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RESEARCH PAPER

The importance of nodule CO_2 fixation for the efficiency of symbiotic nitrogen fixation in pea at vegetative growth and during pod formation

Stephanie Anastasia Fischinger† and Joachim Schulze*

Department of Crop Science, Plant Nutrition, Georg-August-University of Goettingen, Carl-Sprengel-Weg 1, D-37075 Goettingen, Germany

† Present address: Department of Agronomy and Plant Breeding, Organic Farming, Justus-Liebig-University of Giessen, Karl-Gloeckner-Strasse 21 C, D-35394 Giessen, Germany.
* To whom correspondence should be addressed: E-mail: jschulz2@gwdg.de

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Abstract

Nodule CO_2 fixation is of pivotal importance for N_2 fixation. The process provides malate for bacteroids and oxaloacetate for nitrogen assimilation. The hypothesis of the present paper was that grain legume nodules would adapt to higher plant N demand and more restricted carbon availability at pod formation through increased nodule CO_2 fixation and a more efficient N_2 fixation. Growth, N_2 fixation, and nodule composition during vegetative growth and at pod formation were studied in pea plants (*Pisum sativum* L.). In parallel experiments, $^{15}N_2$ and $^{13}CO_2$ uptake, as well as nodule hydrogen and CO_2 release, was measured. Plants at pod formation showed higher growth rates and N_2 fixation per plant when compared with vegetative growth. The specific activity of active nodules was about 25% higher at pod formation. The higher nodule activity was accompanied by higher amino acid concentration in nodules and xylem sap with a higher share of asparagine. Nodule $^{13}CO_2$ fixation was increased at pod formation, both per plant and per $^{15}N_2$ fixed unit. However, malate concentration in nodules was only 40% of that during vegetative growth and succinate was no longer detectable. The data indicate that increased N_2 fixation at pod formation is connected with strongly increased nodule CO_2 fixation. While the sugar concentration in nodules at pod formation was not altered, the concentration of organic acids, namely malate and succinate, was significantly lower. It is concluded that strategies to improve the capability of nodules to fix CO_2 and form organic acids might prolong intensive N_2 fixation into the later stages of pod formation and pod filling in grain legumes.

Key words: Amino acid, $^{13}CO_2$, H_2 evolution, N_2 fixation, nitrogen fixation, nodule CO_2 fixation, PEPC, *Pisum sativum*, xylem sap.

Introduction

Legume nodules fix substantial amounts of CO_2 largely through the combined activity of carbonic anhydrase (CA, EC 4.2.1.1) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) resulting in carboxylation of phosphoenolpyruvate (PEP) (Vance *et al.*, 1983; King *et al.*, 1986; Rosendahl *et al.*, 1990). Nodule CO_2 fixation rates are difficult to measure since any applied labelled CO_2 is diluted by simultaneous intensive nodule respiration. Measurements thus tend to underestimate nodule CO_2 fixation rates. This tendency is further enhanced through the fact, that a large proportion of any fixed CO_2 is rapidly respired. Nevertheless, estimates reveal that nodule CO_2 fixation rates per protein unit can reach those of young maize leaves (Miller *et al.*, 1987) and contributes considerably to the overall nodule carbon turnover (Warembourg and Roumet, 1989). In fact, PEPC levels in nodules are in the range of

Abbreviations: ANA, apparent nitrogenase activity measured as H_2 evolution in an N_2/O_2 mixture (80/20, v/v); TNA, total nitrogenase activity measured as H_2 evolution in an Ar/O_2 mixture (80/20, v/v); EAC=1-ANA/TNA, electron allocation coefficient; AA, amino acids; OA, organic acids.

those in leaves of C_4 plants, and PEPC is expressed in alfalfa nodules about 10–15-fold greater than in other organs and can comprise up to 2% of the soluble protein in the nodules (Vance and Stade, 1984; Vance *et al.*, 1994). Several nodule-enhanced forms of carbonic anhydrase, PEPC, and malate dehydrogenase (MDH, EC 1.1.1.37), have been identified from legume nodules (de la Pena *et al.*, 1997; Miller *et al.*, 1998; Atkins *et al.*, 2001). The fixed carbon in nodules is channelled into malate formation. Malate is taken up by the symbiosome and drives nitrogen fixation. In addition, a substantial portion of the fixed carbon is used for nitrogen assimilation into aspartate and asparagine. Moreover, a possible involvement of malate in the functioning of a putative nodule oxygen diffusion barrier has been proposed (Vance and Heichel, 1991; Galvez *et al.*, 2000), although the precise mechanism is not yet defined.

Nodule CO_2 fixation is apparently tightly bound to nitrogenase activity. In developing nodules PEPC protein occurs alongside nitrogenase protein (Suganuma *et al.*, 1997). A down-regulation of PEPC in nodules impairs nitrogen fixation (Schulze *et al.*, 1998*b*; Nomura *et al.*, 2006). Taking the whole plant into consideration, nodule CO_2 fixation constitutes a carbon-saving mechanism when the fixed carbon is used for N assimilation and N transport to shoots. While any malate channelled into the symbiosome is rapidly respired, the use of oxaloacetate for N assimilation is connected with the carboxylation of PEP. In turn, any use of carbon skeletons from the tricarboxylic acid cycle (TCA cycle) for N assimilation is connected with a preceding decarboxylation of pyruvate. On a root/nodule basis the use of fixed carbon for N transport is connected to a certain energy gain from glycolysis together with CO_2 fixation rather than CO_2 loss.

Although it is commonly accepted that under most conditions nodules are sufficiently supplied with assimilates, various stress conditions affecting photosynthesis (drought, phosphorus deficiency) might alter the situation (Galvez *et al.*, 2005; Schulze *et al.*, 2006). Moreover, during ontogeny the onset of pod formation profoundly changes source–sink relations within plants, in particular, in grain legumes (Salon *et al.*, 2001). Growing pods not only attract a considerably higher amount of nitrogen than emerging leaves, but significant amounts of carbon as well (Voisin *et al.*, 2003*a*, *b*). Thus, nitrogen is in increasing demand from nodules during pod formation and pod filling, which at the same time most probably find themselves in a position of more unreliable assimilate supply. If nodules cannot meet the pods' N requirements, additional nitrogen is attracted from older leaves, inducing progressive senescence (Schiltz *et al.*, 2005). This, in turn, reduces the overall photosynthetic capacity of the plant. Eventually, these interconnections, at least in part, determine the rapidity of ripening and extent of nitrogen yield in pods (Schulze *et al.*, 1998*a*). This might be critical, in particular in a grain legume like semi-leafless pea varieties. In such varieties, breeding efforts for agronomical benefits were connected with a considerable reduction in photosynthetic capacity.

