuals at the following ages: 7, 12, 21, 28, 44, 58, 72, 92, 98, 105, 111, 119, 126, 133, and 140 d. For individuals <28 d old, two groups of four to 10 individuals were pooled to provide estimates of protein synthesis at each age. By the time individuals had reached 28 d of age and were >0.1 g, they were large enough to be sampled as single individuals. Two individuals were sampled at each age.

Estimating Fractional Rates of Protein Synthesis

For individuals >28 d old, total-body protein synthesis rates in each age group was determined by a single injection of L-(2,6-^{3}H)phenylalanine (Amersham Pharmacia Biotech, Rydalmere, New South Wales) at the base of the arms, using the flooding-dose method (Garlick et al. 1980; Carter et al. 2009). Each individual was weighed live immediately before the injection to calculate dosage (1.8 mL/100 g). Following this, each individual was injected with a solution of 150 mM L-phenylalanine and L-(2,6-^{3}H)phenylalanine (Amersham Pharmacia Biotech) in 0.2 μm of filtered seawater at pH 7.4. (Carter et al. 2009) and was immediately returned to aerated seawater at 20°C for 60 min, which is sufficient time for incorporation of L-(2,6-^{3}H)phenylalanine into the tissues (Carter et al. 2009). Individuals younger than 28 d of age weighed <0.1 g and were too small to be injected, and so they were bathed in seawater buffered to pH 8 that contained ^{3}H-phenylalanine at a concentration of 72 kBq per mL seawater (Houlihan et al. 1995*a*). For individuals aged 7, 14, 21, and 28 d, duplicate groups of 10, 10, 4, and 4 individuals, respectively, were bathed for between 125 and 144 min based on the bathing trial. In the bathing trial, groups of eight animals were bathed for 60, 120, and 180 min, and analysis showed that free pools remained stable and there was linear incorporation of ^{3}H-phenylalanine into protein. Validation of bathing is presented for the 7-, 14-, 21-, and 28-d-old animals used in this experiment (but not the bathing trial).

All individuals were killed by decapitation, weighed, and stored in liquid nitrogen. Weighed frozen tissue was homogenized in 2% perchloric acid. In all samples, the phenylalanine-specific radioactivity of the free pool, the free-pool phenylalanine concentration, and protein-bound phenylalanine-specific radioactivity were determined as described elsewhere (Houlihan et al. 1986, 1988; Carter et al. 1993*a*). Total-body fractional rates of protein synthesis (k_s, in percent per d) were calculated as $k_s = 100[(S_b/S_a)(1{,}440/t_1)]$, where S_b is the protein-bound phenylalanine-specific radioactivity at time t_1 (min) and S_a is the free-pool phenylalanine-specific radioactivity (Garlick et al. 1980). The method was validated previously for injected squid (Carter et al. 2009) and validated for the bathed animals used in this experiment: over the time course, incorporation was linear ($S_b = 2.75 \times t + 1{,}369$; $n = 8$, $r = 0.91$, $P < 0.001$) and free pools remained elevated ($n = 8$, $r = 0.41$, $P > 0.05$). Protein concentration was determined using a modification of the folin-phenol method (Lowry et al. 1951), and RNA concentration was estimated using dual-wavelength absorbance with an internal RNA standard, with blanks to confirm that the protocol was working (Ashford and Pain 1986). Fractional rates of protein degradation (k_d, in percent per d) were calculated as $k_d = k_s - k_g$ (Millward et al. 1975). RNA was expressed as both the capacity for protein synthesis (C_s, in mg RNA per g protein) and RNA activity (k_{RNA}, as k_s per g RNA per d; Sugden and Fuller 1991).

Estimating Fractional Rates of Protein Accretion

Whole-body protein accretion rates (k_g, in percent per d) were calculated from the following equation (Ricker 1979; Forsythe and Van Heukelem 1987):

$$\text{protein accretion rate} = \frac{\log W_2 - \log W_1}{t} \times 100,$$

where W_1 is the initial whole-body protein mass (g), W_2 is the final whole-body protein mass (g), and t is the length of the growing period (d). Whole-body protein content was determined for W_2 directly from each live mass and the protein concentration of the whole body of each individual (mg protein per g fresh weight). W_1 was the whole-body protein mass of the individual 7–14 d before death; this was estimated from total wet mass at time 1 and the protein concentration at time 2. The total wet mass of individuals was determined using digital pictures of the dorsal view of the individual in the tank. A relationship between dorsal surface area (digitized from the photograph) and the total mass was determined for known-mass individuals (total wet mass $= 0.03 \times \text{surface area}^2 + 0.84 \times \text{surface area}$, $r^2 = 0.95$, over a mass range of 0.79–8.01 g). Calculating the protein content at W_1 assumed no change in tissue-protein concentration over the previous 7–14-d period. Although there was evidence of a significant relationship between body mass and protein content (see "Results"), the increase of 0.04% protein per 1 g in body mass per d was not considered to be large enough to affect this assumption, and so the final protein content was not adjusted further.

