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Comparison of whole animal costs of protein synthesis among polar and temperate populations of the same species of gammarid amphipod

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Running head: Costs of protein synthesis in *Gammarus oceanicus*

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Abbreviations:

CHX, Cycloheximide
FW, Fresh weight
$\dot{MO}_2$, Rates of oxygen uptake
PEA, $\beta$-phenylethylamine
$k_s$, Whole-animal fractional rates of protein synthesis
$A_s$, Whole-animal absolute rate of protein synthesis
ABSTRACT

Protein synthesis can account for a substantial proportion of metabolic rate. Energetic costs of protein synthesis, should in theory, be the same in marine invertebrates from a range of thermal habitats, and yet direct measurements using inhibitors produce widely differing values, especially in the cold. The present study aimed to remove any potential confounding interspecific effects by determining costs of protein synthesis in two latitudinally separated populations of the same species (amphipod, *Gammarus oceanicus*) living in two different thermal regimes; polar vs cold-temperate. Costs of protein synthesis were determined in summer acclimatised *G. oceanicus* from Svalbard (79˚N) at 5˚C and from Scotland (58˚N) at 13˚C. Amphipods were injected with the protein synthesis inhibitor, cycloheximide (CHX), at 9 mmol l⁻¹ in crab saline to give a tissue concentration of 0.05 mg CHX g⁻¹ FW and left for 60 min before the injection of [³H] phenylalanine. After incubation for 120 min (180 min in total from initial injection), both whole-animal rates of oxygen uptake and absolute rates protein synthesis were significantly reduced in CHX-treated amphipods vs controls injected with saline. Both populations exhibited similar costs of protein synthesis of ~7 μmol O₂ mg⁻¹ protein which is close to the estimated theoretical minimum for peptide bond formation, and similar to the values obtained in cell-free systems. The study demonstrates that in *G. oceanicus*, costs of protein synthesis rates were not elevated in the cold but were fixed among polar and cold-temperate populations.

Keywords: marine, crustaceans, metabolic rate, energetic costs, latitude, temperature.
Introduction

One of the major driving forces of evolution is the growth and development of organisms in order to reach reproductive maturity and pass genetic information on to the next generation (Jobling, 2002). However, little is known about the energetic costs of growth and development, despite their important influence on life history traits (Marsh et al., 2001; Pace and Manahan, 2007). The dominant cost of growth is that of protein synthesis with costs typically within the range of 70-100 µmol ATP mg⁻¹ compared to lipids at 15-25 µmol ATP mg⁻¹ and glycogen at 10-12 µmol ATP mg⁻¹ (Jobling, 1985). In reality the costs of protein synthesis are even higher due to the greater degradation and turnover rates of proteins compared to either lipids or carbohydrates, but the costs of protein synthesis alone can be considerable (Reeds et al., 1985; Houlihan et al., 1995; Fraser and Rogers, 2007; Pace and Manahan, 2007). Costs of protein synthesis, for example, can account for up to half of total metabolic rate with values of 54 and 59% recently reported for haploid and diploid larvae of the temperate oyster, Crassostrea gigas, respectively (Lee et al., 2016). Costs have been determined experimentally in a number of marine species using a specific inhibitor to block cytosolic protein synthesis while simultaneously measuring rates of oxygen uptake (Fraser and Rogers, 2007; Bowgen et al., 2007). The resulting values vary over an unlikely range as demonstrated in polar marine invertebrates where whole-animal values differ over three orders of magnitude from 0.5 µmol O₂ mg⁻¹ in sea urchin embryos, Sterechinus neumayeri (Pace and Manahan, 2007), to 147.5 µmol O₂ mg⁻¹ in the adult isopod, Glyptonotus antarcticus (Whiteley et al., 1996; Table 2). Moreover, costs of protein synthesis have only been determined in a limited number of polar species, all from Antarctica (Whiteley et al., 1996; Marsh et al., 2001; Storch and Pörtner, 2003; Bowgen, et al., 2007; Pace and Manahan, 2007), where marine invertebrates have a number of cold-water specialisations that may be unique to the isolated waters of the Southern Ocean (Pörtner et al., 2007).
Antarctic marine species living at temperatures close to 0±1°C are generally characterised by slow rates of growth and development, and low protein synthesis retention efficiencies at 16-20% as opposed to an average of 52% when compared with tropical and temperate species (Peck et al., 1997; Clarke et al., 2004; Fraser et al., 2007; Peck, 2016). Low retention efficiencies are linked to the observation that proteins are unstable below 5°C leading to increased rates of denaturation (Place et al., 2004; Place and Hofmann, 2005). The associated production of high levels of constitutive heat shock proteins, gene duplication events for hsp70s, and the possibility of increased ubiquitination in Antarctic invertebrates suggests considerable turnover of proteins, and hence high energetic costs (Clark and Peck, 2009; Shin et al., 2012; Peck, 2016). High energetic costs of protein synthesis have been observed in the giant Antarctic isopod, *G. antarcticus* (Whiteley et al., 1996), which was attributed to the theory that costs consist of two components: one that is fixed and independent of synthesis rate and one that is variable but dependent on synthesis rate (Pannevis and Houlihan, 1992). It was argued that the fixed cost dominates at low rates of synthesis and protein is proportionately more energetically expensive to synthesise (Pannevis and Houlihan, 1992; Smith and Houlihan, 1995; Whiteley et al., 1996; Pedersen, 1997). Subsequent experiments, however, have failed to show any differences in protein synthesis costs with change in protein synthesis rate in Antarctic species suggesting that all costs are fixed (Bowgen et al., 2007; Pace and Manahan, 2007). In addition, considerably low costs of protein synthesis have been observed in one Antarctic species during development. In embryos and larvae of the sea urchin, *S. neumayeri*, low costs of protein synthesis were thought to ensure high rates of protein synthesis despite low rates of metabolism permitting comparable rates of protein turnover and development to temperate sea urchins at 15°C (Pace and Manahan, 2007). More recently it has been shown that whole-animal costs of protein synthesis in *C. gigas* larvae are fixed across genotypes, growth rates and the temperatures to
which the oysters were acclimated (Lee et al., 2016). Storch and Pörtner (2003), have also shown that costs of protein synthesis do not vary between the Antarctic and the temperate scallop *Adamussium colbecki* and *Aequipecten opercularis* when measurements are carried out *in vitro* in cell free systems. Clearly further measurements are required to more fully understand the protein synthesis strategies used by marine invertebrates in the cold.