The hypothesis of the present paper was that nitrogen fixation during pod formation versus vegetative growth would be supported through increased nodule CO_2 fixation supplying organic acids and carbon skeletons for N assimilation, thereby achieving a more efficient nitrogen fixation. A semi-leafless variety of pea plants was chosen for this study. Growth, N assimilation, nodule amino acids (AA), organic acids (OA), and free sugar composition were compared between a 14 d growth interval before flowering (vegetative growth), and one during pod formation. Moreover, in additional experiments root/nodule fixation of $^{15}N_2$ and $^{13}CO_2$, as well as evolution of H_2 and CO_2 was measured at both stages of ontogeny.

Materials and methods

Experimental design

The objective of the experiments was the comparison of nitrogen fixation efficiency in pea plants during a growth interval at vegetative growth and at early pod formation to test the hypothesis that increased nodule CO_2 fixation and organic acid formation would support intensive nitrogen fixation at early pod formation. For that purpose data on growth, nodule activity (*in vitro* PEPC and AAT activity, nodule H_2 and CO_2 evolution), progressing nodule senescence, nodule composition (AA, OA, soluble sugars), nodule CO_2 and N_2 fixation (measured through $^{13}CO_2$ and $^{15}N_2$ uptake), and the composition of AA in the xylem sap at the two stages of ontogeny were obtained in four separate experiments. Experiment one was performed with nodulated pea plants in glass pots with quartz sand supplied with an N-free nutrient solution. Consecutive harvests were made at 10, 17, 24, 28, 35, and 42 d after emergence (DAE). The first pods became visible between 26–28 DAE. Since the substrate was N free, the nitrogen increment between consecutive harvests corresponded to nitrogen fixation. In this way it was possible to calculate plant dry matter increment and nitrogen fixation in 14-d growth intervals during vegetative growth (10–24 DAE) and at pod formation (28–42 DAE). Plants at the vegetative growth stage had formed 9–11 nodes at 10 DAE and 17–20 nodes at 24 DAE. Plants at pod formation had formed 21–24 nodes at 28 DAE and 27–28 nodes at 42 DAE. Seed water content was above 85% at 42 DAE indicating that the plants had not yet reached the state of seed abortion at the end of the growth interval (Dumoulin *et al.*, 1994). Plants at the beginning of the respective growth intervals are shown in Fig. 1. The share of senescent nodules at the individual harvest was determined to relate nitrogen fixation to the amount of active nodules. In addition, at the harvests at 24 DAE and 42 DAE *in vitro* nodule PEPC and AAT activity was measured. To determine nodule activity and composition at the end of the respective growth intervals, plants grown exactly as in experiment one were transferred to a nutrient solution culture system at day 17 after emergence. The system allowed measurements of nodule H_2 and CO_2 evolution as well as of $^{13}CO_2$, $^{15}N_2$, and O_2 uptake. Moreover, nodules could easily be harvested for AA, OA, and soluble sugar analyses, and xylem exudates could be obtained at days 23–24 after emergence (vegetative growth) and at days 41–42 (pod formation). In experiment two, nodule H_2 and CO_2 evolution along with O_2 uptake were measured and nodules were harvested for analyses of AA, OA, and soluble sugar composition. In experiment three, xylem exudates was harvested at the respective time of growth and analysed for AA composition. Experiment four was performed to determine root and nodule $^{13}CO_2$ and $^{15}N_2$ uptake. For the experiments, *Pisum sativum* L. cv. '*Davina*' plants were used and inoculated with *Rhizobium leguminosarum* (DSZM 30132).

Experiment one: growth and nitrogen fixation in sand culture

Pisum sativum L. plants were grown in glass pots (diameter=100 mm, h=200 mm) on C- and N-free fine quartz sand amended with basic fertilization as previously described by Adgo and Schulze (2002). Plants were inoculated with 1 ml of *Rhizobium*

Fig. 1. *Pisum sativum* L. plants at the beginning of the growth interval of vegetative growth (left) and generative growth (right).

leguminosarum grown on YEM to an approximate cell density of 10^9 ml^{-1}. One ml of the cell suspension was applied to each plant on the sand at sowing and at days 3 and 6 after emergence. Inoculation led to effective nodulation and N_2 fixation while an uninoculated control died due to N starvation. Plants were kept under controlled conditions in climate chamber with a 16/8 h day/night cycle at 24/16 °C and a photon flux density of about 360 µmol m^{-2} s^{-1} at stem height during light periods. The plants were watered daily with deionized water to 75% of the maximum water-holding capacity of the sand. The maximum water-holding capacity of the sand was 21% of its weight.

Ten days after emergence (DAE), four replicates were harvested and separated into shoots, roots, active (reddish), and inactive (greenish or brownish) nodules (Baudouin *et al.*, 2004). A second and third harvest of a further four replicates was carried out at 17 DAE and 24 DAE, respectively (plants at 24 DAE corresponded to BBCH 36; Feller *et al.*, 1995*b*). The 14 d between the first and third harvest are referred to as 'the growth interval of vegetative growth'. At the third harvest, nodule *in vitro* PEPC and AAT activity was determined on active nodules of plants from six parallel pots.

A further experiment following the same experimental scheme was made with a growth interval from 28 DAE to 42 DAE (pod formation, plants at 42 DAE corresponded to BBCH 71) with harvest at 28, 35, and 42 DAE. This growth interval is subsequently referred to as 'the growth interval during pod formation'. *In vitro* PEPC and AAT activity was determined on active nodules of plants from six parallel pots at 42 DAE.

