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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

$\dot{M}O_2$, 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.

51 **Introduction**

52 One of the major driving forces of evolution is the growth and development of organisms in
53 order to reach reproductive maturity and pass genetic information on to the next generation
54 (Jobling, 2002). However, little is known about the energetic costs of growth and
55 development, despite their important influence on life history traits (Marsh et al., 2001; Pace
56 and Manahan, 2007). The dominant cost of growth is that of protein synthesis with costs
57 typically within the range of 70-100 μmol ATP mg^{-1} compared to lipids at 15-25 μmol ATP
58 mg^{-1} and glycogen at 10-12 μmol ATP mg^{-1} (Jobling, 1985). In reality the costs of protein
59 synthesis are even higher due to the greater degradation and turnover rates of proteins
60 compared to either lipids or carbohydrates, but the costs of protein synthesis alone can be
61 considerable (Reeds et al., 1985; Houlihan et al., 1995; Fraser and Rogers, 2007; Pace and
62 Manahan, 2007). Costs of protein synthesis, for example, can account for up to half of total
63 metabolic rate with values of 54 and 59% recently reported for haploid and diploid larvae of
64 the temperate oyster, *Crassostrea gigas,* respectively (Lee et al., 2016). Costs have been
65 determined experimentally in a number of marine species using a specific inhibitor to block
66 cytosolic protein synthesis while simultaneously measuring rates of oxygen uptake (Fraser
67 and Rogers, 2007; Bowgen et al., 2007). The resulting values vary over an unlikely range as
68 demonstrated in polar marine invertebrates where whole-animal values differ over three
69 orders of magnitude from 0.5 μmol O_2 mg^{-1} in sea urchin embryos, *Sterechinus neumayeri*
70 (Pace and Manahan, 2007), to 147.5 μmol O_2 mg^{-1} in the adult isopod, *Glyptonotus*
71 *antarcticus* (Whiteley et al., 1996; Table 2). Moreover, costs of protein synthesis have only
72 been determined in a limited number of polar species, all from Antarctica (Whiteley et al.,
73 1996; Marsh et al., 2001; Storch and Pörtner, 2003; Bowgen, et al., 2007; Pace and Manahan,
74 2007), where marine invertebrates have a number of cold-water specialisations that may be
75 unique to the isolated waters of the Southern Ocean (Pörtner et al., 2007).

76 Antarctic marine species living at temperatures close to 0±1ºC are generally
77 characterised by slow rates of growth and development, and low protein synthesis retention
78 efficiencies at 16-20% as opposed to an average of 52% when compared with tropical and
79 temperate species (Peck et al., 1997; Clarke et al., 2004; Fraser et al., 2007; Peck, 2016). Low
80 retention efficiencies are linked to the observation that proteins are unstable below 5°C
81 leading to increased rates of denaturation (Place et al., 2004; Place and Hofmann, 2005). The
82 associated production of high levels of constitutive heat shock proteins, gene duplication
83 events for hsp70s, and the possibility of increased ubiquitination in Antarctic invertebrates
84 suggests considerable turnover of proteins, and hence high energetic costs (Clark and Peck,
85 2009; Shin et al., 2012; Peck, 2016). High energetic costs of protein synthesis have been
86 observed in the giant Antarctic isopod, *G. antarcticus* (Whiteley et al., 1996), which was
87 attributed to the theory that costs consist of two components: one that is fixed and
88 independent of synthesis rate and one that is variable but dependent on synthesis rate
89 (Pannevis and Houlihan, 1992). It was argued that the fixed cost dominates at low rates of
90 synthesis and protein is proportionately more energetically expensive to synthesise (Pannevis
91 and Houlihan, 1992; Smith and Houlihan, 1995; Whiteley et al., 1996; Pedersen, 1997).
92 Subsequent experiments, however, have failed to show any differences in protein synthesis
93 costs with change in protein synthesis rate in Antarctic species suggesting that all costs are
94 fixed (Bowgen et al., 2007; Pace and Manahan, 2007). In addition, considerably low costs of
95 protein synthesis have been observed in one Antarctic species during development. In
96 embryos and larvae of the sea urchin, *S. neumayeri*, low costs of protein synthesis were
97 thought to ensure high rates of protein synthesis despite low rates of metabolism permitting
98 comparable rates of protein turnover and development to temperate sea urchins at 15°C (Pace
99 and Manahan, 2007). More recently it has been shown that whole-animal costs of protein
100 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.