Data Analysis

Linear relationships among variables were analyzed and described using least squares linear regression ($n = 25$). For re-

lationships with body mass as the independent variable, allometric relationships (log-log) were provided to allow the mass exponents to be estimated. For all other relationships, the form of the fitted relationship was determined by examining the pattern of residuals around the fitted line. Because age cannot be determined in wild *E. tasmanica* through hard-structure analysis, a multiple regression was used to determine whether biochemical variables and body size could be used as predictors of recent growth rates for individuals in wild populations. The multiple regression used a stepwise function for simultaneous addition and removal of explanatory variables, with a probability of $F = 0.05$ for entry of variables and $F = 0.10$ for removal of variables.

Results

Validation of Bathing to Estimate Protein Synthesis

For individuals <0.1 g (younger than 28 d) that were bathed in seawater containing ^{3}H-phenylalanine, free-pool ^{3}H-phenylalanine in the whole body remained stable; there was no significant linear relationship between specific activity of free-pool phenylalanine (S_a, in dpm/nmol) and time (t, in min; $S_a = 2.75 \times t + 1{,}369$; $n = 8$, $R^2 = 0.17$, $P > 0.05$). Incorporation of labeled phenylalanine into proteins was linear over time; specific activity of protein-bound phenylalanine (S_b) increased over time as described by $S_b = 0.239 \times t - 19.70$ ($n = 8$, $R^2 = 0.83$, $P > 0.001$).

Size at Age

No single model could be generated to fit the size-at-age data across all individuals. Both the von Bertalanffy and the Gompertz models were used to fit a single growth model; however, both models failed to adequately fit the data (Fig. 1*a*). Given that there was a substantial change in the fractional rates of protein synthesis, accretion, and protein synthesis retention efficiency when the individuals were approximately 44 d old, size-at-age relationships were generated separately for individuals ≤44 and >44 d of age. Individuals ≤44 d old grew exponentially (Fig. 1*b*), while older individuals (>44 d old) displayed linear growth (Fig. 1*c*). The change in growth pattern occurred approximately 40 d before gender of individuals could be identified on the basis of gonad identification (Fig. 1).

While growth was exponential (in smaller and younger individuals), fractional rates of protein synthesis (k_s) were on average 1.4% faster than fractional rates of protein degradation (k_d; paired-sample t-test, $t = 4.38$, df = 26, $P < 0.001$). This pattern was still evident for individuals >44 d old when changes in size at age were linear, but k_s in this case was on average 0.4% faster than k_d (paired-sample t-test, $t = 3.44$, df = 18, $P = 0.003$).

Given the relationships of RNA activity, RNA content, and protein content with age and size, these variables were used to generate a predictive relationship for age to be used in wild animals that cannot be aged using hard structures. In a multiple regression, RNA and protein content were strong predictors of age (age = 4.64(SEM 0.39) + 0.03(0.01) × protein concentration − 1.24(0.09) × RNA concentration, $r^2 = 0.90$, $F = 212.96$, df = 2, 24, $P < 0.001$).

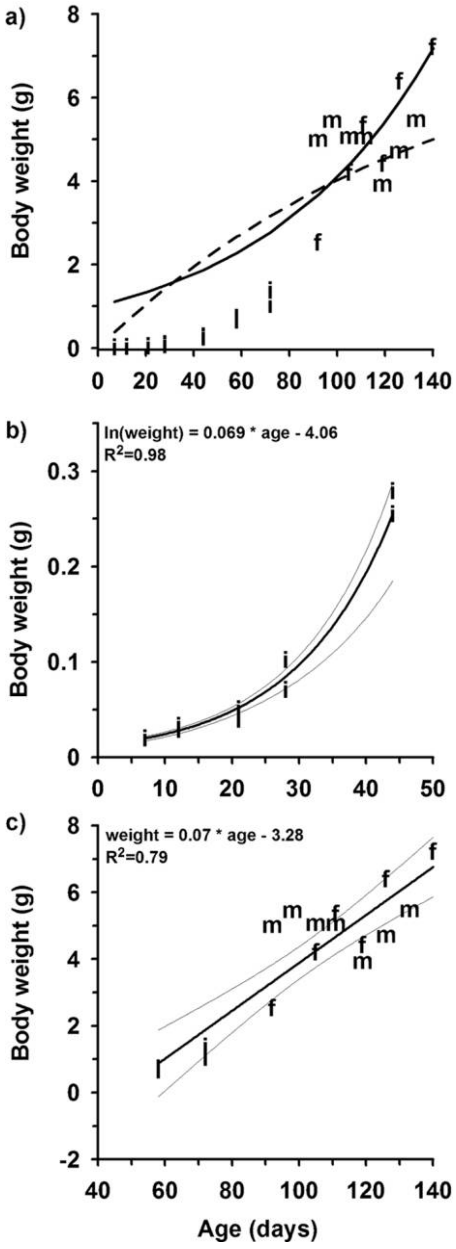

Figure 1. Relationship between total body mass and age for all individuals with a von Bertalanffy (*dashed line*) and a Gompertz (*solid line*) fit (*a*), individuals ≤44 d old with an exponential fit (*b*), and individuals >44 d old with a linear relationship (*c*). Fitted lines with 95% CIs are shown, with the parameter estimates provided in the equations. i = immature, f = female, m = male.

