Energetic cost of synthesizing proteins in Antarctic limpet, Nacella concinna (Strebel, 1908), is not temperature dependent

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The energetic cost of synthesizing proteins is thought to account for a significant proportion of total metabolism. However, attempts to estimate the energetic cost of synthesizing proteins have resulted in surprisingly variable results, particularly for the small number of polar organisms studied, where cost estimates vary by two orders of magnitude. Much of this variability is probably the result of differing methodologies and experimental designs. Here we have used two different, carefully validated methods to measure the costs of protein synthesis in Antarctic limpets. One method, which utilized a specific protein synthesis inhibitor, was used to measure the cost of protein synthesis at two temperatures to test the hypothesis that the cost of protein synthesis varies with temperature. The cost of protein synthesis measured using the inhibitor cycloheximide was 13.95 ± 0.77 μmol O2/mg protein, while correlation of absolute protein synthesis with oxygen consumption suggested the cost of protein synthesis was 19.58 μmol O2/mg protein. Water temperature did not alter the cost of protein synthesis in Nacella concinna (Student’s t-test, P = 0.849, t = 0.19, df = 12). In a meta-analysis of literature values for the cost of protein synthesis there was no significant effect of temperature, but there was a significant relationship between the concentration of cycloheximide used to inhibit protein synthesis and the measured cost.

Protein synthesis is an essential and energetically expensive process, with a minimum cost of between 11 and 42% of total oxygen consumption in a range of endo- and ectotherms (14). As protein synthesis comprises such a significant component of total metabolism and is essential for animal growth, it is important that we have an accurate understanding of the costs, how these vary, and how they relate to the other cellular energy consuming processes. Numerous studies have attempted to determine the energetic cost of protein synthesis, using a variety of methods (e.g., see Refs. 1, 14, 26, 27, 33, and 37), but the results obtained vary over a surprising and unrealistically large range. Nowhere is this more obvious than in Antarctic organisms, where reported costs of protein synthesis range from 0.92 (Stereochirus neumayeri, sea urchin larvae) to 147.5 μmol O2/mg protein (Glyptonotus antarcticus, ispod) (37).

Three methods have been used in the various studies to estimate the energetic cost of protein synthesis. In the first, the absolute rate of protein synthesis is measured and a theoretical cost per unit of protein used to determine the energy allocated to protein synthesis. Generally, it has been assumed that 4ATP (2ATP and 2GTP) equivalents are required per peptide bond, plus a further 1ATP equivalent for transport processes (31). This estimate presumes a mean peptide molecular weight of 110 and that 6 mmol of ATP are synthesized per millimole of O2, resulting in a theoretical minimum cost of 8.3 μmol O2/mg protein synthesized. This method relies on many assumptions, and there exists a large margin for error (31).

The other two experimental methods allow measurement of the absolute costs of protein synthesis, expressed as units of oxygen or ATP required per unit of protein synthesized. The regression slope describing the relationship between absolute protein synthesis and oxygen consumption has been used to estimate protein synthesis costs in a number of species (see MATERIAL AND METHODS and Refs. 12, 18, 24, and 27). This technique relies on the observation that there is a linear relationship between oxygen consumption and protein synthesis and that an increase in protein synthesis necessitates a closely associated increase in oxygen consumption. However, there is little logical reason to believe that a change in metabolism is solely determined by a change in protein metabolism (36). If, for example, an increase in protein synthesis were coupled to increases in membrane turnover or ion pumping, then the metabolic cost of protein synthesis would be overestimated by this technique.

Finally, the cost of protein synthesis can be estimated by the application of a specific protein synthesis inhibitor, typically cycloheximide, which blocks cytosolic protein synthesis. If protein synthesis is measured in untreated control animals and cycloheximide-treated animals, the cycloheximide-sensitive fraction of total protein synthesis can be determined. By measuring the rate of oxygen consumption before and after cycloheximide application, the energetic cost of protein synthesis per unit of protein can be calculated (6, 16, 20, 26). Previous studies have suggested that the use of excessive concentrations of cycloheximide and inaccurate measurement of oxygen consumption may produce erroneous protein synthesis cost estimates (6, 38). It is therefore vital that studies employing this technique are carefully validated.

The cost of protein synthesis in Antarctic organisms has only been reported in four studies, with values differing by two orders of magnitude (24, 27, 35, 37). Authors have alternatively proposed very high, or very low costs of protein synthesis at polar water temperatures (24, 37), and it is not currently clear whether temperature effects the cost of protein synthesis in animals more generally. Energetic costs of protein synthesis reported in non-Antarctic species are also highly variable, ranging from 2 to 495 μmol O2/mg protein (e.g., Refs. 1, 17, 20, 24, 26, 35, 37).
19, 27, and 38). It seems highly unlikely that the energetic cost of protein synthesis really varies by such a large degree in Antarctic organisms, or indeed, in animals generally. Some of the variability in reported protein synthesis costs may be due to differences in methodology and body temperature, but it appears likely that much is the result of experimental design.

