

1 Title: **Nitrite Protects Mitochondrial Structure and Function under Hypoxia**

3 Running head: Nitrite and mitochondrial activity under hypoxia

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14 Subject area: Environmental and stress responses

16 Black and white figures: 5

17 Colour figures: 1

18 Tables: 0

19 Supplementary material: None

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37 Abbreviations: ANOVA, analysis of variance; BN-PAGE, blue native polyacrylamide gel
38 electrophoresis; BSA, bovine serum albumin; DCFDA, 2',7'-dichlorofluorescein diacetate;
39 MDA, malondialdehyde; NO, nitric oxide; ROS, reactive oxygen species; TCA,
40 trichloroacetic acid; TMRM, tetramethyl rhodamine methyl ester.

Abstract

Oxygen deprivation leads to changes in mitochondrial morphology and impaired flow of reducing equivalents through the electron transport chain. The extent of these changes depends on the duration and severity of the treatment as well as on the species and cell type. Nitrate is known to ameliorate these effects in some instances but it is possible that it is nitrite, rather than nitrate, that is the key to the mechanism. To test this, mitochondria were isolated from 21 d old pea (*Pisum sativum*) roots and incubated for 90 min under normoxia or hypoxia in the presence or absence of 0.5 mM nitrite. The supply of nitrite under hypoxia led to nitric oxide production, improved mitochondrial integrity, improved energisation of the inner mitochondrial membrane, increased ATP synthesis, decreased production of reactive oxygen species and decreased lipid peroxidation. It also resulted in higher levels and activities of complex I and the supercomplex I+III₂. It is concluded that nitrite has an important role in maintaining mitochondrial function under hypoxia, and that it achieves this through the reduction of nitrite to nitric oxide by the mitochondrial electron transport chain.

Key words: Hypoxia, mitochondria, nitric oxide, nitrite, *Pisum sativum*, respiration.

61

62 **Introduction**

63

64 Flooding is a common cause of oxygen deprivation in plants, particularly in roots, and
65 the reduced availability of oxygen under such conditions leads to a major reorganisation
66 of primary metabolism (Shingaki-Wells *et al.*, 2014). At the level of the mitochondria,
67 the reduced availability of oxygen under hypoxic conditions, or its complete absence
68 under anoxia, has a major impact on the mitochondrial electron transport chain because
69 the flux through this pathway is largely dependent on using oxygen as the terminal
70 electron acceptor. However mitochondrial metabolism does not cease entirely and one
71 pathway that becomes more important during oxygen deprivation is the mitochondrial
72 pathway for the synthesis of nitric oxide (NO). Mitochondrial reduction of nitrite to NO is
73 considered to be the main source of NO when plants experience low oxygen (Gupta and
74 Igamberdiev, 2011; Gupta *et al.*, 2011; Igamberdiev *et al.*, 2014) and this is important
75 because of the established role for NO in mediating the biochemical response to such
76 conditions. In particular NO has been shown to contribute to the recycling of NADH
77 (Igamberdiev and Hill, 2004; Stoimenova *et al.*, 2007), the synthesis of ATP
78 (Stoimenova *et al.*, 2007), and the regulation of oxygen consumption (Borisjuk *et al.*,
79 2007) under low oxygen. The involvement of NO in these processes is facilitated by the
80 accumulation of nitrite that occurs under hypoxia due to the inhibition of plastidic nitrite
81 reductase (Planchet *et al.*, 2005).

82

83 Marked changes in mitochondrial structure can occur under low oxygen and these effects
84 correlate to some extent with the ability of a plant to survive periods of hypoxia or
85 anoxia (Vartapetian *et al.*, 2003; Shingaki-Wells *et al.*, 2014). For example mitochondria
86 from wheat seedlings, which are anoxia-intolerant, showed rapid changes in
87 mitochondrial morphology following the onset of anoxia and these changes could be
88 reversed provided the duration of the anoxic episode was short (Vartapetian *et al.*,

1985). In contrast anoxia had no effect on the mitochondrial ultrastructure of the anoxia-tolerant *Echinochloa crus-galli* (Kennedy *et al.*, 1980), and only a minor effect on the mitochondria from intact rice coleoptiles (Vartapetian *et al.*, 1976). The difference between anoxia-tolerant and intolerant species is not always observed (Couée *et al.*, 1992), and the extent to which changes in morphology compromise mitochondrial function can be influenced by the growth and treatment conditions of the plant or mitochondria. In this regard several papers have emphasised the protective effect of nitrate on plant mitochondria exposed to low oxygen conditions (Vartapetian and Polyakova, 1999; Polyakova and Vartapetian, 2003; Vartapetian *et al.*, 2003). For example supply of nitrate protected the ultrastructure and integrity of the mitochondria in excised rice coleoptiles (Vartapetian and Polyakova, 1999) and wheat roots (Polyakova and Vartapetian, 2003) under anoxia. This effect, and other protective effects of nitrate under anoxia, have been attributed to nitrate acting as an electron acceptor (Vartapetian *et al.*, 2003) but the interpretation of such observations is complicated by the fact that nitrite, the reduction product of nitrate, can also contribute to the amelioration of the effect of hypoxia on plants. For example, it has been shown that it is nitrite that is responsible for the beneficial effect of nitrate on pH regulation under anoxia (Libourel *et al.*, 2006), and that nitrate reduction itself did not contribute significantly to pH regulation under these conditions. Moreover the absence of nitrate reduction as an NADH-consuming process was unable to explain the greater sensitivity of a tobacco (*Nicotiana tabacum*) transformant lacking root nitrate reductase to oxygen deprivation (Stoimenova *et al.*, 2003).

The anoxic activation of nitrate reductase (Kaiser *et al.*, 1999), and the resulting accumulation of nitrite, are important for plant survival under hypoxia, but to date there is no direct evidence for the protective role of nitrite on mitochondrial structure and function. Accordingly this paper reports an investigation into the effect of nitrite on the structure and function of pea root mitochondria under hypoxia and concludes that there is strong evidence for the presumed protective effect of nitrite.