Such wide ranging differences in the costs of protein synthesis have previously been discussed in terms of differences in thermal habitat, developmental stage and differences in the inhibitor methodology (Storch and Pörtner 2003; Bowgen et al., 2007; Pace and Manahan, 2007; Fraser and Rogers 2007; Whiteley and Fraser, 2009), in addition to the rates of synthesis shown by the species concerned (Pannevis and Houlihan, 1992; Whiteley et al., 1996). In order to further our understanding of the effect of thermal habitat on costs of protein synthesis, an alternative approach was taken in the present study by examining marine invertebrates in the Arctic where polar species are less isolated and there is an opportunity to make comparisons with populations of the same species occupying warmer habitats further south. Such intraspecific comparisons avoid the confounding effects associated with differences in life-style and phylogeny (Whiteley et al., 2011). Previous work on marine gammarid amphipods distributed along the coasts of Western Europe have investigated whole-animal rates of oxygen uptake and of protein synthesis in species and populations with known phylogeny (Rastrick and Whiteley, 2011; 2013; Whiteley et al., 2011). The purpose here was to investigate whether a gammarid amphipod species, *Gammarus oceanicus*, distributed in the low intertidal along the coasts of Western Europe from Scotland to the western coast of Svalbard above the Arctic Circle, exhibits higher costs of protein synthesis in the cold (Whiteley et al., 1996; Whiteley et al., 2011). Two populations were investigated: polar (Svalbard 79˚N) and cold-temperate (Scotland 58˚N) where individuals experience differing habitat temperatures from restricted thermal regimes at temperatures close to
freezing in the north (summer temperatures = 1.5-6°C) to more eurythermal temperatures in
the south (summer temperatures 5-13°C) (Rastrick and Whiteley, 2013). As G. oceanicus has
a relatively recent history on Svalbard, having migrated north after surviving in southern
refugia during the last glacial maxima, and as there is little evidence for local adaptation
among populations (Costa et al., 2009), this cold eurythermal species was used to study the
effects of a reduction in habitat temperature on costs of protein synthesis as opposed to the
adaptive responses shown by cold stenothermal species living in the permanently cold waters
of the Southern Ocean.

Materials and methods

Determination of energetic costs of protein synthesis - experimental

G. oceanicus (Segerstråle, 1947) were collected from the low intertidal at two latitudes to
represent populations living in a polar and a cold-temperate regime. Individuals from the
polar population of G. oceanicus were collected from Ny-Ålesund, Svalbard (78.92˚N-
11.92˚E) at a habitat temperature of 5˚C. Individuals from the cold-temperate population were
collected from the Isle of Skye, Scotland (57.66˚N-5.33˚W) at a habitat temperature of 13˚C.
All collections were made between July and August 2008. Species were identified according
to morphological characteristics detailed by Lincoln (1979). Specifically, the length of the
inner and outer ramus on the peduncle of the third uropod was examined under a hand lens on
the shore before further inspection under a microscope on return to the laboratory. Taxonomic
clarification of the occurrence of G. oceanicus at both sites has previously been performed by
DNA barcoding using individuals from the same populations (Costa et al., 2009). In addition,
G. oceanicus, occupies distinct areas of the shore (lower intertidal) occupied by G. locusta
further south but not at the two sites used in this study (Costa et al., 2009; Rock et al., 2009).
Amphipods were returned to Bangor University, North Wales, within 24 h of collection.
During transit amphipods were maintained between sheets of damp filter paper at the temperature of capture. After transit, amphipods from each population were maintained in tanks (vol = 6 L) of fully aerated seawater at a salinity of 33, in a 12L:12D light regime and at their respective capture temperatures of either 5 or 13°C. All animals were fed ad libitum on algal flakes (TetraVeg®, Tetra GmbH, Germany), but were not fed for 24 h before experimentation. Costs of protein synthesis were determined within 7 days of capture.