151 During transit amphipods were maintained between sheets of damp filter paper at the
152 temperature of capture. After transit, amphipods from each population were maintained in
153 tanks (vol = 6 L) of fully aerated seawater at a salinity of 33, in a 12L:12D light regime and at
154 their respective capture temperatures of either 5 or 13˚C. All animals were fed *ad libitum* on
155 algal flakes (Tetra*Veg*®, Tetra GmbH, Germany), but were not fed for 24 h before
156 experimentation. Costs of protein synthesis were determined within 7 days of capture.
157 For the determination of energetic costs of protein synthesis, individual *G. oceanicus*
158 from the Svalbard (mean body mass = 0.27±0.04 g) and Scotland (mean body mass =
159 0.08±0.01 g) were placed into individual stop-flow respirometers and allowed to settle for 4 h
160 in order to determine resting $\dot{M}O_2$ after Rastrick and Whiteley (2011). A flow of aerated sea
161 water at the respective acclimatisation temperature was maintained to each of the
162 respirometers. After 4 h, the flow to each respirometer was stopped for 30 min to determine
163 baseline resting rates of oxygen uptake as described by Rastrick and Whiteley (2011).
164 Following this each individual amphipod was carefully removed from its respirometer and
165 injected with 2 µl 100 mg^{-1} FW of crab saline (Pantin, 1934) containing 9 mmoles l^{-1} CHXat
166 to give a tissue concentration of 0.05 mg CHX g^{-1} FW. Injections were made directly into the
167 haemolymph via the bulbus arteriosus of the heart using a micro-droplet manipulation system
168 (Rastrick and Whiteley (2013). The concentration of 0.05 mg CHX g^{-1} FW was chosen after
169 preliminary investigations on a closely related amphipod species (*Echinogammarus marinus*)
170 demonstrated that this was the minimum dose to cause a significant decrease in whole animal
171 rates of oxygen uptake ($\dot{M}O_2$) for up to 180 min post-CHX injection when compared with
172 amphipods injected with saline (Rastrick, 2010). In these preliminary experiments, rates of
173 oxygen uptake were used as a proxy for protein synthesis rates as both show a similar trend
174 after addition of the inhibitor in previous studies (Bowgen et al., 2007; Pace and Manahan
175 2007). In addition, a separate group of *G. oceanicus* from Svalbard (mean body mass =

176 0.31±0.03 g) and Scotland (mean body mass = 0.06±0.01 g) were injected with crab saline to
177 act as controls. After injection, individuals were returned to their respective respirometers
178 and left in flow-through recirculated seawater at the appropriate temperature for 60 min.
179 Following the initial 60 min incubation period post CHX or saline injection, individual
180 amphipods were carefully removed from the respirometers and injected with 2 µl 50 mg^{-1} FW
181 of crab saline containing 150 mmol l^{-1} of unlabelled L- phenylalanine and 3.7 MBq ml^{-1} of L-
182 [2,3,4,5,6-^{3}H] phenylalanine (G. E. Healthcare, Specific Activity 4.37 TBq $mmol^{-1}$) for the
183 determination of protein synthesis rates using the flooding dose method (Garlick et al., 1980;
184 modified by Rastrick and Whiteley, 2013). Previous validation of the flooding-dose technique
185 in *G. oceanicus* from Svalbard at 5°C and Scotland at 13°C established that this dose ensured
186 a rapid equilibration of the radiolabel into the free pools and rapid linear incorporation into
187 proteins over an incubation time of 120 min (Rastrick and Whiteley, 2013). In addition,
188 specific activities of the radiolabel remained stable over the 120 min incubation time at both 5
189 and 13°C (Rastrick and Whiteley, 2013). Therefore, amphipods in the present study were
190 returned to their respirometers for a further 120 min to allow for the incorporation of [^{3}H]
191 phenylalanine into proteins. All amphipods were then sacrificed, frozen in liquid nitrogen and
192 stored at -80˚C for the analysis of protein synthesis rates. As the protein synthesis technique is
193 terminal, rate of synthesis were only determined after the 120 min [^{3}H] phenylalanine
194 incorporation period incubation period. $\dot{M}O_2$, however, was determined 30 and 60 min after
195 CHX injection and then subsequently 60 and 120 min after [^{3}H] phenylalanine injection.
196 Simultaneous measurements of rates of oxygen uptake and protein synthesis were only taken
197 at the end of the experiment, 180 min after CHX/saline injection and 120 min after [^{3}H]
198 phenylalanine injection when CHX was still shown to significantly reduce $\dot{M}O_2$ in
199 preliminary experiments (Rastrick, 2010).
200