Changes in Fractional Rates of Protein Synthesis, Degradation, Accretion, and Specific Growth Rate with Size and Age

Significant negative mass allometric relationships were evident for fractional rates of protein synthesis (k_s), protein degradation

(k_d), and protein accretion (k_g; Fig. 2*a*–2*c*). The allometric relationship was strongest for k_s, which was faster in the smaller hatchlings before quickly slowing as animals became larger (Fig. 2*a*). The mass allometric relationship for fractional rates of both protein accretion (k_g) and degradation (k_d) were weak, and there was little evidence that these rates were a function of body mass (Fig. 2*b*, 2*c*). Both k_s and k_g had negative logarithmic relationships with age (Fig. 2*d*, 2*e*); younger individuals had fractional rates that were two and four times faster for k_s and k_g, respectively, than those of older individuals (>44 d of age). In the case of k_g, despite the significant relationship, it appears that after 44 d, k_g is independent of age (Fig. 2*e*). The fractional rate of protein degradation was weakly linearly related with age and, similar to body mass, the rate of change (i.e., the slope of the lines) is very slow (Fig. 2*f*), suggesting that although k_d declined as these animals got older, the difference in the rate between small/young individuals and large/older individuals was not as dramatic as it was for k_s and k_g.

The protein synthesis retention efficiency (k_g/k_s) was weakly negatively allometrically related to body mass (Fig. 3*a*), with only 21% of the variation in protein synthesis retention efficiency explained by body mass, which was largely a result of substantial variation among all individuals that was independent of mass (Fig. 3*a*). The protein synthesis retention efficiency showed a stronger negative logarithmic relationship with age (Fig. 3*b*); however, only 48% of the variation in protein synthesis retention efficiency was explained by age. Efficiencies of younger and smaller individuals (≤44 d old and with body mass <0.1 g) were in the range of 33.1%–56.1%, while older, larger individuals (>44 d and >0.1 g) had efficiencies ranging from 0.7% to 34.5% (Fig. 3). In both groups there was substantial variation that, given the spread of values, did not appear to be attributable to age, size, reproductive status, or sex.

The total-body concentrations of both protein and RNA changed significantly with size and age of *Euprymna tasmanica*, but very differently. Total-body protein concentration increased in a logarithmic relationship with body size and age (Fig. 4*a*, 4*b*), with the greatest concentrations observed in individuals >0.1 g in size and >44 d old. However, age and size individually explained 27%–53% of the variation in the total-body protein concentration. In contrast, the decline in RNA concentration was linear, and both age and size explained approximately 90% of the variation of total-body RNA concentration (Fig. 4*a*, 4*b*). The difference in total-body RNA concentration was threefold between the younger/smaller individuals and the older/larger individuals.

Of the RNA : protein ratio (C_s) and RNA activity (k_{RNA}), both of which are potential measures of the rates of protein synthesis and capacity for growth, only C_s showed any relationship with size or age. C_s decreased logarithmically with body mass (Fig. 5*a*) and linearly with age (Fig. 5*b*). Although the relationships appeared to be very strong, with 84% and 74% of the variation in C_s explained by body mass and age, respectively, there were distinct patterns of individuals around the fitted line. In the case of body mass, immature individuals of intermediate mass (0.2–1.2 g) have consistently greater values of C_s than are predicted for their size (Fig. 5*a*). In the older, mature individuals, there was a pattern around the fitted line that was associated with gender for both mass and age, with females being more likely to have smaller C_s values for their age and mass and males being more likely to have greater C_s values for their age and mass (Fig. 5*a*, 5*b*). In contrast, there was no evidence of k_{RNA} showing an allometric relationship with mass ($R^2 = 0.27$) or a linear relationship with age ($R^2 = 0.33$).

The amount of protein (g) produced per day as *E. tasmanica* grows and the actual amount used for growth can be estimated across the size range given the fractional rates of protein synthesis and accretion, the total amount of protein (g) in the total body mass of individual, and the percentage of synthesized protein retained for growth. As *E. tasmanica* grows, the model estimated that, in individuals over the total body mass range of 0.02–7.2 g, the amount of protein synthesized would increase allometrically by two orders of magnitude (Fig. 6). The amount of protein accreted also increased allometrically with total body mass; however, the exponent was 30% slower than it was for the amount of protein produced (Fig. 6).