Nodule in vitro *PEPC and AAT activity*

To measure *in vitro* nodule PEPC and AAT activity, 100 mg of freshly detached nodules were ground in extraction buffer [100 mM MES-NaOH (pH 6.8), 100 mM sucrose, 2% v/v 2-mercaptoethanol, 15% v/v ethylene glycol, 2 mm PMSF] and centrifuged for 15 min at 10 000 *g* to obtain the soluble protein fraction. Protein content was measured using Bradford's reagent (Bradford, 1976). PEPC and AAT activity were measured in a coupled or direct assay, respectively, monitoring the disappearance of NADH at A_{340} and 20 °C using protocols described in Egli *et al.* (1989). All samples were measured in triplicate.

Dry matter and N concentration

Plant material was dried at 60 °C to a constant weight. Dry shoot, root or nodule material was weighed and ground to a fine powder. N concentration was determined by means of an elementary analyser (Vario EL, Elementar Analysen GmbH, Hanau, Germany). All samples were measured in duplicate.

Experiment two: growth and harvest

In a second experiment, root/nodule gas exchange and nodule AA, OA, and soluble sugar concentration was measured on plants in hydroponic culture (Schulze and Drevon, 2005). The measurements were performed on plants corresponding in age and developmental stage to the end of the growth intervals at vegetative growth (23–24 DAE) and during pod formation (41–42 DAE) in the first experiment. Root/nodule net CO_2 release, O_2 uptake, H_2 evolution in air (apparent nitrogenase activity, ANA) and under argon (total nitrogenase activity, TNA) were measured. Inoculated plants were grown in N-free sand culture as described for experiment one and transferred to a hydroponic system at 17 DAE. The system was designed to allow more detailed root nodule gas exchange measurements. Plant roots were transferred into darkened glass cylinders (diameter=20 mm, h=600 mm) with rubber stoppers at both ends. The stem base of each plant was carefully placed through a hole (diameter=5 mm) in the upper rubber stopper. The cylinder contained 250 ml of the following nutrient solution (mM): KH_2PO_4 (0.06), K_2SO_4 (0.7); $MgSO_4$ (0.5); $CaCl_2$ (0.8); and micronutrients (µM): H_3BO_3 (4.0); Na_2MoO_4 (0.1); $ZnSO_4$ (1.0); $MnCl_2$ (2.0); $Co(NO_3)_2$ (0.2); $CuCl_2$ (1.0), and FeNaEDTA (10). The pH was buffered with MES (0.25 mM) and adjusted to 6.5 in the nutrient solution by applying KOH. Plants were held at their stem bases with a sterilized sponge leaving roots in the nutrient solution. The solution was intensely aerated by a flow of normal air of about 1 vol min^{-1}. The nutrient solution was renewed daily. Solution that had evaporated or passed through the plant was replaced by deionized water 1–3 times per day. After measurements, plants were separated into shoots, roots, and nodules. The plant fractions were dried at 60 °C to a constant weight and dry matter was determined. Roots and nodules of four parallel plants were submerged in liquid nitrogen. Active nodules were harvested and not allowed to melt before extraction for analyses on AA, OA, and soluble sugars.

Root nodule H_2, CO_2, and O_2 exchange

For the H_2 and CO_2 evolution measurement, roots and nodules had to be enclosed under air-tight conditions in gas cylinders allowing the application of a regulated amount of air and to measure the composition of the outflowing gas. For that purpose, the hole in the upper rubber stopper was sealed with a plasticine material with a high beeswax content. The beeswax gave the material a soft, pliable consistency that ensured a tight adherence to the rubber stopper, tubing, and plant stem. The material is non-toxic to plants. Before sealing, stiff tubing to act as an inflow and outflow of the sealed root/nodule compartment was laid through the hole in the upper rubber stopper and also sealed with the plasticine material. The inflow tubing reached down to the lower end of the glass cylinder while the outflow was put above any nodules on the lower side of the upper rubber stopper.

The sealed root/nodule compartment was connected to an open flow gas exchange measurement system that allowed a mixture of N_2/O_2 (80/20, v/v) to be applied. For measurements, the nutrient solution level was lowered to about one-third of the glass cylinder, leaving the lower virtually nodule-free part of the root system in the solution. An airflow of 200 ml min^{-1} (about 1.2 vols min^{-1}) was applied to the root compartment. A sub-sample (100 ml min^{-1}) of the outflowing gas was taken, dried (ice trap and $MgClO_4$) and passed through an H_2 analyser and a CO_2 analyser (S121 Hydrogen Analyser, S161 infrared CO_2 analyser Quibit Systems, Canada). When a stable H_2 and CO_2 outflow from the

root/nodule compartment was reached, the value was taken as ANA (apparent nitrogenase activity) and root nodule net CO_2 release. In order to achieve information about the relative efficiency (electron allocation between N_2 and H^+), the inflow air composition was subsequently switched to Ar/O_2 (80/20, v/v). Argon is inert to nitrogenase and thus the whole electron flow is diverted to H^+. Consequently, H_2 evolution under argon represents total nitrogenase activity (TNA). The peak value, taken approximately 5 min after switching to Ar/O_2, was taken as the TNA value. The electron allocation coefficient (EAC) of nitrogenase activity was calculated as 1–(ANA/TNA). In pre-experiments the validity of the H_2-evolution measurements were tested through parallel $^{15}N_2$-application. O_2 uptake was measured on parallel plants grown in glass containers with a volume of 150 ml. The open flow measurement of O_2 uptake was done as described in Schulze and Drevon (2005). For measurements, the flow through the root/nodule compartment was lowered to 30 ml min^{-1}. The oxygen concentration in the outflowing gas was measured with an Oxynos 100 (Rosamount, Germany).

Nodule amino acid, organic acid, and sugar concentration

For the analysis of free AAs and OAs in nodules, nodules were extracted as previously described (Fischinger *et al.*, 2006). Amino acids were analysed by reverse phase HPLC in the ion suppression mode by precolumn derivatization through *o*-phthaldialdehyde (Chen *et al.*, 1979). OAs were separated through HPLC and were detected by a photodiode array detector. For sugar analysis water extracts were used. Separation was achieved by reverse HPLC and detected by a refractometer. For analytical details see Keutgen and Pawelzik (2008).