201 *Determination of rates of oxygen uptake*

202 Rates of oxygen uptake were determined as described by Rastrick and Whiteley (2011) using
203 stop-flow respirometry (chamber vol = 14 ml) and an OxySense®101 Non-invasive Oxygen
204 Analyzer System (OxySense® Inc., Dallas, Texas, USA). Whole animal rates of oxygen
205 uptake were calculated as the difference in oxygen partial pressure (PO_2) before and after the
206 stop flow period in minutes multiplied by the solubility coefficient for oxygen, adjusted for
207 salinity and temperature (Harvey 1955), and the volume of water within each respirometer in
208 ml. Whole animal values for $\dot{M}O_2$ in µl O_2. h^{-1} were converted into STDP and expressed as
209 nmol O_2. $animal^{-1}$. h^{-1}.

210

211 *Determination of energetic costs of protein synthesis – analysis*

212 Whole animal fractional rates of protein synthesis (k_s) were analysed by first grinding the
213 samples under liquid nitrogen and precipitating the protein fraction in ice-cold 2% perchloric
214 acid (PCA). After centrifugation the resulting supernatant (free-pool fraction) was stored at -
215 20˚C and the protein bound-fraction was washed twice in 2% PCA and solubilised in 0.3 N
216 NaOH for 1 hour at 37˚C. The alkali-soluble protein was determined from 20 µl sub-samples
217 using a modified Lowry method (Peterson, 1977). The remaining protein was precipitated by
218 addition of 12% PCA and hydrolysed in 6 N HCl at 110˚C for 24h, before being re-suspended
219 in citrate buffer (pH=6.3). Phenylalanine levels were determined in both the free-pools and
220 the protein-bound fractions by enzymatic conversion to β-phenylethylamine (PEA) using
221 tyrosine decarboxylase (Worthington Biochemical Corporation, Lakewood, USA) and
222 extraction through heptane into 0.01 N sulphuric acid (Garlick et al., 1980; McCarthy and
223 Fuiman, 2011). PEA levels were measured fluorometrically (Victor^{2TM} Multilabel Counter,
224 Perkin Elmer, Massachusetts, USA) using various dilutions of 150 nmole ml^{-1} PEA as
225 standards. The specific radioactivities of phenylalanine in the intracellular free-pool and

226 protein-bound factions were determined by liquid scintillation (Wallac WinSpectral™, 1414
227 Liquid scintillation counter) and Optiphase 'HiSafe' scintillant at a counting efficiency of
228 37%. Whole-animal fractional rates of protein synthesis (k_s) were calculated using the
229 equation (Garlick et al., 1983):

230 $$k_s = S_b/S_a \times 24/t \times 100$$

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

236 $$A_s = k_s / 100 \times \text{total protein content of each amphipod}$$

237 As wet body mass was significantly higher in the northern population of *G. oceanicus*
238 (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
239 exponent of 0.62 specifically determined for gammarid amphipods (Rastrick and Whiteley,
240 2011). Absolutes rates of protein synthesis were scaled using a weight exponent of -0.2
241 (Houlihan et al., 1990). Whole animal energetic costs of protein synthesis (µmol O_2 mg^{-1}
242 protein) were calculated for the polar and cold-temperate populations of *G. oceanicus* using a
243 modification of the equation from Bowgen et al. (2007):

244

245 $$\text{Costs of protein synthesis} = (\dot{M}O_2^{Saline} - \dot{M}O_2^{CHX}) / (A_s^{Saline} - A_s^{CHX})$$

246

247 Where $\dot{M}O_2^{Saline}$ is the whole-animal rate of oxygen uptake in the control amphipods and
248 $\dot{M}O_2^{CHX}$ is the whole-animal rate of oxygen uptake in the amphipods injected with
249 cycloheximide. The difference between the two represents cycloheximide-sensitive rates of
250 oxygen uptake. A_s^{Saline} is the mean absolute rate of protein synthesis of the control amphipods

251 (mg protein animal^{-1} day^{-1}) and A_s^{CHX} is the absolute rate of synthesis of amphipods injected

252 with cycloheximide. Costs of protein synthesis were expressed as μmol O_2 mg protein^{-1} and as

253 μmol ATP mg protein^{-1}, assuming that one mole of oxygen is equivalent to 6 moles of ATP

254 (Reeds et al., 1985; Houlihan et al., 1995).