Results

Mitochondria were extracted from the roots of 3-4 week old, hydroponically-grown pea (*Pisum sativum*) plants prior to incubation under normoxic or hypoxic conditions in the presence or absence of 0.5 mM nitrite. Two considerations guided the choice of nitrite level. First, nitrite can reach millimolar levels in anaerobic root issues (Miller and Smith, 1996), reflecting the inhibition of nitrite reductase and the availability of nitrate for reduction. Secondly, the K_m for the conversion of nitrite to NO is 175 μ M for anoxic tobacco (*Nicotiana tabacum*) root mitochondria and 210 μ M for anoxic root segments (Gupta *et al.*, 2005).

Nitrite reduction to NO and nitrite protection of mitochondria under hypoxia

It has been previously demonstrated that isolated mitochondria from roots and plant cell suspensions reduce nitrite to NO in a completely oxygen-free environment and that mitochondrial electron transport contributes to this process (Gupta *et al.*, 2005; Planchet *et al.*, 2005). In agreement with these observations Figure 1 shows that isolated pea root mitochondria reduced nitrite to NO under hypoxia (0.4% oxygen, 99.6% nitrogen), with NO production starting after the addition of nitrite and reaching a steady state after approximately 100 min. To test the impact of nitrite reduction on mitochondrial integrity, freshly isolated mitochondria were first incubated with MitoTracker Green for 30 min and then subjected to either hypoxia or normoxia for a further 90 min. As shown in Figure 2, including nitrite in the incubation medium increased the level of MitoTracker Green fluorescence under both normoxia and hypoxia, with the effect under hypoxia being particularly marked (Fig. 2e). Under normoxia, the mitochondria retained their normal size (Fig. 2a,b), but under hypoxia in the absence of nitrite the fluorescent marker appeared to be largely present in minute submitochondrial particles (Fig. 2c). In contrast the mitochondria incubated with nitrite under hypoxia retained their normal size with

larger mitochondrial structures clearly visible (Fig. 2d). Thus nitrite protected mitochondrial structure under hypoxic conditions.

Effect of nitrite on the mitochondrial membrane potential and ATP synthesis

The effects of nitrite and hypoxia on the mitochondrial membrane potential were assessed by monitoring tetramethyl rhodamine methyl ester (TMRM) fluorescence (Fig. 3). Under normoxia, incubation with nitrite had no significant effect on the mitochondrial membrane potential; whereas under hypoxia TMRM fluorescence decreased in the absence of nitrite, indicating a less polarised membrane, but substantially recovered in the presence of nitrite (Fig. 3a). This suggests that the absence of nitrite as a terminal acceptor for the electron transport chain compromises electron transport under hypoxia leading to mitochondrial depolarization. The mitochondrial membrane potential is a critical parameter for the function of the respiratory chain in ATP synthesis and in keeping with the effect of nitrite on the mitochondrial membrane potential (Fig. 3a), nitrite boosted ATP synthesis under hypoxia (Fig. 3b). Nitrite is known to have no effect on mitochondrial respiration under aerobic conditions (Millar and Day, 1996), but under hypoxia, where the mitochondrial production of ATP is very low, nitrite has a beneficial effect on mitochondrial function increasing ATP synthesis by more than 200% (Fig. 3b).

Nitrite supply reduces ROS production and lipid peroxidation under hypoxia

Further evidence for the impact of nitrite on mitochondrial electron transport under hypoxia was obtained by measuring production of reactive oxygen species (ROS) and lipid peroxidation. The conversion of nitrite to NO consumes reducing equivalents, and under hypoxia this might have a significant effect on the redox status of the ubiquinone pool, reducing electron leakage and the concomitant production of ROS (Blokhina *et al.*, 2003). In agreement with this prediction ROS production (Fig. 4a) and lipid peroxidation (Fig. 4b) were significantly elevated under hypoxia in the absence of nitrite. Incubating

hypoxic mitochondria with nitrite restored ROS production and lipid peroxidation to the levels observed in normoxic mitochondria, emphasising the importance of nitrite in maintaining mitochondrial function at low oxygen levels.

Effect of nitrite on mitochondrial electron transport complexes and supercomplexes

Blue native polyacrylamide gel electrophoresis (BN-PAGE) showed that the levels of complexes I, II and IV under hypoxia, as well as the levels of supercomplex I+III₂ and complex V under both normoxia and hypoxia, were higher in the presence of nitrite (Fig. 5). Supercomplexes are known to influence electron flux in the mitochondrial electron transport chain (Lapiente-Brun et al 2013), and the higher levels of several complexes under hypoxia in the presence of nitrite is consistent with the increased ATP production by hypoxic mitochondria incubated with nitrite (Fig. 3b). In parallel with these observations it was found that nitrite increased the activities of both complex I and I+III under hypoxia (Fig. 6).

Discussion

Loss of mitochondrial integrity is a likely consequence for most plants during flooding, and mechanisms that protect the mitochondria and promote their recovery after transient flooding events have an important bearing on the overall sensitivity of plants to oxygen deprivation. Earlier work showed that both ATP (Couée *et al.*, 1992) and nitrate (Vartapetian *et al.*, 2003) have a protective effect on mitochondria under low oxygen. For example providing a supply of exogenous ATP to isolated mitochondria from rice shoots supported protein synthesis under anoxia, implying that mitochondrial protein synthesis could occur in flooded plants given a source of ATP from glycolysis (Couée *et al.*, 1992). The latter depends on an adequate carbohydrate supply, and it has been demonstrated that while the mitochondria in excised rice coleoptiles show more extensive degradation under anoxia than the mitochondria in intact seedlings, the

204 difference can be abolished by adding glucose to the incubation medium (Vartapetian *et*
205 *al.*, 1976). Similarly several papers provide compelling evidence that the provision of
206 nitrate can prevent anoxia-induced loss of mitochondrial integrity (Vartapetian and
207 Polyakova, 1999; Polyakova and Vartapetian, 2003; Vartapetian *et al.*, 2003), and it has
208 been suggested that this effect arises through the action of nitrate as a terminal electron
209 acceptor for the mitochondrial electron transport chain (Polyakova and Vartapetian,
210 2003). Although there is no direct evidence that nitrate can act in this way in plant
211 mitochondria, it is known that nitrite is a substrate for both complex III and complex IV
212 of the mitochondrial electron transport chain (Gupta *et al.*, 2005; Stoimenova *et al.*,
213 2007; Igamberdiev *et al.*, 2014), suggesting that it would be worthwhile to focus on the
214 effect of nitrite on mitochondrial integrity and function under hypoxia.

