

Serotonin and nitric oxide interaction in the control of bioluminescence in northern krill, *Meganctiphanes norvegica* (M. Sars)

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Summary

The role of nitric oxide (NO) in the control of bioluminescence (light production) in the crustacean *Meganctiphanes norvegica* (krill) was investigated using pharmacological and immunohistochemical methods. All nitric oxide donors tested failed to induce bioluminescence *per se* but modulated light production stimulated by 5-hydroxytryptamine (5-HT). NO donors [sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP)] injected in live specimens significantly reduced light production stimulated by 5-HT, whereas inhibition of the enzyme NO synthase (NOS) with L-NAME (N^G-nitro-L-arginine methyl ester) resulted in an enhancement of the 5-HT response. The effects of NO do not seem to be mediated *via* production of cGMP as injections of a cGMP analogue (8-Bromoguanosine 3',5'-cyclic monophosphate) gave inconclusive effects on the 5-HT-stimulated light response. Inhibition of cGMP production with ODQ (1H-

[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) did not affect the light response. Moreover, a few individuals showed a considerably higher response to 5-HT in April and June compared with specimens collected in the autumn and winter. Furthermore, both NOS-like and 5-HT-like materials were detected by immunohistochemistry inside the light organs. NOS-like immunoreactivity was primarily observed in structures associated with vessels inside the light organs, whereas 5-HT-like material was abundant in nerve fibres throughout the whole light organ. The results suggest that NO has a modulatory role at several levels in the control of light production in *M. norvegica* and that NO and 5-HT interact in this regulation.

Key words: bioluminescence, 5-HT, *Meganctiphanes norvegica*, nitric oxide, nitric oxide synthase, krill.

Introduction

Within the crustacean family Euphasiidae (krill) all species have developed the ability to produce light (Bassot, 1966; Herring and Locket, 1978). Various adaptive benefits have been suggested for this phenomenon, including camouflage through counter shading and communication between individuals. Experiments have shown that krill respond to different types of light stimuli with light production of their own (light-induced light reaction) (Mauchline, 1960; Kay, 1965).

The northern krill (*Meganctiphanes norvegica*) produces light from 10 separate light organs (photophores): one on each eyestalk, two pairs on the ventral thorax and four separate organs under the abdomen (Herring and Locket, 1978). The control of light emission in these animals is not well studied, but serotonin (5-hydroxytryptamine, 5-HT) is known to stimulate light production or to increase the sensitivity to light gradients (Kay, 1962; Fregin and Wiese, 2002), although the mechanism for this is unknown. The putative light-producing cells (photocytes) are not innervated; instead, nerves follow capillaries inside the photophore and innervate a sphincter-like

structure at the base of a second cell-type located close to the assumed photocytes in the light-producing structure (lantern) (Fig. 1) (Pettersson, 1968; Herring and Locket, 1978).

The firefly light organ is similar to the krill lantern in its morphological structure, with a well-developed supply of oxygen *via* trachea and a lack of innervation of the photocytes (Greenfield, 2001). Light production in the firefly is initiated when specific cells closely associated to the photocytes are stimulated by octopamine to produce nitric oxide (NO). NO in turn stimulates flashing by inhibiting photocyte respiration, thereby leaving oxygen free for the light reaction (Trimmer et al., 2001). NO has also been suggested to be involved in the control of light production in the fish *Argyropspectus hemigrammus*, where it has a modulatory effect on adrenaline-stimulated bioluminescence (Krönström et al., 2005).

The signalling molecule NO is involved in many different physiological processes, including vasodilation, immune defence, neurotransmission and neuromodulation, in vertebrates as well as invertebrates (Bredt and Snyder, 1992; Bredt and Snyder, 1994; Colasanti and Venturini, 1998; Torilles, 2001). It

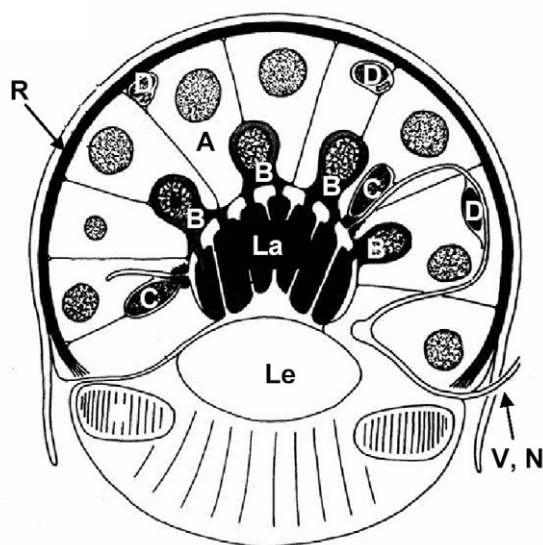


Fig. 1. Schematic picture of a longitudinal section through a ventral photophore from krill. Light is produced in the lantern (La) that is made up by processes from B-cells (possibly photocytes; B) and refractive rods. Light produced in the lantern is reflected against the inside of the organ wall (reflector; R) and passes through a lens (Le) before leaving the photophore. Apart from B-cells, three other cell types are present in the organ – large A-cells (A) and smaller C-cells (C) and D-cells (D). On both sides of the lens, photophore vessels (V) and nerves (N) enter the organ. Capillaries branch off from the arteries and pass both D- and C-cells before they empty in the lantern. Nerves follow the capillaries and end at a sphincter-like structure at the base of the C-cells. Modified from Herring and Locket (Herring and Locket, 1978) with permission.

is a molecule with a short half-life that can diffuse through cell membranes and have different intracellular effects. In many systems, the physiological effects of NO are mediated through the production of cyclic guanosine monophosphate (cGMP), by stimulation of the enzyme guanylyl cyclase in the target cell (Jacklet, 1997). NO can also control physiological mechanisms in the target cell by inhibiting mitochondrial respiration through a cGMP-independent pathway (Brown, 1994). Evidence for the presence of the enzyme nitric oxide synthase (NOS) in the nervous system of different crustacean species has been presented by several authors using antibodies against NOS and/or NADPH-diaphorase histochemistry (Scholz et al., 2002; Zou et al., 2002; Christie et al., 2003).

