The Protein Synthesis Machinery Operates at the Same Expense in Eurythermal and Cold Stenothermal Pectinids

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ABSTRACT

Translationally active cell-free systems from gills of the Antarctic scallop *Adamussium colbecki* and the European scallop *Aequipecten opercularis* were developed, characterised, and optimised for an analysis of translational capacity. The aim was to determine the energetic cost of protein synthesis in the in vitro cell-free system by directly measuring the required energy equivalents in the lysates. Protein synthesis rate in assays conducted with lysates of *A. colbecki* (1.029 ± 0.061 μmol Phe min^{-1} at 15°C; Phe = phenylalanine) were higher compared with lysates of *A. opercularis* (0.087 ± 0.013 μmol Phe min^{-1} at 15°C and 0.156 ± 0.023 μmol Phe min^{-1} at 25°C). This can in part be attributed to the naturally occurring higher RNA content in lysates of *A. colbecki* (0.883 ± 0.037 mg RNA mL^{-1} lysate) compared with *A. opercularis* (0.468 ± 0.013 mg RNA mL^{-1} lysate). There was no significant difference in the energetic costs of protein synthesis in cell-free systems of gill lysates of the cold stenothermal *A. colbecki* with 4.3 ± 0.7 energy equivalents per peptide bond formed and the eurythermal *A. opercularis* with 5.6 ± 0.6 energy equivalents, indicating that there are no differences in the efficiency of the translation machinery. The energetic costs specified for protein synthesis correspond with the generally accepted theoretical value of four energy equivalents per peptide bond formed, especially in gill lysates of *A. colbecki*, whereas the value for gill lysates of *A. opercularis* was slightly higher.

Introduction

Over the last decades, the concept of metabolic cold adaptation, originally used to explain early findings of relatively high metabolic rates in Antarctic fish (Scholander et al. 1953; Wohlschlag 1960), has gradually been disproved by measurements of oxygen uptake in a number of Antarctic and Arctic invertebrates and fish under relatively stress-free conditions (Holeton 1974; White 1975; Ralph and Maxwell 1977*a*, 1977*b*; Peck et al. 1987; Chapelle and Peck 1995; Whiteley et al. 1996; Clarke and Johnston 1999; Marsh et al. 2001). It now appears that living in the permanent cold, especially of the Antarctic, is associated with low metabolic rate and, thus, less costly than living in warmer water. The low rates of oxygen demand at low temperature are likely related to reduced protein turnover, ion pump activity, and other aspects of basal metabolism (Clarke 1991). As a precondition, the stoichiometric costs for all of these processes should remain the same; for example, at reduced rates of protein turnover, ATP cost of protein synthesis should also be reduced.

At present, however, studies of the energetic costs of protein synthesis in marine ectotherms and especially in invertebrates living in the southern ocean have led to controversial results. A high protein turnover associated with low metabolic rate was measured in Antarctic sea urchin embryos in vivo, resulting in a synthesis cost of 0.45 J mg $protein^{-1}$, which is only 4% of the value reported for other marine invertebrates (Marsh et al. 2001). These costs were calculated from a linear regression of total respiration rates versus protein synthesis rates during development. This method produces a global estimate of protein synthesis costs because it includes the energy requirements for overall protein turnover, that is, for transcription, transport of mRNA and amino acids to the location of translation, as well as mRNA and protein degradation. However, this estimate must be considered low because from a strictly biochemical point of view, an average use of four to five ATP molecules per peptide bond is assumed (Buttery and Boorman 1976; Fuery et al. 1998). Neglecting the other costs of protein turnover, the energy cost of 0.45 kJ g^{-1} protein synthesis as determined for Antarctic sea urchin embryos corresponds to a cost of 0.5–0.6 ATPs per peptide bound, depending on the conversion factor used (e.g., five ATPs per peptide bond convert to 3.56 kJ g^{-1} protein synthesised [Aoyagi et al. 1988]; five ATPs convert to 4.5 kJ g^{-1} protein [Fuery et al. 1998]).

Whiteley et al. (1996) found low metabolic rates in the Antarctic isopod *Glyptonotus antarcticus* compared with the temperate *Idotea rescata*. In contrast to Marsh et al. (2001), they observed a low protein synthesis rate associated with relatively high energetic costs of protein synthesis in adult specimens. The ATP requirements were four times higher in *G. antarcticus*

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at 885 ± 141 mmol ATP g^{-1} protein than in the eurythermal *I. rescata* (237 ± 76 mmol ATP g^{-1} protein). In the study of isopods, inhibition of protein synthesis by cycloheximide and its effect on oxygen consumption were used to evaluate the cost of protein synthesis, which was measured by injection of a single dose of a radiolabeled amino acid according to the flooding dose method (Garlick et al. 1980). The application of cycloheximide in vivo has long been considered to yield specific inhibition and to provide the "true" costs of protein synthesis. However, recent work by Wieser and Krumschnabel (2001) distinctively demonstrates the variability of cost estimates depending on the concentration of cycloheximide used. Cycloheximide seems not to be a specific inhibitor since it is known to affect the membrane transport of potassium (Reilly et al. 1970) and glucose (Evans 1971). Furthermore, Whiteley et al. (1996) could not obtain complete inhibition of protein synthesis in *G. antarcticus*; possibly, the injected cycloheximide was not evenly distributed in the whole body. In conclusion, this method likely leads to an overestimation of the energetic costs (Aoyagi et al. 1988; Wieser and Krumschnabel 2001). Assuming an average molecular weight of 110 g mol^{-1} amino acids, the consequential costs would be 97 ATPs per peptide bond for *G. antarcticus* and 26 ATPs per peptide bond for *I. rescata* (Whiteley et al. 1996).

Oxygen consumption is a complex summation of many processes so that the actual rate of ATP turnover measured in whole animals by far exceeds the theoretical value associated with peptide bond formation. Accordingly, there are major problems with the precision of cost estimates based on whole animal or cellular metabolic rate. Differences (up to fivefold) have been found between the energy cost of protein synthesis estimated directly from the stoichiometry of bond formation and the physiological measurements of the global energy cost of protein synthesis (Waterlow and Millward 1989).

