Increase in respiratory cost at high growth temperature is attributed to high protein turnover cost in *Petunia × hybrida* petals

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ABSTRACT

It is widely believed that turnover of nitrogenous (N) compounds (especially proteins) incurs a high respiratory cost. Thus, if protein turnover costs change with temperature, this would influence the dependence of respiration rate on growth temperature. Here, we examined the extent to which protein turnover cost explained differences in N-utilization costs (nitrate uptake/reduction, ammonium assimilation, amino acid and protein syntheses, protein turnover and amino acid export) and in respiration rate with changes in growth temperature. By measurements and literature data, we evaluated each N-utilization cost in *Petunia × hybrida* petals grown at 20, 25 or 35 °C throughout their whole lifespans. Protein turnover cost accounted for 73% of the integrated N-utilization cost on a whole-petal basis at 35 °C. The difference in this cost on a dry weight basis between 25 and 35 °C accounted for 75% of the difference in N-utilization cost and 45% of the difference in respiratory cost. The cost of nitrate uptake/reduction was high at low growth temperatures. We concluded that respiratory cost in petals was strongly influenced by protein turnover and nitrate uptake/reduction, and on the shoot basis, C investment in biomass was highest at 25 °C.

Key-words: nitrate reduction.

INTRODUCTION

Nitrogen (N) utilization processes, including nitrate/ammonium uptake, nitrate reduction, ammonium assimilation, amino acid synthesis, protein synthesis, protein turnover and amino acid export, incur large respiratory costs in plants (Penning de Vries, Brunsting & van Laar 1974; Zerihun, McKenzie & Morton 1998). Among these processes, nitrate reduction and ammonium assimilation are thought to consume high respiratory costs. In fact, the form of N source (e.g. nitrate or ammonium) greatly influences estimates of construction respiration (McDermitt & Loomis 1981; Williams *et al.* 1987). On the other hand,

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maintenance respiration rates are often correlated with protein content, suggesting that protein turnover cost dominates maintenance respiration (Penning de Vries 1975; Amthor 1989; Lambers, Chapin & Pons 1998). The cost of amino acid export during senescence is also claimed to be substantial (Dangl, Dietrich & Thomas 2000). Because these processes consume large respiratory costs, these processes would influence plant growth and production. Thus, it is important to assess the costs of these respective N-utilization processes and their responses to environmental factors, including growth temperature.

On the basis of data from the literature, Zerihun *et al.* (1998) calculated the total respiratory cost of N-utilization processes (N-utilization cost) in bean leaves. They suggested that protein turnover cost dominates the N-utilization cost. However, their calculation was based on data on protein turnover rates in several plant species grown under different conditions. Moreover, these plant materials were at various developmental stages, and protein turnover rate is known to differ markedly depending on the developmental stage (Mae, Makino & Ohira 1983; Barneix *et al.* 1988; Bouma *et al.* 1994). Therefore, their calculation would have been inaccurate, although the approach was valid.

Growth temperature greatly influences plant respiration. This is because growth temperature changes substrate availability and/or demand for ATP (Bunce 2004; Atkin, Scheurwater & Pons 2006). Generally, the specific maintenance respiration rate increases with increasing growth temperature, whereas specific construction respiration is affected little (Amthor 1989; Marcelis & Baan Hofman-Eijer 1995; Van Iersel 2003, 2006). Interestingly, the half-life of protein in wheat roots decreases with increasing temperature because of activity of the ubiquitin proteolytic pathway (Ferguson, Guikema & Paulsen 1990). This indicates that the protein turnover rate increases with temperature.

All of these studies indicate that protein turnover cost is a major component of the N-utilization cost and is likely to increase with growth temperature. Our aims here were to estimate the costs of the respective N-utilization processes – especially that of protein turnover – and to examine the effects of growth temperature on these costs. The quantitative understanding of these components of respiratory costs

will provide us the information to improve plant growth and productivity.

Growth temperature generally changes not only the specific maintenance respiration rate, but the lifespans of plant organs (Kikuzawa 1995; Forbes, Black & Hooker 1997). Therefore, it is difficult to estimate the protein turnover cost from data on one developmental stage of an organ, and analyses that cover the whole lifespan of the organ are preferable. We estimated each of the N-utilization costs in petals of *Petunia × hybrida* grown at one of three temperatures throughout their lifespans. Petals are useful materials, because they have shorter lifespans than leaves and it is easy to determine their developmental stages. Moreover, most petals are non-photosynthetic, and therefore ATP and reducing equivalents are supplied mainly from the respiratory pathway.