Discussion

Quantifying the processes of growth at the level of protein synthesis validates the growth patterns of cephalopods that were obtained from size-at-age data, and it provides a mechanistic and physiological explanation for the observed patterns of nonasymptotic growth. Somatic growth rates and the factors that influence them are a critical part of describing how energy is used and allocated by animals for the biological processes of maintenance and somatic and reproductive growth. The pattern of nonasymptotic growth in our laboratory-reared individuals was similar to that described for many cephalopods (Jackson 1994). Growth was biphasic, as it is in many laboratory-reared cephalopods (Forsythe 1993; Jackson et al. 1993), with exponential growth occurring early in life, followed by linear growth in older individuals. Over the 150 d that individuals were held, there was no evidence that an asymptotic body size was attained, despite growth rates slowing. The change in growth rates of *Euprymna tasmanica* individuals as they got older and larger was found to be a function of decreasing fractional rates of protein synthesis and degradation. As animals got older and larger, there was a decrease in the rates of protein accretion, RNA concentration, and capacity for growth (C_s) but an increase in protein concentration. For larger (>0.1-g) and older (>44-d-old) individuals, the fractional rate of protein synthesis (k_s) slowed but never reached zero, growth (k_g) slowed but also never reached zero, and the protein synthesis retention efficiency across the size range averaged approximately 10%. Such relationships between size and protein synthesis, degradation, and retention would allow continued slower growth in older and larger individuals, and it supports the cephalopod growth model of continuous growth throughout life with individuals failing to reach an asymptotic size.

Fractional rates of protein synthesis and degradation scaled negatively allometrically with body size, supporting the evi-

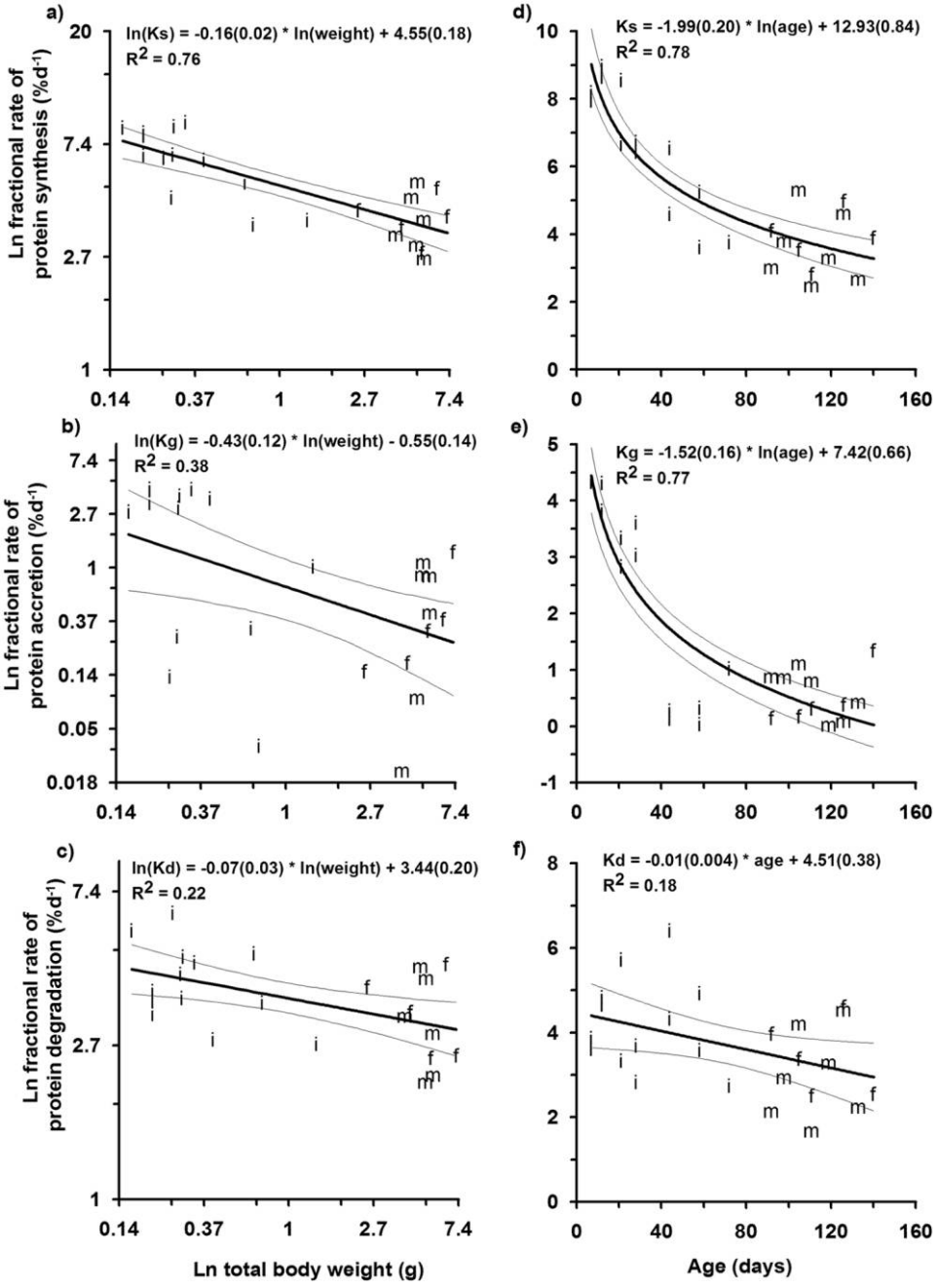

Figure 2. Changes with size in the fractional rates of protein synthesis (*a*), accretion (*b*), and degradation (*c*), and changes with age in the fractional rates of protein synthesis (*d*), accretion (*e*), and degradation (*f*). Fitted lines with 95% confidence intervals are shown, with the parameter estimates provided in the equations. Values in parentheses are the standard errors of the parameter estimates. All regression relationships were significant (df = 1, 23 for all tests, $P < 0.05$). i = immature, f = female, m = male.

