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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*

Abbreviations:

CHX, Cycloheximide

FW, Fresh weight

$\dot{M}O_2$, Rates of oxygen uptake

PEA, β-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 [^{3}H] 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 O_2 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 O_2 mg^{-1} in sea urchin embryos, *Sterechinus neumayeri* (Pace and Manahan, 2007), to 147.5 μmol O_2 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±1ºC 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

101 which the oysters were acclimated (Lee et al., 2016). Storch and Pörtner (2003), have also
102 shown that costs of protein synthesis do not vary between the Antarctic and the temperate
103 scallop *Adamussium colbecki* and *Aequipecten opercularis* when measurements are carried
104 out *in vitro* in cell free systems. Clearly further measurements are required to more fully
105 understand the protein synthesis strategies used by marine invertebrates in the cold.
106 Such wide ranging differences in the costs of protein synthesis have previously been
107 discussed in terms of differences in thermal habitat, developmental stage and differences in
108 the inhibitor methodology (Storch and Pörtner 2003; Bowgen et al., 2007; Pace and Manahan,
109 2007; Fraser and Rogers 2007; Whiteley and Fraser, 2009), in addition to the rates of
110 synthesis shown by the species concerned (Pannevis and Houlihan, 1992; Whiteley et al.,
111 1996). In order to further our understanding of the effect of thermal habitat on costs of protein
112 synthesis, an alternative approach was taken in the present study by examining marine
113 invertebrates in the Arctic where polar species are less isolated and there is an opportunity to
114 make comparisons with populations of the same species occupying warmer habitats further
115 south. Such intraspecific comparisons avoid the confounding effects associated with
116 differences in life-style and phylogeny (Whiteley et al., 2011). Previous work on marine
117 gammarid amphipods distributed along the coasts of Western Europe have investigated
118 whole-animal rates of oxygen uptake and of protein synthesis in species and populations with
119 known phylogeny (Rastrick and Whiteley, 2011; 2013; Whiteley et al., 2011). The purpose
120 here was to investigate whether a gammarid amphipod species, *Gammarus oceanicus*,
121 distributed in the low intertidal along the coasts of Western Europe from Scotland to the
122 western coast of Svalbard above the Arctic Circle, exhibits higher costs of protein synthesis in
123 the cold (Whiteley et al., 1996; Whiteley et al., 2011). Two populations were investigated:
124 polar (Svalbard 79˚N) and cold-temperate (Scotland 58˚N) where individuals experience
125 differing habitat temperatures from restricted thermal regimes at temperatures close to

126 freezing in the north (summer temperatures = 1.5-6°C) to more eurythermal temperatures in
127 the south (summer temperatures 5-13°C) (Rastrick and Whiteley, 2013). As *G. oceanicus* has
128 a relatively recent history on Svalbard, having migrated north after surviving in southern
129 refugia during the last glacial maxima, and as there is little evidence for local adaptation
130 among populations (Costa et al., 2009), this cold eurythermal species was used to study the
131 effects of a reduction in habitat temperature on costs of protein synthesis as opposed to the
132 adaptive responses shown by cold stenothermal species living in the permanently cold waters
133 of the Southern Ocean.
134
135 **Materials and methods**
136 *Determination of energetic costs of protein synthesis - experimental*
137 *G. oceanicus* (Segerstråle, 1947) were collected from the low intertidal at two latitudes to
138 represent populations living in a polar and a cold-temperate regime. Individuals from the
139 polar population of *G. oceanicus* were collected from Ny-Ålesund, Svalbard (78.92˚N-
140 11.92˚E) at a habitat temperature of 5˚C. Individuals from the cold-temperate population were
141 collected from the Isle of Skye, Scotland (57.66˚N-5.33˚W) at a habitat temperature of 13˚C.
142 All collections were made between July and August 2008. Species were identified according
143 to morphological characteristics detailed by Lincoln (1979). Specifically, the length of the
144 inner and outer ramus on the peduncle of the third uropod was examined under a hand lens on
145 the shore before further inspection under a microscope on return to the laboratory. Taxonomic
146 clarification of the occurrence of *G. oceanicus* at both sites has previously been performed by
147 DNA barcoding using individuals from the same populations (Costa et al., 2009). In addition,
148 *G. oceanicus*, occupies distinct areas of the shore (lower intertidal) occupied by *G. locusta*
149 further south but not at the two sites used in this study (Costa et al., 2009; Rock et al., 2009).
150 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 (Tetra*Veg*®, 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 $\dot{M}O_2$ 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} CHXat 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 ($\dot{M}O_2$) 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-^{3}H] 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 [^{3}H] 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 [^{3}H] phenylalanine incorporation period incubation period. $\dot{M}O_2$, however, was determined 30 and 60 min after CHX injection and then subsequently 60 and 120 min after [^{3}H] 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 [^{3}H] phenylalanine injection when CHX was still shown to significantly reduce $\dot{M}O_2$ 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 $\dot{M}O_2$ in μl 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^{2TM} 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^{TM}, 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):