To calculate the costs of nitrate/ammonium uptake, nitrate reduction, ammonium assimilation, amino acid and protein syntheses, and amino acid transport, we used the specific costs of these respective processes in the literature (De Visser, Spitters & Bouma 1992; Boorer & Fischer 1997; Zerihun *et al.* 1998; Amthor 2000; Dangl *et al.* 2000), and multiplied these specific costs by actual changes in the amounts of amino acids and proteins. The cost of protein turnover was estimated by using cycloheximide (CHI), an inhibitor of cytosolic protein synthesis, according to Bouma *et al.* (1994). On the basis of the data obtained, we discuss the contribution of protein turnover cost to N-utilization cost and respiration rate. We also evaluated the effects of different N sources on estimates of N-utilization cost. Furthermore, we calculated the biomass C, N-utilization cost and respiratory cost to the petals on one shoot over 90 d, and we discuss the changes in N-utilization cost and in C investment in petal biomass with growth temperature. Our precise estimations clarified that the costs of nitrate reduction/uptake and protein turnover were large, and that the growth temperature intensively influenced these costs.

MATERIALS AND METHODS

Plant materials and growth conditions

We used *Petunia × hybrida* cv. Surfinia 'White Vein' (Suntry flowers, Tokyo, Japan). We purchased seedlings and removed the shoots other than the main one. We then cultivated the seedlings in vermiculite in pots (diameter, 10.5 cm; depth, 17.5 cm; one seedling per pot). We placed the pots in a growth chamber (Biotron, Nippon Medical & Chemical Instruments Co., Osaka, Japan). Light was supplied by a bank of cool-white fluorescent tubes. The photosynthetically active photon flux density (PPFD) was ca. 450 μmol m^{-2} s^{-1}. The air temperature was 20, 25 or 35 °C, relative humidity was 55%, and day length was 16 h (from 1000 to 0200 h). Twice a week, the plants received 200 mL per pot of a nutrient solution containing 2 mM KNO$_3$, 2 mM Ca(NO$_3$)$_2$, 0.75 mM MgSO$_4$, 0.665 mM KH$_2$PO$_4$, 25 μM ethylenediaminetetraacetic acid (EDTA)-Fe, 5 μM MnSO$_4$, 0.5 μM ZnSO$_4$, 0.5 μM CuSO$_4$, 25 μM H$_3$BO$_3$,

0.25 μM Na$_2$MoO$_4$, 50 μM NaCl and 0.1 μM CoSO$_4$. We cultivated the plants for approximately 1 month. For measurements, we used the new petals on shoots newly developed in the growth chamber at 20, 25 or 35 °C. We measured and sampled the petals between 1400 and 0000 h.

Respiration rate

Respiratory O$_2$ uptake rates of detached petals were measured polarographically with a gas-phase oxygen electrode system (LD2; Hansatech Instruments Ltd, Kings Lynn, Norfolk, UK). The respiratory quotient (RQ) was assumed to be 1.

Protein and amino acid contents

Proteins were extracted from samples frozen in liquid N$_2$ and powdered using a mortar and pestle with a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate [w/v], 7.5% glycerol [v/v], 0.01% bromophenol blue [w/v], 50 mM 1,4-dithiothreitol, and 1 tablet/50 mL Complete proteinase inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The ratio of the sample dry weight (DW) to the sample buffer was 10 to 40 mg/mL. Extracts were incubated at 100 °C for 5 min and centrifuged at 12 000 *g*. The supernatants were used for protein quantification by the modified Lowry method (Peterson 1977).

Amino acids were extracted from the powdered samples with 80% ethanol by the method of Ono, Terashima & Watanabe (1996). Amino acids in the supernatant were quantified by the modified ninhydrin method (Rosen 1957).

Inhibition of protein synthesis with CHI

To estimate the protein turnover cost, we incubated the petals in 40 mM Hepes-KOH buffer (pH 7.2) in the presence or absence of 500 μM CHI for 20 min in the dark at the growth temperature, and then measured the O$_2$ uptake rates.