This study aims to carefully validate the methods available to measure the in vivo cost of protein synthesis in an attempt to minimize methodological artifacts. We also investigate whether temperature affects the cost of protein synthesis in an Antarctic limpet, *Nacella concinna* (Strebbl, 1908), and, in turn, whether the cost of synthesizing proteins is temperature dependent in animals more generally.

**MATERIALS AND METHODS**

Collection of animals and husbandry. *N. concinna* were collected from sublittoral sites at Rothera Point, Adelie Island, Antarctic Peninsula (67°34′07″ S, 68°07′30″ W) by scuba divers, and either held in a through-flow aquarium system at ambient Antarctic temperatures or returned to the United Kingdom in a refrigerated transport aquarium. In the United Kingdom, limpets were maintained in a recirculating flow aquarium, under an automatic 12:12-h light-dark regimen [water temperature 0 ± 0.2°C, salinity 34–36 practical salinity units (PSU)]. The animals were allowed to graze freely on biofilms growing on the tank walls. Experimental work was carried out both in Antarctica (during January 2004) and in the United Kingdom (between June 2004 and July 2005). All experimental work fully complied with regulations governing both institute and United Kingdom animal procedures.

Validation: establishment of the inhibitor dose. To establish the minimum dose of cycloheximide that would significantly suppress cytosolic protein synthesis, groups of limpets were injected with a range of concentrations of cycloheximide, and protein synthesis was measured using the 3H-labeled phenylalanine flooding dose method (5). Establishment of a minimal cycloheximide concentration was important to avoid the use of excessive cycloheximide concentrations that could in turn increase the risk of secondary effects. Cycloheximide (Sigma-Aldrich, St. Louis, MO) was dissolved in ethanol and diluted with saline to produce 10 concentrations ranging between 0.01 mM and 1,000 mM, with an order of magnitude difference between concentrations. After injection (see below) these concentrations resulted in tissue concentrations ranging between 2.8 × 10⁻⁵ and 2.8 mg cycloheximide/g fresh weight (FW). FW is defined as total animal mass less shell mass. The maximum concentration of ethanol in the injected solution was 10% vol/vol.

The shell lengths of 10 groups of limpets, (n = 3 per group, except the control group in which n = 9 per treatment) were measured with vernier calipers (±0.05 mm). The limpets were dried with a tissue, weighed (±1 mg) and labeled individually using small plastic numbers attached to the shell with cyanoacrylate adhesive. Each limpet was then injected into the pedal sinus with a predetermined concentration of cycloheximide (10 mg cycloheximide/g FW). Two control groups were placebo injected with either physiological saline or ethanol/saline (50% vol/vol). Individual specific injection volumes were obtained from a published shell length vs. FW relationship [y = 0.84x - 0.14, where y = FW and x = shell length (5)]. Thirty minutes after the cycloheximide injection, each animal was injected in the pedal sinus with a flooding dose of unlabeled and 3H-labeled phenylalanine (10 μg/g limpet FW of 135 mmol/l [l-2,6-3H]phenylalanine at 3.6 MBq/ml (Amershams, Little Chalfont, UK)) to measure the rate of protein synthesis.

After injection, limpets were placed in a beaker containing 4 liters of clean, aerated seawater maintained at 0 ± 0.1°C. Two hours after the flooding dose injection, the limpets were shucked, killed, and the FW measured before they were homogenized (Status X120 homogenizer) in a known volume of ice-cold 0.2 M perchloric acid (PCA). To allow an estimate of the preinjection phenylalanine concentration, and hence allow calculation of flooding levels, a control group of 10 noninjected limpets were shucked, killed, and their FW measured and homogenized in 0.2 M PCA.

After analysis (see below), the dose-response relationship between cycloheximide concentration and inhibition of protein synthesis was further refined using the same techniques but over a narrower range of cycloheximide concentrations (1 mM to 10 mM, n = 4 per group apart from controls where n = 10). These cycloheximide concentrations resulted in tissue concentrations ranging between 2.8 × 10⁻³ and 0.028 mg cycloheximide-g⁻¹FW⁻¹. In all experiments measuring protein synthesis, the homogenized limpets were refrigerated (−4°C) prior to analysis. All samples were analyzed within 4 wk of collection.

**Protein synthesis: sample analysis.** The whole animal rate of protein synthesis was measured using methods previously described (5, 9, 15). The limpet homogenate was vortex mixed, and a 2-ml aliquot was removed for analysis. This sample was centrifuged (Eppendorf 5810R swing bucket rotor; 3,980 g, 10 min, 4°C) to separate the protein precipitate and RNA from the intracellular free pool (13, 14). The supernatant, containing the free pool, was carefully decanted, and the NaOH soluble protein in the protein pellet was measured using BSA (Sigma-Aldrich) as the standard (21). The total protein mass of the limpet was calculated using the following equation

\[
\text{protein mass} = \left( \frac{\text{FW}}{\text{sample mass}} \right) \times \text{sample protein content}
\]

where protein mass is expressed in milligrams protein per animal and fresh weight, sample mass (within the 2 ml aliquot used for the analysis) and sample protein content in milligrams. The sample mass was calculated by dividing the total FW of the limpet by the volume of PCA used to homogenize the animal and multiplying by two to allow for the fact that a 2-ml aliquot was used.