For the determination of energetic costs of protein synthesis, individual *G. oceanicus* from the Svalbard (mean body mass = 0.27±0.04 g) and Scotland (mean body mass = 0.08±0.01 g) were placed into individual stop-flow respirometers and allowed to settle for 4 h in order to determine resting MO₂ after Rastrick and Whiteley (2011). A flow of aerated sea water at the respective acclimatisation temperature was maintained to each of the respirometers. After 4 h, the flow to each respirometer was stopped for 30 min to determine baseline resting rates of oxygen uptake as described by Rastrick and Whiteley (2011).

Following this each individual amphipod was carefully removed from its respirometer and injected with 2 μl 100 mg⁻¹ FW of crab saline (Pantin, 1934) containing 9 mmoles l⁻¹ CHX at to give a tissue concentration of 0.05 mg CHX g⁻¹ FW. Injections were made directly into the haemolymph via the bulbus arteriosus of the heart using a micro-droplet manipulation system (Rastrick and Whiteley (2013). The concentration of 0.05 mg CHX g⁻¹ FW was chosen after preliminary investigations on a closely related amphipod species (*Echinogammarus marinus*) demonstrated that this was the minimum dose to cause a significant decrease in whole animal rates of oxygen uptake (MO₂) for up to 180 min post-CHX injection when compared with amphipods injected with saline (Rastrick, 2010). In these preliminary experiments, rates of oxygen uptake were used as a proxy for protein synthesis rates as both show a similar trend after addition of the inhibitor in previous studies (Bowgen et al., 2007; Pace and Manahan 2007). In addition, a separate group of *G. oceanicus* from Svalbard (mean body mass =
0.31±0.03 g) and Scotland (mean body mass = 0.06±0.01 g) were injected with crab saline to act as controls. After injection, individuals were returned to their respective respirometers and left in flow-through recirculated seawater at the appropriate temperature for 60 min.

Following the initial 60 min incubation period post CHX or saline injection, individual amphipods were carefully removed from the respirometers and injected with 2 μl 50 mg⁻¹ FW of crab saline containing 150 mmol l⁻¹ of unlabelled L-phenylalanine and 3.7 MBq ml⁻¹ of L-[2,3,4,5,6⁻³H] phenylalanine (G. E. Healthcare, Specific Activity 4.37 TBq mmol⁻¹) for the determination of protein synthesis rates using the flooding dose method (Garlick et al., 1980; modified by Rastrick and Whiteley, 2013). Previous validation of the flooding-dose technique in G. oceanicus from Svalbard at 5°C and Scotland at 13°C established that this dose ensured a rapid equilibration of the radiolabel into the free pools and rapid linear incorporation into proteins over an incubation time of 120 min (Rastrick and Whiteley, 2013). In addition, specific activities of the radiolabel remained stable over the 120 min incubation time at both 5 and 13°C (Rastrick and Whiteley, 2013). Therefore, amphipods in the present study were returned to their respirometers for a further 120 min to allow for the incorporation of [³H] phenylalanine into proteins. All amphipods were then sacrificed, frozen in liquid nitrogen and stored at -80°C for the analysis of protein synthesis rates. As the protein synthesis technique is terminal, rate of synthesis were only determined after the 120 min [³H] phenylalanine incorporation period incubation period. ṀO₂, however, was determined 30 and 60 min after CHX injection and then subsequently 60 and 120 min after [³H] phenylalanine injection. Simultaneous measurements of rates of oxygen uptake and protein synthesis were only taken at the end of the experiment, 180 min after CHX/saline injection and 120 min after [³H] phenylalanine injection when CHX was still shown to significantly reduce ṀO₂ in preliminary experiments (Rastrick, 2010).
Determination of rates of oxygen uptake

Rates of oxygen uptake were determined as described by Rastrick and Whiteley (2011) using stop-flow respirometry (chamber vol = 14 ml) and an OxySense®101 Non-invasive Oxygen Analyzer System (OxySense® Inc., Dallas, Texas, USA). Whole animal rates of oxygen uptake were calculated as the difference in oxygen partial pressure ($PO_2$) before and after the stop flow period in minutes multiplied by the solubility coefficient for oxygen, adjusted for salinity and temperature (Harvey 1955), and the volume of water within each respirometer in ml. Whole animal values for $\dot{MO}_2$ in $\mu$l O$_2$. h$^{-1}$ were converted into STDP and expressed as nmol O$_2$. animal$^{-1}$. h$^{-1}$.