Experiment three: xylem sap amino acid concentration

In a third experiment plants were grown precisely as in experiment two. At 24 DAE and 42 DAE xylem sap from these plants was collected for analyses of AA. For xylem sap collection the shoot was cut directly under the cotyledons. To avoid any contamination, closing the phloem and removing the cell bleeding sap was achieved by rinsing the cut surface of the root part for about 15 s with 1 M $CaCl_2$ solution. The root was then placed in a pressure chamber (Model 600 Pressure Chamber Instrument, PMS Instrument Co, Corvallis, Oregon, USA), where it was subjected to a 300 MPa overpressure. The xylem sap was collected for a period of 10 min. During the whole procedure the xylem sap was kept on ice and was then frozen immediately (–20 °C). AAs were analysed as described above.

Experiment four: nodule $^{13}CO_2$ and $^{15}N_2$ fixation

In a fourth experiment plants were grown precisely as in experiment two. At 22–24 DAE and 40–42 DAE N_2 and CO_2 fixation of roots and nodules was measured through exposure of the root/nodule system to $^{15}N_2$ and $^{13}CO_2$. For $^{15}N_2$ application, the whole tube (root compartment) was filled with nutrient solution and the nutrient solution was subsequently replaced by a $^{15}N_2$ (99 atom%$^{15}N_{exc}$)/O_2 (80/20) mixture. Roots and nodules were exposed to the $^{15}N_2/O_2$ mixture for 30 min (Sulieman and Schulze, 2010). Subsequently, the root/nodule compartment was flushed with an N_2/O_2 mixture enriched with 2000 µl l^{-1} $^{13}CO_2$ (99 atom%) for 15 min in an open flow-through system to avoid a significant dilution of $^{13}CO_2$ by root respiration. The O_2 concentration during $^{15}N_2/O_2$ incubation did not decline below 18% (Fischinger *et al.*, 2010).

Immediately after label application plants were fixed in liquid nitrogen and separated into roots, shoots, and nodules. The plant material was dried at 60 °C to a constant weight. The dried shoot and root fractions were weighed and milled using a pebble mill. Nodules were ground with a mortar and pestle.

For ^{15}N and ^{13}C analysis, subsamples of the dried plant material were measured with a combination of a C/N analyser (Porapak PQS) and a mass spectrometer (Finnigan MAT, model 252). The $^{13}CO_2$ uptake was determined by multiplying the C content of a fraction with the ^{13}C excess of this fraction over the ^{13}C% of an unlabelled reference group:

$$^{13}C_{fixed}[g] = C[g]\left(\frac{^{13}C_{treatment}[g^{13}C]}{100[gC]} - \frac{^{13}C_{reference}[g^{13}C]}{100[gC]}\right)$$

The reference group consisted of plants grown under the same conditions but without any contact to excess $^{13}CO_2$ in the atmosphere.

Results

Growth and nitrogenase activity

Pea plants during pod formation showed higher dry matter formation and N assimilation than pea plants before flowering in a 14 d growth interval (Table 1). N assimilation was measured as the total N increment of plants during the growth intervals. Since the plants received no combined nitrogen, N assimilation corresponded to N_2 fixation. $\Delta N/\Delta dm$ ratios in plants at the end of both growth intervals were not different (Table 1). Total nodule dry matter per plant at the end of the growth intervals was about 180% at pod formation (42 DAE) when compared to the end of the growth interval at vegetative growth (24 DAE) (Fig. 2). However, about one-third of the nodules at 42 DAE showed clear signs of senescence, being either greenish or brownish and soft. Active nodules were selected by their reddish to pink colour indicating intact leghaemoglobin in the infected zone. Measurements (H_2 evolution) on detached active nodules revealed a nitrogen fixation activity of 0.49 mg N g^{-1} fresh weight $nodule^{-1}$ d^{-1}, while nodules selected by their greenish or white colour as inactive displayed zero H_2 evolution. Nodule senescence started between 24 DAE and 28 DAE progressing during pod formation (Fig. 2). The onset of nodule senescence coincided with the development of first pods (between 26–28 DAE). The higher plant productivity during pod formation coincided with about a 25% higher specific activity of nodules calculated on the basis of N-increment in the plants and the mean amount of active nodules during the growth interval (Fig. 3).

Table 1. Dry matter increment and N accumulation of pea plants during a 14 d growth interval at vegetative growth or at pod formation in experiment one

Values in parenthesis are in % of the values at vegetative growth. Data are means of four replicates. An asterisk indicates a statistically significant difference to the growth interval at vegetative growth (*t* test, $P \leq 0.05$).

Parameter	Unit	Growth interval	
		Vegetative growth	Pod formation
Δ dry matter	g 14 d^{-1} $plant^{-1}$	0.94	1.50* (160)
Δ N	mg 14 d^{-1} $plant^{-1}$	26	46* (177)
Δ N/Δ dry matter	mg g^{-1}	28	31 (110)

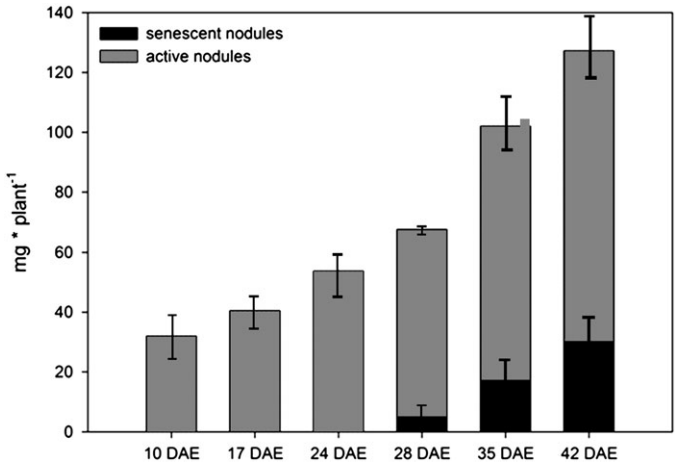

Fig. 2. Amount of active and inactive nodules per plant during ontogeny in experiment one. Nodules were separated according to their colour (red to pink, active; greenish to brown, inactive). Measurements on detached nodules confirmed that inactive nodules had no H_2-evolution activity left, while active nodules showed significant H_2-release. Data are the means of four replicates. Error bars represent standard deviation.

