Occurrence of nitric oxide synthase in *Megoura viciae* Buckton (Homoptera, Aphididae): an histochemical and immunohistochemical localisation

S. Ganassi,[1] D. Tagliazucchi,[2] L. Mola[3]

[1]Department of Animal Biology, University of Modena and Reggio Emilia, Modena; [2]Department of Agricultural Science, University of Modena and Reggio Emilia, Reggio Emilia; [3]Department of Paleobiology Museum and Botanical Garden, Section of Anatomical Museums, University of Modena and Reggio Emilia, Modena, Italy

Nitric oxide (NO) is known to be involved in many physiological reactions of insects. We analysed NOS localisation in aphids of the species *Megoura viciae* by means of histochemical reaction for the NADPH-diaphorase activity and immunohistochemical methods for uNOS, nNOS and iNOS. The obtained data provided a complex and peculiar pattern of NOS distribution in cells and tissue of *M. viciae*. The histochemical reaction for NADPH-diaphorase was an indicative, but not exact marker of NOS localisation in aphids. The use of anti uNOS antiserum (frequently applied in insects) was of limited value in our specimens, whereas more satisfactory results were obtained with anti nNOS and iNOS antisera of human origin. The results of Western blot analysis confirmed the immunohistochemical ones, showing an aphid protein that reacted strongly with the polyclonal antibody anti-iNOS and anti-nNOS while a similar protein band was weakly immunoreactive with the polyclonal antibody anti-uNOS. Our results suggest that NO, prevalently synthesised by calcium/calmodulin-dependent isoform, plays important physiological roles both in adult and embryological stages of aphids. The data of principal interest was NOS presence in bacteriocytes, cells that host symbiotic prokaryotes belonging to the species *Buchnera aphidicola*, and in nuclei of adipocytes and gut cells.

Key words: insects, aphids, NADPH-diaphorase, nervous system, salivary glands, adipocytes, bacteriocytes.

Correspondence: L. Mola,
Dipartimento del Museo di Paleobiologia e dell'Orto Botanico,
Comparto Musei Anatomici, Via Berengario n 14,
41100 Modena, Italy.
Tal: +39.059.2055535.
Fax: +39.059.2055548.
E-mail: mola.lucrezia@unimore.it

Paper accepted on September 23, 2005

European Journal of Histochemistry
2005; vol. 49 issue 3 (Oct-Dec): 385-393

Nitric oxide (NO) is a membrane permeant signalling molecule which leads to the formation of cyclic GMP in target cells. The family of enzymes which form NO from L-arginine are known as nitric oxide synthases (NOS) and NO generation occurs in the presence of NADPH as cofactor. NOS isoforms were originally named after the tissue of origin, i.e. eNOS (endothelial NOS), nNOS (neuronal NOS) and iNOS (inducible, macrophage NOS). The eNOS and nNOS are constitutive calcium/calmodulin-dependent isoforms while iNOS is calcium/calmodulin-independent isoform one detected in different type of tissues.

A number of research provided strong evidence that NO is a signal molecule highly conserved during biological evolution. The cloning of a NOS homologue in *Drosophila melanogaster* (Regulski and Tully, 1995), *Rhodnius prolixus* (Yuda *et al.,* 1996) Anopheles stephensi (Luckhart *et al.,* 1998) *Manduca sexta* (Nighorn *et al.,* 1998) and *Bombix mori* (Imamura *et al.,* 2002) revealed that vertebrates and insects share the same identified NOS functional domain. In particular, the insect NOSs have higher amino acid sequence overlap with mammalian nNOS (mean of 49% identity) than iNOS (44% identity) or eNOS (47% identity) (Luckhart and Rosemberg, 1999). Analysis of NOS sequences in these insect species also reveals extensive regions of 100% identity in the catalytic domains and the co-factor binding sites for calmodulin, FAD, FMN and NADPH (Davies, 2000). Comparing available protein sequences for different phyla, insect NOS sequences form a distinct group, most closely related to human nNOS and eNOS, while iNOS family clusters in a separate group. The high degree of similarity at both the gene and protein level between insect NOS and nNOS over 400 million years of evolution suggests that the nNOS gene is the ancestor of the NOS gene family

(Davies, 2000).

NO is known to be involved in many physiological reactions of insects. NOS is present in the chemosensory and in the visual system, in brain regions implicated in control of distinct motor programmes and in learning and memory (Davies, 2000; Bicker, 1998; 2001). Moreover, NOS isoforms have also been found in the salivary glands of blood-sucking bugs with vasodilatatory action, in malpighian tubules, in midgut and in haemocytes of some species of insects (for a review see Müller, 1997 and Davies, 2000). NO is also involved in the control of synaptogenesis during nervous system development of insects (Truman *et al.,* 1996; Bicker, 1998) and in regulation of balance between cell proliferation and cell differentiation in imaginal discs of *Drosophila* (Bicker, 1998).

We analysed NOS localisation in aphid of the species *Megoura viciae*. Aphids could represent a good model due to a complex of biological traits of great interest. In fact, parthenogenesis and viviparity give rise to *telescopic generations*, namely females that have embryos at different stages of development inside the ovarioles (Tremblay, 1981). This peculiar feature allows the simultaneous cytological observations on mothers and embryos. Lastly, aphids have symbiotic mutualistic associations with intracellular prokaryotes belonging to the species *Buchnera aphidicola* (Munson *et al.,* 1991), harboured in vesicles within specialised cells called bacteriocytes. This allows to verify whether NO also acts as a molecular signal in coupled transorganism (host-symbiont) communications.