215
216 The experiments reported here show that the supply of nitrite to isolated pea root
217 mitochondria under hypoxia leads to NO production (Fig. 1), improved mitochondrial
218 integrity (Fig. 2), improved energisation of the inner mitochondrial membrane (Fig. 3a),
219 increased ATP synthesis (Fig. 3b), decreased ROS production (Fig. 4a) and decreased
220 lipid peroxidation (Fig. 4b). All of these observations are consistent with the conclusion
221 that nitrite has an important role in maintaining mitochondrial function under hypoxia,
222 and that it achieves this through its reduction to NO by the mitochondrial electron
223 transport chain. While much previous work has focused on the contribution of nitrate to
224 the promotion of plant survival during oxygen deprivation, nitrite levels are known to
225 increase under anoxia, leading to readily detectable nitrite efflux from roots (Lee, 1979;
226 Morard *et al.*, 2004; Libourel *et al.*, 2006). This arises because of the activation of
227 nitrate reductase under anoxia (Kaiser *et al.*, 1999; Allègre *et al.*, 2004), and a decrease
228 in the activity of the enzyme that would normally convert nitrite to ammonium, nitrite
229 reductase (Botrel *et al.*, 1996). Thus nitrite availability increases when plants are
230 exposed to nitrate under anoxia, facilitating the maintenance of mitochondrial function *in*
231 *planta* through the mitochondrial reduction of nitrite to NO.

The observations on the nitrite-dependent energisation of the inner mitochondrial membrane, ATP synthesis, ROS production and lipid peroxidation are all consistent with a fully functional electron transport chain under both normoxia and hypoxia. BN-PAGE confirmed the presence of all four electron transport complexes under both conditions, and it also showed that the levels of both complex I and supercomplex I+III₂ were higher under hypoxia in the presence of nitrite (Fig. 5). The higher total complex I level may be significant because it has been reported that the cytoplasmic male sterile mutant of *Nicotiana sylvestris*, which lacks a functional complex I, shows very low NO production (Shah *et al.*, 2013), suggesting that the higher complex I activity seen here (Figs 5, 6) could contribute directly to the maintenance of mitochondrial function under hypoxia by promoting the reduction of nitrite to NO. The higher level of supercomplex I+III₂ in the presence of nitrite (Fig. 5) may also be important for enhanced mitochondrial function and integrity under hypoxia, given the evidence that supercomplexes improve the efficiency of electron transport and that their formation is promoted by correct folding of the mitochondrial cristae (Cogliati *et al.*, 2016). Supercomplex formation is a dynamic process that responds to multiple signals (Genova and Lenaz, 2014) and prolonged hypoxia (36 h) has been shown to alter supercomplex activity in potato mitochondria (Ramirez-Aguilar *et al.*, 2011). The much shorter hypoxic treatment used here had no effect on I+III activity (Fig. 6b), but there was a significantly higher activity under hypoxia in the presence of nitrite providing evidence for the importance of nitrite as a determinant of mitochondrial function.

While nitrite reduction is the most likely explanation for the nitrite-dependent maintenance of mitochondrial integrity and function under hypoxia, other possible explanations for some of the observations could be a direct regulatory effect of nitrite itself, or more plausibly, regulatory effects of the NO produced in the reduction step. Given the established role of NO in mediating the response of plants to low oxygen (Gupta *et al.*, 2011) it could be useful to examine the effect of NO on mitochondrial integrity and activity under hypoxia in the absence of nitrite. This could be done either

by supplying NO as a gas (Millar and Day, 1996) or by using an NO donor such as S-nitrosoglutathione, assuming this to be a substrate for the mitochondrial thioredoxin pathway. However the known inhibitory effect of NO on cytochrome oxidase (Millar and Day 1996) would appear to be inconsistent with the improved energisation of the mitochondria and increased ATP synthesis observed under hypoxia in the presence of nitrite.

In conclusion nitrite plays a crucial role in maintaining mitochondrial integrity and function under hypoxia. Mitochondrial reduction of nitrite to NO can act as an alternative to fermentation, recycling NADH, generating ATP and decreasing ROS production. Efficient use of energy resources is critically important for survival under hypoxia and the availability of nitrite allows some contribution from the mitochondria at the greatly reduced level of the usual terminal electron acceptor.

Material and methods

Plant materials and growth conditions

Pea seeds (*Pisum sativum* cv. Ambassador) were surface-sterilized with a mixture of Tween-20 detergent (0.05%) and 10% NaOCl and washed four times with autoclaved distilled water. Seeds were germinated on sterilized wet filter paper for 48 h and then grown for a further 3-4 weeks in 2 l hydroponic pots. The full-strength nutrient solution contained: 5 mM NH₄NO₃, 1 mM CaCl₂, 1 mM MgSO₄, 0.025 mM NaFe-EDTA, 1 mM K₂HPO₄, 2 mM KH₂PO₄, and trace elements according to Johnson *et al.* (1957). Plants were gradually adapted (1/10, 1/4, 1/2, and 3/4 of full-strength) to the full-strength nutrient solution over the first four days of hydroponic growth. The nutrient solution was adjusted to pH 6.3 with KOH and the medium was flushed continuously with air using needle diffusers. The pots were placed in growth chambers with artificial illumination (HQI 400 W, Schreder, Winterbach, Germany) at a photon flux density of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (PAR)

and a day length of 16 h. The day/night temperature regime of the chamber was 25/20°C respectively.