As a solution, the actual cost of protein synthesis can be measured directly in in vitro systems, as was done in this study. In investigations of the more mechanistic aspects, in vitro models have the advantage of ensuring standardised, physiological conditions for between-species comparisons of the isolated translation machinery. It has long been accepted that the protein synthesis in cell-free extracts is regulated in a way very similar, if not identical, to intact cells (Jackson 1982; Simon 1987). In vitro rates of protein synthesis can be set to levels similar to or in excess of those found in vivo through extensive optimisation of reaction parameters (Patnaik and Swartz 1998). Lopo et al. (1989) described cell-free systems for the sea urchin *Strongylocentrotus purpuratus* that displayed many features and characteristic responses typical of intact eggs and embryos.

In this study, we developed, characterised, and optimised translationally active cell-free systems from gills of the Antarctic scallop *Adamussium colbecki* L. and the European scallop *Aequipecten opercularis* (Smith). The aim was to determine the energetic cost of protein synthesis in the optimised in vitro cell-free system by directly measuring the required energy equivalents in the crude lysates, a newly developed approach that has, in general, not yet been used to study the cost of protein synthesis.

The cold stenothermal *A. colbecki* lives in a narrow temperature range between −1.8° and +2.5°C, whereas the eurythermal *A. opercularis* is found between temperatures of 8° and 24°C. These species have been chosen for their close phylogenetic relationship (Canapa et al. 2000) and equal lifestyle (Stockton 1984) but different ranges of thermal tolerance. The Antarctic *A. colbecki* is particularly interesting because this most conspicuous and circumpolar species is a relatively fast-growing bivalve (Berkman 1990; Chiantore et al. 2001). The translation system has been isolated from the gill because in aquatic invertebrates, gills are among the most active tissues with respect to protein turnover (for review, see Lyndon and Houlihan 1998). Especially the protein synthesis machinery of the gill tissue of this fast-growing, cold stenothermal scallop with its long evolutionary history should be highly adapted to the very low "operating temperatures" in Antarctica and should allow identification of functional differences and differences in the energy requirements of protein synthesis between temperate and polar species. This study demonstrates that the energetic costs to synthesise protein are equal in gill tissues of both pectinids despite different temperatures, which suggests that cold adaptation occurs at the level of increased protein stability, leading to a decrease in overall turnover rates at no increase in the stoichiometric costs of protein synthesis.

Material and Methods

Animals

Aequipecten opercularis were caught at 80 m water depth around Roscoff (48°51′00N, 3°54′00W) in the Western English Channel by fishermen of the Station Biologique de Roscoff in October 1999. After a short period of aquarium maintenance, animals were returned to the Alfred Wegener Institute in Germany, where they were kept in well-aerated, recirculating seawater at 10° ± 1°C and 34 practical salinity units (PSU) for at least 6 d before experimentation. *Adamussium colbecki* were collected from 50–80 m water depth at Road Bay (74°41′91S, 164°07′72E) near Terra Nova Bay, Antarctica, in February 2000. They were maintained in running seawater aquaria at 0.0° ± 0.5°C and 34 PSU at Terra Nova Bay station. They were allowed an acclimation period of 6 d before experiments. Both species were not fed during aquarium maintenance. All experiments were carried out within 3 wk after catching. *Aequipecten opercularis* specimens had a mean size of 4.30 ± 0.15 cm in length and 4.28 ± 0.13 cm in height and *A. colbecki* of 4.42 ± 0.21 cm in length and 4.65 ± 0.17 cm in height.

Preparation of Gill Lysates

The preparation of lysates from gill tissue of *A. opercularis* and *A. colbecki* followed the same procedure. Adductor muscles were dissected, and gills were quickly excised and weighed. Gills of two animals were pooled to provide enough tissue for all measurements. Half of the gill tissue was freeze clamped in liquid N_2 by means of a precooled Wollenberger clamp (Wollenberger et al. 1960) for later examination of the amino acid (AA) composition in tissue protein. The other half was immediately used for the preparation of cell-free protein synthesis systems. Approximately 500–800 mg of gill tissue was transferred to a loosely fitting 2-mL glass homogeniser and homogenised with five strokes in 1 volume of ice-cold extraction buffer containing 100 mM Hepes, 880 mM sucrose, 120 mM potassium acetate, 10 mM magnesium acetate, 7 mM 2-mercaptoethanol, and 30 mM dithiothreitol (DTT). The extraction buffer had been adjusted to pH 7.13 at 25°C. DTT and mercaptoethanol are reductive agents that prevent inhibition of initiation in lysates and counteract the oxidation of certain critical −SH groups to disulphide bonds (Jackson 1982; Kawarasaki et al. 1998). The homogenate was centrifuged at 16,000 *g* for 30 min at 0°C, and the resulting supernatant was carefully separated from the pellet and used as the lysate. Only freshly prepared lysates were used and were kept on ice before starting the in vitro translation assays. Aliquots of the lysate were frozen in liquid nitrogen for later determination of RNA and endogenous phenylalanine (Phe).

For fully active lysates, work was consistently carried out in a scrupulously clean environment (under a clean bench if available) with the use of baked glassware, sterile, autoclaved plastic ware, and sterile solutions. Water required for the preparation of lysate and assay solutions was treated to inactivate RNAse activity by adding 1 mL of diethylpyrocarbonate (DEPC) per litre of distilled water, stirring for half an hour, and then autoclaving.

Cell-Free in Vitro Translation Assays

The buffer composition and osmolarity of the cell-free system was created to compensate for the dilution occurring on lysis of the gill tissue and to mimic the intracellular fluid of marine invertebrates. Translation was measured in assays containing 30 μL of lysate and 120 μL of assay medium. The final volume of 150 μL contained assay buffer (concentration in the assay: 100 mM Hepes, sodium salt, 85 mM taurine, 150 mM glycine, 120 mM potassium acetate, and 5 mM magnesium acetate, adjusted to pH 7.13 at 25°C), 5 mM DTT, 0.2 mM spermidine, all amino acids except Phe at 0.1 mM, 60 μCi of [2,3,4,5,6-^{3}H] Phe (Amersham, 116 Ci mmol^{-1}, 1 mCi = 37 MBq), 4.9 units arginine kinase, and 100 units RNasin ribonuclease inhibitor (Promega) with a fivefold dilution of further tissue ions. The ubiquitously distributed polyamine spermidine has been found in cells of marine bivalves (Gasparini and Audit 2000) and has a stimulatory effect on polypeptide synthesis in vitro and in vivo (Giannakouros et al. 1990; Monnier et al. 2001). Apart from spermidine, it was important to add labeled and unlabeled amino acids and the energy equivalents phospho-L-arginine (PLA), ATP, and GTP to avoid substrate limitation and to yield high protein synthesis rates.