This study was performed both with histochemical reaction for the NADPH-diaphorase activity and with immunohistochemical method because the bulk of the evidences suggests that NADPH activity is a good marker for insect NOS (Bullerjahn and Pflüger, 2003), as known for vertebrates.

Materials and Methods

Animals

The aphids used belong to the species *Megoura viciae* Buckton (mean length 3 mm). The original stock was provided by Prof. S. Barbagallo of the University of Catania (Italy). For the present study specimens were reared for several generations in the laboratory. The aphids were maintained on broad bean plants in a thermostatic chamber at 20°C under L16:D8 photoperiod. Under these conditions only parthenogenetic females were obtained. Analyses were carried out on wingless adult morphs.

Forty specimens were fixed overnight in freshly depolymerized 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4 at 4°C. The specimens were then cryoprotected in 30% sucrose for 48 hours at 4°C, cut into a 18-20 μm frontal and transverse sections with a cryostat (Reichert-Jung) and mounted on gelatin coated slides. All slides were air-dried and processed for the following methods.

Histochemistry

The histochemical demonstration of NADPH-diaphorase was performed according to the method of Li and Furness (1993). After a brief wash in 0.1M Tris buffer (pH 7.6) the slides were incubated at room temperature in a medium containing 0.25 mg/mL Nitroblue Tetrazolium (NBT), 1 mg/mL β-NADPH and 0.2% Triton X-100 in the same Tris buffer. They were then incubated for 4h, renewing the mixture after 2h. The specificity of the staining was tested by omission of the substrate reaction. All reagents were obtained from Sigma (St. Louis, MO., U.S.A.). The reaction was stopped by immersion in the same buffer, and the slides were mounted with gelatinated coverslips.

Immunohistochemistry

The following rabbit polyclonal antibodies were used:

- antibody (anti uNOS) raised against a synthetic peptide corresponding to aminoacid 1113-1122 of murine iNOS and nNOS (1:100 dilution) (PRB, Affinity Bioreagents, Inc, U.S.A.);
- antibody (anti nNOS) raised against a recombinant protein corresponding to aminoacid 2-300 mapping at the aminoterminus of NOS1 (H-299) of human origin (1:150 dilution) (Santa Cruz Biotechnology, Inc., U.S.A.);
- antibody (anti iNOS) raised against a recombinant protein corresponding to aminoacid 2-175 mapping at the amino terminus of NOS2 (H-174) of human origin (1:300 dilution) (Santa Cruz Biotechnology, Inc., U.S.A.).

For Biotin-Avidin System (BAS) technique the slides were rinsed three times in PBS 0.01M, pH 7.4 and then incubated for 30 min in 0.3% H2O2 to block endogenous peroxidase activity. They were then placed in PBS containing 0.3% Triton X-100

and blocked with 5% normal goat serum (DAKO, Denmark) for 30 min. The slides were incubated overnight with the primary antibody (titer 1:400) in a humid chamber at 4°C. After rinsing with PBS, they were incubated for 30 min at room temperature with the secondary antibody goat anti-rabbit (titer 1:300) (DAKO, Denmark). After rinsing in PBS and Tris 0.1M pH 7.6, they were incubated with ABC (Vector, Vectastain, Burlingame, CA) in Tris for 45 min at room temperature. The reaction was visualised with 3,3′-diaminobenzidine tetrachloride (10 mg each 15 mL Tris) (Sigma). Immunostaining was allowed to develop for 10-15 min with 12 μL 30% H_2O_2. The specificity of immunostainings was always checked by incubating sections with normal serum instead of specific antisera or incubating sections with antiserum preabsorbed in liquid phase with the respective antigen (50 μg/mL diluted antiserum). The preabsorbtion procedures were carried out overnight at 4°C. All sections were rinsed in Tris and *aqua fontis*, dehydrated and mounted in Eukitt, and observed at a Zeiss Axioscop microscope.

Western Blot analysis

Animals were homogenized with Ultra-Turrax (Heidolph DIAX 900, Germany) in an ice-cold buffer containing 50 mM Tris-HCl, 320 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL antipain and 10 μg/mL bestatin brought to pH 7.0 at 20°C with HCl. The homogenate was centrifuged at 20,000 xg for 30 min at 4 °C, and the supernatant was collected for western blot analysis.

Briefly, samples were subjected to electrophoresis by using 7.5% polyacrylamide gel and then blotted onto a PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules CA, U.S.A.). Membranes were blocked with 5% non-fat milk and incubated with the same three rabbit policlonal antibodies utilised for immunohistochemistry and before cited. For Western blot analysis the dilution of three antibodies was 1:1000 dilution.

After extensive washing, a biotinylated goat anti-rabbit IgG (1:2000 dilution) (Calbiochem, Merck Biosciences, Germany) was added. After washing a streptavidin-horseradish peroxidase conjugate (1:2000 dilution) (Calbiochem, Merck Biosciences, Germany) were added. Proteins recognized by the antibody were revealed by reaction with 4-chloro-1-naphtol (Calbiochem, Merck Biosciences, Germany). All the other reagent were obtained from Bio-Rad (Hercules CA, U.S.A.).

Results

Histochemistry

In adult specimens of *M. viciae* all neuropilar fibres of the protocerebrum with optic lobes (Figure 1a), deutocerebrum and tritocerebrum (Figure 1b), suboesophageal and thoracic ganglia (Figure 1b) showed positivity to NADPH-diaphorase reaction. The positivity revealed a gradient of decreasing intensity from the margin towards the central regions of the neuropile in suboesophageal and thoracic ganglia (Figure 1b). The rhabdoms of eyes were also stained (Figure 1d).