Isolation of mitochondria

Mitochondria were isolated at 4°C from 25-30 g FW of 21 d old pea roots using a procedure similar to that described elsewhere (Vanlerberghe *et al.*, 1995; Gupta *et al.*, 2005). Roots were sliced into 1-2 cm segments and washed four times. Root segments were homogenized using a laboratory blender containing 250 ml of homogenization medium (0.3 M sucrose, 100 mM HEPES, 0.1% (w/v) bovine serum albumin (BSA), 0.6% polyvinylpyrrolidone (w/v), 1 mM EDTA, 2 mM MgCl₂, 4 mM cysteine, 5 mM KH₂PO₄, pH 7.6) and a ready-to-use protease cocktail ('Complete', Roche, Mannheim, Germany; 1 tablet in 100 ml medium). The homogenate was filtered through two layers of miracloth (GE Healthcare) and two layers of cheesecloth. The filtered cell extract was centrifuged at 1000g for 15 min with a Beckman ultra-centrifuge, and the supernatant was centrifuged again at 10,000g for 20 min. The resulting pellet was suspended in 4 ml of suspension medium containing 20 mM HEPES pH 7.6, 0.3 M sucrose, 0.1% (w/v) BSA, 2 mM MgCl₂, 1 mM EDTA, and 0.5 mM KH₂PO₄. The mitochondria were further purified on a discontinuous Percoll gradient composed of the following steps (bottom to top): 3 ml of 60% (v/v), 4 ml of 45% (v/v), 4 ml of 28% (v/v), 4 ml of 5% (v/v), all containing 250 mM sucrose, 20 mM HEPES pH 7.6, and 0.1% BSA. The mitochondria (4 ml) were layered on top, and the gradient was centrifuged at 30,000g for 40 min. The mitochondrial fraction appeared at the interface between the 45% and 28% (v/v) layers and was collected, diluted in 16 ml suspension medium, centrifuged (27,000g, 10 min), and resuspended twice in 8 ml, in order to remove Percoll. The final mitochondrial pellet was suspended in 2 ml medium. The protein content of the resuspended mitochondrial pellet was determined by the Bradford assay and was typically 2-3 mg protein/ml.

Hypoxic and normoxic treatment of mitochondria

Immediately after isolation, or in some cases following incubation for 30 min with MitoTracker Green, mitochondria (0.5 mg protein) in 1 ml suspension medium supplemented with 0.5 mM ADP, 0.5 mM NADH, and in some experiments 0.5 mM potassium nitrite, were transferred to an open Eppendorf tube that was placed in a plastic box (40x10x10 cm) fitted with inlet and outlet tubes. The box was flushed with either air or 0.4% oxygen/99.6% nitrogen for 90 min at 25°C. Mitochondria were then used immediately for confocal microscopy and blue native PAGE, or they were frozen for subsequent analysis of ATP, ROS, lipid peroxidation, and assays of complex I and complex I+III.

Nitric oxide production under hypoxia

Mitochondrial NO production at 25°C was measured by the chemiluminescence method (Gupta et al., 2005; Gupta and Kaiser, 2010) using a detector (CLD 700 AL Eco-Physics, Dürnten, Switzerland) with a detection limit of 0.1 ppb and a time resolution of 1 s. Mitochondrial suspensions (2 mg protein in 20 ml suspension medium plus 0.5 mM ADP, 0.5 mM NADH and 0.5 mM potassium nitrite) were placed in a 1 l flask which was bubbled with a 0.4% oxygen, 99.6% nitrogen gas mixture (BOC, UK). A constant flow of gas was passed from the cuvette to the detector through the action of a vacuum pump connected to an ozone destroyer. Calibration was carried out with NO-free air (0 ppt NO) and with NO gas (1000 ppb). The chemiluminescence data were logged into a computer and processed using customized 'Visual designer' based software (Intelligent Instrumentation Inc., Tucson, USA).

ATP measurements

ATP was measured with a luciferase assay system (Promega) using a procedure similar to that described elsewhere (Stoimenova et al., 2007). Aliquots of frozen mitochondria (0.1 mg protein) were homogenised in 1 ml 10% trichloroacetic acid (TCA), neutralized to pH 7.0, and centrifuged at 10,000g for 5 min. The supernatant was diluted 1:10 with 100 mM HEPES-KOH (pH 7.4) and 100 µl was placed in a multiwell plate containing 20

mM HEPES-KOH pH 7.4, 3 mM MgCl₂ and 10 µl of luciferin-luciferase assay to a total volume of 200 µl. Luminescence was recorded over a period of 20 s using a Beckman Coulter DTX880 multimode detector.

Lipid peroxidation assay

Lipid peroxidation was measured by the colorimetric assay of malondialdehyde (MDA) (Jambunathan, 2010). Aliquots of frozen mitochondria (1 mg protein) were homogenized in 4 ml 0.1% TCA and centrifuged at 10,000g for 15 min. Supernatant (1 mL) was mixed with 2 mL of 20% TCA and 2 mL of 0.5% thiobarbituric acid and heated at 95°C for 30 min. After cooling on ice the absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated from ($A_{532} - A_{600}$) using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

ROS estimation

Total ROS levels were determined using 2',7'-dichlorofluorescein diacetate (DCFDA) (Jambunathan, 2010). Mitochondria (0.2 mg protein) were suspended in 1 mL of 10 mM Tris-HCl, pH 7.2, freeze-thawed and then centrifuged at 12,000g for 20 min at 4°C. The extract was incubated in the dark for 10 min with 10 µM DCFDA and the fluorescence was measured using a Beckman plate reader.