Unless stated otherwise, substrates of the translation process were used in the following optimised concentrations: 8 μM Phe (including [2,3,4,5,6-^{3}H] Phe), 15 mM PLA, 1 mM ATP, and 0.5 mM GTP. Before starting the translation assay with 30 μL lysate, the reaction mixture was preincubated for 5 min in a water bath set at the experimental temperature of 15°C for *A. colbecki* and 15°C or 25°C for *A. opercularis*. After the addition of lysate, which was considered as time zero, the assay was briefly mixed on a Vortex mixer, quickly subdivided into aliquots of 20 μL, and returned to the water bath, which was set to 15°C or 25°C, respectively. Reactions were run for given time periods and were terminated by adding 2 μL pancreatic RNAse (25 units mL^{-1}), followed by an additional 5 min of incubation at the experimental temperature. Then assays were placed on ice. Subsequently, 19-μL samples were pipetted onto Phe-saturated, semiwet Whatman GF/C filters (quenched in 5 mM Phe for 1 h to minimise nonspecific binding of [2,3,4,5,6-^{3}H] Phe). Filters were placed in ice-cold 10% TCA, containing 5 mM Phe, for 5 min to precipitate the synthesised protein onto the filter surface. The filters were then washed in ice-cold 10% TCA for 10 min and were washed twice in ice-cold 5% TCA for 15 min, followed by one wash in 95% ethanol at room temperature. The filters were allowed to dry in air before dissolving them in 5-mL scintillation cocktail (Packard, 57% tritium counting efficiency).

Radioactivity in the precipitated protein was determined by liquid scintillation counting. Also, a zero control, which contained RNAse right from the beginning of the assay to prevent protein synthesis, was used to correct for background due to nonspecific binding of [2,3,4,5,6-^{3}H] Phe to components of the lysate. The background averaged to 550 ± 50 disintegrations per minute (DPM) for lysates prepared from *A. colbecki* and to 400 ± 47 DPM for *A. opercularis*. There was no nonspecific incorporation over time, and background values were subtracted from the total counts obtained at each time point. Results were expressed as [2,3,4,5,6-^{3}H] Phe incorporated into trichloroacetic acid–precipitable protein (DPM 19 μL assay^{-1}). With each radioactive translation assay, two nonradioactive assays were run simultaneously under identical conditions with nonradioactive Phe. After the given time periods, samples were immediately frozen in liquid nitrogen. One of the nonradioactive assays was run with and one without RNAse from the outset of the translation assay. PLA, ATP, and GTP concentrations were monitored over time. These data sets were used to quantify the energy demand of protein synthesis and to correct for background ATP consumption within the assay.

Analytical Methods

The concentrations of PLA, ATP, and GTP in samples collected from the nonradioactive assays were measured using a capillary electrophoresis method described by Casey et al. (1999). Total RNA in lysates was hydrolysed by alkaline exposure according to the Schmidt-Tannhauser procedure and was quantified by the dual-wavelength procedure of Munro and Fleck (1966). For relating the RNA concentration to the absorbance at these two wavelengths, the modified, more suitable formula, RNA (μg mL^{-1}) = 32.9 × A260 − 6.11 × A232, was used as suggested by Ashford and Pain (1986). The free, endogenous Phe levels in lysates obtained from both species were measured by RP-HPLC in the lab Dr. Haase-Aschoff (Bad Kreuznach, Germany). Total AA composition of tissue protein was determined by acidic hydrolysis followed by HPLC analysis (Dr. Wiertz-Dipl. Chem. Eggert-Dr. Joerissen GmbH, Hamburg, Germany).

Calculations

For accurate determination of the energetic costs, protein synthesis rates were elevated by increasing the temperature to 15°C above the acclimatization temperatures of the two species. Thus, calculations of AA incorporation rates and PLA consumption rates were made using the initial, linear intercept of the curves at 15°C for *A. colbecki* and at 25°C for *A. opercularis*. AA incorporation rates were expressed as DPM 19 μL $assay^{-1}$ and PLA consumption rates as mM^{-1}. All AA incorporation rates were corrected for differences in [2,3,4,5,6-^{3}H] specific activity and expressed as pmol Phe 19 μL $assay^{-1}$ min^{-1}. AA incorporation rates depending on various PLA, ATP, and GTP levels were further converted to percent fractional values of the respective optimised concentration in order to facilitate comparison between different translation efficiencies of assay mixtures due to variable tissue pulping.

The energetic costs of AA incorporation were determined for each individual translation assay and were calculated from the measured absolute concentrations of high-energy phosphates. Therefore, the specific radioactivity, expressed as Bq pmol Phe^{-1} (1 DPM = 1/60 Bq), of each assay was calculated from the amount of added radioactive and nonradioactive Phe in the assay plus the measured endogenous Phe of the lysates. The specific radioactivity was used to convert incorporation rates of Phe (DPM min^{-1}) into mM Phe min^{-1}. PLA consumption rates due to protein synthesis were calculated from the difference between rates found in the two nonradioactive sets. Energy equivalents consumed per peptide bond formed were calculated from the ratio of PLA consumption (mM PLA min^{-1}) to Phe incorporation (mM Phe min^{-1}). Subsequently, the values were normalised for differences in total Phe content in tissue protein of both species and then expressed as PLA consumed per AA.

Statistics

All data are expressed as means ± SE unless stated otherwise. Numbers (n) of determinations are given in parentheses or figure legends. Statistical differences at the 5% level were tested using ANOVA or ANCOVA followed by the Student-Newman-Keuls post hoc test for independent samples. The slopes of regression lines of the ribonuclease treated and untreated assays were compared by use of an F-test.

Results

Characterization of Gill Protein and Lysates

The amino acid composition of gill protein from *Aequipecten opercularis* and *Adamussium colbecki* is specified in Table 1. There was no significant difference between species in protein contents of gill tissues (4.44 ± 0.27 g 100 g^{-1} in *A. opercularis* and 4.43 ± 0.22 g 100 g^{-1} in *A. colbecki*) and the percentage of Phe within the protein (3.83% ± 0.18% and 3.84% ± 0.04%). Despite identical protocols used for both species, endogenous Phe levels and RNA content in lysates of *A. colbecki* (0.88 ± 0.04 mg RNA mL^{-1}; 0.29 ± 0.05 mM Phe) were significantly higher than in lysates of *A. opercularis* (0.47 ± 0.01 mg RNA mL^{-1}; 0.11 ± 0.01 mM Phe; Table 2). This indicates higher levels of RNA and free Phe in the Antarctic species.