Moreover, principal and accessory salivary glands (Figure 1a, b), bacteriocytes and gut (Figure 1c) showed enzymatic activity. This positivity was uniformly strong in accessory salivary glands and in gut epithelium.

Embryos inside the maternal abdomen showed positivity, in neuropilar fibres throughout the nervous system, bacteriocytes, gut, pericardic cells and oenocytes (Figure 1e).

All the control sections resulted negative (Figure 4a).

Immunohistochemistry

The immunohistochemical reaction carried out with antibodies anti universal NOS (uNOS), frequently used in insects for detect both nNOS and iNOS, were positive in bacteriocytes of mothers and embryos (Figure 2a).

Antiserum against nNOS showed a broader immunoreactivity (IR); the neuron soma in the protocerebrum (Figure 3a), with optic lobes (Figure 3c), deutocerebrum and tritocerebrum, in suboesophageal and thoracic ganglia of mothers (Figure 3b) and embryos were immunopositive. The rhabdoms of eyes were also immunostained (Figure 3c). iNOS–IR was also detected in cell bodies of optic lobes, protocerebrum, suboesophageal and thoracic ganglia (Figure 2b).

IR to nNOS was observed in the mother (Figure 3d) and embryo bacteriocytes, in the accessory salivary glands and in some posterior regions of the maternal principal salivary glands (Figure 3e).

IR to iNOS antiserum was also found in the mother and embryo bacteriocytes, in the accessory salivary glands and in some posterior regions of the

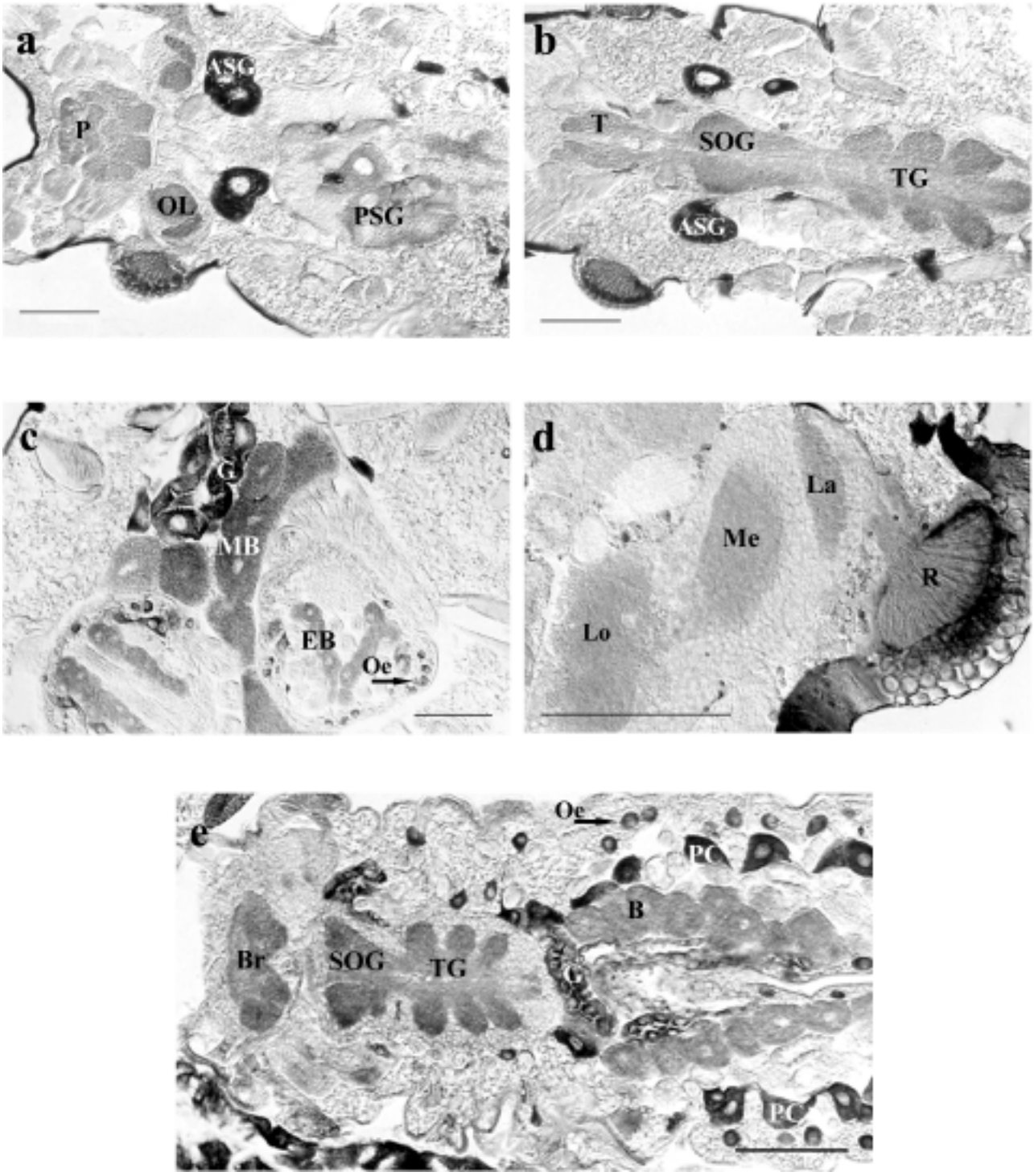

Figure 1. Frontal sections of *Megoura viciae* specimens treated with NADPH-diaphorase reaction. a-d: adult specimens; e: embryo inside maternal abdomen. a) Positivity in protocerebrum (P), optic lobe (OL), accessory salivary glands (ASG) and principal salivary glands (PSG). b) Staining in tritocerebrum (T), suboesophageal ganglia (SOG), thoracic ganglia (TG) and accessory salivary glands (ASG). c) Enzymatic activity in maternal bacteriocytes (MB), embryonal bacteriocytes (EB), oenocytes (Oe) and gut (G). d) NADPH-d positivity in lobula (Lo), medulla (Me) and lamina (La) of optic lobe and in rhabdoms (R) of eyes. e) The embryo inside maternal abdomen shows staining in brain (Br), suboesophageal ganglia (SOG), thoracic ganglia (TG), gut (G) bacteriocytes (B), pericardic cells (PC) and oenocytes (Oe). Bar = 100 μm.