Subsequently, the protein pellet was washed twice with 0.2 M PCA and hydrolyzed in 6 M HCl for 18 h at 110°C. The acid was removed from the hydrolyzed protein residue using repeated washes of distilled water with rotary evaporation (Buchi R114) to dryness between washes. The residue was resuspended in 0.5 M sodium citrate buffer (pH 6.3), and the phenylalanine concentrations of the hydrolyzed proteins, the injection solution, and the free-pool were measured fluorometrically after enzymatic conversion of the phenylalanine to β-phenylethylamine (9). For a full description of these methods see Ref. 14.

To assess the enzymatic conversion efficiency of phenylalanine to β-phenylethylamine, known concentrations of phenylalanine were also converted and analyzed as described above. The specific radioactivities of the free pools, protein pellets, and injection solutions were measured by scintillation counting (Wallac 1409 LSC; Packard Bioscience Hionic-Fluor) and expressed as disintegrations per min (dpm) per nanomoles phenylalanine, after correcting for counting efficiency (34%).

The following equation was used to calculate the fractional (kₙ) rate of protein synthesis (10)

\[
kₙ = \frac{Sₙ}{S₀} \times \frac{100}{t} \times 1,440
\]

where kₙ = %protein mass synthesized per day (%/day), S₀ = specific radioactivity of protein-incorporated radiolabel (dpm/nmol phe), Sₙ = specific radioactivity of the intracellular free-pool (dpm/nmol phe), t = time since injection of radiolabel in minutes, and 1,440 = the number of minutes in a day.

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The absolute rate of protein synthesis was calculated using the following equation (5)

\[ A_i = \frac{k_i}{10^6} \times \text{protein mass} \]

where \( A_i \) is expressed as milligrams of protein synthesized per animal per day and protein mass is expressed as milligram protein per animal.

The flooding dose methodology has previously been validated for *N. concinna* (5). Therefore, no further time course validation was performed in this study. However, to ensure the animals were sufficiently flooded with phenylalanine, the level of phenylalanine was measured in preinjection control animals and postinjection experimental animals.

**Reversibility of cycloheximide induced protein synthesis inhibition.** The inhibitory effects of cycloheximide were measured over an extended time period, by injecting a solution of 3 mM cycloheximide at 10 \( \mu \)L/mg FW, which resulted in a tissue concentration of 8.4 \( \times 10^{-3} \) mg cycloheximide/g FW. This cycloheximide concentration was selected on the basis of the previously described dose response trials (see RESULTS). Limpets were measured, labeled, and injected as previously described. Thirty minutes after the cycloheximide injection, the first group of animals were injected with a flooding dose of \( ^3\text{H} \)-labeled phenylalanine (n = 4). A control group of animals (n = 4) that had been injected with saline was also injected with a flooding dose. Single groups of animals (n = 4) were then injected with \( ^3\text{H} \)-labeled phenylalanine at 2-h intervals up to 10 h after the cycloheximide injection. Two further groups of animals, one of which was a control group that had been injected with saline but not cycloheximide (n = 4) and a final experimental group were injected with the flooding dose 22 h after the cycloheximide injection. Immediately after injection, all limpets were placed in a beaker containing 4 liters of clean aerated seawater (temperature \( -0.2 \pm 0.1\)C over the course of the incorporation). Two hours after the flooding dose injection, animals were shucked, their FW measured, and homogenized into 0.2 M ice-cold PCA.

**The effect of a placebo injection on the rate of protein synthesis.** To determine whether injecting the animals affected the rate of protein synthesis, groups of limpets were injected into the pedal sinus with physiological saline (34 PSU), ethanol/saline (50% vol/vol) or left noninjected (control), before protein synthesis was determined using the flooding dose method as previously described. Limpets were weighed, measured, and labeled and then injected in the pedal sinus with the relevant solution, or left noninjected (control). The limpets were then placed in a beaker containing 4 liters of clean aerated seawater for 30 min (temperature 0.2 \( \pm 0.1\)C) before receiving a flooding dose injection. The animals were returned to the beaker immediately after injection. Two hours later the limpets were shucked, their FW was measured, and they were homogenized in 0.2 M PCA prior to analysis.

**Measurement of oxygen consumption.** Oxygen consumption rates in *N. concinna* have been shown to be independent of water oxygen concentration down to levels that are 80% of those measured in control respirometers containing no animals (4). Limpets were placed in 80 ml Perspex respirometers for periods of 2–5 h (depending on size), resulting in a 5–15% decrease in oxygen content during the measurement period. The respirometers were placed in a recirculating aquarium to maintain a constant water temperature identical to that of the maintenance tank. At each sampling time point, the chamber was gently inverted several times to ensure mixing, and a 25-\( \mu \)L sample of water was removed for analysis. The oxygen content of the water sample was measured using coulooximetry (28, 29). The oxygen consumed by the limpet was calculated by subtracting the oxygen content in the experimental chambers from that of a control chamber containing no limpet.