Definition of petal age and lifespan

Day 0 denoted the day when the petal started to unfold. Negative days were those on which the petals were still folded. We measured the folded petal lengths of *P. × hybrida* everyday at each temperature ($n \geq 30$ on each day at each growth temperature) and plotted length against the day number. The age of the folded petal (in days) was estimated from the folded petal length by polynomial approximation ($R^2 \geq 0.99$). We defined the petal lifespan as the number of days for which the size and respiratory activity of a single detached petal were sufficient for us to measure the respiration rate (see Fig. 1).

Definition of respiratory cost

Respiratory cost was taken to include not only C in the CO$_2$ emitted by the respiratory pathway to supply ATP and
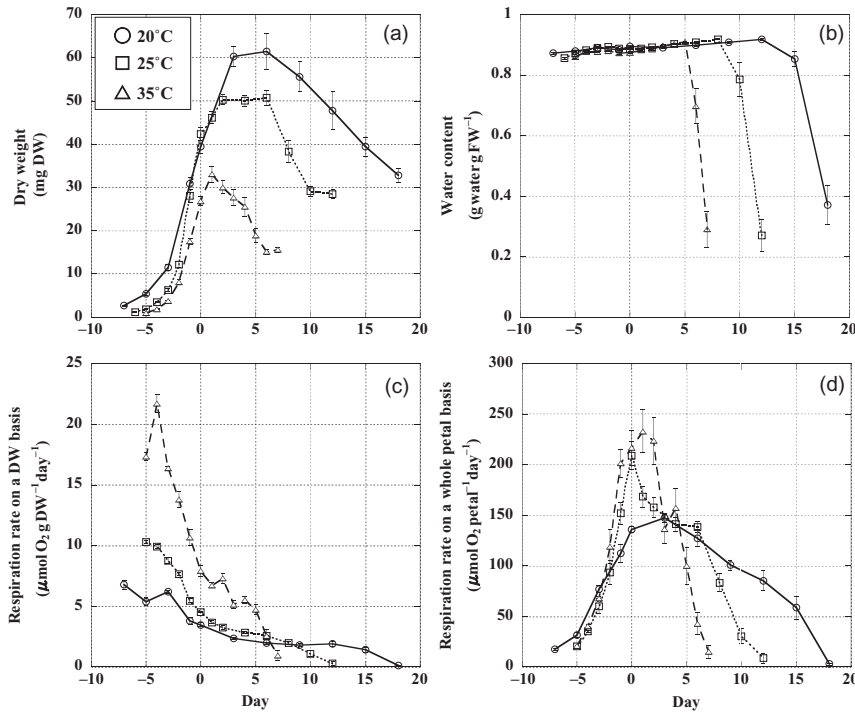
Figure 1. Changes in dry weight (DW) (a), water content on a fresh weight (FW) basis (b), respiration rate on a DW basis (c) and respiration rate on whole-petal basis (d) during development of *Petunia × hybrida* petals at each growth temperature (20 °C, circles; 25 °C, squares; 35 °C, triangles). Day 0 was the first day on which the petal began to unfold. Vertical bars represent standard error of the mean (SEM) ($n \geq 4$).

reducing equivalents, but also the C in CO_2 emitted in other biosynthetic pathways (see Amthor 2000).

Efficiency of respiratory ATP production

ATP production efficiencies from sucrose and reducing equivalents were estimated according to Amthor (1994). He assumed that oxidation of 1 mol of sucrose via pyruvate produces 58 mol of ATP, and that all sucrose is degraded by invertase (for details, see Amthor 1994). The number of ATP produced via mitochondrial oxidation of reducing equivalents (P_{ATP}; mol ATP) was estimated as

$$P_{ATP} = \frac{[H^+_{III,IV}] \times ([NADH_{cyt}] + [NADPH_{cyt}] + [FADH_2]) + [H^+_{I,III,IV}] \times [NADH_{mit}]}{(1 + [H^+_{ATP}])}$$

(1)

where $[H^+_{III,IV}]$ and $[H^+_{I,III,IV}]$ are the numbers of protons pumped into the inter-membrane space when a pair of electrons flows through Complex III/IV and Complex I/III/IV, respectively. We adopted 6 for $[H^+_{III,IV}]$ and 10 for $[H^+_{I,III,IV}]$ (Amthor 2000). $[NADH_{cyt}]$, $[NADPH_{cyt}]$, $[FADH_2]$ and $[NADH_{mit}]$ are the concentrations of the corresponding reductants, and the subscripts 'cyt' and 'mit' indicate the cytosol and mitochondrial matrix, respectively, and indicate the compartments where these reductants are produced. In the respiratory oxidation of sucrose via pyruvate, $[NADH_{cyt}]$, $[NADPH_{cyt}]$, $[FADH_2]$ and $[NADH_{mit}]$ are given the values 4, 0, 4 and 16, respectively (Amthor 2000). $[H^+_{ATP}]$ is the number of protons moving through H^+-ATPase per ADP that undergoes phosphorylation; a value of 3 was used for the calculation (Amthor

2000). The 1 in the denominator is the number of protons co-transported with phosphate into the matrix to compensate for the deficit in phosphate.