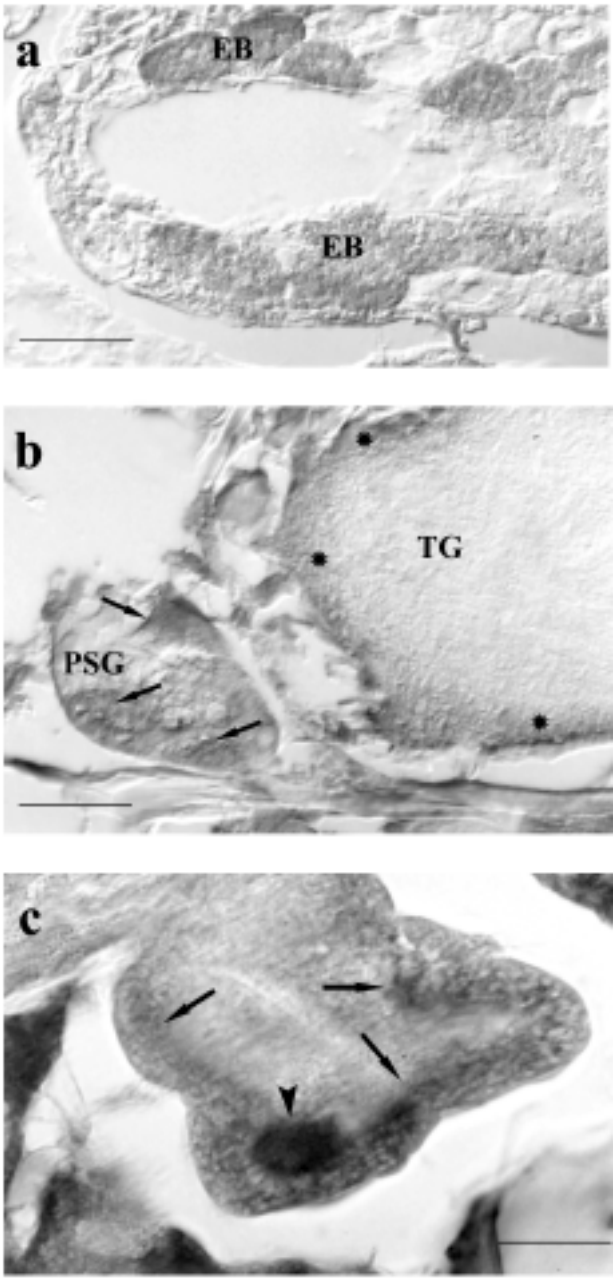

Figure 2. Frontal sections of *M. viciae* specimens. a) Immunopositivity to anti uNOS in embryonal bacteriocytes (EB). b) Immunopositivity to anti iNOS in cells bodies of thoracic ganglia (TG) indicated by asterisk, in principal salivary glands (PSG) indicated by arrows. c) Gut epithelial cells showing cytoplasmic (arrows) and nuclear (arrowhead) immunoreactivity. Bar = 50 μm.

maternal principal salivary glands (Figure 2b). Interestingly, IR to iNOS antiserum was also observed in nuclei of epithelial gut cells (Figure 2c). Moreover, adipocytes showed cytoplasmic and nuclear staining with nNOS (Figure 3f) and iNOS antibodies. All the control sections were negative (Figure 4b,c).

Western blot analysis

Western blots revealed an aphid protein with an apparent molecular mass of approximately 158 KDa that reacted strongly with the polyclonal antibody anti-iNOS and anti-nNOS (Figure 5). A similar protein band with an identical molecular weight was also observed with the polyclonal antibody anti-uNOS but with lower immunoreactivity (Figure 5). The apparent molecular mass of the aphid protein is similar to mammalian neuronal NOS (with a molecular mass of approximately 160 KDa) and *Drosophila dNOS* (with a molecular mass of approximately 152 KDa).

Discussion

The obtained data illustrate a complex and peculiar pattern of NOS distribution in cells and tissue of aphid *M. viciae*.

Firstly, the results of this study permit some methodological comments. The histochemical reaction for NADPH-diaphorase detection was an indicative, but not exact marker of NOS in aphids. In the salivary glands and bacteriocytes the histochemical results were similar to the immunohistochemical ones obtained with both nNOS and iNOS antisera. On the contrary, colocalization was not observed in the nervous system; infact the positivity for NADPH-diaphorase activity was located in the neuropilar fibres, wehereas immunoreactivity for nNOS was observed in cell bodies of neurons. The only overlap of positivity was in the rhabdoms of the eyes. The differences in staining distribution cannot be attributed to fixation, because all specimens were treated in the same way. Thus, since much evidences suggest that NADPH activity is a good marker for the presence of insect NOS (Conforti *et al.,* 1999; Zayas *et al.,* 2000; Bullerjahn and Pflüger, 2003), as known for vertebrates, the aphids could represent a special case. Indeed, it is not absolutely certain whether the histochemical NADPH diaphorase reaction accounts for all NOS activities or if it detects enzyme activities other than NOS (Muller, 1997). In any case, we intend in the future to test the relevance of NADPH-diaphorase staining to NOS localization in the aphid *M. viciae* by conducting *in vitro* NADPH assays in the presence and absence of NOS inibitor, such as L-NAME or L-NIO.