**Effect of handling stress on oxygen consumption.** The effect of handling stress on the rate of oxygen consumption in *N. concinna* was investigated to determine whether oxygen consumption rates were artificially elevated by handling during placement of the limpets in the respirometers. Limpets were placed in respirometers, and oxygen consumption was measured after 2, 5, and 7 h (n = 6). The respirometers were then opened and flushed with clean seawater before being loosely sealed with plastic mesh to prevent the limpets from escaping. The limpets were left undisturbed until 22 h after they were initially handled, when the respirometers were resealed and respiration rates remeasured. The water temperature over the course of the entire experiment was \( -0.2 \pm 0.1\)C.

**Effect of placebo injections on oxygen consumption.** The effect on oxygen consumption of injecting the animals was determined by measuring oxygen consumption in three groups of limpets for 2 h (n = 6 per group) as previously detailed. The limpets were then injected in the pedal sinus with physiological saline (34 PSU), ethanol/saline (50% vol/vol) or left noninjected (control). The limpets were replaced in respirometers, and after a further 5 h oxygen consumption was remeasured.

**Measurement of the energetic cost of protein synthesis using cycloheximide inhibition of protein synthesis.** The limpets used in the work detailed below had been acclimated to either 0 ± 0.2°C or 3 ± 0.1°C for at least 28 days prior to use. All other environmental conditions were as previously described. The 0°C and 3°C experimental work was carried out in the United Kingdom in November 2004 and June 2005, respectively. Limpets were placed in respirometers, and oxygen consumption was measured over 2 h as described previously (n = 18). The respirometers were opened, and the animal’s length was measured. A known volume of 3 mM cycloheximide (8.4 \( \times 10^{-3} \) mg cycloheximide/g FW) was injected into the pedal sinus, and the limpets were placed in a beaker containing 4 liters of clean, aerated seawater (temperature 0 ± 0.2°C or 3 ± 0.2°C for the 0°C and 3°C acclimation groups, respectively) for 5 h. The limpets were then replaced in the respirometers, and oxygen consumption was measured over the following 2 h. The animals were removed from the respirometers, injected in the pedal sinus with \( ^3\text{H} \)-labeled phenylalanine and placed in a beaker containing aerated seawater (temperature 0 ± 0.2°C and 3 ± 0.2°C). After 2 h, the limpets were shucked, their FW was measured, and they were homogenized in ice-cold 0.2 M PCA. Protein synthesis was also measured in a parallel group of limpets that had not been injected with cycloheximide.

The cost of protein synthesis was calculated using the following equation

\[
\text{Cost of protein synthesis} = \frac{\text{MO}_2^{\text{ctrl}} - \text{MO}_2^{\text{chx}}}{A_i^{\text{ctrl}} - A_i^{\text{chx}}}\]

Where \( \text{MO}_2^{\text{ctrl}} \) is the rate of oxygen consumption in animals prior to injection with cycloheximide (\( \mu \)mol \( O_2/d\)ay) and \( \text{MO}_2^{\text{chx}} \) is the rate of oxygen consumption after injection. While \( A_i^{\text{ctrl}} \) is the absolute rate of protein synthesis in control animals (mg protein·animal\(^{-1}\)·day\(^{-1}\)) and \( A_i^{\text{chx}} \) is the absolute rate of protein synthesis in animals injected with cycloheximide.

**Measurement of the cost of protein synthesis using the correlative method.** The rates of oxygen consumption and protein synthesis were measured in limpets maintained in the British Antarctic Survey (BAS) aquarium in Cambridge and a second group of limpets collected and maintained at the BAS Rothera Research station. All animals were maintained under an automated 12:12-h light-dark regimen and water temperatures of 0.94 ± 0.02°C (Antarctic) and −0.4 ± 0.1°C (Cambridge). Limpets were measured, individually labeled, and placed in respirometers (temperature −0.1 ± 0.2°C for the Cambridge aquarium animals, 1.4°C for the animals maintained in the Antarctic). Oxygen consumption was measured over 2 h as described previously. The animals were then removed from the respirometers and injected into the pedal sinus with a flooding dose of \( ^3\text{H} \)-labeled phenylalanine, before being placed in beakers containing seawater [temperature 0 ± 0.2°C (UK) and 0.8 ± 0.2°C (Rothera)]. After 2 h the animals were
shucked and killed, their FW was measured, and they were homogenized in 0.2 M PCA. Sample analysis was as previously described. Statistical analysis. All data are expressed as means ± SE. Statistical analysis was carried out using Minitab 14 (Minitab, Coventry, UK). Prior to statistical analysis, data were checked for normality and homogeneity of variances using the Anderson-Darling test and Levene’s test (34). Parametric data were analyzed using ANOVA and Tukey’s honestly significant difference tests, while nonparametric data were analyzed using the Kruskal-Wallis test.