Confocal microscopy

For MitoTracker Green loading, freshly isolated mitochondria (1 mg protein/ml) were incubated in 500 nM MitoTracker Green in a medium that contained 0.3 M sucrose, 10 mM TES, pH 7.2 (adjusted with NaOH) in the dark for 30 min at 4°C. Mitochondria were immobilized onto round glass cover slides by centrifugation (2,000g, 5 min, 4°C) in suspension medium. Immediately before imaging, mitochondria were energized by addition of medium containing 0.5 mM NADH and labelled with 100 nM TMRM (Schwarzlander *et al.*, 2012). Microscopy was performed using a Zeiss LSM510 META confocal microscope (Carl Zeiss MicroImaging) equipped with laser lines for 488 and 543

nm excitation. Images were collected with a 60× lens (Zeiss 60× 1.4 N.A. Plan-Apochromat oil-immersion lens) in multi-track mode with line switching between channels. Excitation/emission wavelengths were selected for TMRM at 543/565-615 nm and MitoTracker Green at 488 nm/505-530 nm. The pinhole diameter was 1.2-1.4 Airy units depending on the emission band-pass filter, but the optical slice was kept constant at 1.0 µm for all channels.

Blue native PAGE

BN-PAGE was performed according to Eubel *et al.* (16). In brief, isolated mitochondria were solubilized in solubilization solution (5 mg digitonin per mg protein, 150 mM potassium acetate, 30 mM HEPES (pH 7.4)) for 20 min. Following a brief centrifugation (20,000g for 10 min), the pellet was discarded and 1 µl of Serva blue solution (5% (w/v) Coomassie Blue in 750 mM aminocaproic acid) was added for every 20 µl of the supernatant. Samples were then loaded onto a continuous 1.5-mm-thick polyacrylamide gradient gel (consisting of a 4% stacking gel and a 4.5-12% separating gel) in a Hoefer SE400 Vertical Electrophoresis System. Electrophoresis was carried out for 13-15 h at 4°C with a current of 9 mA per gel and a maximal voltage of 500 V.

Complex I activity (NADH: ubiquinone oxidoreductase, EC 1.6.5.3)

Complex I activity was measured in freeze-thawed mitochondria using the procedure described by van Bergen *et al.* (2014). Mitochondrial protein (50 µg) was added to a 1 ml quartz cuvette containing 500 µl sucrose/Tris buffer (250 mM sucrose, 100 mM Tris-HCl, 2 mM EDTA), pH 7.4, 1 mM decylubiquinone and 0.1 mM KCN. The volume was made up to 950 µl with distilled water, and after checking the absorbance at 340 nm, the reaction was initiated by adding 50 µl of 1 M NADH solution. The oxidation of the added NADH was measured at 340 nm for 1 min. The assay was repeated in the presence of 1 µg rotenone to correct for the small (<5%) contribution from the alternative NADH dehydrogenases.

Complex I+III assay (NADH-cytochrome c oxidoreductase)

Complex I+III activity was measured in freeze-thawed mitochondria using the procedure described by van Bergen *et al.* (2014). Mitochondrial protein (50 µg) was added to a 1 ml quartz cuvette containing 500 µl of 40 mM potassium phosphate buffer, pH 7.4, 50 µM NADH, 300 µM cytochrome-c and 0.1 mM KCN. The volume was made up to 1 ml with distilled H₂O and cytochrome-c reduction was measured at 550 nm for 3 min. The assay was repeated in the presence of 1 µg rotenone to correct for the small (<5%) contribution from the alternative NADH dehydrogenases.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS 21.0. All data were tested for normality and homogeneity of variance. Comparisons for which P<0.05 were considered to be significantly different.

Funding

This work was supported by a Marie Curie Intra-European Fellowship for Career Development within the 7th European Community Framework Programme (K.J.G. and R.G.R.). C.P.L. was supported by a European Molecular Biology Organization (EMBO) long-term fellowship (ALTF 1140-2011).

Disclosures

The authors have no conflicts of interest to declare.

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Figure legends

Fig. 1 Nitric oxide production by pea root mitochondria under hypoxia. Mitochondria (0.1 mg protein ml⁻¹) were incubated in hypoxic suspension medium supplemented with 0.5 mM nitrite 0.5 mM ADP, and 0.5 mM NADH. NO emission was measured continuously by gas phase chemiluminescence (n = 2±SD).

Fig. 2 Effect of nitrite on normoxic and hypoxic pea root mitochondrial integrity. Representative MitoTracker Green fluorescence images from two independent experiments after a 90 min incubation under normoxia (a, b) or hypoxia (c, d) in the absence (a, c) or presence (b, d) of 0.5 mM nitrite. (e) Intensity of MitoTracker Green fluorescence in the presence (+) or absence (-) of nitrite (n=4±SD). Means with different letters are significantly different (one-way ANOVA, *P* <0.01).

Fig. 3 Effect of nitrite pea root mitochondrial membrane potential and ATP production. (a) TMRM fluorescence of isolated mitochondria was quantified with Image J after a 90 min incubation under normoxia or hypoxia in the presence (+) or absence (-) of 0.5 mM nitrite (n = 4±SD). Means with different letters are significantly different (one-way ANOVA, *P* < 0.01). (b) ATP in mitochondrial suspensions was measured with luciferase after a 90 min incubation under hypoxia in the presence (+) or absence (-) of 0.5 mM nitrite (n = 3±SD). Means with different letters are significantly different (t-test, *P* < 0.001).

Fig. 4 Effect of nitrite on ROS levels and lipid peroxidation in normoxic and hypoxic pea root mitochondria. (a) Total ROS levels in mitochondria were measured by DCFDA fluorescence after a 90 min incubation under normoxia or hypoxia in the presence (+) or absence (-) of 0.05 mM nitrite (n = 3±SD). Means with different letters are significantly different (one-way ANOVA, *P* < 0.001). (b) Lipid peroxidation in mitochondria was quantified by the colorimetric MDA assay after a 90 min incubation under normoxia or

hypoxia in the presence (+) or absence (-) of nitrite ($n = 3 \pm \text{SD}$). Means with different letters are significantly different (one-way ANOVA, $P < 0.05$).