Determination of energetic costs of protein synthesis – analysis

Whole animal fractional rates of protein synthesis ($k_s$) were analysed by first grinding the samples under liquid nitrogen and precipitating the protein fraction in ice-cold 2% perchloric acid (PCA). After centrifugation the resulting supernatant (free-pool fraction) was stored at -20°C and the protein bound-fraction was washed twice in 2% PCA and solubilised in 0.3 N NaOH for 1 hour at 37°C. The alkali-soluble protein was determined from 20 μl sub-samples using a modified Lowry method (Peterson, 1977). The remaining protein was precipitated by addition of 12% PCA and hydrolysed in 6 N HCl at 110°C for 24h, before being re-suspended in citrate buffer (pH=6.3). Phenylalanine levels were determined in both the free-pools and the protein-bound fractions by enzymatic conversion to β-phenylethylamine (PEA) using tyrosine decarboxylase (Worthington Biochemical Corporation, Lakewood, USA) and extraction through heptane into 0.01 N sulphuric acid (Garlick et al., 1980; McCarthy and Fuiman, 2011). PEA levels were measured fluorometrically (Victor$^{\text{TM}}$ Multilabel Counter, Perkin Elmer, Massachusetts, USA) using various dilutions of 150 nmole ml$^{-1}$ PEA as standards. The specific radioactivities of phenylalanine in the intracellular free-pool and
protein-bound factions were determined by liquid scintillation (Wallac WinSpectral™, 1414 Liquid scintillation counter) and Optiphase ‘HiSafe’ scintillant at a counting efficiency of 37%. Whole-animal fractional rates of protein synthesis ($k_s$) were calculated using the equation (Garlick et al., 1983):

$$k_s = \frac{S_b}{S_a} \times \frac{24}{t} \times 100$$

where $k_s$= percentage protein mass synthesised per day (% day$^{-1}$); $S_a$= specific radioactivity of phenylalanine in the intracellular free-pools (dpm nmol$^{-1}$); $S_b$= specific radioactivity of phenylalanine bound to protein (dpm nmol$^{-1}$); $t$ = incubation time in hours. Absolute rates of protein synthesis ($A_s$) were expressed as mg of protein synthesised day$^{-1}$ and calculated for each sample by using the following equation:

$$A_s = k_s / 100 \times \text{total protein content of each amphipod}$$

As wet body mass was significantly higher in the northern population of *G. oceanicus* (t-test, $t=8.5, P<0.001$), all MO$_2$ data were standardised for a fresh mass of 1g using a weight exponent of 0.62 specifically determined for gammarid amphipods (Rastrick and Whiteley, 2011). Absolute rates of protein synthesis were scaled using a weight exponent of -0.2 (Houlihan et al., 1990). Whole animal energetic costs of protein synthesis (μmol O$_2$ mg$^{-1}$ protein) were calculated for the polar and cold-temperate populations of *G. oceanicus* using a modification of the equation from Bowgen et al. (2007):

$$\text{Costs of protein synthesis} = \frac{(\dot{M}O_2^{\text{Saline}} - \dot{M}O_2^{\text{CHX}})}{(A_{s}^{\text{Saline}} - A_{s}^{\text{CHX}})}$$

Where $\dot{M}O_2^{\text{Saline}}$ is the whole-animal rate of oxygen uptake in the control amphipods and $\dot{M}O_2^{\text{CHX}}$ is the whole-animal rate of oxygen uptake in the amphipods injected with cycloheximide. The difference between the two represents cycloheximide-sensitive rates of oxygen uptake. $A_s^{\text{Saline}}$ is the mean absolute rate of protein synthesis of the control amphipods.
(mg protein animal\(^{-1}\) day\(^{-1}\)) and \(A_{s}^{\text{CHX}}\) is the absolute rate of synthesis of amphipods injected with cycloheximide. Costs of protein synthesis were expressed as \(\mu\text{mol O}_2\) mg protein\(^{-1}\) and as \(\mu\text{mol ATP mg protein}^{-1}\), assuming that one mole of oxygen is equivalent to 6 moles of ATP (Reeds et al., 1985; Houlihan et al., 1995).

\[\text{Statistical analysis}\]

All data were tested for normality using Kolmgorov-Smirnov tests and the Levene’s test for homogeneity of variances. Variations in mass adjusted \(\text{MO}_2\) across the incubation period between the control (Saline) and CHX-injected animals for both populations were tested using a repeated measures general linear mixed model (GLMM). Further comparisons of mass adjusted \(\text{MO}_2\) between control and CHX-injected animals at specific time points were analysed by F-tests based on pairwise comparisons generated from the estimated marginal means of the GLMM. At 180 min post CHX/saline injection all whole-animal \(K_s, A_s\) and \(\text{MO}_2\) data used for determining costs of protein synthesis in \(G.\ oceanicus\) were parametric. Means between control and treatment groups at this time point were compared using independent samples t-tests. All statistical analyses were performed using SPSS software (SPSS INC., Chicago, IL, USA). All values are means ± SEM with the number of observations in parentheses.

\[\text{Results}\]

\[\text{Changes in } \dot{\text{MO}}_2 \text{ during incubation in response to CHX}\]