RESULTS

Unless stated otherwise, there were no significant differences in limpet FW or lengths between experimental groups. There was no detectable effect of handling stress (ANOVA, \( P = 0.849, F = 0.04 \)) or injection (ANOVA, \( P = 0.767, F = 0.09 \)) on respiration rates. The rate of protein synthesis in groups of limpets injected with either saline or ethanol/saline (50% vol/vol) did not differ significantly from noninjected control animals (ANOVA, \( P = 0.767, F = 0.09 \)).

Validation of the flooding dose. Injection of the limpets resulted in an average 4.2-fold increase in phenylalanine concentrations above the measured baseline (0.30 nmol/mg fresh mass). After a flooding dose injection of 1.35 nmol/mg FW, phenylalanine levels should have increased to 1.65 nmol/mg. Phenylalanine levels increased to between 85 and 100% of the theoretical postinjection concentration, suggesting that the flooding dose was successful.

Establishment of the required inhibitor dose. In the first trial, protein synthesis was significantly inhibited in limpets injected with concentrations of cycloheximide greater than or equal to 0.028 mg cycloheximide/g FW (Fig. 1, ANOVA, \( P < 0.001, F = 65.27 \)). Increasing the cycloheximide concentration above this did not elicit a significant further decrease in \( A_s \). A second dose-response trial revealed that injecting a cycloheximide concentration of \( 8.4 \times 10^{-3} \) mg cycloheximide/g FW reduced \( A_s \) significantly below control values (Fig. 2, ANOVA, \( P < 0.001, F = 71.33 \)). The calculations used to determine the cost of protein synthesis in the present study do not require that \( k_s \) is completely inhibited, but rather that there is a significant reduction in \( k_s \) and oxygen consumption; therefore a concentration of \( 8.4 \times 10^{-3} \) mg cycloheximide/g FW was utilized as the cycloheximide dose in this study.

Reversibility of cycloheximide inhibition of protein synthesis. The rate of \( A_s \) in control limpets that were not injected with cycloheximide did not differ significantly over the time course, i.e., from 2 to 24 h. \( A_s \) in cycloheximide-injected animals was significantly lower than control animals over a 24-h period, but the level of protein synthesis suppression did not differ significantly with time (Fig. 3, ANOVA, \( P < 0.05, F = 4.81 \)).

The energetic cost of protein synthesis. Cycloheximide inhibition of protein synthesis. For both temperature groups, the rate of oxygen consumption was significantly reduced after the application of cycloheximide [Table 1, ANOVA, \( P < 0.001, F = 44.45 \) (0°C), ANOVA, \( P < 0.001, F = 254.73 \) (3°C)]. The measured absolute rate of protein synthesis in each temperature group was also significantly reduced below control animals after the application of cycloheximide [Table 1, ANOVA, \( P < 0.001, F = 109.39 \) (0°C), ANOVA, \( P < 0.001, F = 117.22 \) (3°C)]. There was no significant difference in the cost of protein synthesis in animals acclimated to 0°C (14.17 ± 1.82 \( \mu \)mol O2/mg protein) or 3°C (13.8 ± 0.50 \( \mu \)mol O2/mg protein; ANOVA, \( P = 0.820, F = 0.05 \)).

Correlation of oxygen consumption and absolute protein synthesis. There was a significant linear relationship between \( A_s \) and oxygen consumption (Fig. 4, \( y = 19.58x + 0.574, F = 78, r^2 = 65.8\%, P < 0.001, n = 44 \)). The slope of 19.58 suggests the cost of synthesizing 1 mg of protein was 19.58 \( \mu \)mol O2.

DISCUSSION

The primary aims of the current study were first, to carefully validate the use of cycloheximide to measure the costs of...
protein synthesis in N. concinna and second, to investigate whether the cost of synthesizing proteins is dependent on body temperature.

**Validation.** Estimates of the energetic cost of protein synthesis made using cycloheximide vary by several orders of magnitude (Table 2). This technique assumes that the effects of the inhibitor are specific, which has recently been disputed (6, 38). In fact, some studies have demonstrated that the degree to which cycloheximide suppresses metabolism maybe dose dependent, suggesting that there may be nonspecific effects that increase with dose size (6, 38). Other factors, such as handling and injection, may also affect both protein synthesis and oxygen consumption.

Therefore, the initial part of this study aimed to establish a minimal concentration of cycloheximide that would significantly inhibit protein synthesis and investigate whether handling and injecting an animal had a significant effect on the rate of protein synthesis and oxygen consumption. Any alteration in protein synthesis, independently of oxygen consumption, or vice versa, will, in turn, effect the estimate of the cost of protein synthesis. A cycloheximide concentration of \(8.4 \times 10^{-3}\) mg cycloheximide/g FW was found to significantly reduce protein synthesis; therefore this concentration was used throughout the study to minimize the potential nonspecific effects of cycloheximide. The concentration of cycloheximide used in the current study contrasts sharply with that used in many other studies, some of which have used concentrations as high as 5 mg/g FW, 600-fold higher (Table 2, Ref. 37). It would seem prudent, that in any study utilizing cycloheximide to measure the cost of protein synthesis, the concentration of cycloheximide used should be minimized to reduce potential nonspecific effects. As long as protein synthesis and oxygen consumption are measurably reduced, there is no requirement to terminate all of cytosolic protein synthesis, indeed, even in studies utilizing very high concentrations of cycloheximide some residual protein synthesis still occurs (1, 10).