Fig. 5 Effect of nitrite on the levels of respiratory complexes in normoxic and hypoxic pea root mitochondria. (a) Protein complexes from mitochondria incubated for 90 min under normoxia or hypoxia in the presence (+) and absence (-) of 0.5 mM nitrite were separated by BN-PAGE and visualized by Coomassie staining. The gel is representative of three independent experiments and the identity of the bands follows Eubel *et al.* (2003): I, NADH dehydrogenase; II, succinate dehydrogenase; III₂, cytochrome c reductase; IV, cytochrome oxidase; V, ATP synthase, F₁, the F₁ component of the ATP synthase, I+III₂, a supercomplex of complexes I and III. (b - g) Bands were quantified by Image J ($n = 3 \pm \text{SD}$) and means with different letters are significantly different (one-way ANOVA, $P < 0.05$).

Fig. 6 Effect of nitrite on the activities of complex I and complex I + III in normoxic and hypoxic pea root mitochondria. Specific activity of (a) complex I and (b) complex I + III from mitochondria incubated for 90 min under normoxia or hypoxia in the presence (+) or absence (-) of 0.5 mM nitrite ($n = 3 \pm \text{SD}$). Means with different letters are significantly different (one-way ANOVA, $P < 0.05$).

Figure 1

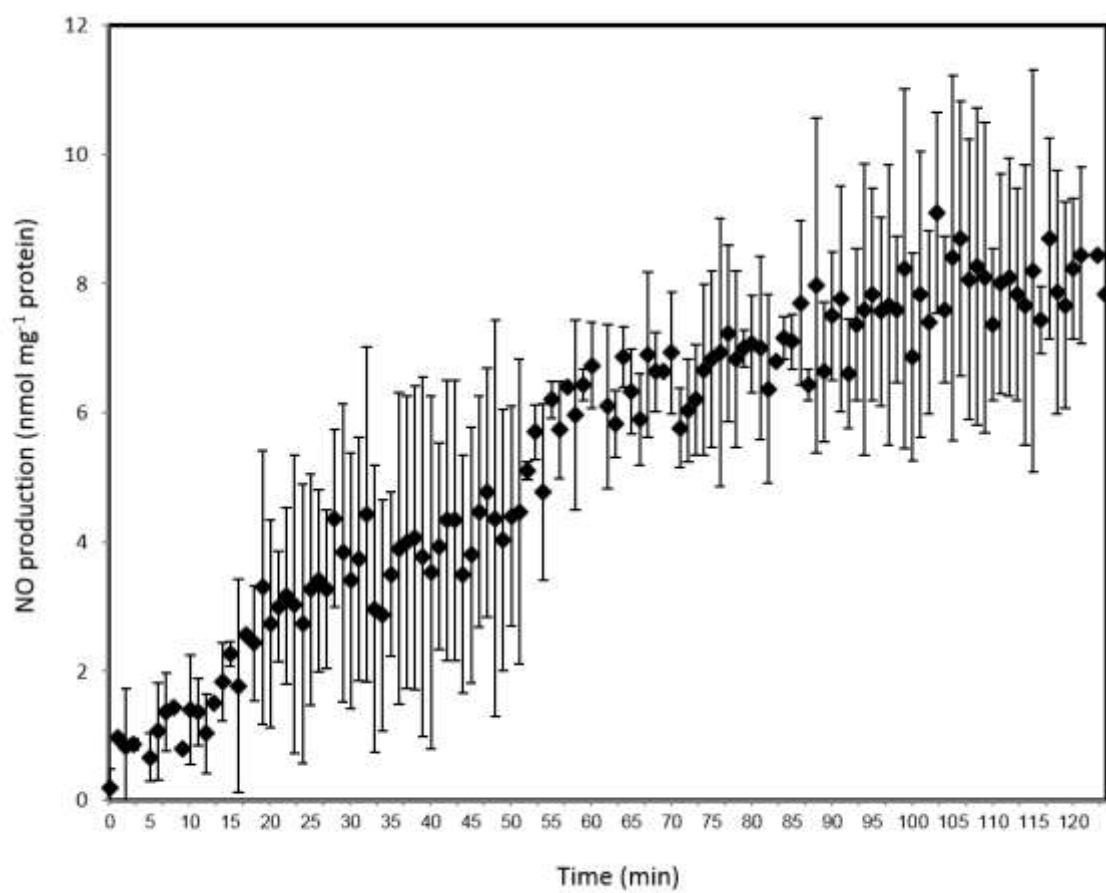


Figure 2

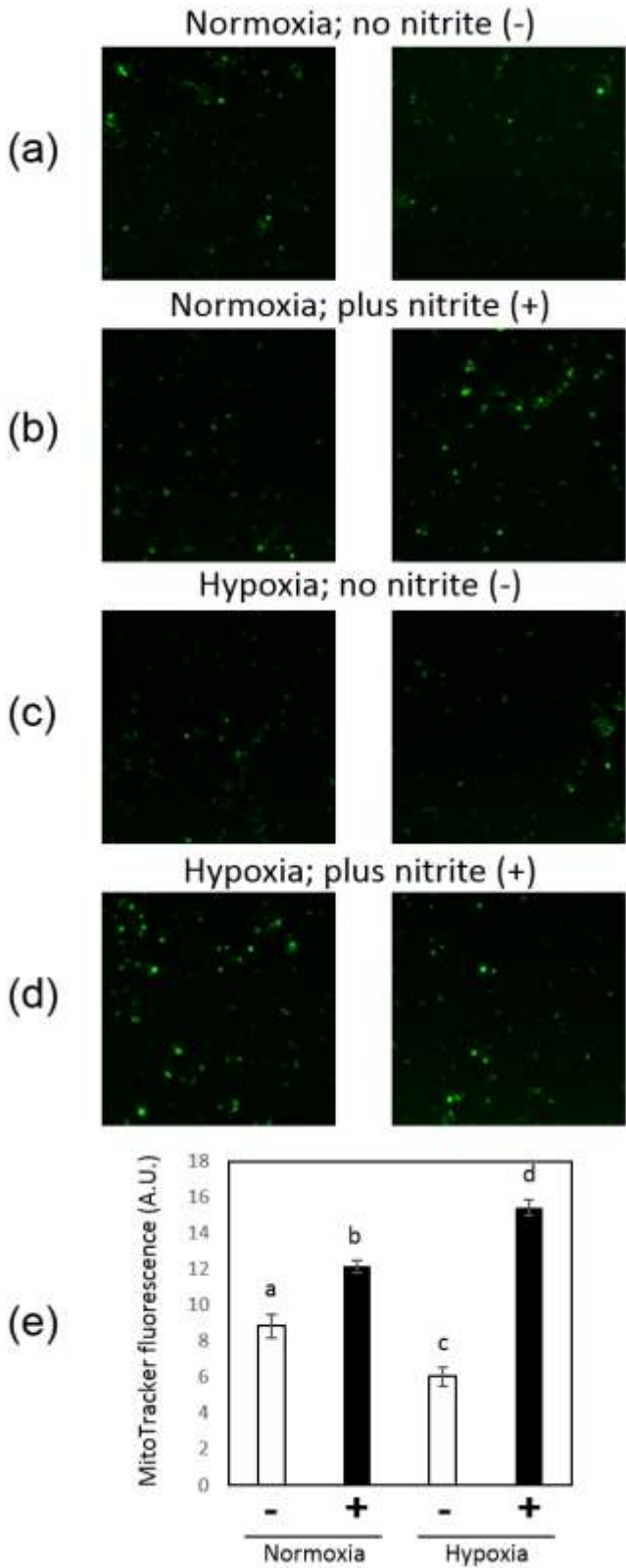


Figure 3

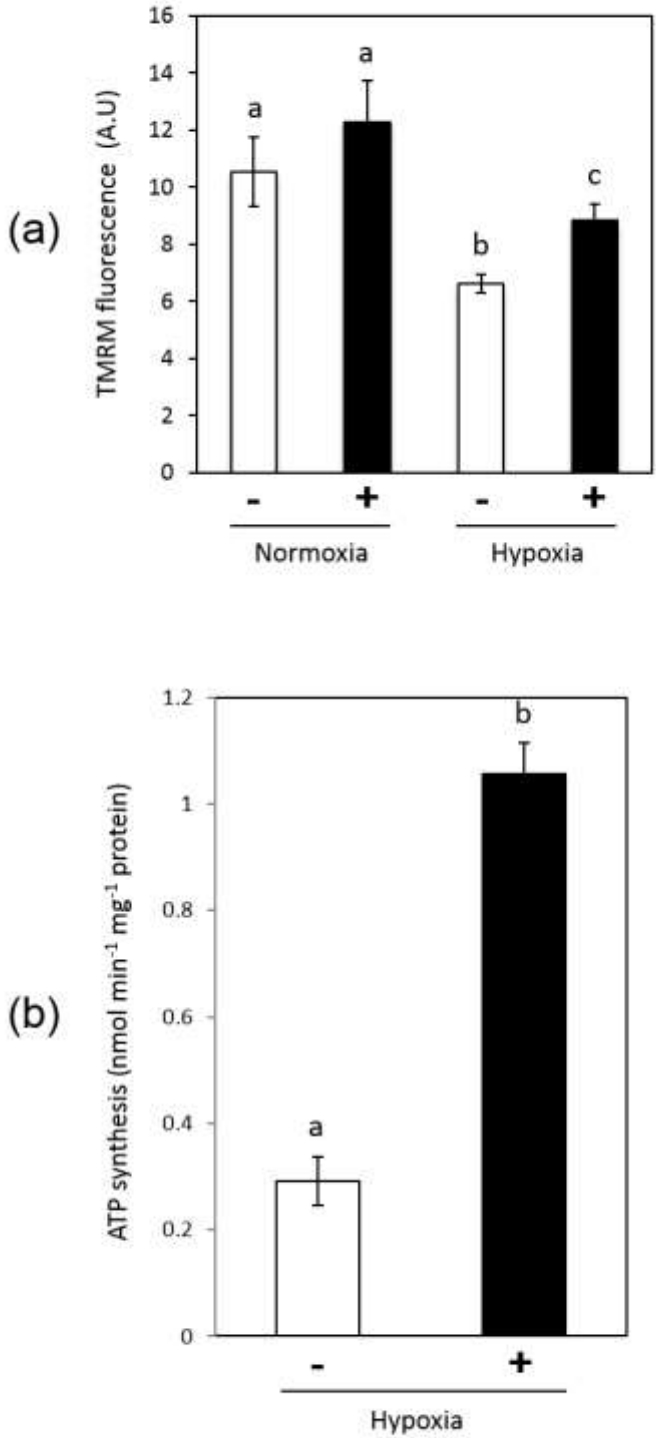
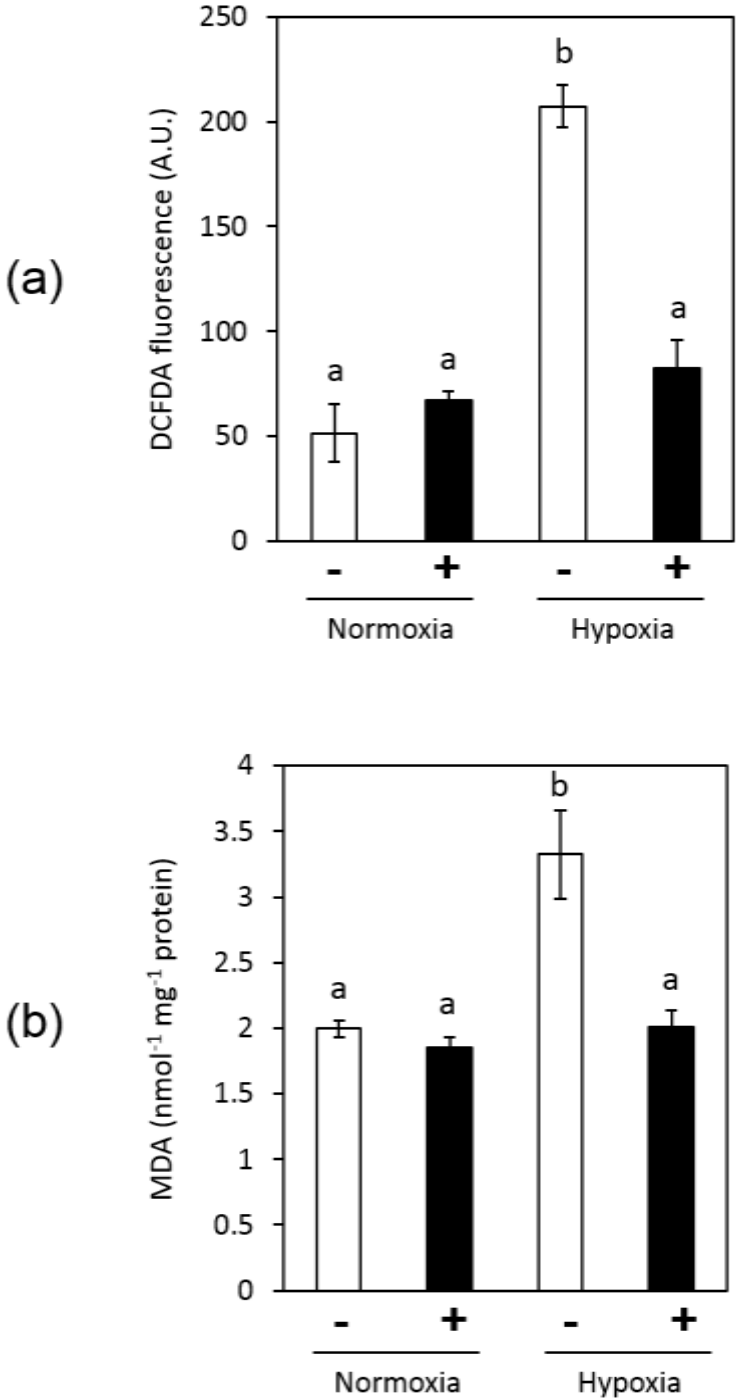
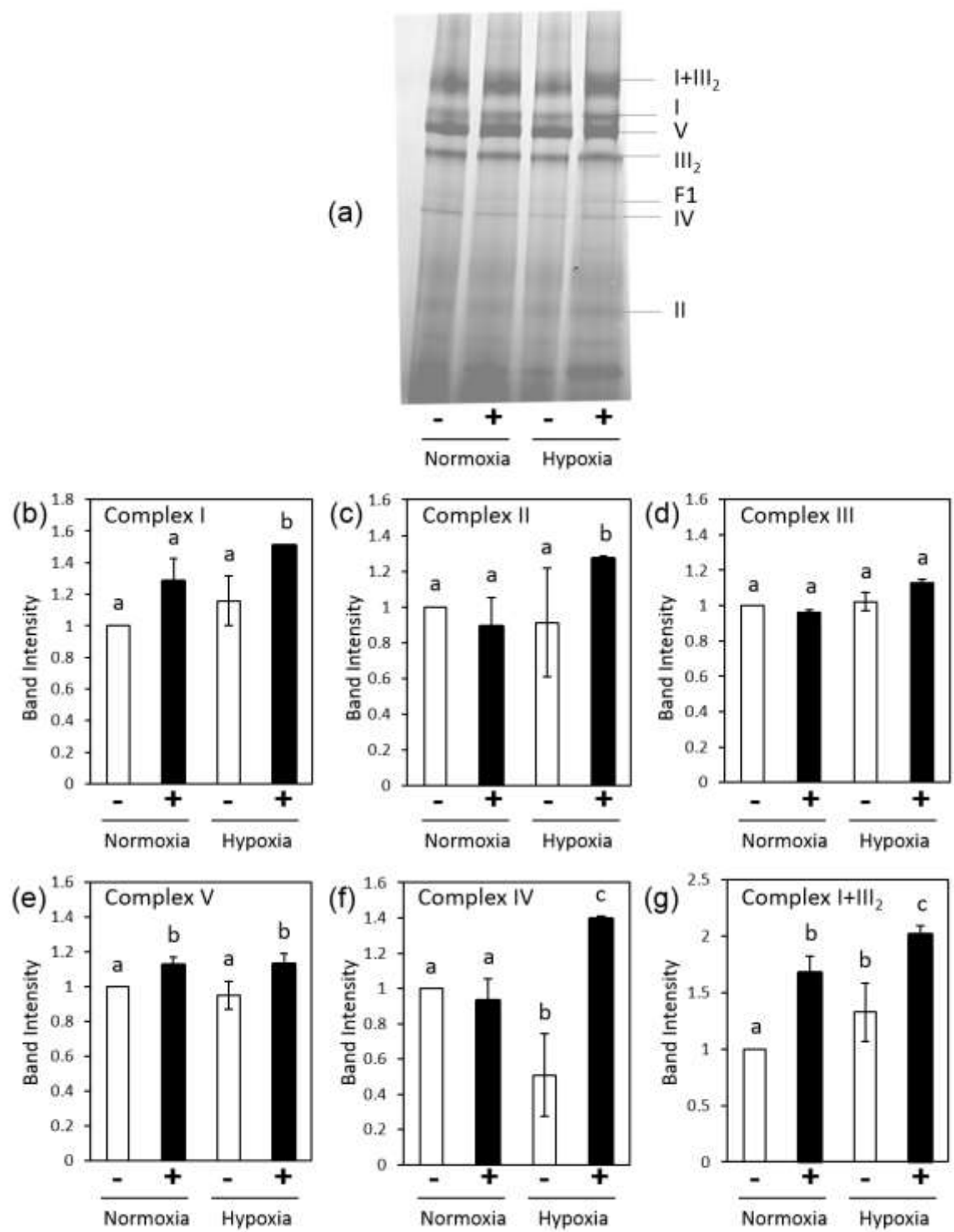


Figure 4



642 Figure 5

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Figure 6