The use of anti uNOS antiserum (frequently utilised for insects) was of limited value in our spec-

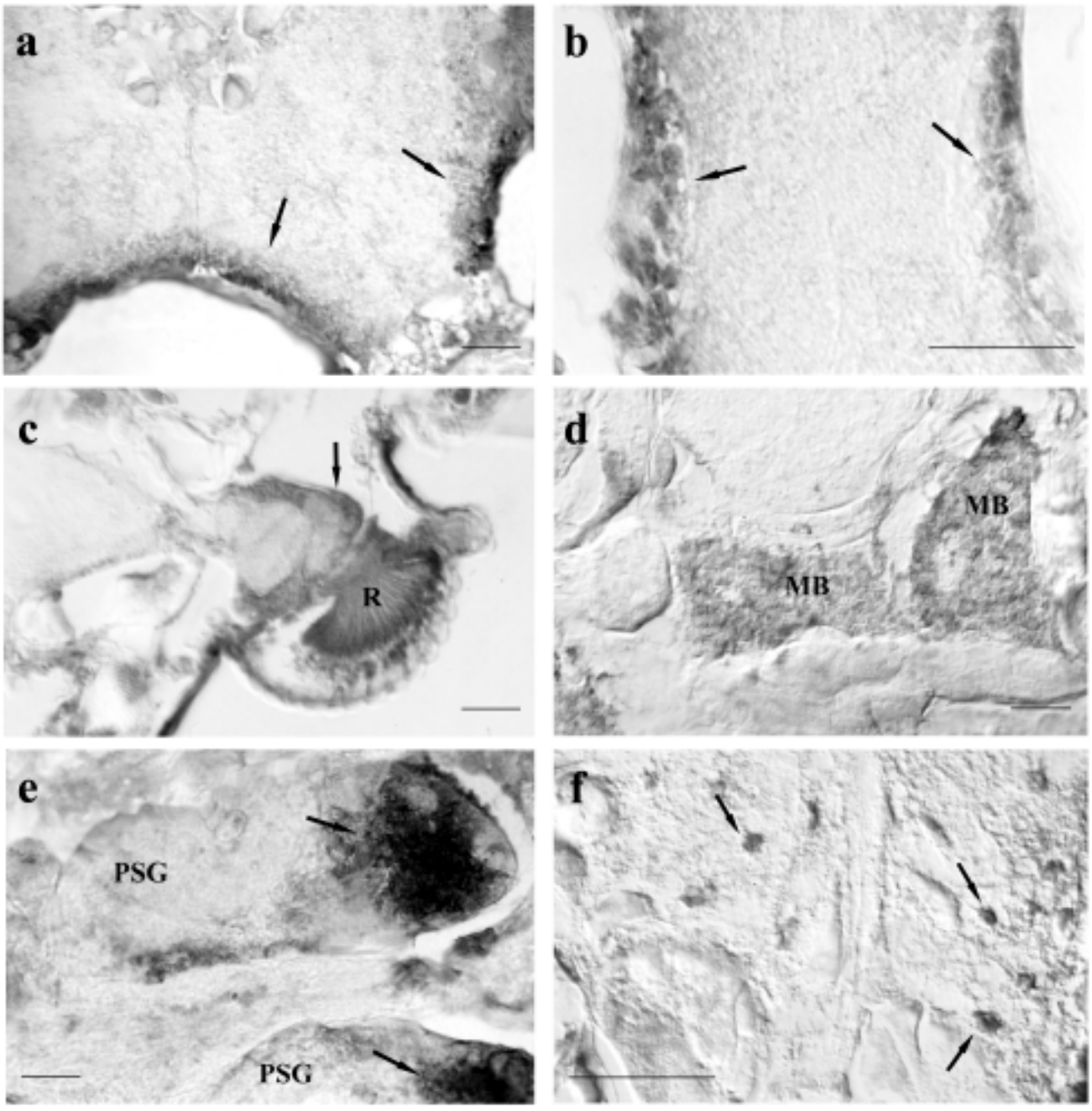

Figure 3. Frontal sections of *M. viciae* specimens treated with anti nNOS. a) Immunoreactivity in cell bodies of protocerebrum (arrows). b) Immunoreactivity in cells bodies of thoracic ganglia (arrows). c) Cell bodies of optic lobe (arrow) and rhabdoms (R) immunopositive. d) Maternal bacteriocytes (MB) show immunoreactivity. e) Maternal principal salivary glands (PSG) with posterior regions immunopositive indicated by arrows. f) Adipocytes with immunopositive nuclei (arrows). Bar = 30 μm.

imens, whereas more satisfactory results were obtained with anti nNOS and iNOS antisera of human origin. The results of Western blot analysis confirmed the immunohistochemical ones, showing an aphid protein (about 158 KDa) that reacted strongly with the polyclonal antibody anti-iNOS and anti-nNOS while a similar protein band was weakly immunoreactive with the polyclonal antibody anti-uNOS. The anti uNOS antibodies are raised against a very short synthetic peptide corre-