Based on the morphological similarities of the krill and the firefly light organs as well as the properties of the NO molecule outlined above, we hypothesized that NO might be involved in the control of light production in krill. This possibility was investigated both by functional studies of light production in living animals and with immunohistochemistry using antibodies raised against NOS.

Materials and methods

Collection and maintenance of animals

Krill, *Meganyctiphanes norvegica* (M. Sars 1856), were collected in the Gullmar fjord, Sweden using an Isaacs-Kidd midwater trawl. The animals were kept in deep-sea seawater (6°C) at Kristineberg Marine Research station, in a dark and

temperature-controlled room (6°C), and fed regularly with *Artemia salina*. Collection of animals and experiments were performed in September, November and December 2004, and April, June and September 2005. Experiments were started the day after collection at the earliest.

Immunohistochemistry

Method

Abdominal photophores from 21 krill were dissected out and fixed for 1–24 h in Zamboni's fixative (15% picric acid, 2% formaldehyde in phosphate-buffered saline) (PBS, 0.9% NaCl, pH 7.2) or 1–4 h in paraformaldehyde (PFA) [3.7% PFA in phosphate buffer (PB), pH 7.2]. Zamboni-fixed preparations were washed repeatedly in 80% ethanol, and the tissue was dehydrated (95% and 99.5% ethanol, 30 min each), which allows penetration of xylene. Treatment with xylene was made (30 min) to remove tissue fats, and the preparations were then rehydrated (99.5%, 95%, 80% and 50% ethanol, PBS, 30 min each) for storage. Preparations fixed in PFA were rinsed for 30 min in PBS. All preparations were stored overnight in PBS with 30% sucrose, before they were embedded in OCT (Sakura, Zoeterwude, The Netherlands) or agarose (Sigma Chemical Company, St Louis, MO, USA) and quick frozen in isopentane chilled with liquid nitrogen. Frozen samples that were not cut immediately were stored at –40°C until use.

A cryostat microtome (Zeiss Microm International GmbH, Walldorf, Germany) was used to cut 10–20 µm sections, which were captured on chrome alum gelatine-coated slides and left overnight to dry. The slides were stored at –20°C until use.

Antibodies

Antibodies against all (mammalian) isoforms of NOS [neuronal, inducible and endothelial NOS (nNOS, iNOS and eNOS); Table 1] were used in preliminary tests to detect NOS-immunoreactive material in sections of abdominal photophores. Antibodies against nNOS and the universal NOS antibody revealed NOS-like immunoreactivity (NOS-LI IR) in structures inside the photophores in similar, but not identical, patterns of labelling. No staining was detected with the antibodies against iNOS and eNOS.

The antigen sequences against which the different primary antibodies are raised were compared with the most related NOS sequence available, i.e. that of a crustacean, *Gecarcinus lateralis* (Kim et al., 2004), using the program ClustalW to establish the theoretical fit to a possible krill NOS. The antigen sequences for all the antibodies that showed staining in our experiments show significant alignments with *G. lateralis* NOS. The best agreement (100%) was obtained with the universal NOS (uNOS)-antibody (PA1-039), which is also most commonly used in studies of invertebrates (Scholz et al., 2002; Christie et al., 2003). The uNOS antibody was therefore judged to be the most likely to show genuine NOS IR, and results from further tests are reported from this antibody only.

An antibody against 5-HT was used to establish the presence of structures containing 5-HT in the photophores (Table 1).

To reduce non-specific staining, sections were preincubated with normal donkey serum (10% in PB with 2% NaCl, 0.1% bovine serum albumin, 0.2% NaN₃ and 0.2% Triton X-100) for 30–60 min. Primary antibodies (Table 1) were applied and the

Table 1. Antibodies used for immunohistochemistry

	Antibody	Host	Working dilution	Antigen (peptide sequence in brackets)	Source
Primary	eNOS N30030	Rabbit	1:500	Human eNOS c-terminal (1030-1209)	1
	iNOS N32030	Rabbit	1:1000	Mouse iNOS c-terminal (961-1144)	1
	nNOS N31030	Rabbit	1:100	Human nNOS c-terminal (1095-1289)	1
	nNOS SC1025	Rabbit	1:150	Human/mouse nNOS n-terminal	2
	uNOS PA1-039	Rabbit	1:100	Murine iNOS and nNOS c-terminal (1113-1122)	3
	nNOS B220	Rabbit	1:400	Rat nNOS c-terminal	4
	Ser5545	Rabbit	1:4000	Serotonin	5
Secondary	DaR-biotin 711-065-152	Donkey	1:400		6
	DaR-FITC 711-095-152	Donkey	1:100		6

All the NOS antibodies are polyclonal. eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; uNOS, universal NOS – i.e. antibody reacting to both iNOS and nNOS. Sources: (1) BD Biosciences (Transduction Laboratories), San Jose, CA, USA; (2) Santa Cruz Biotechnology, Santa Cruz, CA, USA; (3) Affinity BioReagents, Golden, CO, USA; (4) Euro-Diagnostica, Malmö, Sweden; (5) Sigma Chemical Company, St Louis, MO, USA; (6) Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

sections incubated for 24 h. The sections were rinsed (3×5 min in PB with 2% NaCl) and incubated for 60 min with the secondary antibody conjugated either to a fluorophore or to biotin (Table 1). All incubations were made in a humid chamber at room temperature ($\sim 20^\circ\text{C}$).