dence of those few studies on ectotherms that quantified scaling relationships for fractional rates of protein synthesis and degradation (Houlihan et al. 1986, 1995*a*). It is expected that metabolic rate and fractional rates of protein synthesis should scale similarly with mass, as has been observed in mammals (Waterlow 2006) and fish (Houlihan et al. 1995*b*) and is likely for mussels (Hawkins 1985). There are no data for oxygen consumption in *Euprymna*, but the mass exponent for protein synthesis for *E. tasmanica* (0.16) was very similar to mass-specific exponents for oxygen consumption in octopus (*Octopus cyanea*; −0.167; Maginniss and Wells 1969), which is also a benthic cephalopod. Squid have a faster mass-specific exponent for oxygen consumption (−0.21 for *Sepioteuthis lessoniana*; Segawa 1991), which may be a function of the greater activity

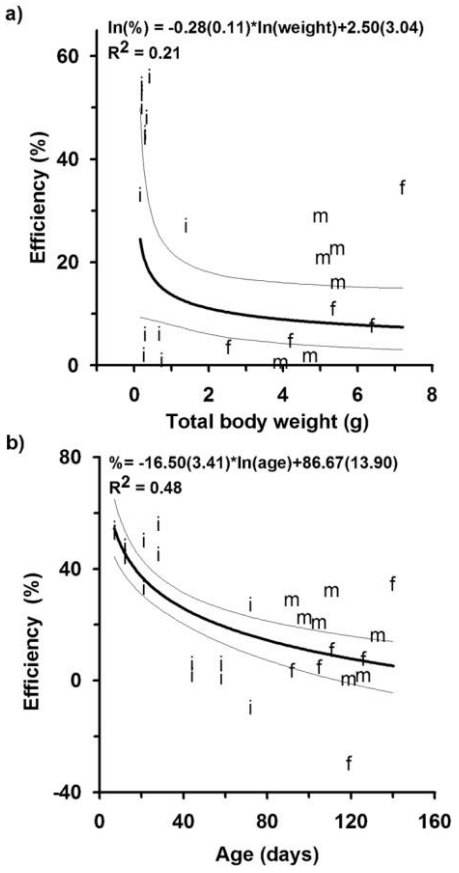

Figure 3. Changes in the protein synthesis retention efficiency with size (*a*) and age (*b*). Fitted lines with 95% confidence intervals are shown, with the parameter estimates provided in the equations. Values in parentheses are the standard errors of the parameter estimates. All regression relationships were significant (df = 1, 23 for all tests, $P < 0.05$). i = immature, f = female, m = male.

levels of squid compared with those of the benthic octopus and of sepiolid species. The amount of protein synthesized per day, when scaled, was linear but almost isometric (Fig. 6; $b = 0.89$), suggesting that *E. tasmanica* fits a Type II scaling of metabolic rates, as observed in cephalopods (Glazier 2005). Therefore, our data support the hypothesis that the energetic demands of protein synthesis are large enough for *E. tasmanica* to strongly influence overall metabolic rates and have similar patterns (Houlihan 1991).

It is possible that the negative allometric relationships for protein degradation may be more important; the total-body mass exponent for protein degradation in *Euprymna* was more than one-half of that calculated for protein synthesis and as much as seven times slower than the total-body mass exponent for protein degradation for teleosts (Houlihan et al. 1986; Carter and Houlihan 2001). It is very possible that the continuous growth observed in many cephalopods may be a function of a slow mass-specific rate of protein degradation in healthy animals. But why it is so slow is not clear. Protein is hypothesized to be a storage product for many cephalopods, and in a few squid species it is used as an energy substrate for reproduction (Jackson and Mladenov 1994), although most squid appear to fuel reproduction directly from ingested food (Hatfield et al. 1992; Moltschaniwskyj 1995).