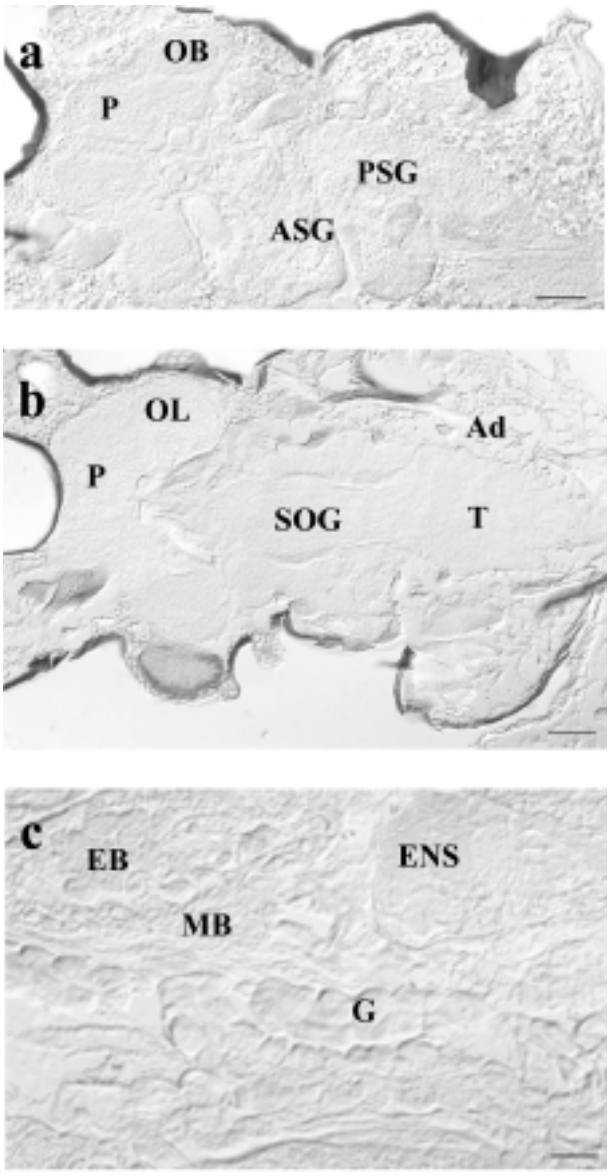

Figure 4. Frontal sections of *M. viciae* specimens. a) control section treated with NADPH-diaphorase reaction after omission of the substrate; protocerebrum (P), optic lobe (OL), principal salivary glands (PSG), accessory salivary glands (ASG). b) control section treated with anti nNOS preabsorbed in liquid phase with the respective antigen (50 μg/mL diluted antiserum); protocerebrum (P), optic lobe (OL), suboesophageal ganglia (SOG), thoracic ganglia (TG), adipocytes (AD). c) control section treated with anti iNOS preabsorbed in liquid phase with the respective antigen (50 μg/ml diluted antiserum); gut (G), embryonal bacteriocytes (EB), maternal bacteriocytes (MB), embryonal nervous system (ENS). Bar = 50 μm.

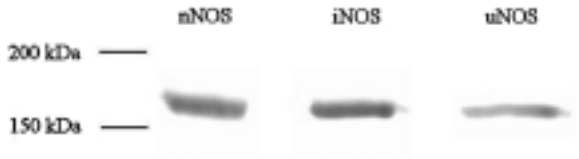

Figure 5. Western blots showing an approximately 158 KDa aphid protein. The protein is strongly reactive with antibody anti-iNOS and anti-nNOS of human origin and weakly reactive with antibody anti-uNOS.

sponding to aminoacid 1113-1122 of murine iNOS and nNOS and are suitable for many insects (Bullerjahn and Pflüger, 2003). Indeed, this epitope is conserved in all known insect NOS (Pollock *et al.,* 2004). However, in literature are not works on Aphids NOS, thus, it could be hypothesised that the NOS of aphids shows some differences in this short sequence; if this was true, the anti nNOS and iNOS antisera raised against two recombinant proteins of human origin might be more suitable. However, insect NOS and vertebrate nNOS have a high degree of similarity at both the gene and protein level (Davies, 2000).

The cell bodies of the entire brain, suboesophageal and thoracic ganglia of *M. viciae* showed IR to nNOS. The majority of works on nNOS-IR localisation in the insect nervous system describes a larger nervous systems dissected from larger insects, and so, an exact and detailed comparison of our data with those in literature is not straightforward. However, our results are in line with other previous research. NOS is known to be present in the visual system of *Drosophila* (Gibbs and Truman, 1998), *Ceratitis capitata* (Conforti et al, 1999; 2002) and *Schistocerca gregaria* (Elphick *et al.,* 1996), in antennal lobes of *S. gregaria* (Elphick *et al.,* 1995; Muller and Bicker, 1994); in suboesophageal ganglion of *Locusta migratoria* (Bullerjahn and Pfluger, 2003) and in the thoracic ganglia of *S. gregaria* (Ott and Burrows, 1998). NO has been correlated with the chemosensory and visual functions and with the control of distinct motor programmes and learning and memory (Davies, 2000; Bicker, 1998; 2001).

It has also been detected in cell bodies of *M. viciae* brain iNOS–IR and it is known in *Drosophila* and *Apis*, in which 5-10% of the total NOS is independent from calcium (Muller, 1997). Nevertheless, the existence of distinct isoform of insect NOS is still uncertain. The only NOS activity induced has been described in *Anopheles* upon infection (Davies, 2000).