Rates of oxygen uptake (adjusted to a 1 mg individual) in control and CHX-injected \(G.\ oceanicus\) from Svalbard and Scotland are given in Figure 1. Injection of 0.05 mg g\(^{-1}\) FW of CHX significantly decreased \(\text{MO}_2\) across the incubation compared to saline injected animals in both populations (\(F_{45,4}= 2.664, P<0.05\)). This CHX induced responses in \(\dot{\text{MO}}_2\) across the
incubation period was also not significantly different between the polar and cold-temperate populations ($F_{29,9}= 0.674, P=0.725$). 30 min after CHX injection mass adjusted $\dot{MO}_2$ was 28% lower in polar animals ($P<0.05$) and 23% lower in cold temperate animals ($P<0.05$) compared to saline controls. In the polar population, mass adjusted $\dot{MO}_2$ in CHX-injected animals remand significantly reduced compared to saline controls at 60 min (34% reduction, $P=0.01$) 120 min (22% reduction, $P=0.05$) and 180 min (25% reduction, $P=0.001$). In the cold-temperate population, mass adjusted $\dot{MO}_2$ in CHX-injected animals remand significantly reduced compared to saline controls at 60 min (23% reduction, $P=0.001$) 120 min (32% reduction, $P=0.05$) and 180 min (26% reduction, $P=0.001$).

Energetic costs of protein synthesis

After 180 min incubation at a CHX dose of 0.05 mg g$^{-1}$ FW, mean whole-animal $\dot{MO}_2$ and whole-animal absolute rates of protein synthesis ($A_s$) used to estimate energetic costs of protein synthesis, as well as, whole-animal fractional rates of protein synthesis ($k_s$) were significantly lower than controls in *G. oceanicus* from Svalbard and from Scotland (Table 1). In the polar population, whole-animal $\dot{MO}_2$ was 25% lower in the amphipods injected with CHX versus controls (t-test $P<0.05$), and in the cold-temperate population $\dot{MO}_2$ was 26% lower (t-test $P<0.01$). Cycloheximide inhibited mean whole–animal $A_s$ by 91% in the polar population and by 89% in the cold-temperate population (t-test, $P<0.05$). Energetic costs of protein synthesis were relatively low in both populations of *G. oceanicus* at around 7 μmol O$_2$ mg protein$^{-1}$ (Table 1), and not significantly different (t-test, $P=0.89$). Costs of protein synthesis expressed as ATP equivalents were 40.4±5.7(7) μmol ATP mg protein$^{-1}$ in the polar population at 5°C, and 42.1±12.4(8) μmol ATP mg protein$^{-1}$ in the temperate population at 13°C.
Discussion

In the present study, comparison of costs of protein synthesis in natural populations of the same species, acclimatised to different thermal regimes, has resulted in two key observations:

(a) costs of synthesising proteins in *G. oceanicus* were relatively low at ~ 7 µmol O₂ mg⁻¹; and (b) costs were the same in both polar and temperate populations. The energetic costs of protein synthesis measured in *G. oceanicus* are comparable to the minimum costs associated with peptide bond formation derived from theoretical estimates by Reeds et al. (1985) at 8.3 µmol O₂ mg⁻¹, and the values measured in cell free systems by Storch and Pörtner (2003) at 7 and 9 µmol O₂ mg⁻¹. In cell-free systems, the inhibitor-based methodology should only measure the costs associated with specific peptide bonds as transport costs and protein deposition are not involved (Pannevis and Houlihan, 1992). In addition, cycloheximide inhibits protein synthesis by interfering with translational elongation and therefore the formation of peptide bonds, but secondary effects can lead to an overestimation of costs (Wieser and Krumschnabel, 2001; Bowgen et al., 2007). In whole-animals, costs of protein synthesis are generally considered to exceed the costs of peptide bond formation due to the additional costs associated with protein and RNA turnover, protein deposition, amino acid transport and metabolic regulation (Waterlow and Millward, 1989; Wieser and Krumschnabel, 2001; Storch and Pörtner, 2003). Indeed, the costs reported here for *G. oceanicus* were lower than the costs summarised in Table 2 for two Antarctic species: the Antarctic limpet *Nacella concinna* (Bowgen et al., 2007); and the giant Antarctic isopod, *Glyptonotus antarcticus* (Whiteley et al., 1996); and for three temperate species: the isopod crustaceans *Idotea rescata* and *Ligia oceanicus* (Whiteley et al., 1996); and the sea urchin, *Lytechinus pictus* (Pace and Manahan, 2006). The costs in *G. oceanicus*, however, were considerably higher than those reported during early life stages of the Antarctic echinoderm
Stereochinus neumayeri (embryos and larvae) (Table 2; Pace and Manahan, 2007), and the larvae of the temperate species, C. gigas and S. purpuratus (Pan et al., 2015; Lee et al., 2016).