Respiratory costs of nitrate/ammonium uptake, nitrate reduction, ammonium assimilation, amino acid synthesis and protein synthesis

Nitrate and ammonium ions are taken up from the xylem into the symplast across the plasma membrane, with stoichiometries of $2H^+/NO_3^-$ and H^+/NH_4^+, respectively (Cannell & Thornley 2000; Kronzucker *et al.* 2001). We assumed that the number of membranes to be crossed was one (Kurimoto *et al.* 2004). The uptake of nitrate and ammonium as N sources requires additional specific respiratory costs of proton motive force formation, at 1.24 and 0.62 mol C mol^{-1} amino acid residue, respectively (Table 1). The processes of unloading of sucrose and amide into the petal are assumed to occur only via the symplastic pathway and thus require no respiratory costs (Cannell & Thornley 2000).

The respiratory costs of nitrate reduction, ammonium assimilation [glutamine (Gln) synthesis], and synthesis of amino acids other than Gln were calculated from Zerihun *et al.* (1998) and the AraCyc metabolic map (Zhang *et al.* 2005). The elemental composition of amino acid residues was considered to be the average of the 20 amino acids, with a molecular weight of 118.9 g mol^{-1} ($C_{5.35}H_{7.95}O_{1.45}N_{1.45}S_{0.10}$). Carbon skeletons and reducing equivalents for amino acid synthesis were assumed to be supplied by Gln and sucrose. The pathway for each amino acid synthesis (see Appendix) was based on the AraCyc metabolic pathway database for

Table 1. Specific respiratory costs of nitrate reduction, ammonium assimilation, other amino acid synthesis and nitrate/ammonium uptake

Process basis	Equation	Specific cost on amino acid residues [mol C mol^{-1} amino acid residues]
(1) Nitrate reduction	$NO_3^- + NADH_{cyt} + 3NADPH_{cyt} \rightarrow NH_3 + OH^- + H_2O$[a] (a) $0.05 Sucrose \rightarrow 0.6CO_2 + NADH_{cyt} + 0.2FADH_{2cyt} + 0.4ATP$ (b) $0.125 Sucrose + 0.25ATP \rightarrow 1.5CO_2 + 3NADPH_{cyt}$	3.05
(2) Ammonium assimilation	$2NH_3 + 0.5 Sucrose + ATP \rightarrow {}_L\text{-glutamine} + CO_2 + NADH_{cyt} + 2NADH_{mit}$[b] (c) $\alpha\text{-ketoglutarate} + 2NH_3 + 2ATP + NADH_{cyt} \rightarrow {}_L\text{-glutamine}$ (d) $0.5 Sucrose \rightarrow \alpha\text{-ketoglutarate} + 2NADH_{mit} + 2NADH_{cyt} + ATP + CO_2$	0.73
(3) Other amino acid synthesis	$14.5_L\text{-glutamine} + 9.95 Sucrose + 2SO_4^{2-} + 8NADPH_{cyt} + 19ATP$[c] $\rightarrow 20C_{5.35}H_{7.95}O_{1.45}N_{1.45}S_{0.10} + {}_D\text{-glyceraldehyde-3-phosphate}$ $+ 10.5\alpha\text{-ketoglutarate} + Succinate + 2Fumarate + 2Acetate + 12.21CO_2$ $+ 25.5NADH_{cyt} + 7NADH_{mit}$	0.81
(4) NO_3^- uptake		1.24
(5) NH_4^+ uptake		0.62