Aphids have two pairs of salivary glands, the principal and the accessory glands. Principal glands are divided into two parts, an anterior membranous and

a posterior glandular part. Secretion of salivary proteins by aphids is a complex event which occurs in different phases during the separate stages of plant penetration by mouthparts (Cherqui and Tjallingii, 2000). The salivary glands of *M. viciae* showed positivity to the histochemical reaction; this staining was particularly strong in the accessory salivary glands. The same glands also have nNOS- and iNOS-IR, uniform in the accessory salivary glands and localised to the posterior glandular region only in the principal glands. The presence of NO with vasodilatatory action is known in the salivary glands of the blood-sucking bug *R. prolixus* (Müller 1997; Davies, 2000; Nussenzveig *et al.,* 1995). NO detected in the salivary glands might also play an important role in insects, for instance in aphids, sucking the plant sap. Our data may provide a further knowledge in the field of aphid-plant interactions.

Aphids have symbiotic mutualistic associations with intracellular prokaryotes belonging to the species *B. aphidicola* (Munson *et al*., 1991), harboured in vesicles within specialised cells called bacteriocytes. These cells contain the normal complement of cellular organelles in addition to the symbiotic microrganisms distributed throughout the cytoplasm (Griffiths and Beck, 1974). These symbionts cannot be grown outside the host and are maternally transmitted (Buchner, 1965; Blackman, 1987). Aphids are dependent on endosymbionts, and their elimination by antibiotics or other treatments leads to sterility and eventual death (Douglas, 1989; 1992; Ishikawa, 1989; Ohtaka and Ishikawa, 1991). It has been suggested that aphids use amino acids and other nitrogenous compounds synthesised by *B. aphidicola* (Douglas, 1998). Bacteria have been also implicated in non-nutritional functions, such as promotion of aphid transmission of circulative viruses and aphid stylet penetration of plant tissue (Douglas, 1998).

The positive results we obtained, both with the histochemical reaction and all antisera utilised, in bacteriocytes, clearly indicate the presence of NO in these cells. These data suggest that NO might represent a molecule involved regulating the close aphid-Buchnera interaction. Even if less is known about the role of NO in the establishment and maintenance of beneficial animal-microbes interactions, a recent finding shows that NO play a role in the interactions between a host (the squid *Euprymna scolopes*) and its beneficial bacterial partner (*Vibrio fischeri*) during symbiotic colonization (Davidson *et al.,* 2004).

Adipocytes of *M. viciae* showed cytoplasmic and, surprisingly, nuclear positivity to nNOS and iNOS antibodies. It is known that NO is involved in thermogenesis and vasodilatation and affects the respiratory rate of isolated brown adipocytes mitochondria by an autocrine action (Koivisto *et al.,* 1997). In rat brown adipocytes, cytoplasmic and nuclear staining for eNOS and iNOS have been found and these results were confirmed by immunoblotting analyses of subcellular fraction (Giordano *et al.,* 2002). The hypothesis that a possible function of NO produced directly in the nucleus is the modulation of gene expression (Giordano *et al.,* 2002), could also be valid for aphid adipocytes. This way of regulating gene expression might also occur in epithelium gut cells of *M. viciae*, whose nuclei were intensely positive to iNOS antibodies.

The brain, bacteriocytes, and salivary glands of embryos inside maternal abdomen showed an histochemical and immunohistochemical pattern comparable to those of same cells and organs in adults. This suggests that the functional roles of NO be assumed early in aphids.

In conclusion, our results suggest that NO, prevalently synthesised by the calcium/calmodulin-dependent isoform, plays important physiological roles both in adult and during embryological stages of aphids. Further and more in depth studies with other methodological approaches will clarify some peculiar features, such as the role of NO in host-symbiont interactions and in the regulation of gene expression of adipocytes and gut cells. For example, the direct method to measure amperometrically the release of nitric oxide in real time, recently described (Stefano *et al.,* 2002, 2004; Mantione and Stefano, 2004), could be represent a valid help for clarify these points.

References

Bicker G. NO news from insect brains. TINS 1998; 21: 349-55.

Bicker G. Sources and targets of nitric oxide signalling in insect nervous systems. Cell Tissue Res 2001; 303: 137-46.

Bullerjahn A, Pflüger HP. The distribution of putative nitric oxide releasing neurones in the locust abdominal nervous system: a comparison of NADPHd histochemistry and NOS-immunocytochemistry. Zoology 2003; 106: 3-17.

Blackman, RL. Reproduction, cytogenetics and development. In: Minks AK, Harrewijn P, eds. Aphids: their biology, natural enemies and control, vol. 2A. Elsevier Biomedical Press, Amsterdam, 1987, pp.163-95.

Buchner P. Endosymbiosis of animals with plant microorganisms. Interscience Publishers, Inc., New York, 1965.

Cherqui A, Tjallingiì WF. Salivary proteins of aphids, a pilot study on

identification, separation and immunolocalisation. J Insect Physiol 2000; 1177-86.

Conforti E, Torti C, Malacrida AR, Bernocchi G. Mature and developing visual system of *Ceratitis capitata* (Diptera, Tephritidae): histochemical evidence of nitric oxide synthase in the wild type and the white eye mutant strains. Brain Res 1999; 843: 1-11.

Conforti E, Pisu MB, Guioli S, Malacrida AR, Bernocchi G. Nitric oxide synthase-like and calmodulin-like immunoreactivity in the visual system of wild type and white eye mutant strains of *Ceratitis capitata* (Diptera, Tephritidae). Ital J Zool 2002; 69: 285-94.

Davies S-A. Nitric oxide signalling in insects. Insect Biochem Mol Biol 2000; 30: 1123-38.

Davidson SK, Koropatnick TA, Kossmehl R, Sycuro L, McFall-Ngai MJ. NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association. Cell Microbiol 2004; 6:1139-51.

Douglas AE. Mycetocyte symbiosis in insects. Biol Rev 1989; 64: 409-34.