The similarity between energetic costs of protein synthesis and the values observed in cell-free systems, and the theoretical minimum indicate that theoretical estimates are either over estimates for G. oceanicus acclimatised to summer conditions, and/or that any non-specific effects of cycloheximide were minimised. It is possible that the estimated minimum costs of protein synthesis vary among species as this value is based on many assumptions leading to potential sources of error (Reeds et al., 1985). Minimal costs of protein synthesis are typically taken as 4 ATP (2ATP and 2GTP) equivalents per peptide bond (Reeds et al., 1985; Fraser and Rogers, 2007), but this is based on a mean peptide molecular weight of 110 and the assumption that 6 mmol ATP is synthesised per mmol O₂ (Reeds et al., 1985; Houlihan et al., 1995). Differences in amino acid composition and average molecular weight, for example, could alter the theoretical minimum costs as demonstrated by Pace and Manahan (2007). Variations in cellular conditions might also influence energy availability from ATP causing further variation in theoretical costs among species. In addition, species can show differing responses to the reduction of ATP production, with a considerable down-regulation of protein synthesis in hepatocytes from anoxia-sensitive trout vs maintenance of protein synthesis capacity in anoxia-tolerant goldfish (Wieser and Krumschnabel, 2001). The physiology of the species under examination is therefore important. As cycloheximide has a direct effect on translation, it is unlikely to affect any of the associated costs, although interference with other processes, such as RNA synthesis has been demonstrated in cell cultures (McMahon 1975). Given the low costs of synthesis reported here it is unlikely that cycloheximide at the present dose influenced vital cell processes other than translation. G. oceanicus has a relatively brief history at Arctic latitudes, and is normally referred to as a subarctic species as it occupies habitats subject to warming where it is increasing in
abundance (Węsławski, et al., 2010). On the west coast of Svalbard, however, this species experiences habitat temperatures of <6°C (mean winter temperature = 1°C, and mean summer temperature = 3°C). At these temperatures it shows physiological similarities to Antarctic benthic marine invertebrates as acclimatised whole-animal rates of metabolism and protein synthesis are relatively low and remain uncompensated despite the cold (Rastrick and Whiteley 2011; 2013). Further clarification on the relationship between costs of synthesis and turnover with thermal experiences, however, is required by conducting experiments under controlled conditions of temperature and food availability.

The discrepancy between the relatively low costs of protein synthesis determined here in *G. oceanicus* and the values reported in some other marine species in Table 2 could also be explained by differences in inhibitor concentration and incubation times (Fraser and Rogers, 2007; Bowgen et al., 2007). Bowgen et al. (2007), for example, demonstrated a weak but significant positive correlation between costs of protein synthesis and inhibitor concentration in their analysis of the inhibitor technique with higher concentrations associated with higher costs. The current study used a cycloheximide concentration of 0.05 mg g⁻¹ FW which is 100 times lower than that used to estimate costs in two isopod species by Whiteley et al. (1996) (Table 2). The higher dose of 5 mg g⁻¹ FW administered to the Antarctic isopod, *Glyptonotus antarcticus*, and the temperate isopod, *Idotea rescata* probably caused secondary effects, inhibiting a wide range of metabolic processes, including RNA synthesis, leading to an overestimation of protein synthesis costs (Ellis and MacDonald, 1970; McMahon, 1975; Fraser and Rogers, 2007). It is also possible that variations in incubation times can contribute to variations in protein synthesis costs among studies. Timing between the first and the second cycloheximide injections range from 7 h in *N. concinna*, giving a total incubation time of 9 h (Bowgen et al., 2007) to 2 min in the isopod, *G. antarcticus*, resulting in a total incubation time of 2 h (Whiteley et al., 1996). In the current study, *G. oceanicus* acclimatised at 5 and
13°C were left for 180 min after cycloheximide injection which was a compromise between allowing the inhibitor to have an effect, avoiding any handling effects on $\dot{MO}_2$ and incorporating the validated incubation time of 120 min required for the protein synthesis measurements. At a CHX concentration of 0.05 mg g$^{-1}$ FW, whole-animal $\dot{MO}_2$ and $A_s$ in *G. oceanicus* were significantly lower than the values in controls after 180 min, which is a key requirement for the success of the inhibitor technique (Bowgen et al., 2007).

The similarity in protein synthesis costs between populations of polar and cold-temperate populations of *G. oceanicus* supports the argument that costs of protein synthesis are fixed and are not influenced by thermal habitat (Bowgen et al., 2007; Pace and Manahan 2006; 2007; Lee et al., 2016). Moreover, costs were the same regardless of a nearly 8-fold difference in fractional protein synthesis rates previously determined in acclimatised *G. oceanicus* from the same populations (Rastrick and Whiteley, 2013). Consequently, there was no evidence of the involvement of a fixed vs a variable component in protein synthesis costs in *G. oceanicus*, matching observations in both the temperate (*L. pictus*) and Antarctic sea urchin *S. neumayeri* during development where costs remained the same despite considerable increases in protein synthesis rates (Pace and Manahan, 2006; 2007). Similar responses have also been reported in the Antarctic limpet, *N. concinna*, where costs remained the same even though protein synthesis rates varied significantly between 0.91 mg protein day$^{-1}$ at 0°C and 1.16 mg protein day$^{-1}$ at 3°C (Bowgen et al., 2007), and the lack of any seasonal differences in costs in the same species despite a 10-fold decrease in food consumption and an associated decrease in protein synthesis during the winter (Fraser et al., 2002). Collectively, these studies show that costs of protein synthesis are independent of rates of synthesis (Pace and Manahan, 2006; Bowgen et al., 2007).