Handling and injecting N. concinna were shown to have no significant effect on either oxygen consumption or protein synthesis in this study. Any alteration in oxygen consumption or protein synthesis after the injection of cycloheximide should therefore be directly due to cycloheximide-induced changes in the rate of protein synthesis rather than an experimental artifact. The approach used in this study requires that cycloheximide suppresses protein synthesis over the experimental period (~7 h) during which protein synthesis and oxygen consumption are measured. Our data demonstrate that the dose of cycloheximide used (8.4 \(\times 10^{-3}\) mg cycloheximide/g FW) suppresses protein synthesis over at least 24 h (Fig. 3). Most previous studies have suggested that protein synthesis is irrevocably suppressed by cycloheximide, although in the ciliate, Tetrahymena thermophila, it appears the effects of cycloheximide may be reversible (3, 11, 32). A phenylalanine flooding

<table>
<thead>
<tr>
<th>Initial MO2, μmol O2/day</th>
<th>Inhibited MO2, μmol O2/day</th>
<th>Baseline Aₘ, mg prot. an/day</th>
<th>Inhibited Aₘ, mg prot. an/day</th>
<th>CHX Inhibited Energetic Cost of Protein Synthesis, μmol O2/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C 13.89±0.04</td>
<td>5.93±0.61</td>
<td>0.91±0.03</td>
<td>0.36±0.03</td>
<td>14.17±1.82</td>
</tr>
<tr>
<td>3°C 17.19±0.35</td>
<td>9.15±0.37</td>
<td>1.16±0.06</td>
<td>0.56±0.02</td>
<td>13.80±0.50</td>
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</tbody>
</table>

All values are means ± SE. Initial and inhibited rates of oxygen consumption differed significantly within temperature groups [ANOVA, \(P < 0.001, F = 44.45\) (0°C), ANOVA, \(P < 0.001, f = 254.73\) (3°C)], as did rates of uninhibited Aₘ and cycloheximide-inhibited Aₘ, [ANOVA, \(P < 0.001, F = 109.39\) (0°C), ANOVA, \(P < 0.001, F = 117.22\) (3°C)].

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heximide inhibition was 13.95

hand, should include associated processes (for example, RNA protein synthesis (31). The correlative method, on the other synthesis accounts only for the direct synthesis and transport costs of current study. The minimum theoretical cost of protein synthe-

produce a significant correlation, and this was the case in the range of protein synthesis rates and oxygen consumptions to dependent upon the animals selected having a wide enough

respectively, than the theoretical minimum cost of protein synthesis measured using a variety of experimental methods

Table 2. The energetic cost of protein synthesis in a range of ectotherms and endotherms measured using a variety of experimental methods

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Temperature, °C</th>
<th>CHX Conc., mg/g FW</th>
<th>Energetic Cost of Protein Synthesis, μmol O2/mg protein</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick</td>
<td>Whole body</td>
<td>40.8</td>
<td>5.06</td>
<td>11.05</td>
<td>1</td>
</tr>
<tr>
<td>Oncorhynchus mykiss</td>
<td>BF-2 cell line</td>
<td>23.5</td>
<td>0.2</td>
<td>10.9±0.2</td>
<td>33</td>
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<tr>
<td>O. mykiss</td>
<td>Macrophages</td>
<td>10.9</td>
<td>2</td>
<td>45.8±8.9</td>
<td>33</td>
</tr>
<tr>
<td>O. mykiss</td>
<td>RTG-2 cell line</td>
<td>23.5</td>
<td>2</td>
<td>133.2±76.7</td>
<td>33</td>
</tr>
<tr>
<td>O. mykiss</td>
<td>Scale cells</td>
<td>10.9</td>
<td>2</td>
<td>217.2±50.4</td>
<td>33</td>
</tr>
<tr>
<td>Chrysemys picta bellii</td>
<td>Hepatocytes</td>
<td>25</td>
<td>0.14</td>
<td>8.67</td>
<td>19</td>
</tr>
<tr>
<td>Juvenile Oreochromis mossambicus</td>
<td>Whole body</td>
<td>27</td>
<td>2.5</td>
<td>6.2</td>
<td>16</td>
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<tr>
<td>C. picta bellii</td>
<td>Hepatocytes</td>
<td>22</td>
<td>0.028</td>
<td>3.9</td>
<td>20</td>
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<td>Clapea harengus larvae</td>
<td>Whole body</td>
<td>8</td>
<td>1</td>
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<td>Glyptonotus antarcticus</td>
<td>Whole body</td>
<td>0</td>
<td>5.06</td>
<td>147.5</td>
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<td>25.4±7.5*, 23.5±8.8†</td>
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<tr>
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<td>Not reported</td>
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<td>12.2±12.1‡</td>
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Where necessary, previously reported costs of protein synthesis were converted from ATP equivalents, or kilojoules per milligram of protein to micromoles O2 per milligram protein to allow comparison. In doing so, we have assumed an energetic conversion factor of 484 kJ of energy per mole of O2. CHX concentrations (CHX conc.) were converted to milligrams CHX per gram fresh weight (FW), presuming uniform distribution of the CHX in injected animals and the tissue concentration of CHX being the same as the bathing solution in bathed animals and cells. The study number refers to the publication source in the reference list. *Estivating; †nonestivating; ‡starved; §fed.