255

256 *Statistical analysis*

257 All data were tested for normality using Kolmgorov-Smirnov tests and the Levene's test for

258 homogeneity of variances. Variations in mass adjusted $\dot{M}O_2$ across the incubation period

259 between the control (Saline) and CHX-injected animals for both populations were tested using

260 a repeated measures general linear mixed model (GLMM). Further comparisons of mass

261 adjusted $\dot{M}O_2$ between control and CHX-injected animals at specific time points were

262 analysed by F-tests based on pairwise comparisons generated from the estimated marginal

263 means of the GLMM. At 180 min post CHX/saline injection all whole-animal K_s, A_s and $\dot{M}O_2$

264 data used for determining costs of protein synthesis in *G. oceanicus* were parametric. Means

265 between control and treatment groups at this time point were compared using independent

266 samples t-tests. All statistical analyses were performed using SPSS software (SPSS INC.,

267 Chicago. IL, USA). All values are means ± SEM with the number of observations in

268 parentheses.

269

270 **Results**

271 *Changes in $\dot{M}O_2$ during incubation in response to CHX*

272 Rates of oxygen uptake (adjusted to a 1 mg individual) in control and CHX-injected *G.*

273 *oceanicus* from Svalbard and Scotland are given in Figure 1. Injection of 0.05 mg g^{-1} FW of

274 CHX significantly decreased $\dot{M}O_2$ across the incubation compered to saline injected animals

275 in both populations ($F_{45,4}$= 2.664, P<0.05). This CHX induced responses in $\dot{M}O_2$ across the

276 incubation period was also not significantly different between the polar and cold-temperate

277 populations ($F_{29,9}$= 0.674, P=0.725). 30 min after CHX injection mass adjusted $\dot{M}O_2$ was 28%

278 lower in polar animals (P<0.05) and 23% lower in cold temperate animals (P<0.05) compared

279 to saline controls. In the polar population, mass adjusted $\dot{M}O_2$ in CHX-injected animals

280 remand significantly reduced compared to saline controls at 60 min (34% reduction, P=0.01)

281 120 min (22% reduction, P=0.05) and 180 min (25% reduction, P=0.001). In the cold-

282 temperate population, mass adjusted $\dot{M}O_2$ in CHX-injected animals remand significantly

283 reduced compared to saline controls at 60 min (23% reduction, P=0.001) 120 min (32%

284 reduction, P=0.05) and 180 min (26% reduction, P=0.001).

285

286 *Energetic costs of protein synthesis*

287 After 180 min incubation at a CHX dose of 0.05 mg g^{-1} FW, mean whole-animal $\dot{M}O_2$ and

288 whole-animal absolute rates of protein synthesis (A_s) used to estimate energetic costs of

289 protein synthesis, as well as, whole-animal fractional rates of protein synthesis (k_s) were

290 significantly lower than controls in *G. oceanicus* from Svalbard and from Scotland (Table 1).

291 In the polar population, whole-animal $\dot{M}O_2$ was 25% lower in the amphipods injected with

292 CHX versus controls (t-test P<0.05), and in the cold-temperate population $\dot{M}O_2$ was 26%

293 lower (t-test P<0.01). Cycloheximide inhibited mean whole–animal A_s by 91% in the polar

294 population and by 89% in the cold-temperate population (t-test, P<0.05). Energetic costs of

295 protein synthesis were relatively low in both populations of *G. oceanicus* at around 7 μmol O_2

296 mg $protein^{-1}$ (Table 1), and not significantly different (t-test, P=0.89). Costs of protein

297 synthesis expressed as ATP equivalents were 40.4±5.7(7) μmol ATP mg $protein^{-1}$ in the polar

298 population at 5˚C, and 42.1±12.4(8) μmol ATP mg $protein^{-1}$ in the temperate population at

299 13˚C.