Optimum Assay Conditions

It was important to use identical assay conditions for the two species to compare the energetic costs of in vitro protein synthesis of gill. Therefore, the same assay buffer was used. Hepes is a commonly used buffer substance in cell-free protein synthesis and is particularly suitable because it causes pH in the assay buffer to change according to the preferred value of −0.015 pH $°C^{-1}$. According to the alpha-stat hypothesis, such a pH shift ensures a constant net charge of protein at the different assay temperatures of 15°C for *A. colbecki* and 15°C and 25°C for *A. opercularis* (Cameron 1989).

Cell-free translation systems of both species were optimised for maximum protein synthesis rates and more accurate calculations of energy demand at 15°C for *A. colbecki* and 25°C for *A. opercularis*. Various Phe, PLA, ATP, and GTP levels were applied in assays conducted with lysates of *A. opercularis* at 25°C and subsequently tested for lysates of *A. colbecki* at 15°C (insets in Figs. 1, 2). In vitro protein synthesis rates of both cell-free systems reached saturation at 3 μM Phe and remained constant up to 8 μM Phe (Fig. 1). Figure 2 depicts the Phe incorporation rate into protein as a function of PLA (Fig. 2*A*), ATP (Fig. 2*B*), and GTP (Fig. 2*C*). Protein synthesis rate was gradually stimulated up to a concentration of 15 mM PLA and was rapidly inhibited at higher PLA levels. A significant loss of protein synthesis rates by 35% occurred at a PLA concentration of 30 mM in both cell-free systems. Due to the presence of

Table 1: Concentrations of amino acids in protein obtained from gill tissue of *Aequipecten opercularis* and of *Adamussium colbecki*

	Gill Protein (g 100 g^{-1} gill tissue)	
Amino Acids	*Aequipecten opercularis*	*Adamussium colbecki*
Cysteine acid	ND	.03 ± .01
Aspartate	.39 ± .03	.39 ± .01
Hydroxy-proline*	.09 ± .01	.04 ± .01
Threonine	.19 ± .01	.20 ± .02
Serine	.17 ± .02	.18 ± .01
Glutamate	.65 ± .06	.64 ± .03
Proline	.31 ± .04	.29 ± .02
Glycine	.55 ± .02	.60 ± .07
Alanine	.21 ± .01	.21 ± .01
Valine	.22 ± .02	.22 ± .01
Cysteine*	.04 ± .00	.02 ± .01[a]
Methionine*	.11 ± .01	.09 ± .01
Isoleucine	.19 ± .00	.20 ± .02
Leucine	.27 ± .02	.28 ± .01
Tyrosine*	.15 ± .02	.12 ± .01
Phenylalanine	.17 ± .01	.17 ± .01
Hydroxy-lysine	.02 ± .01	.03 ± .01[b]
Ornithin	.01[c]	.01[c]
Lysine	.29 ± .02	.29 ± .01
Histidine	.11 ± .01	.11 ± .01
Arginine	.30 ± .02	.31 ± .03
Total	4.44 ± .27	4.43 ± .22
% fraction of Phe	3.83 ± .18	3.84 ± .04

Note. The percent fraction of Phe within the protein was calculated from the ratio of phenylalanine content over the sum of amino acids multiplied by 100. Data are given as means ± SD (n = 5). ND = not detectable.

[a] Only three detectable values out of five determinations.

[b] Only four detectable values out of five determinations.

[c] Only one detectable value out of five determinations.

* Indicates a significant difference ($P < 0.05$) between species.

endogenous PLA in the lysates, the absolute concentration of PLA in optimised assays was 17.49 ± 0.35 mM in *A. colbecki* and 16.43 ± 0.43 mM in *A. opercularis*. When either ATP or GTP was omitted, a reduced incorporation rate of Phe into protein was observed (Fig. 2*B*, 2*C*). Protein synthesis rates in both cell-free systems were maximised in the presence of 0.75 mM ATP and 0.5 mM GTP, respectively. On the basis of these results, further cell-free translation assays were conducted using 8 μM Phe, 15 mM PLA, 1 mM ATP, and 0.5 mM GTP. The average specific radioactivity calculated from the amount of endogenous and added Phe was 0.242 ± 0.032 Bq $pmol^{-1}$ Phe (n = 5) for assays conducted with lysates from *A. colbecki* and 0.512 ± 0.043 Bq $pmol^{-1}$ Phe (n = 5) for assays of *A. opercularis*.

In Vitro Phenylalanine Incorporation

Figure 3 shows the time course of [2,3,4,5,6-^{3}H] Phe incorporation into protein at 15°C by lysates prepared from *A. colbecki* and at 15°C and 25°C by lysates prepared from *A. opercularis* under optimised conditions. Lysates prepared from *A. colbecki* exhibited constant rates of incorporation for the first 5–6 min. The reaction slowed down thereafter and approached completion in an asymptotic manner. In contrast, amino acid incorporation rates by lysates from *A. opercularis* at 15°C remained constant for 26 min and at 25°C for about 17 min, followed by a progressive reduction. Furthermore, the in vitro protein synthesis rate of gill lysates prepared from *A. colbecki* gills (284 ± 27 DPM 19 μL $assay^{-1}$ min^{-1}, n = 5) measured at 15°C was 5.5-fold higher compared with gill lysates from *A.*

Table 2: Characteristics of cell-free lysates prepared from the gills of *Aequipecten opercularis* and of *Adamussium colbecki*

Species	Tissue	Total RNA* (mg mL^{-1})	Free Phe* (mM)
Aequipecten opercularis	Gill	.47 ± .01 (n = 6)	.11 ± .01 (n = 5)
Adamussium colbecki	Gill	.88 ± .04 (n = 6)	.29 ± .05 (n = 5)

Note. All values are means ± SE.