level of 4.2-fold in this study is similar to that reported previously in studies of N. concinna protein synthesis (5).

Costs of protein synthesis. The energetic cost of protein synthesis measured using the correlative technique was 19.58 μmol O2/mg protein, and the mean cost measured via cyclo-
heximide inhibition was 13.95 ± 0.77 μmol O2/mg protein. These values appear realistic and are ~235 and 168% higher, respectively, than the theoretical minimum cost of protein synthesis (8.3 μmol O2/mg protein). The correlation method is dependent upon the animals selected having a wide enough range of protein synthesis rates and oxygen consumptions to produce a significant correlation, and this was the case in the current study. The minimum theoretical cost of protein synthesis accounts only for the direct synthesis and transport costs of protein synthesis (31). The correlative method, on the other hand, should include associated processes (for example, RNA synthesis and peptide transport) and would therefore be expected to be higher than the minimum theoretical value. Although at present we cannot directly quantify most of these associated costs, RNA synthesis has been shown to account for up to 14% of oxygen consumption in tumor cells; therefore, these associated costs are likely to be significant (25). However, some caution in interpretation of the correlative results is important, as we have no evidence that changes in oxygen consumption are solely determined by changes in protein synthesis and its associated processes (36). The correlative method could over- or underestimate the apparent costs of protein synthesis if changes in oxygen consumption are the result of processes not associated with protein synthesis, or the rates of unassociated processes alter due to changes in protein synthesis or oxygen consumption. The intraspecific variability in A0 and oxygen consumption in Fig. 4 is the result of differences in limpet mass and nutritional status.
Estimates of the costs of protein synthesis made using cycloheximide, should be intermediate to the theoretical cost of protein synthesis and the correlative method, because cycloheximide only blocks peptide bond formation (37). In this study, protein synthesis cost estimates made using the correlative method are indeed higher than those made using cycloheximide inhibition. However, in many previously reported studies, this has not been the case (see Table 2).

Reported energetic costs of protein synthesis have varied widely, from 0.92 μmol O2/mg protein in larvae of the Antarctic echinoid Sterechinus neumayeri, to 495 μmol O2/mg protein in the fish Oncorhynchus mykiss (see Table 2 and Refs. 24 and 38). It might be expected that the cost of protein synthesis will vary slightly according to the measurement method used (see above), for example, reported energetic costs of protein synthesis measured using cycloheximide vary between 3.9 and 495 μmol O2/mg protein, and from 0.92 to 72 μmol O2/mg protein using the correlative method (16, 18, 24, 38). However, it seems highly unlikely that protein synthesis costs vary by two orders of magnitude in different species or in the same species under different environmental conditions. In particular, estimated costs of protein synthesis that fall well below the theoretical minimum value require explanation (24). The huge variability in reported costs of protein synthesis are, in all likelihood, at least partly a result of some studies failing to sufficiently validate methodologies, for example, by not minimizing the concentration of cycloheximide used, or ensuring handling or injecting the animals does not effect either protein synthesis rates or oxygen consumption.

The cost of protein synthesis in N. concinna reported here using the correlative method (19.58 μmol O2/mg protein) is over 20-fold higher than that reported for an Antarctic sea urchin embryo (0.92 μmol O2/mg protein), and over 10-fold higher than that reported for an Antarctic starfish embryo (1.9 μmol O2/mg protein) (24, 27). Currently, there are no other correlative estimates of the cost of protein synthesis in Antarctic organisms, although this method has been used in a number of nonpolar ectotherms (Table 2). Reported values have ranged from 5.34 μmol O2/mg protein to 39 μmol O2/mg protein. The cost of protein synthesis in the marine mussel Mytilus edulis was similar to that reported here (23.5 μmol O2/mg protein) (12). Among endotherms, protein synthesis was reported to cost between 7.4 and 31.6 μmol O2/mg protein in the pig (8, 30) and from 24 to 72 μmol O2/mg protein in the rat (2).