The preparations with fluorophore-conjugated secondary antibodies were rinsed again as described above and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA), then examined using a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DMX1200 digital camera. Captured images were processed with Adobe Photoshop.

Sections treated with biotinylated secondary antibodies were incubated with avidin-biotinylated peroxidase complex (ABC Elite PK 6100 standard; Vector Laboratories) for 30 min and either subsequently developed in Vector Nova red substrate kit (SK 4800; Vector Laboratories) for 3 min or incubated with fluorophore-conjugated streptavidine (SA-488, working dilution 1:2000; Molecular Probes, Eugene, OR, USA) for 1 h. The sections were mounted in 50% glycerol and 50% carbonate buffer (pH 8.5) or Vectashield mounting medium and examined as described above.

Controls performed to confirm the specificity of the secondary antibodies, by omission of the primary antibody, did not reveal non-specific staining with any of the secondary antibodies in the test.

Pharmacology

Chemicals

Stock solutions of 8-Bromoguanosine 3',5'-cyclic monophosphate sodium salt monohydrate (8BrGMP), 5-hydroxytryptamine hydrochloride (5-HT), N^G -nitro-L-arginine methyl ester (L-NAME) and sodium nitroprusside (SNP) (all purchased from Sigma Chemical Company) were prepared in crustacean saline (Holmes et al., 1999) containing (in mM l^{-1}): 478.0 NaCl; 12.74 KCl; 13.69 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 20.47 $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$ and 3.9 Na_2SO_4 (all purchased from Merck KGaA, Darmstadt, Germany), and 5.0 HEPES (Sigma); pH was adjusted to 7.45. S-nitroso-N-acetylpenicillamine (SNAP; Sigma) was prepared in distilled water and (1H-

[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) (ODQ; Sigma) in dimethyl sulfoxide (DMSO).

Method

Live krill were used in the pharmacological experiments. Individual specimens were placed in plastic vials containing 10 ml of deep-sea seawater and exposed to light ($\sim 12 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$, LI 1000 Datalogger; LI-COR, Lincoln, NE, USA) for 1 h. This was done to standardize the light exposure of each specimen prior to the experiments. Earlier studies have shown that *M. norvegica* is stimulated to produce light both by short light flashes and more prolonged exposure to light (Mauchline, 1960; Kay, 1965).

After light pretreatment, the vial containing the krill was placed in a luminometer [Berthold FB12 (Pforzheim, Germany) calibrated with a standard light source, 470 nm (Betelight, SRB Technologies, Winston-Salem, NC, USA)] and the light-stimulated light response was recorded. When the response had subsided the animal was injected in the pleon (the tail musculature) as described by Fregin and Wiese (Fregin and Wiese, 2002) with test substance (5 μl) using a Hamilton syringe and the subsequent response measured. All test substances were injected except ODQ, which was dissolved in DMSO (Merck, Germany) and applied, giving a final concentration of 0.1% DMSO in the vial containing the krill.

Temperature during the measurements was kept low either by performing the experiments in a temperature-controlled room (at 6°C) or by changing the water in the vial before injecting the test substance. Responses to 5-HT (L_{tot} , see below for definition), which were used as control, did not differ significantly between the methods.

Calculations and statistics

Each light response after injecting either 5-HT or 5-HT in combination with an NO donor or a NOS inhibitor was characterized by three parameters calculated as constants of a Gompertz equation of an asymmetrical growth curve estimated by least-square method from the original data (Fig. 2). The calculated parameters were L_{tot} , the total amount of light in quanta (q s^{-1}); RLP_{max} , maximum rate of light production (q s^{-2})

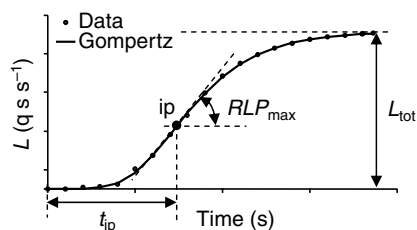


Fig. 2. Parameters used to characterize the light response. An asymmetrical growth curve (Gompertz equation) was fitted to the accumulated original data from the luminometer recordings. L , cumulative value of light emitted ($q\ s^{-1}$); L_{tot} , the total amount of light ($q\ s^{-1}$); RLP_{max} , rate of light production ($q\ s^{-2}$) at inflection point (ip) and t_{ip} , time to the inflection point (s).

(the maximum growth rate of the curve at inflection point, ip); t_{ip} , time to the ip in seconds:

$$L_{tot} = L_{tot,\infty} \times e^{-RLP_{max} \times (t - t_{ip})}$$

Data that did not converge to the Gompertz model were excluded from the analysis. Non-converging data were either a total lack of response or a response divided in several parts. Divided responses are probably because of a changed position of the photophores during the measurements. Three animals out of 69 tested were non-responsive to 5-HT. The 5-HT concentration used in this study ($\sim 0.03\ \text{mmol}\ l^{-1}$) is in the lower range for triggering light production. Earlier studies show that concentrations lower than $0.01\ \text{mmol}\ l^{-1}$ in the haemolymph induced a feeble or no light response in *M. norvegica* (Fregin and Wiese, 2002).