* Indicates a significant difference ($P < 0.05$) between species.

opercularis (51 ± 3 DPM 19 μL $assay^{-1}$ min^{-1}, n = 5) at the same temperature, which was threefold higher than in *A. colbecki* at 25°C (91 ± 7 DPM 19 μL $assay^{-1}$ min^{-1}, n = 5). Finally, when we consider the specific radioactivity of 0.242 ± 0.032 Bq pmol Phe^{-1} and 0.512 ± 0.043 Bq pmol Phe^{-1} (1 DPM = 1/60 Bq), respectively, the discrepancy between Phe incorporation rates results are even higher, with 1.029 ± 0.061 μmol Phe min^{-1} (n = 5) in assays conducted with *A. colbecki* lysates and 0.087 ± 0.013 μmol Phe min^{-1} (n = 5) for *A. opercularis* lysates at 15°C (0.156 ± 0.023 μmol Phe min^{-1}, n = 5, at 25°C).

Cost of in Vitro Protein Synthesis

Further experiments were conducted to determine the energy requirements of amino acid incorporation into protein. PLA and arginine kinase were used in both cell-free systems to compensate for ATP and GTP hydrolysis over time. While ATP is directly generated from PLA by arginine kinase, GTP is resynthesised at the expense of ATP in the presence of endogenous nucleoside monophosphate kinases or nucleoside diphosphate kinases. Kawarasaki et al. (1995) demonstrated in a cell-free system using wheat germ extract that GTP is regenerated as long as the ATP level is kept at its initial concentration by the ATP regeneration system phospho-L-arginine and arginine kinase. The efficiency of the PLA regeneration system is demonstrated by constant ATP and GTP concentrations in ribonuclease treated and untreated assays (Fig. 4*A*, 4*B*). Thus, energy requirements could be calculated from the decrease in PLA levels. However, it is well known that, in addition to the energy-consuming protein synthesis reactions, endogenous protein kinase and phosphatase activities are responsible for further use of ATP in translation assays (Nakano et al. 1996; Kim and Swartz 2000). For adequate consideration of this background ATP turnover, it was required to measure PLA depletion in parallel assays conducted with and without ribonuclease. In both species, addition of ribonuclease resulted in significantly lower rates of PLA degradation than in assays where protein synthesis was not inhibited (Fig. 5*A*, 5*B*). Also, ribonuclease caused complete inhibition of [2,3,4,5,6-^{3}H] Phe incorporation in both cell-free systems (data not shown). Therefore, the difference in PLA use between ribonuclease treated and untreated assays could be exclusively attributed to protein-synthesizing reactions.

Protein synthesis accounted for 59% ± 5% of total PLA degradation (0.971 ± 0.142 mM PLA) in cell-free translation assays of *A. colbecki*, whereas in assays of *A. opercularis* only 20% ± 2% of total PLA use (0.586 ± 0.072 mM PLA) could be ascribed to protein synthesis. Translation-dependent PLA degradation in *A. colbecki* and *A. opercularis* assays amounted to 0.115 ± 0.022 mM min^{-1} and 0.023 ± 0.003 mM min^{-1}, respectively (see also regression slopes in Fig. 5*A*, 5*B*). The energy consumed by protein synthesis was calculated for each lysate from the ratio of PLA degradation per minute and Phe incorporation per minute and then corrected for the fraction of Phe within the protein (*A. colbecki* at 15°C: 0.115 ± 0.022 mM PLA min^{-1}/1.029 ± 0.061 × 10^{-3} mM Phe min^{-1} × 0.0384; *A. opercularis* at 25°C: 0.023 ± 0.003 mM PLA min^{-1}/0.156 ± 0.023 × 10^{-3} mM Phe min^{-1} × 0.0383). The same calculation procedure

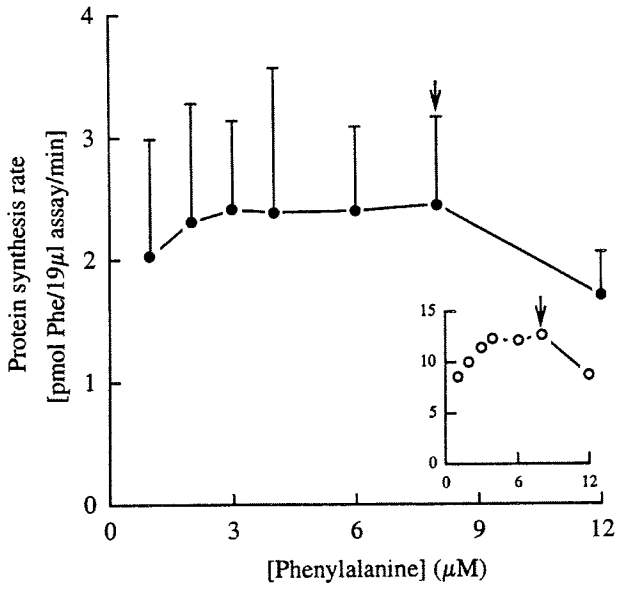

Figure 1. Rates of [2,3,4,5,6-^{3}H] Phe incorporation into protein by cell-free lysates prepared from gills of *Aequipecten opercularis* at 25°C (*filled symbols*) and *Adamussium colbecki* at 15°C (*open symbols*, inset) as a function of Phe. Incorporation rates are expressed as pmol Phe 19 μL $assay^{-1}$ min^{-1}. The arrow identifies the optimal concentration (8 μM). Each data point represents the mean ± SE (n = 3 for *A. opercularis*; n = 1 for *A. colbecki*).