**Conclusion**
Costs of protein synthesis in a marine amphipod species, *G. oceanicus*, were relatively low when compared with most whole-animal costs determined to date using the inhibitor method, but similar to the estimated theoretical minimum for peptide bond formation. Amphipods from both polar and cold-temperate populations shared a common cost of synthesising proteins despite differences in habitat temperatures and acclimatised fractional rates of protein synthesis. The relatively low costs of synthesis measured in acclimatised *G. oceanicus* are likely to be related to reductions in non-specific effects of the inhibitor which was administered at concentrations that were 100 fold lower than those previously used in crustaceans. Further experiments are required on a broader range of cold-water species to increase our understanding of the influence of cold temperatures on the costs of protein turnover in marine invertebrates.

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The authors wish to thank Prof Deri Tomos for providing lab space and use of his micro-droplet manipulation system. We are also grateful to the staff at the Kings Bay Marine Laboratory, Ny-Ålesund for the use of facilities. The collection of amphipods form Svalbard was funded by European Centre for Arctic Environmental Research (ARFAC V European Commission). SPSR was supported by a NERC studentship (NER/S/A/2005/13424).

References


Table 1. Effect of saline and cycloheximide on whole-animal rates of oxygen uptake (\(\dot{\text{MO}_2}\)) as well as absolute (\(A_s\)) and fractional (\(k_s\)) rates of protein synthesis in a polar (\(n = 7\)) and temperate (\(n = 8\)) population of \(G.\ oceanicus\). Costs of protein synthesis were calculated as described in the text. All values are means ± SEM. Any statistical differences between groups are represented by: * = \(P<0.05\); ** = \(P<0.01\) (independent sample t-test).

<table>
<thead>
<tr>
<th>Latitude</th>
<th>Temperature</th>
<th>Variable (whole-animal)</th>
<th>Saline (\dot{\text{MO}_2}) (μmol (\text{O}_2) mg(^{-1}) protein (\text{day}^{-1}))</th>
<th>Saline (A_s) (mg protein (\text{day}^{-1}))</th>
<th>Saline (k_s) (% protein (\text{day}^{-1}))</th>
<th>Saline [Cycloheximide] (0.05 mg g(^{-1}) FW)</th>
<th>Costs (μmol (\text{O}_2) mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>79°N</td>
<td>5°C</td>
<td>(\dot{\text{MO}_2})</td>
<td>36.89±1.87</td>
<td>1.48±0.45</td>
<td>1.93±0.62</td>
<td>27.79±1.28*</td>
<td>6.74±0.94</td>
</tr>
<tr>
<td>79°N</td>
<td>5°C</td>
<td>(A_s)</td>
<td>27.79±1.28*</td>
<td>0.13±0.02*</td>
<td>0.19±0.03</td>
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<td></td>
</tr>
<tr>
<td>79°N</td>
<td>5°C</td>
<td>(k_s)</td>
<td>6.74±0.94</td>
<td>0.19±0.03*</td>
<td>0.19±0.01</td>
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<td></td>
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<tr>
<td>58°N</td>
<td>13°C</td>
<td>(\dot{\text{MO}_2})</td>
<td>33.93±1.07</td>
<td>24.99±1.80**</td>
<td>7.01±1.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58°N</td>
<td>13°C</td>
<td>(A_s)</td>
<td>24.99±1.80**</td>
<td>0.17±0.06*</td>
<td>0.19±0.01</td>
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<tr>
<td>58°N</td>
<td>13°C</td>
<td>(k_s)</td>
<td>7.01±1.87</td>
<td>0.19±0.01*</td>
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</tbody>
</table>
Table 2. Summary of the energetic costs of protein synthesis measured to date in marine invertebrates using the inhibitor methodology. For the experiments using cycloheximide (CHX), the inhibitor was injected into the circulation. For the remainder, the inhibitor was added to the bathing solution. Costs marked with an asterisk represent values converted from energy equivalents in J mg\(^{-1}\) into \(\mu\)mol O\(_2\) mg\(^{-1}\) using 484 kJ = mol\(^{-1}\) O\(_2\) from Gnaiger (1983) based on oxyenthalpic values of lipids and proteins (Pace and Manahan, 2006). Mean values given ± SEM.