In treatments without 5-HT (saline control or nitroergic drugs) the integrated light production for 10 minutes at the peak of response was used for comparisons between treatments.

Each mean value is expressed with its standard error of the mean (mean \pm s.e.m.); analysis of variance (ANOVA), t -test, Dunnet and Bonferroni tests were used to determine significant differences between the groups. All methods were designed under the assumption that the data are normally distributed. Shapiro and Wilk statistics were used to check that the data were a random sample from a normal distribution (Shapiro and Wilk, 1965). When data were not normally distributed or when unequal variances occurred, a log transformation of data was performed as indicated by Sokal and Rohlf (Sokal and Rohlf, 1995).

Analyses were performed using SAS/STAT[®] software's capabilities (SAS Institute Inc., 1990) or GraphPad Prism, version 4.

Results

Immunohistochemistry

The abdominal photophores of *M. norvegica* are bell-shaped, globular organs with a highly organized inner structure and a bright-red surface (Fig. 1). The inner structures of the organ, including the light-producing tissue (lantern), are enclosed by a reflector. Light produced inside the organ is directed through a well-defined lens on the ventral, transparent side of the organ. Photophore arteries together with nerves enter at two sites laterally, beside the lens. The photophore arteries form a ring vessel around the lens from which capillaries branch, spread in

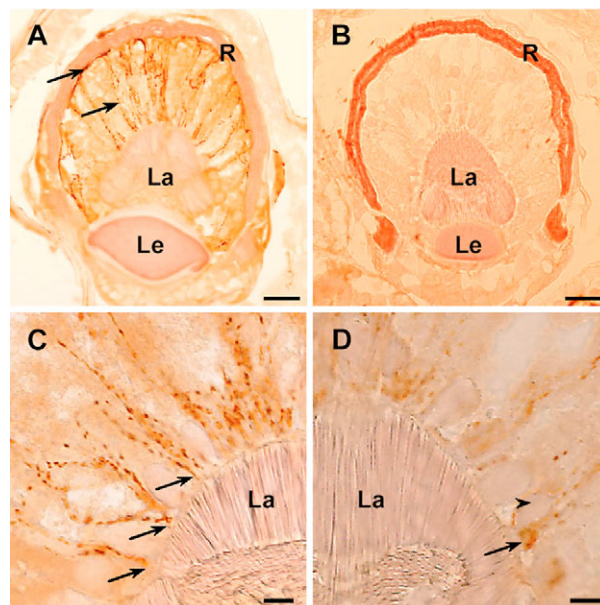


Fig. 3. Longitudinal sections of ventral photophores from *M. norvegica*. 5-HT-IR fibres are found throughout the light organ (A; for example, at the arrows) following capillaries and ending at the lantern (C; for example, at the arrows). Staining is abundant at the junction (D, arrow) between C-cells (D, arrowhead) and capillaries where the capillaries open into the lantern. Controls without primary antibody do not show any non-specific binding of the secondary antibodies (B). La, lantern; Le, lens; R, reflector. Scale bars, $50\ \mu\text{m}$ in A,B; $10\ \mu\text{m}$ in C,D.

the organ and apparently empty into the lantern, which is a hollow structure. Haemolymph drains from the lantern into two lacunae located perpendicular to the entry point of each photophore artery. Four different cell types (A-, B-, C- and D-cells) have been identified in the organ. Most of the space inside the photophore is occupied by the large A-cells. They extend from the reflector to the edge of the lantern. Among the A-cells, wrapped around the capillaries, smaller D-cells are situated. C-cells guard the opening of the vessels into the lantern with a sphincter-like structure at the base of the cell. The lantern consists of processes from B-cells (the assumed photocytes) and refractive rods (Pettersson, 1968; Herring and Locket, 1978).

5-HT

5-HT-like IR is abundant in varicose nerves running along the whole length of the capillaries between the A-cells, and along the inside of the reflector (Fig. 3A). 5-HT-IR fibres end with the capillaries at the edge of the lantern (Fig. 3C,D). The connection between C-cells and vessels seems to be innervated by nerves containing 5-HT-IR (Fig. 3D). In cross-section this area is seen as a characteristic ring-shaped structure.

NOS

The universal NOS antibody revealed NOS-like IR of vessels or the area around vessels, primarily at the border of the lantern where the vessels connect to the C-cells before draining into the lantern (Fig. 4A), but also in the periphery of the light organ (Fig. 4B). Staining is most clear in cross-sections of the C-cells, as described above. Moreover, staining is detected beside the

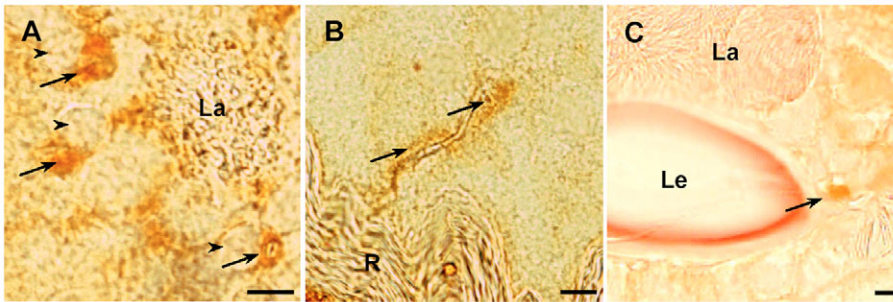


Fig. 4. Transversal (A,B) and longitudinal (C) sections of ventral light organs from *M. norvegica*. NOS-like IR is detected around capillaries at the base of C-cells (A: staining at arrows, C-cells at arrowheads). IR against NOS is also detected along the capillaries in the peripheral parts of the light organ (B, arrows) and at both sides of the lens (C, arrows), possibly where the photophore arteries enter the light organ. La, lantern; Le, lens; R, reflector. Scale bars, 10 μm .

lens, possibly at the entry point for the photophore arteries (Fig. 4C).