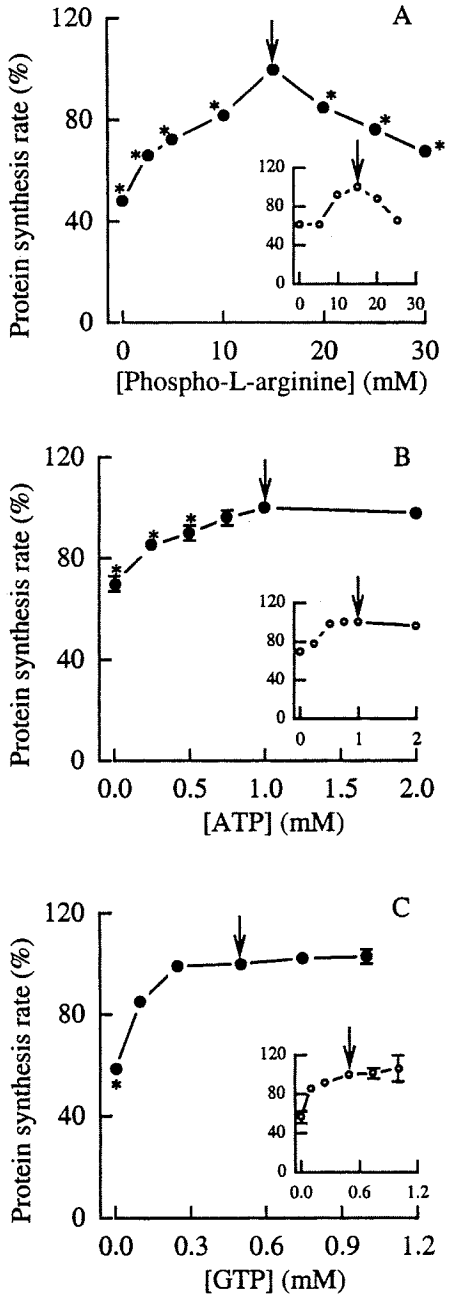

Figure 2. Rates of [2,3,4,5,6-^{3}H] Phe incorporation by cell-free lysates prepared from gills of *Aequipecten opercularis* at 25°C (*filled symbols*) and *Adamussium colbecki* at 15°C (*open symbols*, inset) as a function of phospho-L-arginine (*A*), ATP (*B*), and GTP (*C*). Incorporation rates are expressed as a percent fraction of the optimal concentration (*A*, 15 mM PLA; *B*, 1 mM ATP; *C*, 0.5 mM GTP) identified by the arrow. Each data point represents the mean ± SE (*A–C*, $n = 3$ for *A. opercularis*; *A*, *B*, $n = 1$ for *A. colbecki*; *C*, $n = 3$ for *A. colbecki*). An asterisk indicates a significant difference ($P < 0.05$) from the optimal concentration.

as for PLA was carried out for ATP and GTP (Fig. 4*A*, 4*B*). There was no significant difference between ribonuclease treated and untreated assays and, thus, no ATP and GTP consumption per peptide bond formed if compared with PLA (Table 3). With 5.6 ± 0.6 molecules PLA consumed per peptide bond formed in *A. opercularis* and 4.3 ± 0.7 molecules PLA in *A. colbecki*, there was no significant difference in the costs for protein synthesis between the two species.

Discussion

Methodology

The aim of this study was to investigate the energy requirements for protein synthesis in two pectinid species adapted to different ambient temperatures. In order to overcome the difficulties to estimate protein synthesis costs from whole-organism oxygen consumption rates, the translation machinery was isolated and energy equivalents used per peptide bond formed were directly determined. Crude, postmitochondrial lysate was used because the plethora of low molecular weight compounds utilised for protein synthesis, which are eventually specific to the translation apparatus of the two pectinids and, thus, could change the energetic costs, had to be retained unchanged.

High initial protein synthesis rates have been achieved in cell-free translation systems of both pectinid species by elevating the temperature and optimising the assay composition. There was no thermal deterioration of protein synthesis capacity at the experimental temperatures as shown in a study on the temperature dependence of in vitro protein synthesis (D. Storch

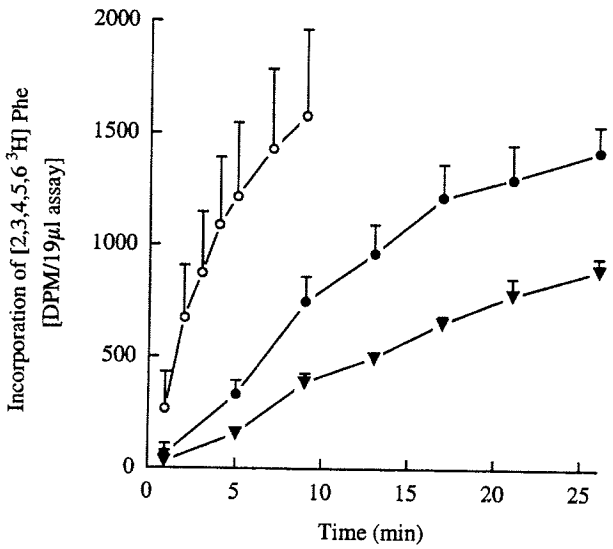

Figure 3. Time course of incorporation of [2,3,4,5,6-^{3}H] Phe into trichloroacetic acid–precipitable protein by cell-free lysates prepared from gills of *Aequipecten opercularis* (*filled circles*, 25°C; *filled triangles*, 15°C) and *Adamussium colbecki* (*open circles*, 15°C). Each data point represents the mean ± SE ($n = 5$).

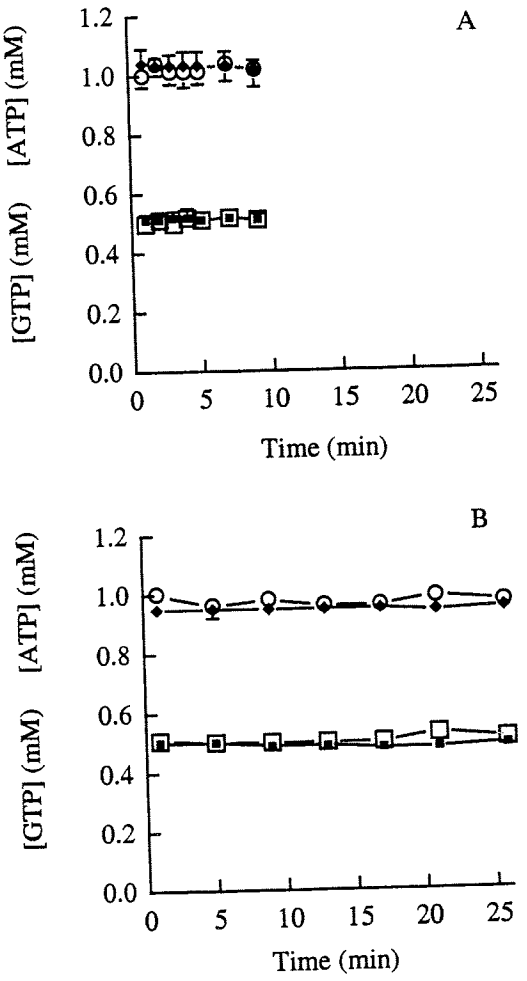

Figure 4. Concentrations of ATP and GTP as a function of time in a cell-free protein synthesis assay prepared from gills of *Adamussium colbecki* at 15°C (*A*) and of *Aequipecten opercularis* at 25°C (*B*). *Open circles*, ATP in assays active in protein synthesis; *filled rhombuses*, ATP in assays treated with ribonuclease; *open squares*, GTP in assays active in protein synthesis; *filled squares*, GTP in assays treated with ribonuclease. Data are presented as means ± SE (n = 5). No significant changes occurred over time.

in assays conducted with lysates of *Adamussium colbecki* compared with lysates of *Aequipecten opercularis* can be attributed at least in part to the naturally occurring higher RNA content in lysates of *A. colbecki*.