<table>
<thead>
<tr>
<th>Species</th>
<th>Life cycle</th>
<th>Inhibitor</th>
<th>Climate</th>
<th>Temp (°C)</th>
<th>Costs (\mu)mol O(_2) mg(^{-1}) protein</th>
<th>Inhibitor concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molluscs</strong></td>
<td></td>
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<tr>
<td><em>Nacella concinna</em></td>
<td>Adult</td>
<td>CHX</td>
<td>Antarctic</td>
<td>0 - 3</td>
<td>13.95±0.77</td>
<td>8.4x10(^{-3}) mg g(^{-1}) FW</td>
<td>Bowgen et al., 2007</td>
</tr>
<tr>
<td><em>Adamussium colbecki</em></td>
<td>Adult (Cell free system)</td>
<td>RNasin ribonuclease</td>
<td>Antarctic</td>
<td>0 (assay 15)</td>
<td>7.00</td>
<td>3.33 units µl(^{-1}) gill lysate</td>
<td>Storch and Pörtner, 2003</td>
</tr>
<tr>
<td><em>Aequipecten opercularis</em></td>
<td>Adult (Cell free system)</td>
<td>RNasin ribonuclease</td>
<td>Temperate</td>
<td>10 (assay 25)</td>
<td>9.00</td>
<td>3.33 units µl(^{-1}) gill lysate</td>
<td>Storch and Pörtner, 2003</td>
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<tr>
<td><em>Crassostrea gigas</em></td>
<td>Larvae</td>
<td>Emetine</td>
<td>Temperate</td>
<td>20 &amp; 25</td>
<td>4.38±0.42*</td>
<td>25 µmol l(^{-1}) sea water</td>
<td>Lee et al., 2016</td>
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<td><strong>Echinoderms</strong></td>
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<td><em>Lytechinus pictus</em></td>
<td>Embryos/ Larvae</td>
<td>Emetine</td>
<td>Temperate</td>
<td>15</td>
<td>16-21*</td>
<td>100-150 µmol l(^{-1}) seawater</td>
<td>Pace and Manahan, 2006</td>
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<tr>
<td><em>Strongylocentrotus purpuratus</em></td>
<td>Embryos/ Larvae</td>
<td>Emetine</td>
<td>Temperate</td>
<td>15</td>
<td>4.96±0.43*</td>
<td>100 µmol l(^{-1}) sea water</td>
<td>Pan et al., 2015</td>
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<tr>
<td><em>Stereochinus neumayeri</em></td>
<td>Embryos (Blastulae)</td>
<td>Anisomycin</td>
<td>Antarctic</td>
<td>-1.0</td>
<td>0.50±0.06*</td>
<td>5-100 µmol l(^{-1}) seawater</td>
<td>Pace and Manahan, 2007</td>
</tr>
<tr>
<td><em>Stereochinus neumayeri</em></td>
<td>Embryos (Gastrulae)</td>
<td>Anisomycin</td>
<td>Antarctic</td>
<td>-1.0</td>
<td>1.32±0.10*</td>
<td>5-100 µmol l(^{-1}) seawater</td>
<td>Pace and Manahan, 2007</td>
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<td><strong>Crustaceans</strong></td>
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<td><em>Idotea rescata</em></td>
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<td>CHX</td>
<td>Temperate</td>
<td>4</td>
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<td>5 mg g(^{-1}) FW</td>
<td>Whiteley et al., 1996</td>
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<tr>
<td><em>Ligia oceanica</em></td>
<td>Adult</td>
<td>CHX</td>
<td>Temperate</td>
<td>5</td>
<td>44.00</td>
<td>5 mg g(^{-1}) FW</td>
<td>Faulkner, 2002</td>
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<tr>
<td><em>Gammarus oceanicus</em></td>
<td>Adult</td>
<td>CHX</td>
<td>Temperate</td>
<td>13</td>
<td>7.01±1.87</td>
<td>0.05 mg g(^{-1}) FW</td>
<td>This study</td>
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<tr>
<td><em>Gammarus oceanicus</em></td>
<td>Adult</td>
<td>CHX</td>
<td>Subarctic</td>
<td>5</td>
<td>6.74±0.94</td>
<td>0.05 mg g(^{-1}) FW</td>
<td>This study</td>
</tr>
<tr>
<td><em>Glyptonotus antarcticus</em></td>
<td>Adult</td>
<td>CHX</td>
<td>Antarctic</td>
<td>0</td>
<td>147.50</td>
<td>5 mg g(^{-1}) FW</td>
<td>Whiteley et al., 1996</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. Rates of oxygen uptake (ṀO₂), adjusted for an individual body mass of 1 mg, in G. oceanicus injected with either saline (controls, closed circles) or cycloheximide (open circles) at a dose of 2 µl 100 mg⁻¹ FW of 9 mmol l⁻¹ CHX in crab saline to give a tissue concentration of 0.05 mg g⁻¹ FW. ṀO₂ values are given for G. oceanicus from both populations: Svalbard at 79°N and an acclimatisation temperature of 5°C, and Scotland at 58°N and an acclimatisation temperature of 13°C. Resting values refer to ṀO₂ measured pre-CHX injection. All values are means ± SEM (at 79°N, saline n=7, 0.05 mg CHX g⁻¹ FW n=7; at 58°N, saline n=8, 0.05 mg CHX g⁻¹ FW n=8). Any statistical differences between groups are represented by: *= P<0.05, **= P<0.01, ***= P<0.001 (F-test).
Figure 1

![Graph showing the mass adjusted rate of oxygen uptake (nmol O₂, 1 mg individual⁻¹ h⁻¹) at 79°N and 58°N. The graph compares the response to saline (closed circles) and CHX 0.05 mg g⁻¹ (open circles) at different time points (resting, injection (CHX/saline), injection (radiolabel)). The graph includes statistical significance markers (*, **, ***).]