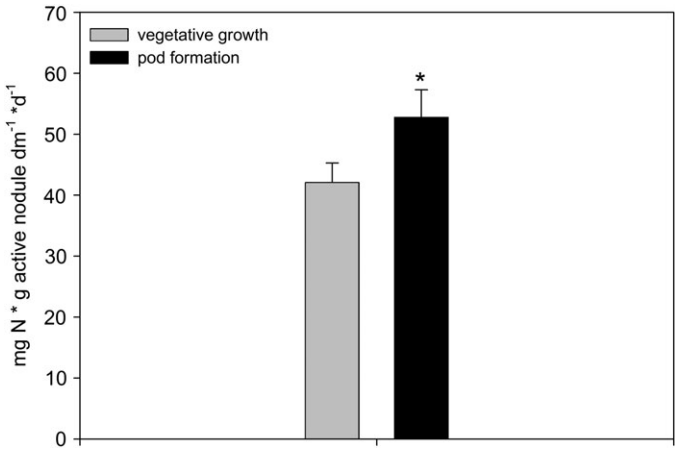

Fig. 3. Specific activity of active nodules over a 14 d growth interval in experiment one. Plants were grown in N-free quartz sand. Nitrogen increment in plants over a 14 d growth interval is related to the mean amount of active nodules during that period of time. Data are the means of four replicates. Error bars represent standard deviation.

Amino acid concentrations in active nodules and xylem sap

The higher specific activity of nodules during pod formation is reflected in a higher total concentration of AA in xylem sap and nodule tissue when compared with plants at the vegetative growth stages (Figs 4, 5A). In addition, in both nodules and xylem sap the increased total AA concentration was largely a result of more asparagine. Apart from changes in asparagine concentration in xylem sap and nodules, no significant shifts in the concentration of any of the other detected AAs were found.

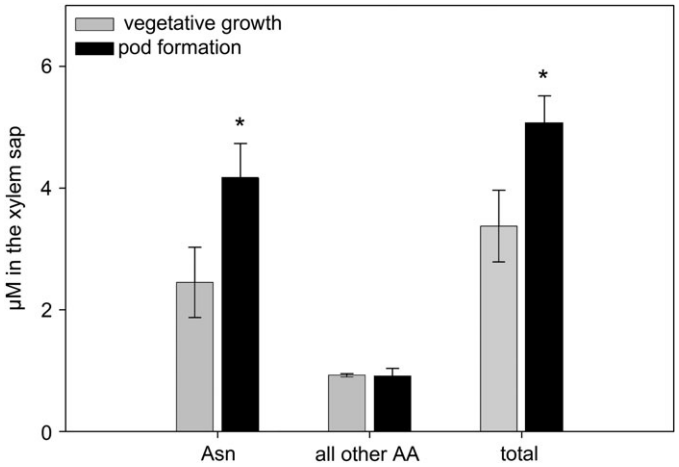

Fig. 4. Xylem sap AA concentration of plants at vegetative growth (24 DAE) and at pod formation (42 DAE) in experiment three. Further detected AAs were Tyr, Ser, Asp, Gly, Val, Thr, Gln, and Glu. Data are the means of four replicates. Error bars represent standard deviation. An asterisk indicates a statistically significant difference between the two growth stages (*t* test, $P \leqslant 0.05$).

Free sugar and organic acid concentrations in active nodules

Total sugar concentration in active nodules showed a tendency to be higher at pod formation, but at statistically non-significant levels (Fig. 5B). In addition to sucrose and galactose, significant concentrations of fructose in active nodules were detected during pod formation. Overall, the detected concentrations in free sugars varied strongly between replicates within both growth intervals.

By contrast, OA concentration was significantly higher in nodules at vegetative growth (Fig. 5C). This was a result of more malate and succinate, both known to be taken up by the symbiosome and to support N_2 fixation (Vance and Heichel, 1991; White *et al.*, 2007). In fact, succinate was not detectable in nodules of plants at the end of the growth interval during pod formation. The levels of tartrate in nodules were equal at both growth intervals. Fumarate was only detected in negligible concentrations.

O_2 and CO_2 exchange of nodulated roots

Net CO_2 release per unit root/nodule dry matter was much lower at pod formation (Table 2). CO_2 release per unit of reduced nitrogen was equal between the growth intervals, while the O_2 uptake per unit of reduced nitrogen was about 50% higher at pod formation, which resulted in a significantly lower respiratory coefficient of nodulated roots. Thus lower CO_2 release per unit of nodulated root, in addition to higher oxygen uptake per unit of fixed nitrogen and a significantly lower apparent respiratory coefficient, coincides with nodules of higher specific activity and increased N_2 fixation per plant.