Pharmacology

The effects of the NO donors SNP and SNAP, the NOS inhibitor L-NAME, a membrane-permeable cGMP-analogue (8BrcGMP) and OEQ, an inhibitor of guanylyl cyclase, on the light production from *M. norvegica* photophores were investigated. 5-HT was used as a positive control and to induce light production. Saline was used as a negative control.

Controls: 5-HT and saline

Injections of 5-HT (resulting in $\sim 0.03 \text{ mmol l}^{-1}$ in the haemolymph) stimulated a light response, which typically lasted at least 15 min. The accumulated total amount of light emitted (L_{tot}) in response to 5-HT seems to vary over the year. In April and June the average L_{tot} appeared to be higher than in the autumn and winter months because of a few specimens with a very high response (Fig. 5). Below, the results from treatments with NO donors and NOS inhibitors are compared with 5-HT controls performed at the same time of year. Injections of saline only occasionally evoked a minor light response (Table 2).

NO donors and NOS inhibitors

None of the NO donors or NOS inhibitors *per se* (i.e. applied without simultaneous addition of 5-HT) had an effect on the

light production of *M. norvegica*, as compared with the saline controls (Table 2).

NO donors

In all further experiments with NO donors and NOS inhibitors, the injections of the nitroergic drug were in combination with 5-HT in a solution producing approximately 0.03 mmol l^{-1} of 5-HT in the haemolymph, as in the 5-HT controls. The effects of two NO donors on the 5-HT-stimulated light response were tested. Both SNP and SNAP ($\sim 0.3 \text{ mmol l}^{-1}$ in the haemolymph) significantly reduced the total light production, L_{tot} , as compared with the 5-HT control (Fig. 6A). SNAP also reduced the maximum rate of light production (RLP_{max} , Fig. 6B) at the peak of the response (ip) and delayed the response by prolonging the time to reach the peak (t_{ip}) (Fig. 6C).

NOS inhibitors

The NOS inhibitor L-NAME ($\sim 3 \text{ mmol l}^{-1}$ in the haemolymph) significantly enhanced the response to 5-HT (L_{tot}) (Fig. 7A). 3 mmol l^{-1} L-NAME also delayed the time to reach the peak of response (Fig. 7B) and produced a higher RLP_{max} compared with the 5-HT control in April (Fig. 7C). The same kinetic effects were observed as a response to 0.3 mmol l^{-1} L-NAME in September. The lowest tested concentration of L-NAME ($\sim 0.03 \text{ mmol l}^{-1}$ in the haemolymph) did not affect any of the parameters (Fig. 7A–C).

Effects on the cGMP pathway

A membrane-permeable cGMP analogue (8BrcGMP) and an inhibitor of soluble guanylyl cyclase (OEQ) were used to

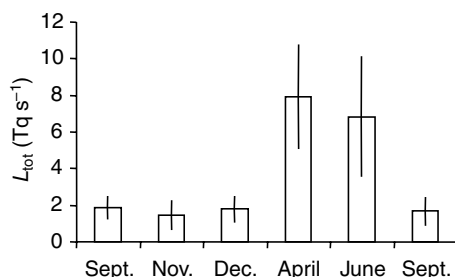


Fig. 5. Comparison of the total amount of light (L_{tot}) produced, as a response to 5-HT (0.03 mmol l^{-1}), between the different months when experiments were performed. Some specimens tested in April and June were very bright, hence the large mean value of L_{tot} and the large variation in response in these months. Number of replicates (non-converging data in brackets, not included in the analysis): Sept., $N=8$ (1); Nov., $N=9$ (1); Dec., $N=10$; Apr., $N=14$ (4); June, $N=10$ (1); Sept., $N=10$ (2); error bars indicate s.e.m.

Table 2. Integrated light production, saline controls and nitroergic drugs

	Saline (Mq s ⁻¹)	Treatment (Mq s ⁻¹)
SNP (0.3 mmol l^{-1})	19.6 ± 13.3 ($N=8$)	18.5 ± 9.6 ($N=5$)
SNP (0.03 mmol l^{-1})	19.6 ± 13.3 ($N=8$)	7.2 ± 6.0 ($N=5$)
SNAP (0.3 mmol l^{-1})	3.1 ± 2.1 ($N=10$)	348.5 ± 338.9 ($N=11$)
L-NAME (3 mmol l^{-1})	4.2 ± 3.7 ($N=12$)	3.1 ± 1.4 ($N=12$)
cGMP (0.03 mmol l^{-1})	1.1 ± 0.6 ($N=10$)	2.1 ± 1.1 ($N=10$)

Integrated light production, 10 min; Mq, megaquanta.

None of the NO donors or NOS inhibitors had a significant effect *per se* on the light production compared with the saline control. The high variation in the SNAP response is caused by one specimen responding to the treatment.