The period of initially high activity remains short followed by a progressive reduction of the translation rate. This is a general phenomenon occurring in cell-free systems and is usually accounted for by the quick depletion of substrates such as ATP, GTP, and amino acids and/or RNA degradation (Spirin

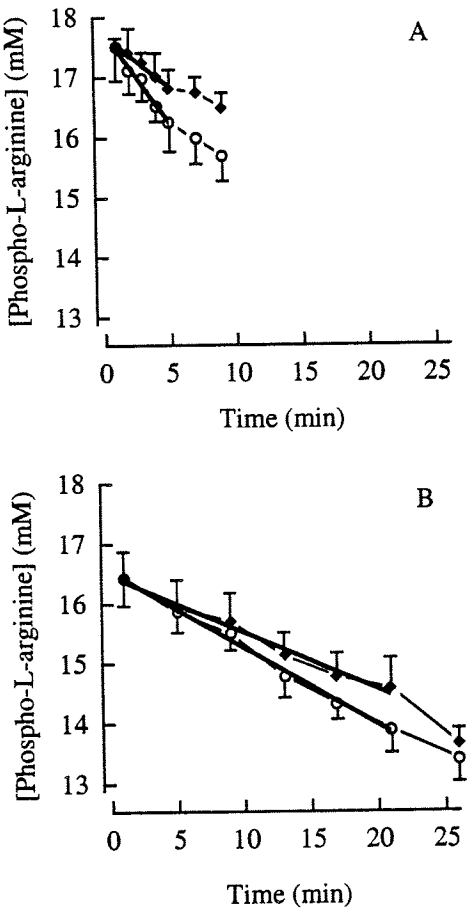

Figure 5. Concentrations of phospho-L-arginine (PLA) in a cell-free protein synthesis assay prepared from gills of *Adamussium colbecki* at 15°C (*A*) and of *Aequipecten opercularis* at 25°C (*B*). *Open circles*, PLA in assays active in protein synthesis; *filled rhombuses*, PLA in assays treated with ribonuclease. Data are presented as means ± SE (n = 5). Regression slopes are for (*A*) *A. colbecki*: $m = -0.079 \pm 0.015$ mM PLA min^{-1} (with ribonuclease), $m = -0.194 \pm 0.028$ mM PLA min^{-1} (without ribonuclease). The level of translation-dependent PLA degradation results as $-0.079 \pm 0.015 - (-0.194 \pm 0.028) = 0.115 \pm 0.022$ mM min^{-1}. For (*B*) *A. opercularis*: $m = -0.094 \pm 0.013$ mM PLA min^{-1} (with ribonuclease), $m = -0.117 \pm 0.014$ mM PLA min^{-1} (without ribonuclease). Translation-dependent PLA degradation results as $-0.094 \pm 0.013 - (-0.117 \pm 0.014) = 0.023 \pm 0.003$ mM min^{-1}.

and H.O. Pörtner, unpublished observation). Isolating the translation machinery with its endogenous mRNA level is a snapshot of the cell. An increase of temperature in the in vitro system merely accelerates the velocity of biochemical reactions until a temperature optimum is reached. Brosnan et al. (1976) also found that amino acid incorporation directed by endogenous mRNA of postmitochondrial supernatant from skate liver was maximal at a temperature 15°C above ambient. Thermal stability has also been demonstrated for other cell-free systems, for example, prepared from liver of Antarctic fish (Haschemeyer and Williams 1982) or from different organs of rainbow trout (Simon 1987). The higher protein synthesis rate

Table 3: Calculated energy requirements for protein synthesis in cell-free translation assays prepared from gills of *Aequipecten opercularis* and of *Adamussium colbecki*

Energy Equivalents	*Aequipecten opercularis*	*Adamussium colbecki*
PLA	5.6 ± .6	4.3 ± .7
ATP	.3 ± .5	−.3 ± .4
GTP	−.3 ± .1	.0 ± .0

Note. Values represent molecules consumed per peptide bond formed. All values are means ± SE (n = 5).

et al. 1988; Kawarasaki et al. 1995, 1998; Kang et al. 2000). Furthermore, it has been shown that the accumulation of inorganic phosphate due to net depletion of high energy phosphates has an inhibitory effect on in vitro translation (Nakano et al. 1996; Kim and Swartz 2000).

In this study, substrate depletion is not likely the reason for the progressive decrease of protein synthesis rates because ATP and GTP levels remained high and addition of higher Phe levels could not prolong protein synthesis decisively. Some RNA degradation is inevitable but will also not be the reason for the distinct decrease of protein synthesis rate after a short time. For the protection of endogenous RNA, the common and effective ribonuclease inhibitor RNAsin was included in the assay (Lopo et al. 1989; Moreno et al. 1991; Hofmann and Hand 1994).

The early decline of translational activity may be a result of decreasing free magnesium levels in the assays. Magnesium is of primary importance in cellular function because only the Mg^{2+}-adenylate complex forms the true substrate with most enzymes (Aikawa 1981; Pörtner 1990). Furthermore, it plays an important role in the stability and activity of the components of cell-free systems (Araya and Krauskopf 1976; Amthauer and Krauskopf 1979). According to some authors, progressively enhanced Mg^{2+} complexation in cell-free systems during the course of in vitro protein synthesis induces a halt of protein synthesis (Giannakouros and Georgatsos 1988; Kim and Swartz 2000). A deprivation below a threshold value results in a decrease of translation efficiency. By addition of exogenous ATP to the assay, some free magnesium ions will be bound to ATP. A bigger loss of free magnesium occurs by generation of the insoluble magnesium ammonium phosphate hexahydrate (Stratful et al. 2001). Ammonium was introduced via the addition of arginine kinase, which was stabilised in suspension with $(NH_4)_2SO_4$ buffer. The liberation of phosphate due to protein synthesis or other reactions in the assay likely stimulated precipitation of $MgNH_4PO_4 \cdot 6H_2O$. As phospho-L-arginine was consumed, inorganic phosphate should have been produced in similar quantities, but a large fraction of the expected free phosphate was missing in assays conducted with either of the pectinid lysates (data not shown), likely due to magnesium precipitation.