Protein-specific growth rates were strongly negatively related to both size and age, with younger, smaller individuals displaying specific growth rates that were as much as five times faster than those of larger, older individuals. The difference in protein-specific growth was a function of younger, smaller individuals having faster rates of protein synthesis, greater protein synthesis retention efficiency, and more RNA. But it is worth noting that fractional rates of protein degradation appeared to have no contribution in determining protein-specific growth rates in *Euprymna*. Faster specific growth rates in *Octopus* were also achieved by greater protein synthesis retention efficiency coupled with fast rates of protein synthesis and slow rates of protein degradation (Houlihan et al. 1990). Although the smaller, younger *Euprymna* individuals achieved rates of protein synthesis that are comparable to adult *Octopus* (Houlihan et al. 1990), their protein synthesis retention efficiency values were substantially lower. The greatest protein synthesis retention efficiency value for *Euprymna* was 56%, which is at the low end of values observed for larval fish (49.7%–93.9%) but within the range of values for adult fish (44.7%–71.7%; Carter and Houlihan 2001). In contrast to other molluscs, *Euprymna* had values that were well below the nearly 100% protein synthesis retention efficiency value recorded for *Octopus* (Houlihan et al. 1990). It is worth noting that *Octopus* and *Euprymna* have dramatically different life-history characteristics: *Octopus* are terminal spawners (Semmens et al. 2004), while *Euprymna* are multiple spawners (Steer et al. 2004). The faster specific growth rate (SGR) observed in the younger/smaller individuals compared with in the older/larger individuals was a function of greater protein synthesis retention efficiency, faster fractional rates of protein synthesis, and, to a lesser extent, the concentration of RNA in the tissues. We found no evidence that the activity of RNA was related to SGR. This relationship between faster growth rates and greater protein synthesis retention efficiency is evident in fish (Houlihan 1991). Furthermore, rates of all of these processes declined with age and size: older, larger individuals had slower specific growth rates compared with those of the younger/smaller individuals, but they also showed greater variability.

Our data for *Euprymna tasmanica* did not support the "molluscan model" developed for the relationship between fractional rates of protein synthesis and degradation and the protein synthesis retention efficiency generated from studies of *Octopus vulgaris* (Houlihan et al. 1990) and *Mytilus edulis* (Hawkins 1985). In the first instance, although the relationship between SGR and fractional rates of protein synthesis for *Euprymna* was positive, the relationship between SGR and fractional rates of protein degradation was constant, and not positively linear, as it was for fish (Houlihan et al. 1988), or negatively linear, as it was for *Octopus* and *Mytilus* (Hawkins 1985; Houlihan et al. 1990). Although the range of SGRs for *Euprymna* in this study was comparable with that of values recorded for *Octopus*, it is

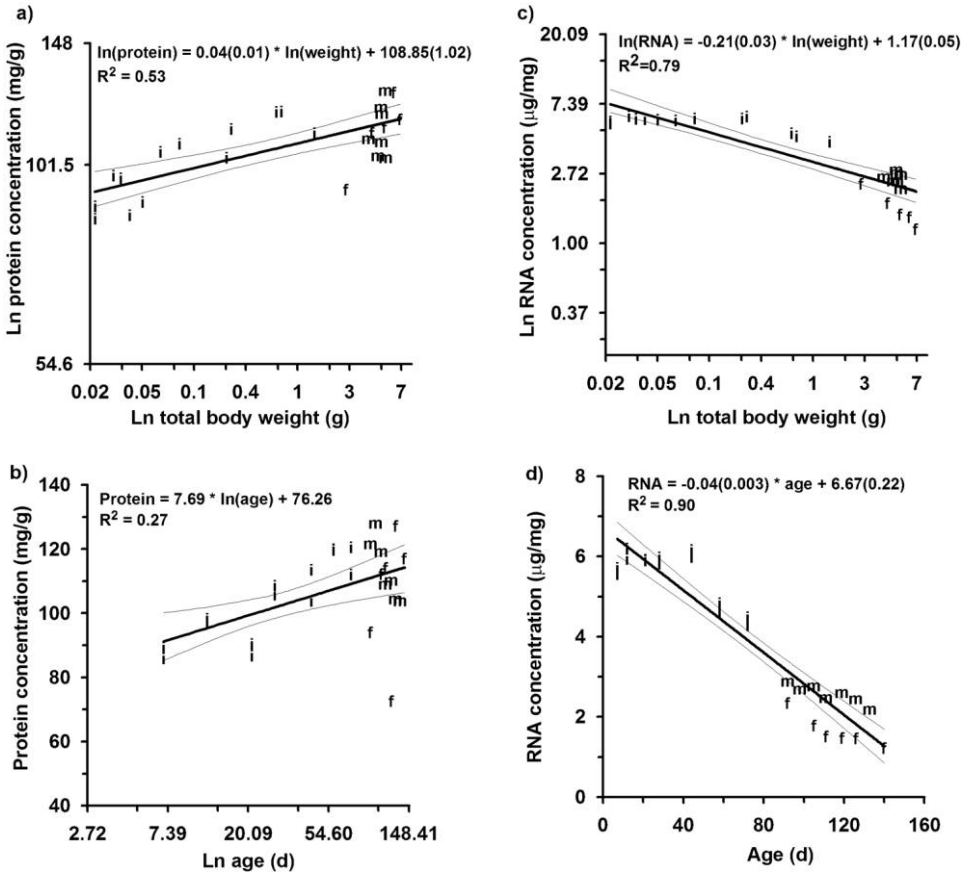

Figure 4. Changes with size in protein concentration (*a*) and RNA concentration (*b*), and changes with age in protein concentration (*c*) and RNA concentration (*d*). Fitted lines with 95% confidence intervals are shown, with the parameter estimates provided in the equations. Values in parentheses are the standard errors of the parameter estimates. All regression relationships were significant (df = 1, 23 for all tests, $P < 0.05$). i = immature, f = female, m = male.

critical that the highest fractional rates of both synthesis and SGR were measured in juveniles, who are expected to have the slowest rates of protein turnover (equivalent to k_d; Houlihan et al. 1995*b*).