Each specific cost is estimated on the basis of the equations shown.
[a]Equations (a) and (b) denote pathways from which NADH and NADPH are supplied, respectively. NADH and NADPH are produced via the glycolysis and tricarboxylic acid cycle, and the pentose phosphate pathway, respectively.
[b]Equation (c) denotes $_L$-Gln synthesis via glutamine synthetase and glutamine: 2-oxoglutarate amidotransferase reactions. α-ketoglutarate is produced from pathway (d). ATP is produced via the oxidation of additional $NADH_{cyt}$ or $NADH_{mit}$ in mitochondrial electron transport.
[c]Average amino acid ($C_{5.35}H_{7.95}O_{1.45}N_{1.45}S_{0.10}$) is assumed to be the average of 20 amino acids. Pathways for 20 amino acids are summarized in the Appendix. NADPH is supplied from the pentose phosphate pathway, as shown in Eqn (b). ATP is produced via the oxidation of additional $NADH_{cyt}$ or $NADH_{mit}$ in mitochondrial electron transport.

plant research (Zhang *et al.* 2005). The specific respiratory costs of nitrate reduction, ammonium assimilation and other amino acid synthesis are listed in Table 1. The respiratory cost of pH regulation was neglected, because little information was available (Zerihun *et al.* 1998). To estimate the respiratory costs of nitrate/ammonium uptake, nitrate reduction, ammonium assimilation and other amino acid synthesis, we multiplied the rate of increase in amino acid residues on a DW basis (ΔA; mol amino acid residue g DW^{-1} d^{-1}) by the specific respiratory costs in Table 1. The rate of increase in amino acid residues was estimated as follows:

$$\Delta A = \frac{(A_n - A_{n-1})}{(T_n - T_{n-1})} \times \frac{1}{DW_{n-1}} \quad (2)$$

where A_n is the amount of amino acid residues in amino acids and proteins on a whole-petal basis on day n (mol amino acid residues petal^{-1}), T_n is the sampling time (day n), and DW_{n-1} is the dry weight on day n–1.

The specific respiratory costs of protein synthesis processes are listed in Table 2. The respiratory cost of protein synthesis (R_{cpro}; mol C g DW^{-1} d^{-1}) was calculated according to the following equations:

$$R_{cpro} = \Delta P \times E_S \times \frac{1}{(1 - F_{org})} \quad (3)$$

and

$$\Delta P = \frac{(P_n - P_{n-1})}{(T_n - T_{n-1})} \times \frac{1}{DW_{n-1}} \times \frac{1}{118.9} \quad (4)$$

where ΔP is the rate of increase in amount of amino acid residues in the proteins on a DW basis (mol amino acid residues g DW^{-1} d^{-1}), E_S is the specific respiratory cost of protein synthesis (mol C mol^{-1} amino acid residue) in Table 2 (we used the mean value in Table 2 for the calculation), and F_{org} is the amount of protein synthesis or turnover in the organelles (e.g. mitochondria and plastids) as a ratio of that in the whole cell. F_{org} was assumed to be 0%, because (1) fewer than 10% of all mitochondrial proteins are encoded by mitochondrial DNA and synthesized in the mitochondria and (2) the petals of *P. × hybrida* do not have green tissues and include little ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco), which accounts for a large proportion of the proteins synthesized in the leaf chloroplasts. P_n is the amount of protein on a whole-petal basis on day n (g petal^{-1}). T_n is the sampling time (on day n). DW_{n-1} is the dry weight on day n–1, and 118.9 (g mol^{-1}) is the average molecular weight of the amino acid residues as noted.

Respiratory cost of protein turnover

From the information provided by De Visser *et al.* (1992), Bouma *et al.* (1994), Zerihun *et al.* (1998) and Noguchi *et al.* (2001), we assumed that the total specific respiratory cost of protein turnover included the costs of protein biodegradation and protein biosynthesis (see Table 2). The expected effects of CHI on protein turnover and the specific respiratory costs of these processes are also shown in Table 2. The respiratory cost of protein turnover (R_{mpro}: mol C g DW^{-1} d^{-1}) was as follows:

Table 2. Specific respiratory costs for each process of protein synthesis, protein turnover and amino acid export on the basis of amino acid residues