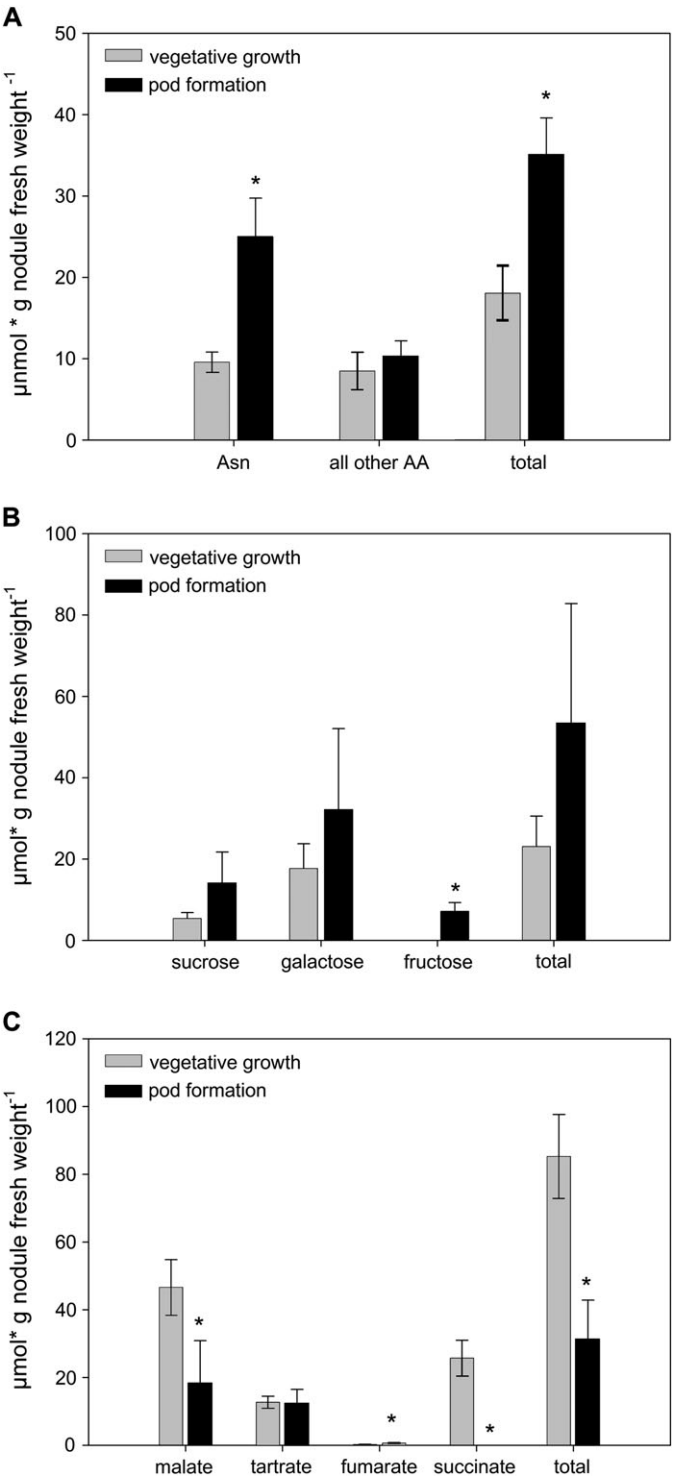

Fig. 5. (A) Nodule AA concentration of plants at vegetative growth (24 DAE) and at pod formation (42 DAE) in experiment two. Further detected AAs were Tyr, Ala, Ser, Leu, Asp, Gly, Val, Arg, His, Phe, Ile, Thr, Gln, Glu, Met, and Lys. Data are the means of four replicates. Error bars represent standard deviation. An asterisk indicates a statistically significant difference between the two growth stages (*t* test, $P \leqslant 0.05$). (B) Nodule sugar concentration of plants at vegetative growth (24 DAE) and at pod formation (42 DAE) in experiment two. Data are the means of four replicates. Error bars represent standard deviation. An asterisk indicates a statistically significant difference between the two growth stages. Fructose and total sugar concentration was compared using the Mann-Whitney Rank Sum Test ($P \leqslant 0.05$), while sucrose and galactose was compared by the *t* test ($P \leqslant 0.05$). (C) Nodule OA concentration of plants at vegetative growth (24 DAE) and at pod formation (42 DAE) in experiment two. Data are the means of four replicates. Error bars represent standard deviation. An asterisk indicates a statistically significant difference between the two growth stages (*t* test, $P \leqslant 0.05$).

Nodule PEPC and AAT activity

Nodule phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and aspartate aminotransferase (AAT, EC 2.6.1.1) activity was about one-third higher during pod formation when compared with nodules from plants at the vegetative growth stage (Fig. 6).

Nodule CO_2 fixation

Nodule CO_2 fixation was directly measured through $^{13}CO_2$ application. Nodule CO_2 fixation per plant was significantly increased at pod formation (Table 3). While nodule CO_2 fixation per plant was increased 30-fold compared to vegetative growth, nodule CO_2 fixation per nodule dry matter was about four times higher. Thus the higher CO_2 fixation per plant during pod formation was a result of both increased nodule mass and a higher specific CO_2 fixation activity. Alongside measurements of CO_2 fixation, nodule N_2 reduction was determined through $^{15}N_2$ application. There was a slight non-significant tendency towards more specific N_2 fixation during pod formation. However, no distinction between active and inactive nodules was made in this experiment. When nodule CO_2 fixation was related to the amount of fixed nitrogen, the calculated value was increased about three times at pod formation when compared with nodules at the vegetative growth stage.

Nitrogenase efficiency

A measurement of nodule activity in terms of higher H_2 evolution confirmed a higher N_2 fixation at pod formation measured by N accumulation and $^{15}N_2$ application (Table 4). However, the electron allocation coefficient (EAC) was significantly lower at pod formation, indicating an increased share of electron flow onto H^+ versus N_2, and thus a decreased relative efficiency of nitrogenase.

Discussion

Our data clearly demonstrate a more intensive nitrogen fixation in pea plants at pod formation in comparison to vegetative growth. Nitrogen fixation peaks during that period, presumably due to newly developing nitrogen attraction throughout pod formation and pod filling (Peat *et al.*, 1981). Pea pods grow quickly and have a high tissue N concentration (Salon *et al.*, 2001). In our experiment the increasing N requirements of the growing pods are met by

Table 2. CO_2 release and O_2 consumption of roots and nodules per fixed N and apparent root/nodule respiratory coefficient in experiment two

N_2 fixation was calculated from H_2 evolution (ANA and TNA) according to Schulze *et al.* (2006). Data are means of four replicates. An asterisk indicates a statistically significant difference compared to the measurement at vegetative growth (*t* test, $P \leq 0.05$).

Parameter	Unit	Time of measurement	
		Vegetative growth 23–24 DAE	Pod formation 41–42 DAE
CO_2 release of nodulated roots per N fixed	mol CO_2 mol^{-1} N	9.1	9.6
Net CO_2 release of nodulated roots	nmol CO_2 mg dm^{-1} h^{-1}	253.4	152.1*
O_2 uptake of nodulated roots per N fixed	mol O_2 mol^{-1} N	10.6	15.4*
Apparent respiratory quotient of nodulated roots	mol CO_2 mol^{-1} O_2	0.86	0.62*

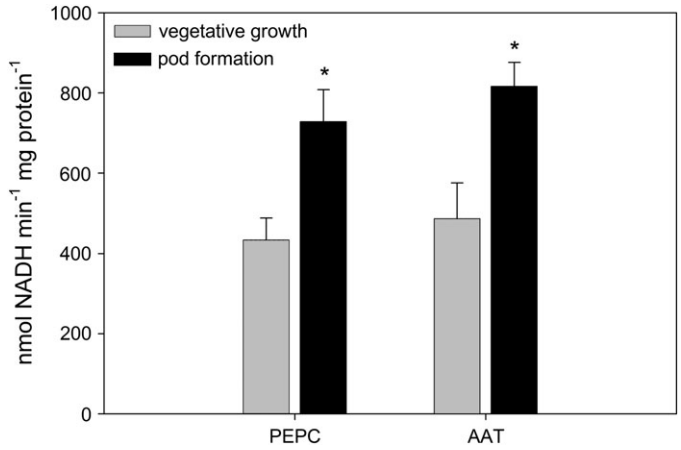

Fig. 6. Nodule PEPC and AAT activity of plants at vegetative growth (24 DAE) and at pod formation (42 DAE) in experiment one. Data are the means of six replicates with standard deviations as error bars. An asterisk indicates a statistically significant difference between the two growth stages (*t* test, $P \leq 0.05$).