$$k_s = S_b/S_a \times 24/t \times 100$$

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 $\dot{M}O_2$ 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). Absolutes 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):

$$\text{Costs of protein synthesis} = (\dot{M}O_2^{Saline} - \dot{M}O_2^{CHX}) / (A_s^{Saline} - A_s^{CHX})$$

Where $\dot{M}O_2^{Saline}$ is the whole-animal rate of oxygen uptake in the control amphipods and $\dot{M}O_2^{CHX}$ 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^{Saline} is the mean absolute rate of protein synthesis of the control amphipods

(mg protein animal^{-1} day^{-1}) and A_s^{CHX} is the absolute rate of synthesis of amphipods injected with cycloheximide. Costs of protein synthesis were expressed as μmol O_2 mg protein^{-1} and as μmol 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).

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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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.

Results

Changes in $\dot{M}O_2$ 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 $\dot{M}O_2$ across the incubation compered to saline injected animals in both populations ($F_{45,4}$= 2.664, P<0.05). This CHX induced responses in $\dot{M}O_2$ 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ was 25% lower in the amphipods injected with CHX versus controls (t-test P<0.05), and in the cold-temperate population $\dot{M}O_2$ 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 at ~ 7 μmol O_2 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 O_2 mg^{-1}, and the values measured in cell free systems by Storch and Pörtner (2003) at 7 and 9 μmol O_2 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

Sterechinus 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 O_2 (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 (Węslawski, et al., 2010). On the west coast of Svalbard, however, this species experiences habitat temperatures of <6ºC (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 over estimation 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 $\dot{M}O_2$ 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 $\dot{M}O_2$ 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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526 Wieser, W., Krumschnabel, G., 2001. Hierarchies of ATP-consuming processes: direct
527 compared with indirect measurements, and comparative aspects. Biochem. J. 355, 389-395.

528 Table 1. Effect of saline and cycloheximide on whole-animal rates of oxygen uptake ($\dot{M}O_2$) as
529 well as absolute (A_s) and fractional (k_s) rates of protein synthesis in a polar (n = 7) and
530 temperate (n = 8) population of *G. oceanicus*. Costs of protein synthesis were calculated as
531 described in the text. All values are means ± SEM. Any statistical differences between groups
532 are represented by: *= P<0.05; **= P<0.01 (independent sample t-test).
533