Calculation of the Energetic Costs

One technical problem to calculate the energetic costs of protein synthesis was the high proportion of other energy-consuming processes by using crude cell-free systems. Therefore, it was important to accurately differentiate between the energy demand of protein synthesis and energy consumed by other reactions. This was achieved by total inhibition of protein synthesis by pancreatic ribonuclease A (RNAse A). The basic assumptions accepted in this study are as follows: first, RNAse A should have no effect on energy-dependent reactions other than protein synthesis; second, when the formation of peptide bond synthesis ceases, amino acid acylation must not proceed.

RNAse A is a highly specific endonuclease for single-stranded RNA and splits the bond of the phosphate residue at C-3′ in a pyrimidine nucleotide to C-5′ in the next nucleotide in sequence (for review, see Davidson 1972; Raines 1998). Since the single-stranded 3′ end of all tRNAs terminates with the sequence CCA, with the terminal A being the point of covalent attachment of the amino acid, amino acylation is arrested immediately after addition of RNAse A. The detachment of radioactive Phe from tRNA after inhibition of protein synthesis was also ensured by RNAse A and was essential because Phe-tRNA otherwise precipitates onto the GFC filters and leads to an overestimation of the protein synthesis rate and, thus, an underestimation of the energetic costs for protein synthesis.

Furthermore, the tRNA population in the lysates should be completely acylated when starting the in vitro translation so that tRNAs are only newly acylated when amino acids are incorporated into protein. This precondition is very likely met because amino acylation is a highly exogenous, almost irreversible reaction. Allen et al. (1969) suggested that the tRNA population in rat liver was nearly 100% aminoacylated even in starving animals. Shenoy and Rogers (1977, 1978) reached a similar conclusion.

Comparison of the Two Species

There was no significant difference in the energetic costs of protein synthesis between in vitro cell-free systems of gill lysates of the cold stenothermal *A. colbecki* and the eurythermal *A.*

opercularis, indicating that there are no major differences in the efficiency of the translation machinery. The energetic costs specified for protein synthesis in gill lysates of *A. colbecki* almost correspond with the generally accepted theoretical value of four energy equivalents per peptide bond, whereas the value for gill lysates of *A. opercularis* was slightly higher. The theoretical costs result from two ATPs required for activation of each amino acid and an additional two GTPs needed for each peptide bond formation (Haselkorn and Rothman-Denes 1973; Aoyagi et al. 1988; Weijland and Parmeggiani 1993), including peptide bond synthesis and translocation. Some authors even assume a cost of five ATP equivalents per peptide bond, with the additional ATP used for unknown costs such as production of RNA or amino acid transport (Waterlow et al. 1978*a*, 1978*b*; Reeds et al. 1985). These costs are irrelevant in the in vitro translation assay because the RNA pool remains unchanged and amino acid transport is not involved.

It has to be considered, however, that, in addition to amino acylation and elongation, there are other energy-consuming reactions associated with the synthesis of polypeptides in vitro and in vivo, such as translation initiation and termination and, furthermore, the posttranslational modification of newly synthesised polypeptide chains. These events might differ between the examined pectinid species. The migration of 40S ribosomal subunits on mRNA and formation of ternary complexes has been demonstrated to be ATP dependent (Kozak 1980; Tahara et al. 1983), but the exact stoichiometry of ATP hydrolysis per initiation event remains enigmatic. Jackson (1991) demonstrated that the use of ATP for the formation of the initiation complex differed quite substantially between different mRNAs. Higher energy requirements per peptide bond due to initiation and termination will be more significant if only short polypeptides are synthesised de novo and terminated in due course. Costs for initiation will also be more preponderant if polypeptide synthesis has not been finished in vitro. There might be differences between the cell-free translation systems of the two pectinids in the capacity to initiate translation. Moreno et al. (1991) found that 45%–60% of the incorporation of radioactive amino acid into protein in a cell-free system from *Artemia* embryos was due to new initiation and the rest was due to elongation of preformed polypeptide chains. The amount of existing polysomes in the lysates might differ. Therefore, costs for initiation become minimal at higher levels of preformed polysomes in the lysates.

In assays capable of synthesising protein, posttranslational phosphorylation will occur to some extent. In animal cells, serine, threonine, and tyrosine are the amino acids subject to phosphorylation. Serine and threonine contents in gill protein of both species were found to be equal, but tyrosine content was significantly higher in gill protein of *A. opercularis* compared with gill protein of *A. colbecki*. The importance of tyrosine phosphorylation is profound. As an example, the activity of numerous growth factor receptors is controlled by tyrosine phosphorylation.

As a corollary, further improvement of the presented approach might result from the use of specific inhibitors of initiation. Further inhibitors might minimise the high proportion of energy-consuming processes other than protein synthesis (Moreno et al. 1991).

Overall, our observation of similar stoichiometries in the two species and the fact that these are close to theoretical values for peptide bond formation indicate that the costs of initiation and phosphorylation represent only a small fraction of the total costs of protein synthesis. The slightly higher value for the energetic costs of protein synthesis predicted for *A. opercularis* compared with the theoretical value might involve a higher initiation capacity of the lysates combined with the de novo synthesis and termination of only short polypeptides as well as a higher level of posttranslational phosphorylation.

In this study, there was no evidence that the cost of protein synthesis in the cold stenothermal *A. colbecki* is as high as calculated for an Antarctic isopod (Whiteley et al. 1996) or as elusively low as determined for Antarctic sea urchin embryos (Marsh et al. 2001) when compared with confamilial species living in warmer waters. Analysis of the contribution of protein synthesis to metabolic rate or overall oxygen consumption and the estimation of associated energetic costs appear to produce very imprecise results, regardless of which method will be used. Respiration represents a summation of many processes, including protein turnover, ion pump activity, basal metabolism, and reproduction. Protein turnover in turn includes protein synthesis as well as degradation, RNA turnover, amino acid transport, and metabolic regulation. All of these processes may react differently to temperature; thus, misinterpretations appear possible. For precise estimates of the energy requirements of protein synthesis, separate analysis of this process appears preferable.

In summary, low protein turnover rates seen in many polar stenotherms indicate that cold adaptation presumably occurs at the level of increased protein stability but at no increase in stoichiometric costs. However, the capacity of translation is significantly cold compensated. The validity of these results remains to be tested in other ectotherms adapted to different environmental temperature regimes.

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