a higher N_2 fixation per plant. The higher nitrogen fixation rate per plant was, in part, the result of more nodules; however, in addition the active share of nodules had a much higher specific activity. These two factors led to N_2 fixation rates per plant that could support the altered growth rate of the plants (Δdm) at pod formation, resulting in an unchanged $\Delta N/\Delta dm$ ratio when compared to the vegetative growth stage. A significant amount of nodules already showed clear signs of senescence at pod formation. With regard to the physiological background of more intensive nitrogen fixation at pod formation, knowledge should be extended on the following.

(1) While nodules at pod formation still had sufficient sugars available, the reserves of organic acids were depleted. This applied in particular to malate and succinate, both known to support N_2 fixation energetically and, in the case of malate, also to feed the carbon skeletons into N assimilation and N transport.

(2) More intensive nitrogen fixation is connected with strongly increased nodule CO_2 fixation both per plant and per unit of reduced nitrogen. Nodule CO_2 fixation feeds nodule malate supply; the low malate level at pod formation therefore indicates that the nodule's ability to transform sugars into malate is at its limits by the simultaneous intensive use of the product to support N_2 fixation.

Table 3. Nodule CO_2 fixation per plant, specific N_2, CO_2 fixation per nodule mass and specific CO_2 fixation per N fixed in experiment four

Data are means of four or two replicates from plants at vegetative growth or at pod formation, respectively. An asterisk indicates a statistically significant difference compared to the measurement at vegetative growth (*t* test, $P \leq 0.05$).

Parameter	Unit	Time of measurement	
		Vegetative growth 23–24 DAE	Pod formation 41–42 DAE
^{13}C fixation through nodules	µg h^{-1} plant^{-1}	0.5	14.6*
Nodule specific ^{13}C fixation	mg nodule dm^{-1} h^{-1}	33	120*
Nodule specific $^{15}N_2$ fixation	mg N nodule dm^{-1} h^{-1}	1.45	1.88
CO_2 fixation per N_2 reduced	g C g N^{-1}	0.04	0.11*

Table 4. Nodule H_2 evolution in an N_2/O_2 mixture (80/20, v/v) and an Ar/O_2 (80/20, v/v) at vegetative growth and at pod formation in experiment two

Data are means of four replicates. An asterisk indicates a statistically significant difference compared to the measurement at vegetative growth (*t* test, $P \leq 0.05$).

Parameter	Unit	Time of measurement	
		Vegetative growth 23–23 DAE	Pod formation 41–43 DAE
ANA	µmol H_2 plant^{-1} h^{-1}	5.4	13.8*
TNA	µmol H_2 plant^{-1} h^{-1}	17.2	33.7*
EAC		0.69	0.59*

(3) Intensive CO_2 fixation at pod formation results in clearly lower net CO_2 release per unit roots and nodules. This is even more surprising as nodule O_2 uptake and thus respiration was increased by more than 50%. Both facts resulted in an unusually low apparent respiratory quotient of roots and nodules at pod formation.

(4) Intensive N_2 fixation at pod formation was combined with a lower relative efficiency of nitrogenase. This may be explained by the possible insufficient availability of carbon skeletons for nitrogen assimilation, to which the enzyme might react with increased electron allocation to H^+, thereby avoiding excessive ammonium accumulation. When nodule malate formation is suppressed through a PEPC antisense construct, transgenic plants react with a clearly lower EAC compared to untransformed plants (Schulze *et al.*, 1998*b*).

There are several reports that nitrogen fixation in grain legumes peaks at early pod formation and shows a subsequent, occasionally steep, decline (Peat *et al.*, 1981; Jensen, 1987; Imsande, 1989; Vikman and Vessey, 1993*a*). This decline is often connected to the briskly progressing senescence of leaves. Under optimal experimental conditions, i.e. sufficient water, nutrients, and light, intensive nitrogen fixation often remains stable into the later stages of pod formation and pod filling (Vikman and Vessey, 1993*b*). This was the case in our experiments, which lasted about 2 weeks into pod formation although per plant nitrogen fixation showed a brisk decline after that period. During the time of our measurements, plants kept green leaves and almost no leaf senescence was visible at the end of the experimental growth interval. Contrary to this, a significant share of nodules showed clear signs of senescence indicated by a greenish colour. The progressing nodule senescence might be connected to the measured increased oxygen uptake of the nodules and the presumably related oxidative stress (Puppo *et al.*, 2005). Moreover, in senescing nodules of soybean, an increased activity of isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) was detected (Fargeix *et al.*, 2003). Increased activity of isocitrate lyase in the larger share of senescent and overall older nodules at pod formation circumvent a decarboxylation process in the TCA cycle and thus might have contributed to the observed low respiratory quotient and lower net carbon release of the root/nodule system.

In our experiment increased nitrogen fixation at pod formation was clearly connected with higher CO_2 fixation and improved use of refixed carbon for nitrogen assimilation. This is supported by the measured increased nodule $^{13}CO_2$ uptake and the larger share of asparagine in nodules and xylem sap at pod formation. These facts are supported by higher *in vitro* activity of PEPC and AAT, although a direct connection between *in vitro* and *in vivo* enzyme activity has to be considered with caution. *In vitro* activity represents potential activity. These data suggest that, at pod formation, more N assimilation is supported through the carbon skeletons from PEP carboxylation. Figure 7 demonstrates asparagine formation on the basis of carbon skeletons from that source versus drainage of keto-acids from the TCA cycle that constitutes a carbon-saving mechanism for roots and nodules. In the case of PEP carboxylation, one atom of carbon is gained for N transport while organic acids from the TCA cycle are eventually formed combined with a previous loss of carbon from pyruvate. In this way, increased CO_2 fixation improves the overall root/nodule carbon balance for N turnover and N transport to shoots. In fact, various data achieved on different grain legumes yielded consistently lower specific root/nodule carbon loss for driving N_2 fixation during pod formation and pod filling versus vegetative growth (Schulze *et al.*, 2000; Adgo and Schulze, 2002). The data in this report suggest that nodule CO_2 fixation and the use of the resulting carbon skeletons for N transport is a major mechanism for carbon-efficient N_2 fixation.