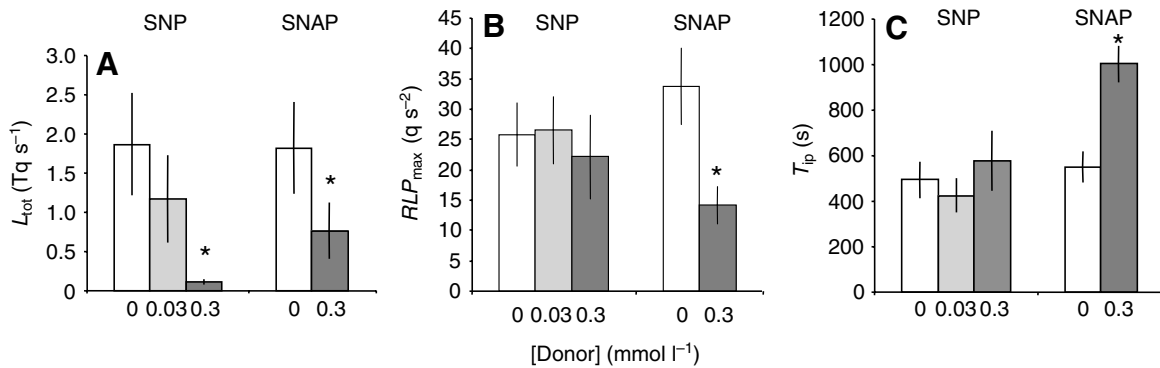


Fig. 6. Effect of the NO donors SNP and SNAP on the 5-HT-stimulated light response. White bars are 5-HT controls (0.03 mmol l⁻¹ 5-HT). Injection of either 5-HT and SNP (0.3 mmol l⁻¹) or 5-HT and SNAP (0.3 mmol l⁻¹) results in a significantly lower L_{tot} than injection of 5-HT *per se* (A, $P < 0.05$). 5-HT controls from September (2004) and December (2004) were combined in the SNAP analysis. Injection of 5-HT and SNAP (0.3 mmol l⁻¹) results in a significantly lower RLP_{max} (B) and a significantly higher t_{ip} (C) than injection of 5-HT *per se* ($P < 0.05$). SNP did not affect these parameters. Number of replicates (non-converging data in brackets, not included in the analysis): SNP treatment: 5-HT, $N = 8$ (1); 5-HT+SNP (0.03 mmol l⁻¹), $N = 5$; 5-HT+SNP (0.3 mmol l⁻¹), $N = 6$ (2); SNAP treatment: 5-HT L_{tot} , $N = 18$ (1); 5-HT RLP_{max} , t_{ip} , $N = 10$; 5-HT+SNAP, $N = 8$ (1); error bars indicate s.e.m.

investigate whether the effects of NO on the 5-HT-stimulated light response are mediated through production of cGMP. Applying ODQ (0.01 mmol l⁻¹ in the test vial) had no effect on the 5-HT-stimulated light response (Fig. 8A–C), whereas injections of 8BrcGMP (~0.3 mmol l⁻¹ in the haemolymph) together with 5-HT resulted in a reduced RLP_{max} of the light response as compared with the 5-HT control. Neither t_{ip} nor L_{tot} was affected by 8BrcGMP (Fig. 9A–C).

Discussion

In this study, conducted to investigate the possible role of NO in the control of bioluminescence in krill (*Meganyctiphanes norvegica*), results suggest that NO indeed has a role in the control of light production. However, rather than influencing

bioluminescence by direct action, NO appears to modulate the effects of 5-HT: all nitric drugs tested, including the NO donors SNP and SNAP, failed to induce bioluminescence *per se* but modulated light production stimulated by 5-HT. Furthermore, both NOS-like and 5-HT-like materials were detected by immunohistochemistry in structures inside the light organs.

Immunohistochemistry

5-HT

This study shows for the first time that 5-HT-like IR is present and abundant in the light organs of krill, in nerve fibres terminating at the sphincter-like structures at the base of the C-cells. The innervation of these structures by 5-HT fibres agrees