300

301 **Discussion**

302 In the present study, comparison of costs of protein synthesis in natural populations of the
303 same species, acclimatised to different thermal regimes, has resulted in two key observations:
304 (a) costs of synthesising proteins in *G. oceanicus* were relatively low at at ~ 7 µmol O_2 mg^{-1};
305 and (b) costs were the same in both polar and temperate populations. The energetic costs of
306 protein synthesis measured in *G. oceanicus* are comparable to the minimum costs associated
307 with peptide bond formation derived from theoretical estimates by Reeds et al. (1985) at 8.3
308 µmol O_2 mg^{-1}, and the values measured in cell free systems by Storch and Pörtner (2003) at 7
309 and 9 µmol O_2 mg^{-1}. In cell-free systems, the inhibitor-based methodology should only
310 measure the costs associated with specific peptide bonds as transport costs and protein
311 deposition are not involved (Pannevis and Houlihan, 1992). In addition, cycloheximide
312 inhibits protein synthesis by interfering with translational elongation and therefore the
313 formation of peptide bonds, but secondary effects can lead to an overestimation of costs
314 (Wieser and Krumschnabel, 2001; Bowgen et al., 2007). In whole-animals, costs of protein
315 synthesis are generally considered to exceed the costs of peptide bond formation due to the
316 additional costs associated with protein and RNA turnover, protein deposition, amino acid
317 transport and metabolic regulation (Waterlow and Millward, 1989; Wieser and
318 Krumschnabel, 2001; Storch and Pörtner, 2003). Indeed, the costs reported here for *G.*
319 *oceanicus* were lower than the costs summarised in Table 2 for two Antarctic species: the
320 Antarctic limpet *Nacella concinna* (Bowgen et al., 2007); and the giant Antarctic isopod,
321 *Glyptonotus antarcticus* (Whiteley et al., 1996); and for three temperate species: the isopod
322 crustaceans *Idotea rescata* and *Ligia oceanicus* (Whiteley et al., 1996); and the sea urchin,
323 *Lytechinus pictus* (Pace and Manahan, 2006). The costs in *G. oceanicus*, however, were
324 considerably higher than those reported during early life stages of the Antarctic echinoderm

325 *Sterechinus neumayeri* (embryos and larvae) (Table 2; Pace and Manahan, 2007), and the
326 larvae of the temperate species, *C. gigas* and *S. purpuratus* (Pan et al., 2015; Lee et al., 2016).
327 The similarity between energetic costs of protein synthesis and the values observed in
328 cell-free systems, and the theoretical minimum indicate that theoretical estimates are either
329 over estimates for *G. oceanicus* acclimatised to summer conditions, and/or that any non-
330 specific effects of cycloheximide were minimised. It is possible that the estimated minimum
331 costs of protein synthesis vary among species as this value is based on many assumptions
332 leading to potential sources of error (Reeds et al., 1985). Minimal costs of protein synthesis
333 are typically taken as 4 ATP (2ATP and 2GTP) equivalents per peptide bond (Reeds et al.,
334 1985; Fraser and Rogers, 2007), but this is based on a mean peptide molecular weight of 110
335 and the assumption that 6 mmol ATP is synthesised per mmol O_2 (Reeds et al., 1985;
336 Houlihan et al., 1995). Differences in amino acid composition and average molecular weight,
337 for example, could alter the theoretical minimum costs as demonstrated by Pace and Manahan
338 (2007). Variations in cellular conditions might also influence energy availability from ATP
339 causing further variation in theoretical costs among species. In addition, species can show
340 differing responses to the reduction of ATP production, with a considerable down-regulation
341 of protein synthesis in hepatocytes from anoxia-sensitive trout vs maintenance of protein
342 synthesis capacity in anoxia-tolerant goldfish (Wieser and Krumschnabel, 2001). The
343 physiology of the species under examination is therefore important. As cycloheximide has a
344 direct effect on translation, it is unlikely to affect any of the associated costs, although
345 interference with other processes, such as RNA synthesis has been demonstrated in cell
346 cultures (McMahon 1975). Given the low costs of synthesis reported here it is unlikely that
347 cycloheximide at the present dose influenced vital cell processes other than translation. *G.*
348 *oceanicus* has a relatively brief history at Arctic latitudes, and is normally referred to as a
349 subarctic species as it occupies habitats subject to warming where it is increasing in

350 abundance (Węslawski, et al., 2010). On the west coast of Svalbard, however, this species
351 experiences habitat temperatures of <6ºC (mean winter temperature = 1°C, and mean summer
352 temperature = 3°C). At these temperatures it shows physiological similarities to Antarctic
353 benthic marine invertebrates as acclimatised whole-animal rates of metabolism and protein
354 synthesis are relatively low and remain uncompensated despite the cold (Rastrick and
355 Whiteley 2011; 2013). Further clarification on the relationship between costs of synthesis and
356 turnover with thermal experiences, however, is required by conducting experiments under
357 controlled conditions of temperature and food availability.