Latitude	Temperature	Variable (whole-animal)	Saline	[Cycloheximide] (0.05 mg g^{-1} FW)	Costs (μmol O_2 mg^{-1} protein)
79°N	5°C	$\dot{M}O_2$ (μmol O_2. $individual^{-1}$. day^{-1})	36.89±1.87	27.79±1.28*	6.74±0.94
79°N	5°C	A_s (mg protein. $individual^{-1}$. day^{-1})	1.48±0.45	0.13±0.02*	
79°N	5°C	k_s (% protein. $individual^{-1}$. day^{-1})	1.93±0.62	0.19±0.03*	
58°N	13°C	$\dot{M}O_2$ (μmol O_2. $individual^{-1}$. day^{-1})	33.93±1.07	24.99±1.80**	7.01±1.87
58°N	13°C	A_s (mg protein. $individual^{-1}$. day^{-1})	1.50±0.59	0.17±0.06*	
58°N	13°C	k_s (% protein. $individual^{-1}$. day^{-1})	2.23±0.01	0.19±0.01*	

534

535 Table 2. Summary of the energetic costs of protein synthesis measured to date in marine invertebrates using the inhibitor methodology. For the
536 experiments using cycloheximide (CHX), the inhibitor was injected into the circulation. For the remainder, the inhibitor was added to the
537 bathing solution. Costs marked with an asterisk represent values converted from energy equivalents in J mg^{-1} into µmol O_2 mg^{-1} using 484 kJ =
538 mol^{-1} O_2 from Gnaiger (1983) based on oxyenthalpic values of lipids and proteins (Pace and Manahan, 2006). Mean values given ± SEM .
539

Species	**Life cycle**	**Inhibitor**	**Climate**	**Temp (°C)**	**Costs µmol O_2 mg^{-1} protein**	**Inhibitor concentration**	**Reference**
Molluscs							
Nacella concinna	Adult	CHX	Antarctic	0 - 3	13.95±0.77	$8.4x10^{-3}$ mg g^{-1} FW	Bowgen et al., 2007
Adamussium colbecki	Adult (Cell free system)	RNasin ribonuclease	Antarctic	0 (assay 15)	7.00	3.33 units $µl^{-1}$ gill lysate	Storch and Pörtner, 2003
Aequipecten opercularis	Adult (Cell free system)	RNasin ribonuclease	Temperate	10 (assay 25)	9.00	3.33 units $µl^{-1}$ gill lysate	Storch and Pörtner, 2003
Crassostrea gigas	Larvae	Emetine	Temperate	20 & 25	4.38±0.42*	25 µmol l^{-1} sea water	Lee et al., 2016
Echinoderms							
Lytechinus pictus	Embryos/ Larvae	Emetine	Temperate	15	16-21*	100-150 µmol l^{-1} seawater	Pace and Manahan, 2006
Strongylocentrotus purpuratus	Embryos/ Larvae	Emetine	Temperate	15	4.96±0.43*	100 µmol l^{-1} sea water	Pan et al., 2015
Sterechinus neumayeri	Embryos (Blastulae)	Anisomycin	Antarctic	-1.0	0.50±0.06*	5-100 µmol l^{-1} seawater	Pace and Manahan, 2007
Sterechinus neumayeri	Embryos (Gastrulae	Anisomycin	Antarctic	-1.0	1.32±0.10*	5-100 µmol l^{-1} seawater	Pace and Manahan, 2007
Crustaceans							
Idotea rescata	Adult	CHX	Temperate	4	39.50	5 mg g^{-1} FW	Whiteley et al., 1996
Ligia oceanica	Adult	CHX	Temperate	5	44.00	5 mg g^{-1} FW	Faulkner, 2002
Gammarus oceanicus	Adult	CHX	Temperate	13	7.01±1.87	0.05 mg g^{-1} FW	This study
Gammarus oceanicus	Adult	CHX	Subarctic	5	6.74±0.94	0.05 mg g^{-1} FW	This study
Glyptonotus antarcticus	Adult	CHX	Antarctic	0	147.50	5 mg g^{-1} FW	Whiteley et al., 1996