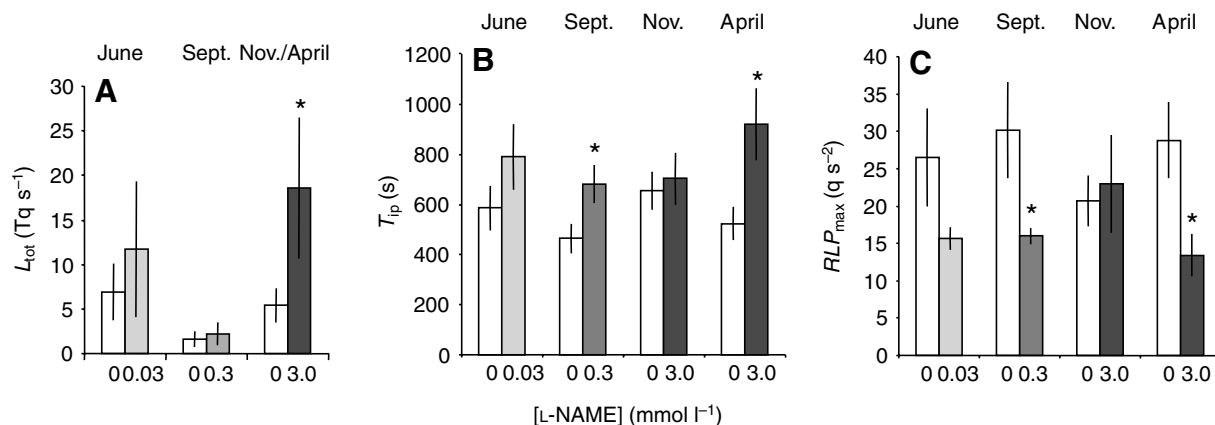


Fig. 7. Effect of the NOS inhibitor L-NAME on the 5-HT-stimulated light response. White bars are 5-HT controls (0.03 mmol l⁻¹ 5-HT). Injection of L-NAME (3.0 mmol l⁻¹) together with 5-HT gave a significantly higher L_{tot} ($P < 0.05$), compared with the 5-HT control (A). As this treatment was performed in two different months (November and April) a two-factor ANOVA with 'treatment' and 'month' as factors was used to determine the effect of L-NAME. Month had no significant effect on L_{tot} . The lower concentrations of L-NAME used had no effect on the L_{tot} of the 5-HT response. In September and April the 5-HT and L-NAME treatment (0.3 and 3.0 mmol l⁻¹ L-NAME, respectively) gave a slower response with longer t_{ip} (B) and a higher RLP_{max} (C) compared with the 5-HT control ($P < 0.05$). Number of replicates (non-converging data in brackets, not included in the analysis): 5-HT, June, $N = 10$ (1); 5-HT+L-NAME, June, $N = 8$; 5-HT, Sept., $N = 10$ (2); 5-HT+L-NAME, Sept., $N = 9$; 5-HT, Nov., $N = 9$ (1); 5-HT+L-NAME, Nov., $N = 9$ (2); 5-HT, Apr., $N = 14$ (4); 5-HT+L-NAME, Apr., $N = 15$; error bars indicate s.e.m.

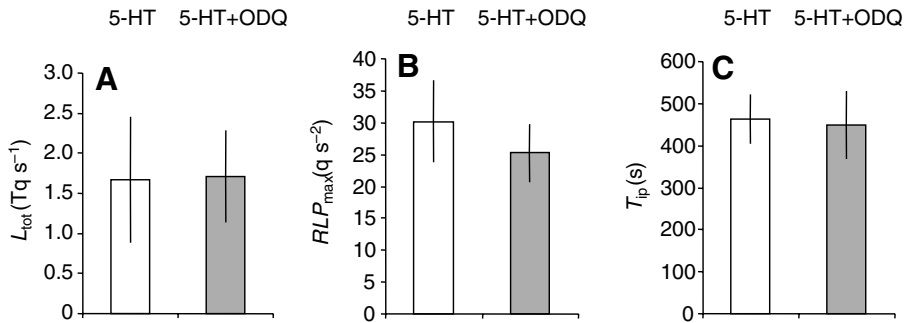


Fig. 8. Effects of the guanylyl cyclase inhibitor ODQ (0.01 mmol l^{-1}) on the 5-HT-stimulated light response (A, L_{tot} ; B, RLP_{max} ; C, t_{ip}). ODQ had no effect on the 5-HT-stimulated light response. Numbers of replicates: 5-HT, $N=10$; 5-HT+ODQ, $N=11$; error bars indicate s.e.m.

with earlier studies using electron and light microscopy, which describe nerve endings with synaptic vesicles connecting to C-cells but not to any other cell type in the organ (Petersson, 1968; Herring and Locket, 1978). It is not yet determined whether the 'sphincters' are contractile, even though they consist of filaments that resemble muscle filaments when studied with electron microscopy (Harvey, 1977; Herring and Locket, 1978). A serotonergic innervation of the sphincter-like structures, where capillaries empty into the lantern sinus, might suggest that 5-HT can regulate the flow of haemolymph, and thereby the flow of oxygen to the lantern.

Transmitters may be released not only from the nerve endings, and the observed 5-HT-IR axons may thus influence other cells and cell functions in the photophores. This is corroborated by the varicose appearance of these axons. For example, 5-HT-IR axons follow capillaries between the large A-cells, which have been described as supportive cells (Herring and Locket, 1978) but have also been proposed to be involved in the luminescence reaction (Bassot, 1966). It is furthermore possible that released 5-HT diffuses into the haemolymph through the capillary walls and is transported as a neurohormone to an effector site. However, a direct stimulation of the photocytes by circulating 5-HT, through 5-HT receptors on the photocytes, is unlikely as isolated photophores do not respond to 5-HT (Herring and Locket, 1978) (and J.K., personal observations).

NOS

Interestingly, NOS-like IR occurred primarily in non-neuronal structures associated with capillaries inside the photophores. NO is a potent vasodilator in mammalian circulatory systems (Bredt and Snyder, 1992; Bredt and Snyder, 1994; Torilles, 2001) and relaxes smooth muscle in general in both vertebrates and invertebrates (Olsson and

Holmgren, 1996; Elphick and Melarange, 1998). However, the open circulatory system of crustaceans usually lacks contractile vessels (Wilkins, 1997; Wilkins, 1999). Instead, in some species, the distribution of haemolymph can be regulated by muscular valves positioned, for example, at the exit of arteries from the heart or at major branching points of arteries in the periphery of the circulatory system (Davidson et al., 1998; McMahon, 2001).

The sphincter-like structures at the base of the C-cells provide a similar opportunity for the regulation of haemolymph flow. The presence of NOS-like material in the area around these 'sphincters' may indicate that NO is involved in the regulation of haemolymph supply to the lantern (as speculated for serotonin, above). This may lead to a modulated oxygen supply and a change in the pH, both of which may affect light production (see below).