358 The discrepancy between the relatively low costs of protein synthesis determined here
359 in *G. oceanicus* and the values reported in some other marine species in Table 2 could also be
360 explained by differences in inhibitor concentration and incubation times (Fraser and Rogers,
361 2007; Bowgen et al., 2007). Bowgen et al. (2007), for example, demonstrated a weak but
362 significant positive correlation between costs of protein synthesis and inhibitor concentration
363 in their analysis of the inhibitor technique with higher concentrations associated with higher
364 costs. The current study used a cycloheximide concentration of 0.05 mg g^{-1} FW which is 100
365 times lower than that used to estimate costs in two isopod species by Whiteley et al. (1996)
366 (Table 2). The higher dose of 5 mg g^{-1} FW administered to the Antarctic isopod, *Glyptonotus*
367 *antarcticus*, and the temperate isopod, *Idotea rescata* probably caused secondary effects,
368 inhibiting a wide range of metabolic processes, including RNA synthesis, leading to an over
369 estimation of protein synthesis costs (Ellis and MacDonald, 1970; McMahon, 1975; Fraser
370 and Rogers, 2007). It is also possible that variations in incubation times can contribute to
371 variations in protein synthesis costs among studies. Timing between the first and the second
372 cycloheximide injections range from 7 h in *N. concinna*, giving a total incubation time of 9 h
373 (Bowgen et al*.,* 2007) to 2 min in the isopod, *G. antarcticus*, resulting in a total incubation
374 time of 2 h (Whiteley et al., 1996). In the current study, *G. oceanicus* acclimatised at 5 and

375 13°C were left for 180 min after cycloheximide injection which was a compromise between

376 allowing the inhibitor to have an effect, avoiding any handling effects on $\dot{M}O_2$ and

377 incorporating the validated incubation time of 120 min required for the protein synthesis

378 measurements. At a CHX concentration of 0.05 mg g^{-1} FW, whole-animal $\dot{M}O_2$ and A_s in *G.*

379 *oceanicus* were significantly lower than the values in controls after 180 min, which is a key

380 requirement for the success of the inhibitor technique (Bowgen et al., 2007).

381 The similarity in protein synthesis costs between populations of polar and cold-

382 temperate populations of *G. oceanicus* supports the argument that costs of protein synthesis

383 are fixed and are not influenced by thermal habitat (Bowgen et al., 2007; Pace and Manahan

384 2006; 2007; Lee et al., 2016). Moreover, costs were the same regardless of a nearly 8-fold

385 difference in fractional protein synthesis rates previously determined in acclimatised *G.*

386 *oceanicus* from the same populations (Rastrick and Whiteley, 2013). Consequently, there was

387 no evidence of the involvement of a fixed vs a variable component in protein synthesis costs

388 in *G. oceanicus*, matching observations in both the temperate (*L. pictus)* and Antarctic sea

389 urchin *S. neumayeri* during development where costs remained the same despite considerable

390 increases in protein synthesis rates (Pace and Manahan, 2006; 2007). Similar responses have

391 also been reported in the Antarctic limpet, *N. concinna*, where costs remained the same even

392 though protein synthesis rates varied significantly between 0.91 mg protein day^{-1} at 0˚C and

393 1.16 mg protein day^{-1} at 3˚C (Bowgen et al*.*, 2007), and the lack of any seasonal differences in

394 costs in the same species despite a 10-fold decrease in food consumption and an associated

395 decrease in protein synthesis during the winter (Fraser et al., 2002). Collectively, these studies

396 show that costs of protein synthesis are independent of rates of synthesis (Pace and Manahan,

397 2006; Bowgen et al., 2007).