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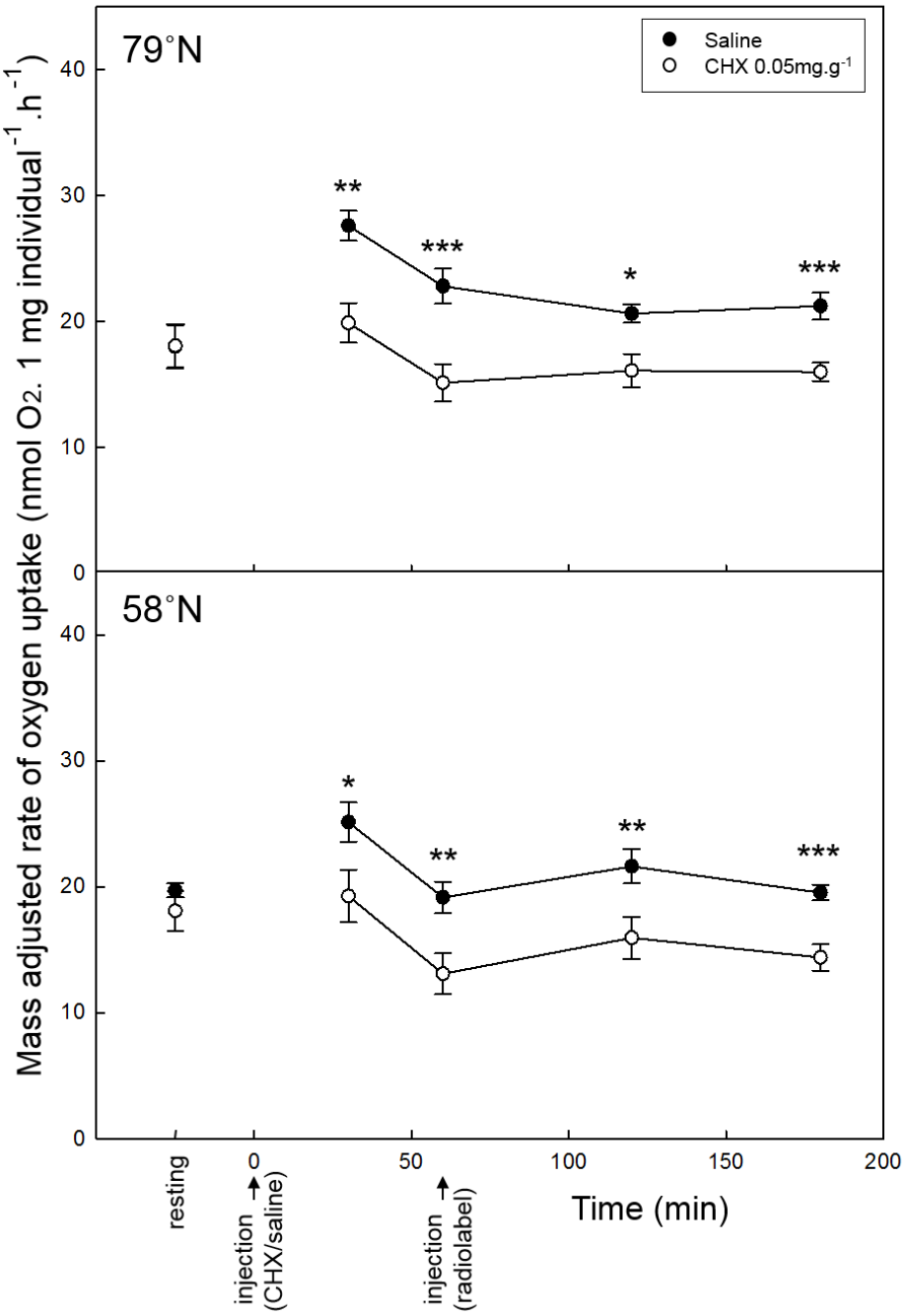
Figure legends

Figure 1. Rates of oxygen uptake ($\dot{M}O_2$), 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. $\dot{M}O_2$ 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 $\dot{M}O_2$ 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).

573 **Figure 1**



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