In the second instance, *Euprymna* did not display the greater efficiencies of synthesized protein retention seen in *Octopus* and *Mytilus*, even in the faster-growing juveniles. However, there are several substantial and significant differences in the energy demands and requirements of this species that differ from those of *Octopus* and *Mytilus*. *Euprymna tasmanica* has a symbiotic relationship with a light-producing *Vibrio* species (Jones et al. 2006), populations of which are maintained in kidney-shaped organs close to the ink sac. The squid provides the *Vibrio* with a nutritional environment that is rich in amino acids, most likely present as peptides or proteins (Graf and Ruby 1998); as a result, there is an energetic cost in maintaining the *Vibrio* population that is unique. Critically, this energy demand is continuous, as every 24 h, shortly after first light, over 90% of the *Vibrio* in the organs are vented; over the next 8–10 h, the *Vibrio* populations enter a rapid, logarithmic phase of growth, returning population density to its morning value (Visick and McFall-Ngai 2000). There are no data that quantify the energetic cost of this relationship, as it is logistically difficult to maintain *Euprymna* that are isolated from *Vibrio*; individuals obtain *Vibrio* from the surrounding water early in their post-hatching development (Visick and McFall-Ngai 2000). However, if we assume that, like *E. coli*, the mass of each *Vibrio* cell is $\sim 2.8 \times 10^{-13}$ g, and that each of the two chambers reaches a density of 10^9 *Vibrio* cells at the start of each night (McFall-Ngai 2000), then this means that an adult squid supports the growth of 0.56 mg dry weight of *Vibrio* spp. across the two chambers per day. Again, if we assume that *Vibrio* spp. is growing aerobically and, like *E. coli*, achieves 10 g dry mass per mol ATP, then across the two chambers this costs the squid 5.6×10^{-2} mmol ATP per 24 h. It has been estimated that a minimum energetic cost of protein synthesis is 40 mmol ATP equivalents per g protein synthesized (Reeds et al. 1985). On the basis of our estimates, supporting this symbiosis costs an adult *E. tasmanica* 1.4 mg protein per d, which is 4.7%–7.0% of its daily synthesized protein.

A second potential source of energy demand that is unique to the nocturnal dumpling squid is the production of a highly