398

399 *Conclusion*

400 Costs of protein synthesis in a marine amphipod species, *G. oceanicus*, were relatively low
401 when compared with most whole-animal costs determined to date using the inhibitor method,
402 but similar to the estimated theoretical minimum for peptide bond formation. Amphipods
403 from both polar and cold-temperate populations shared a common cost of synthesising
404 proteins despite differences in habitat temperatures and acclimatised fractional rates of protein
405 synthesis. The relatively low costs of synthesis measured in acclimatised *G. oceanicus* are
406 likely to be related to reductions in non-specific effects of the inhibitor which was
407 administered at concentrations that were 100 fold lower than those previously used in
408 crustaceans. Further experiments are required on a broader range of cold-water species to
409 increase our understanding of the influence of cold temperatures on the costs of protein
410 turnover in marine invertebrates.
411
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418
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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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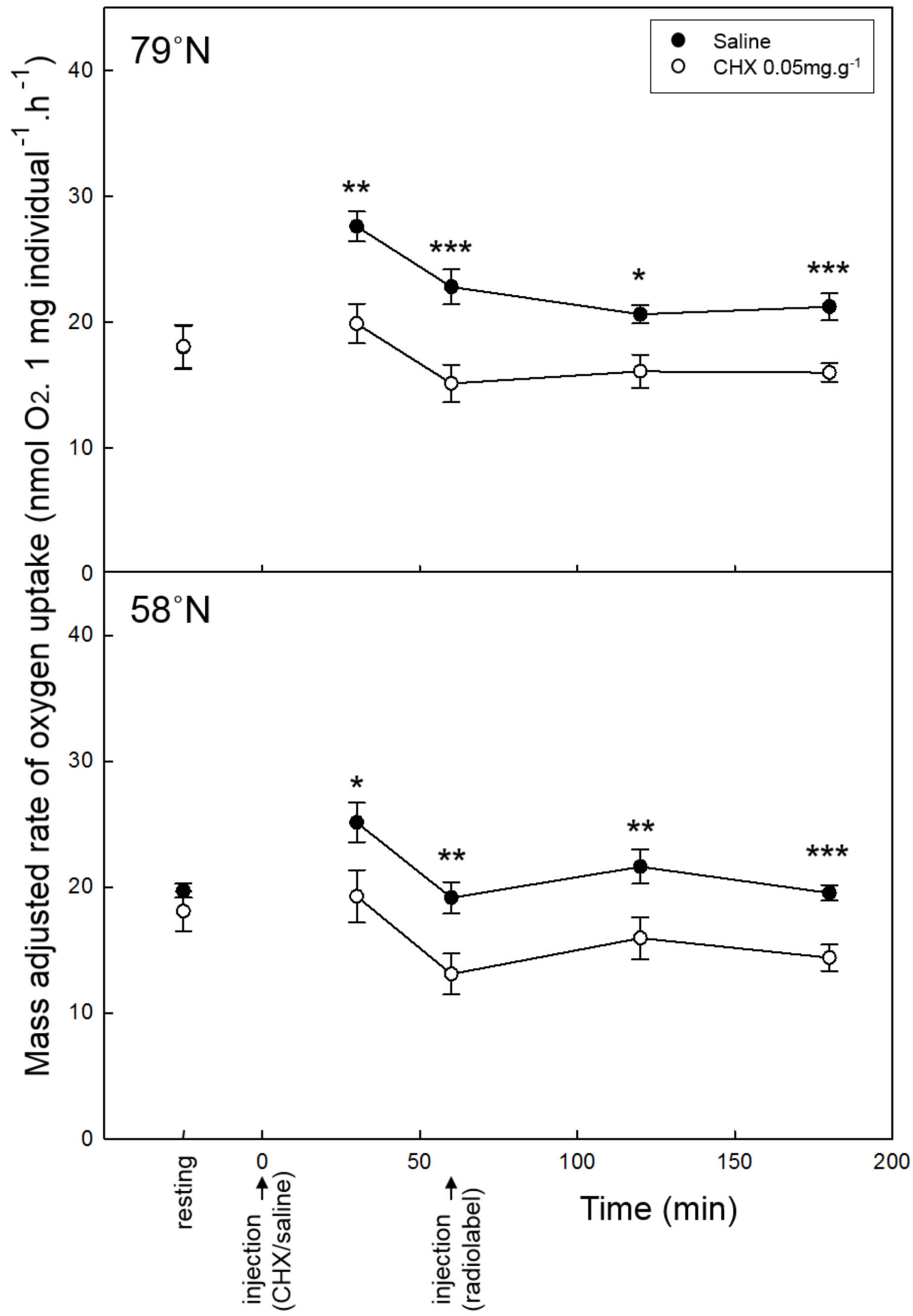
Figure legends

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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