Assimilate shortage could not be detected in active nodules during pod formation. However, it cannot be ruled out that a certain depletion of background reserves like starch or poly-hydroxybutyrate had already occurred and

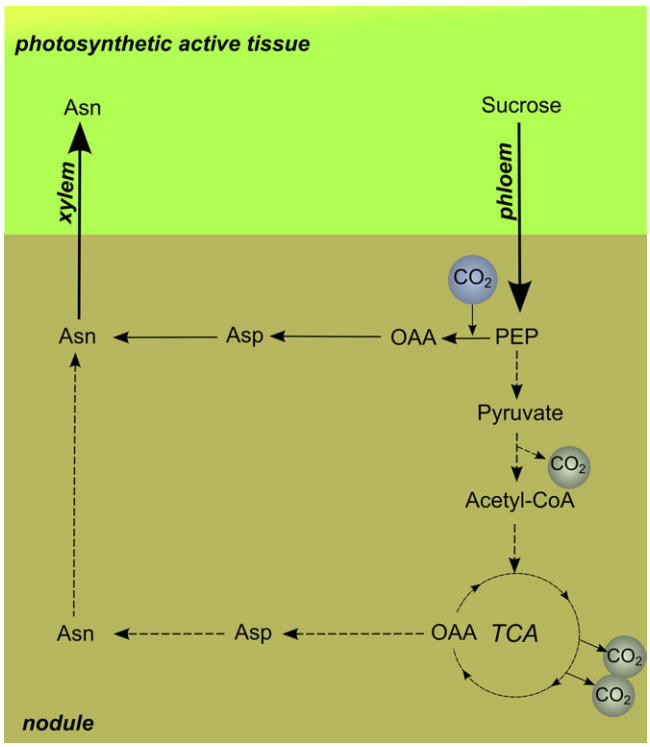

Fig. 7. Alternative ways of carbon skeleton provision for asparagine formation in indeterminate legume nodules. PEP in nodules can either be decarboxylated and feed the TCA cycle, or can become carboxylated and serve directly as carbon skeleton (oxaloacetate, OAA) for N assimilation and N transport to the shoot. In comparison, on root/nodule basis, the use of OAA from carboxylation for N assimilation and N transport is connected with a carbon gain through CO_2 fixation, while OAA formation in the TCA cycle occurs with CO_2 release on root/nodule basis. The use of OAA from carboxylation of PEP for N assimilation and N transport necessitates no further reductive step and thus energy loss for the nodules.

would result in unstable assimilate availability during pod filling. The high variability of our data with respect to nodule sugar concentration might be an indication of this. A higher concentration of fructose and a tendency of higher total sugar concentration at pod formation in conjunction with higher respiration and N assimilation might indicate an increased allocation of assimilates to nodules at that growth stage. Various reports indicate that although assimilate supply does not usually limit nodule activity (Vance and Heichel, 1991; Schulze, 2004), it might be critical during pod formation when growing pods not only induce high activity in nodules but, in addition, attract huge amounts of carbon. For example, the use of hup^+ versus hup^- *Rhizobium* strains show effects on nitrogen fixation and growth but not before the onset of pod growth (Bergersen *et al.*, 1995). Provision of additional assimilates through sugar spraying on leaves had no effect on pea plants during vegetative growth while it significantly improved nitrogen fixation at pod formation and pod filling (Schulze *et al.*, 1994). The interdependence between pea plant photosynthetic capacity and assimilate supply of nodules during pod formation and pod filling might be particularly critical in semi-leafless varieties like the one used in our experiments.

Stronger nodule CO_2 fixation is also connected with emerging P deficiency in legumes (Schulze *et al.*, 2006). P deficiency impairs photosynthetic activity and assimilate supply to nodules. Consequently, a carbon-saving mechanism like CO_2 fixation might be of vital importance for adaptation to low P availability. Christeller *et al.* (1977) suggested that nodule N assimilation had to be almost completely supported by oxaloacetate from PEPC activity, a fact that would result in an up to 25% proportion of nodule fixed carbon in N transport. Otherwise, a significant drainage of keto-acids out of the TCA cycle pool would impair acetic acid influx into the cycle due to insufficient oxaloacetate availability (Walker, 1962). Overall our data support this hypothesis, in that the ability to convert sugar into organic acids was limited or at least on the edge of limitation at pod formation. In particular, the virtual 'disappearance' of succinate in nodules at pod formation suggests that the TCA cycle is significantly drained for N assimilation. This might constitute an additional factor in overstretching nodule activity and adding to emerging nodule senescence at pod formation, as the gap between increasing energy demand and impaired ability for energy provision widens.

In conclusion, the capacity of nodules to fix CO_2 is of crucial importance for their efficiency. Nodule CO_2 fixation provides a mechanism for saving carbon when the fixed carbon is used for N assimilation and N transport. The ability of nodules to fix CO_2 can be influenced through the use of agronomic measures, breeding, and genetic techniques. High CO_2 around nodules support nitrogen fixation (Yamakawa *et al.*, 2004; Fischinger *et al.*, 2010). There are reports that straw application positively affects nitrogen fixation in a subsequent crop (Shivashankar and Vlassak, 1978; Evans *et al.*, 1997). This might be the result of higher CO_2 concentration in the soil atmosphere. Selection for high PEPC activity in nodules of alfalfa plants improved their growth performance. Various nodule-enhanced forms of key enzymes of the biochemical pathways have been identified (Suganuma *et al.*, 1997; Miller *et al.*, 1998; Fedorova *et al.*, 1999). While down-regulating of PEPC, for example, reduces nitrogen fixation (Nomura *et al.*, 2006), over-expression of MDH increases nodule specific activity (Schulze *et al.*, 2002). Our data suggest that an improvement of nodule capability to channel assimilates into oxaloacetate and malate formation through CO_2 fixation might prolong intensive nitrogen fixation in grain legumes into the later stages of ontogeny.

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