The mean cost of protein synthesis measured in N. concinna using cycloheximide was 13.95 μmol O2/mg protein and was independent of temperature. In the only other whole organism Antarctic study, a cost of 147.5 μmol O2/mg protein was reported in the isopod Glyptonotus antarcticus, considerably higher than either theoretical values, the results reported in this study, or those reported for most other nonpolar organisms (37). A cost of 7 μmol O2/mg protein has been reported in the Antarctic scallop (Adamussium colbecki) (35). However, this study examined in vitro protein synthesis in a cell-free system, and the results would be expected to be lower than those in whole cells or organisms, as the costs of amino acid transport for example, are not included (35). In summary, the protein synthesis costs estimated in the current study using two different methods appear to be similar to those reported in many nonpolar organisms and the single in vitro study of an Antarctic organism.

Reported estimates of the cost of protein synthesis measured using cycloheximide appear to vary to an unrealistic degree (Table 2). To examine whether the measured costs of protein synthesis were affected by the concentration of cycloheximide used in these studies, cycloheximide concentrations were standardized to the same units and plotted against the estimated protein synthesis costs (Table 2, Fig. 5). There was a significant relationship, suggesting a significant part of the considerable inter- and intraspecific variation observed in protein syn-
thesis costs is due to the use of excessive concentrations of cycloheximide (Fig. 5). The mechanism by which high cycloheximide concentrations further reduce oxygen consumption rates is currently unclear, but the most likely pathway is via secondary effects on other major cellular processes. This finding would strongly suggest that in studies investigating the cost of protein synthesis using cycloheximide, concentrations of the inhibitor should be kept to a minimum, and detailed validation studies should be carried out. It should also be noted that in any study that uses inhibitors, there is the possibility that other physiological processes could alter their utilization of ATP after the inhibitor blocks the targeted process. In turn, this could affect oxygen consumption and hence the cost estimate of the measured process (38).

The minimal theoretical costs of protein synthesis were used to estimate the proportion of oxygen consumption utilized for protein synthesis in N. concinna. Using the theoretical cost (8.3 μmol O₂/mg protein) of peptide bond formation, the percentage of oxygen consumption allocated to protein synthesis in N. concinna was 55 ± 2.1% and did not differ significantly between temperature groups. The proportion of oxygen consumption allocated to protein synthesis in different seasons has been previously estimated in N. concinna (5). The values obtained (February, 34%; July, 36%; October, 35%; December, 40%) were lower than in the present study, and varied little with season. In the cod (Gadus morhua), 44% of oxygen consumption is allocated to protein synthesis (23). It is likely that the proportion of oxygen consumption utilized for protein synthesis by an animal will vary according to age, sex, and nutritional status of the organism; in turn, this may explain much of the variation in these studies.

In the present study, groups of limpets were acclimated to two temperatures, 0°C and 3°C, the former representing a typical summer water temperature experienced by the animals, while the latter temperature is only likely to be experienced by limpets living in very shallow water, or intertidally (4, 5). There were no significant differences in the cost of protein synthesis between these temperature groups (Table 1), demonstrating that, over a limited range of temperatures, and in the single species employed in this study, the cost of synthesizing proteins appears independent of temperature.

Although the fraction of total oxygen consumption utilized for protein synthesis might be expected to vary seasonally, the cost of synthesis itself should be largely independent of temperature. There is no known mechanism for altering the stoichiometry of ATP use per peptide bond synthesized, although the cost of transport and other associated processes may vary slightly. We have therefore undertaken a meta-analysis of published data (Fig. 6). The analysis demonstrates that for the available protein synthesis cost data, estimated either via the correlative or inhibitor method, there is no significant effect of temperature. It therefore appears that whether an animal lives at polar water temperatures, or maintains an endothermic body temperature, the cost of making a unit of protein should not be affected by body temperature. However, it should be noted that there is considerable variability in the plotted data, and, as previously highlighted, some of the values plotted in this analysis should perhaps be interpreted with some caution. We feel further research is required in a range of species to conclusively establish whether the costs of protein synthesis are indeed independent of temperature in all animals. It is worth noting that the high levels of variability present in the analyzed data set will reduce the likelihood of detecting a significant relationship between temperature and protein synthesis.

In conclusion, we have rigorously validated the use of cycloheximide as an inhibitor to study the cost of protein synthesis. We have shown that the concentration of cycloheximide required to inhibit protein synthesis is considerably lower than that used in most studies and that high cycloheximide concentrations may affect processes other than protein synthesis. In turn, the use of reduced concentrations of cycloheximide should markedly reduce nonspecific effects. We have demonstrated that the measured cost of protein synthesis in an Antarctic limpet varies to a small degree and in the expected fashion, depending on the methodology used. The cost of protein synthesis measured using cycloheximide was less than that measured by the correlation of oxygen consumption and absolute protein synthesis. It appears that the energetic cost of synthesizing proteins in this polar ectotherm is neither greatly elevated nor proceeds at a level of greatly enhanced stoichiometric efficiency, as suggested by workers on other Antarctic species (37, 24). Recently, other authors using in vitro methods have also suggested that the cost of protein synthesis at polar water temperatures is not significantly different to that seen at other latitudes, and our data support this assertion (35). Furthermore, we have demonstrated within the constraints of the available data that the cost of protein synthesis appears to be independent of temperature across a wide range of animal groups.

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