Process	Specific cost on an amino acid residues basis [mol C mol^{-1} amino acid residues]			
	Min	Max	Mean	CHI effect
Protein biodegradation				
(1) Protein biodegradation (E_{deg}) (a)	0.21	0.21	0.21	–
Protein biosynthesis				
(2) Amino acid activation (b)	0.41	0.41	0.41	+
(3) Editing for misaminoacylation of tRNA (c)	0.00	0.03	0.02	+
(4) Peptide bond formation and translocation (a)	0.41	0.41	0.41	+
(5) Signal sequences (c)	0.04	0.21	0.13	+
Tool maintenance				
(6) Amino acid turnover[a] (a,b)	0.36	0.50	0.43	–
(7) mRNA turnover (c)	0.03	0.07	0.05	–
Amino acid export				
(8) Gln synthesis (E_{syn})[b] (d)	0.21	0.21	0.21	–
(9) Membrane transport (E_{tra}) (e)	0.21	0.21	0.21	–
Protein synthesis [E_S: (2) + (3) + (4) + (5) + (6)]	0.86	1.06	0.96	
Protein turnover [E_{SP}: E_S + (7) + (8)]	1.46	1.84	1.65	
Expected CHI effect ($E_{\text{CHI}} = E_S$)	0.86	1.06	0.96	

'CHI effect' denotes the expected effect of cycloheximide (CHI) on the process. It was assumed that oxidation of 1 mol sucrose produced 58 mol ATP. Letters denote references for estimation of the specific cost of each process (a: Zerihun *et al*. 1998, b: De Visser *et al*. 1992, c: Amthor 2000, d: Dangl *et al*. 2000, e: Boorer & Fischer 1997).
[a]Specific respiratory cost of amino acid turnover was estimated on the assumption that 30 to 50% of amino acids are recycled.
[b]Gln was assumed to be transported across one membrane for its export.

$$R_{\text{mpro}} = (R_{\text{CHI}} - R_{\text{cpro}}) \times \frac{E_{\text{SP}}}{E_{\text{CHI}}} \times \frac{1}{(1 - F_{\text{org}})} \quad (5)$$

where R_{CHI} is the decrease in respiration rate (mol C g DW^{-1} d^{-1}) in response to CHI application, E_{SP} is the specific respiratory cost of protein turnover (mol C-mol^{-1} amino acid residue) and E_{CHI} is the specific respiratory cost of the processes blocked by CHI (mol C mol^{-1} amino acid residue). We used the mean value in Table 2 for the estimation.

Respiratory cost of amino acid export from the petal

We defined amino acid export as a series of processes from the biodegradation of proteins into amino acids to the loading of amino acids into the phloem. We assumed that amino acids were transported in the form of Gln. ATP-requiring processes were assumed to be protein biodegradation, Gln synthesis and transport across the plasma membrane.

In protein biodegradation, amino acids are assumed to be converted to Glu by specific transaminases (Dangl *et al*. 2000). The specific cost of Gln synthesis from Glu by Gln synthetase is 1 mol of ATP per mol Gln produced (Table 2). A certain fraction of amino acids from the protein degradation is considered to be degraded into NH$_4^+$ and α-keto acids by deaminase or Glu dehydrogenase (Dangl *et al*. 2000). If NH$_4^+$ for Gln synthesis is derived from the

deamination of amino acids, 2 mol of amino acids will be required for 1 mol of Gln synthesis. We assumed that Gln is exported from the petal via the apoplastic pathway. The export of 1 mol of protons is coupled to that of the equivalent mole of amino acid, in accordance with the stoichiometry of the *Arabidopsis thaliana* amino acid transporters AAP1 and AAP5 (Boorer & Fischer 1997; Table 2).

The minimum and maximum respiratory costs ($R_{\text{amino,min}}$ and $R_{\text{amino,max}}$; mol C g DW^{-1} d^{-1}) of amino acid export from the petal were calculated using Eqns 6 and 7. To calculate the minimum cost, we assumed that half the NH$_4^+$ was derived from the pool in the same cell and half was derived from the deamination of amino acids. To calculate the maximum cost, we assumed that all NH$_4^+$ was derived from that pool:

$$R_{\text{amino min}} = \frac{12}{58} \times \left(E_{\text{deg}} \times NPDR + \frac{1}{2} \times E_{\text{syn}} \times NPDR + E_{\text{tra}} \times NAER_{\text{min}} \right) \quad (6)$$

and

$$R_{\text{aminomax}} = \frac{12}{58} \times (E_{\text{deg}} \times NPDR + E_{\text{syn}} \times NPDR + E_{\text{tra}} \times NAER_{\text{max}}) \quad (7)$$

where E_{deg}, E_{syn} and E_{tra} denote the specific respiratory costs of protein degradation, Gln synthesis and membrane transport, respectively (Table 2). *NPDR* (mol amino acid residues g DW^{-1} d^{-1}) and *NAER* (mol amino acids g DW^{-1} d^{-1})

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