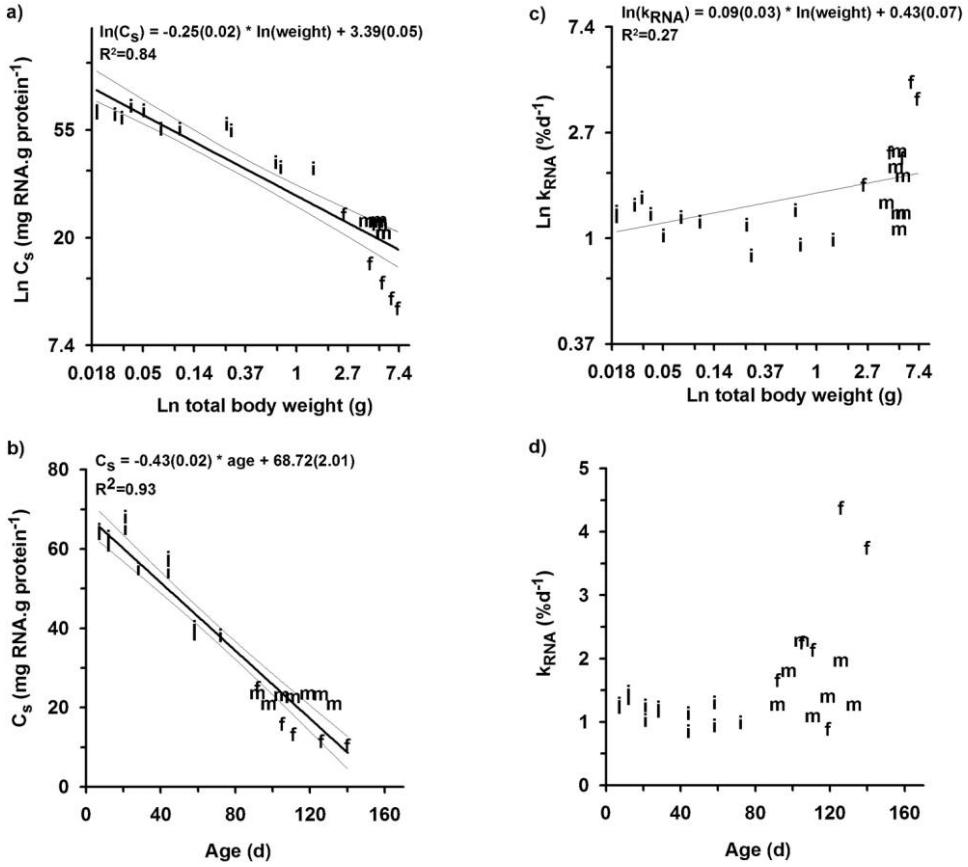

Figure 5. Changes in RNA : protein ratio (C_s) with size (*a*) and age (*b*). Fitted lines with 95% confidence intervals are shown, with the parameter estimates provided in the equations. Values in parentheses are the standard errors of the parameter estimates. All regression relationships were significant (df = 1, 23 for all tests, $P < 0.05$). i = immature, f = female, m = male.

sulfated mucopolysaccharide mucus (Packard 1988) that is associated with the production of a "glue" that is used to stick sand grains to the skin of the individuals during the day; at night, secretion of another substance is thought to dissolve the glue, allowing the sand to drop off (Norman and Lu 1997). Although in our study the animals were not provided with sand to allow this behavior to be expressed, it is not known whether secretion of these mucus products occurs independently of the behavior. The composition of this adhesive mucus is not known, but a secreted mucus in limpets contains equal dry-mass quantities of protein and carbohydrate (Smith and Morin 2002), suggesting that there is a cost to *Euprymna* associated with producing and secreting the mucus coat; this may further explain the reduced protein synthesis retention efficiency relative to both *Octopus* and mussels.

Food quality, particularly amino acid composition, has a significant effect on protein synthesis retention efficiency (Carter and Houlihan 2001). Although our study animals were provided with food ad lib., mysids may not be the preferred prey type, and so this possibly compromised growth rates. Age estimates for wild *Euprymna* are not available, as the hard structures do not lend themselves to age determination (Moltschaniwskyj and Cappo 2009); however, small cephalopod species typically live for approximately 3–4 mo (Jackson 1989; Tracey et al. 2003). Field collections of *E. tasmanica* gathered over 2 yr at two locations in Tasmania found individuals with masses that were in

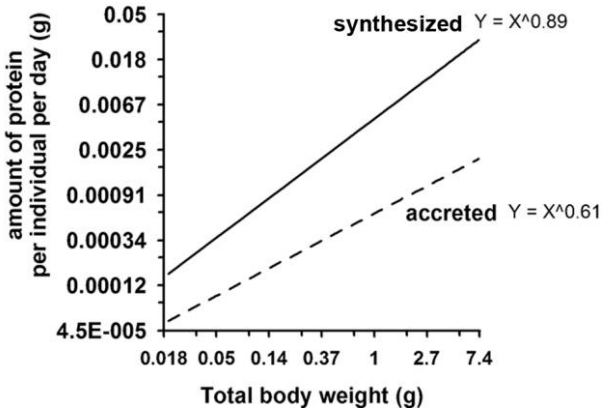

Figure 6. Relationship between body mass and the amount of protein synthesized and accreted.

excess of 11 g (Sinn et al. 2010), which is 25% heavier than our largest individual. However, 95% of the individuals collected in that field study had masses that were <8.5 g, which is comparable to the sizes attained by the individuals in our study, suggesting that the individuals that we grew in captivity were not severely compromised in their growth.

The process of senescence is poorly understood in most ectotherms and is nonexistent for cephalopods. The short life spans of cephalopods in general and the relatively short time between final egg deposition and death in females suggests that processes leading to death are extremely rapid. Variation in protein synthesis retention efficiency could be explained by a breakdown in the cellular processes associated with senescence. While negative relationships of protein synthesis and protein accretion with age may be an expression of senescence processes (Houlihan 1991), fractional rates of protein synthesis in *Euprymna* were consistently greater than fractional rates of protein degradation. It is hypothesized that slow rates of protein degradation in *Octopus vulgaris* may occur because the loss of function associated with retaining damaged protein is outweighed by the advantage of increased size and reproductive capacity, thereby allowing optimum use of proteins for accretion over their short life spans (Houlihan et al. 1990). *Euprymna tasmanica* did not appear to have similarly slow rates of protein degradation, but unlike *O. vulgaris*, *Euprymna* is not a terminal spawner: a female produces multiple batches of eggs throughout her lifetime (Steer et al. 2004). Therefore, the faster rates of protein degradation and less efficient protein retention in *Euprymna* may be a mechanism to extend tissue function. There was evidence in measures of capacity for growth (RNA concentration and C_s) of differences between mature males and females, suggesting that the allocation of energy to reproduction may be affecting growth and, potentially, senescence.

Estimating the cost of protein synthesis requires estimates of both oxygen consumption and protein synthesis for each individual. These data are still not available for any cephalopod. However, to explain the observed patterns of whole-body growth and the unique life-history characteristics of cephalopods, it is necessary to quantify the cost of protein synthesis during each critical phase of the life history, particularly early development, reproductive maturation, and finally, senescence.

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