Douglas AE. Requirement of pea aphid (*Acyrtosiphon pisum*) for their symbiotic bacteria. Entomol Exp Appl 1992; 65: 195-98.

Douglas AE. Nutritional interactions in insect-microbial symbiose: aphids and their symbiotic bacteria. Annu Rew Entomol 1998; 43: 17-37.

Elphick MR, Rayne RC, Riveros-Moreno V, Moncada S, O'Shea M. Nitric oxide synthesis in locust olfactory interneurons. J Exp Biol 1995; 198: 821-29.

Elphick MR, William L, O'Shea M. New features of the locust optic lobe: evidence of a role for nitric oxide in insect vision. J Exp Biol 1996; 2395-407.

Gibbs SM, Truman JW. Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of *Drosophila*. Neuron 1998; 20: 83-93.

Giordano A, Tonello C, Bulbarelli A, Cozzi V, Cinti S, Carruba MO, Nisoli E. Evidence for a functional nitric oxide synthase system in brown adipocyte nucleus Febs Lett 2002; 514: 135-40.

Griffiths GW, Beck SD. Effects of antibiotic*s on intracellular symbiotes in the pea aphid,* Acyrthosiphon pisum. Cell Tiss Res 1974; 148: 287-300.

Imamura M, Yang J, Yamakawa M. cDNA cloning, characterization and gene expression of nitric oxide synthase from the silkworm, *Bombyx mori*. Insect Mol Biol 2002; 11: 257-65.

Ishikawa H. Biochemical and molecular aspects of endosymbiosis in insects. Int Rev Cytol 1989; 116: 1-45.

Li ZS, Furness JB. Nitric oxide synthase in the enteric nervous system of the rainbow trout, *Salmo gairdneri*. Arc Istol Cytol 1993; 56: 185-93.

Koivisto A, Matthias A, Bronnikov G, Nedergaard J. Kinetics of the inhibition of mitochondrial respiration by NO. Febs Lett 1997; 417: 75-80.

Luckhart S, Rosenberg R. Gene structure and polymorphism of an invertebrate nitric oxide synthase gene. Gene 1999; 232: 25-34.

Luckhart S, Vodovotz Y, Cui L, Rosenberg R. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proc Natl Acad Sci U.S.A. 1998; 95: 5700-5.

Mantione K J, Stefano GB. A sub-nanomolar real-time nitric oxide probe: *in vivo* nitric oxide release in heart. Med Sci Monit 2004; 10: MT47-49.

Müller U. The nitric oxide system in insects. Prog Neurobiol 1997; 51: 363-81.

Muller U, Bicker G. Calcium-activated release of nitric oxide and cellular distribution of nitric oxide-synthetizing neurons in the nervous system of the locust. J Neurosci, 1994; 14: 7521-8.

Munson MA, Baumann P, Kinsey MG. *Buchnera gen*. nov. and Buchnera aphidicola sp. nov., a taxon consisting of the mycetocyte-associated, primary endosymbionts of aphids. Int J Syst Bacteriol 1991; 41: 566-8.

Nighorn A, Gibson NJ, Rivers DM, Hildebrand JC, Morton DB. The nitric oxide-cGMP pathway may mediate communication between sensory afferents and projecton neurons in the antennal lobe of *Manduca sexta*. J Neurosci 1998; 18: 7244-55.

Nussenzveig RH, Bentley DL, Ribeiro JMC. Nitric oxide loding of the salivary nitric-oxide-carrying hemoproteins (nitriphorins) in the blood-sucking *Rhodnius prolixus*. J Expl Biol 1995; 198: 1093-8.

Ohtaka C, Ishikawa H. Effects of heat treatment on the symbiotic system of an aphid mycetocyte. Symbiosis 1991; 11: 19-30.

Ott SR, Burrows M. Nitric oxide synthase in the thoracic ganglia of the locust: distribution and morphology of neurones. J Comp Neurol 1998; 395: 217-30.

Pollock VP, McGettigan J, Cabrero P, Maudlin IM, Dow JAT, Davies SA. Conservation of capa peptide-induced nitric oxide signalling in Diptera J Exp Biol 2004; 207: 4135-45.

Regulski M, Tully T. Molecular and biochemical characterization of dNOS-a *Drosophila* Ca2+ calmodulin-dependent nitric oxide synthase Proc Natl Acad Sci U.S.A. 1995; 92: 9072-6.

Stefano GB, Salzet M, Magazine HI. Cyclic nitric oxide release by human granulocytes and invertebrate ganglia and immunocytes: Nano-technological enhancement of amperometric nitric oxide determination. Med Sci Monit 2002; 8: BR199-204.

Stefano GB, Kim E, Liu Y, Zhu W, Casares F, Mantione KJ, Jones DA, Cadet P. Nitric oxide modulates microglial activation. Med Sci Monit 2004; 10: BR17-22.

Yuda M, Hirai M, Miura K, Matsumura H, Ando K, Chinzei Y. cDNA cloning, expression and characterization of nitric-oxide synthase from the salivary glands of the blood-sucking insect *Rhodnius prolixus*. Eur J Biochem 1996; 242: 807-12.

Tremblay E. Entomologia applicata. vol 2, Liguori, Napoli, 1981, pp. 104-19.

Truman JW, De Vente J, Ball EE. Nitric oxide-sensitive guanylate cyclase activity is associated with the maturational phase of neuronal development in insects. Development 1996; 122: 3949-58.

Zayas RM, Quzi S, Morton DB, Trimmer BA. Neurons involved in nitric oxide-mediated cGMP signalling in the tobacco hornworm, *Manduca sexta*. J Comp Neurol 2000; 419: 422-38.