NOS-like IR was also seen along the capillaries in the periphery of the light organ and beside the lens, where the entry point for the photophore arteries and the ring vessel circling the lens are located. As these parts of the vessels are not believed to be contractile, this may suggest that NO has additional functions (Herring and Locket, 1978). NO might be produced along the vessels, released to the haemolymph and transported bound to haemocyanin (Jacklet and Koh, 2001). NO bound to haemocyanin will reduce the oxygen-binding capacity of the haemocyanin, and thereby decrease the oxygen level in the lantern, and thus quench the light reaction. NO has furthermore been shown to alter the excitability of some cell types. In skeletal muscle from the isopod *Idotea baltica*, NO stimulates outward potassium currents from the muscle cells, resulting in a hyperpolarization of the cell (Hermann and Erxleben, 2001). Hyperpolarization of the photocytes by NO in the haemolymph would result in less responsiveness to excitatory stimuli and consequently less light production.

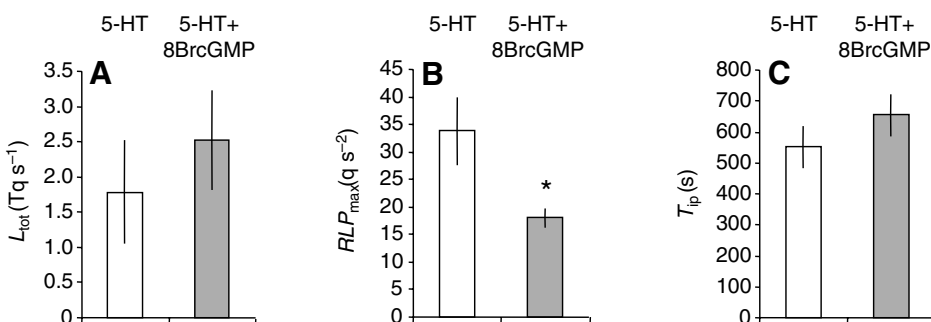


Fig. 9. Effects of the cGMP analogue 8BrcGMP (0.3 mmol l^{-1}) on the 5-HT-stimulated light response (A, L_{tot} ; B, RLP_{max} ; C, t_{ip}). Responses of specimens injected with both 5-HT and 8BrcGMP had a significantly lower RLP_{max} compared with the 5-HT control (B, $P<0.05$). Numbers of replicates: 5-HT, $N=10$; 5-HT+8BrcGMP, $N=10$; error bars indicate s.e.m.

*Pharmacology**5-HT*

Several authors have shown that 5-HT stimulates light production in krill (Kay, 1962; Kay, 1965; Doyle, 1966; Fregin and Wiese, 2002). This was confirmed in the present study. In addition, we report a possible seasonal variation in the response to 5-HT, mainly expressed in a few specimens as a remarkably high response to 5-HT in April and June. In the mouse brain, seasonal and diurnal changes in sensitivity to serotonin have been correlated to corresponding changes in the uptake, release and number of binding sites for serotonin (Rovescalli et al., 1989; Weiner et al., 1992). Seasonal fluctuations in krill bioluminescence and the behavioural implications have been reported and discussed in the literature (Mauchline, 1960), but the physiological mechanisms underlying this variation have not been investigated. The high response to 5-HT in some specimens sampled in April and June in the present study may be connected to the elevated levels of ambient light in the spring and summer.

Another possibility is that a particular social behaviour during this season [i.e. mating, which will start in March in the Gullmar fjord krill population (Thomasson, 2003)] involves increased light production in krill. Some specimens may be in a different physiological state, causing an elevated sensitivity. However tempting it is to suggest that the variability is caused by mating behaviour, no obvious correlation with sex or size could explain the sensitivity to 5-HT in the very highly responsive individuals in this study (J.K., personal observations).

NO donors

In contrast to the stimulating effect of NO in fireflies (Trimmer et al., 2001), both NO donors (SNP and SNAP) used in this study suppressed the total amount of light (L_{tot}) produced after stimulation with 5-HT. This may be compared to the effect of NO in hatchetfish, which is predominantly inhibitory on adrenaline-induced light production. However, the stimulatory effect of NO observed in weakly luminescing hatchetfish was never observed in krill (Krönström et al., 2005).

The two NO donors affected the kinetic parameters, t_{ip} and RLP_{max} , differently. SNAP delayed the 5-HT response significantly, whereas SNP did not. This dissimilarity might be because of the different chemical composition of SNP and SNAP and different modes of generation of NO in the tissue. The degradation of SNAP can be catalysed by membrane components, whereas SNP generates NO mainly in the medium (Kowaluk and Fung, 1990; Ohta et al., 1997).

NOS inhibitors

In krill, the average L_{tot} is higher for individuals injected with both 5-HT and the NOS inhibitor L-NAME, compared with 5-HT controls. The fact that L-NAME (3 mmol l⁻¹) enhanced the effect of 5-HT indicates a nitrenergic suppressing tonus on light production. Lower concentrations of L-NAME gave inconclusive results. There is a large individual variation in the response to the 5-HT and L-NAME treatment. This may indicate that NO is only produced in certain situations and that the specimens we have tested could be in different physiological states.

Taken together, the effects of NO donors and NO blockade support the idea that total light production is reduced by NO.

However, the longer t_{ip} and lower RLP_{max} after L-NAME treatment as well as after treatment with NO donors appear contradictory. The effects cannot be explained by a simple model for an inhibitory control by NO, but suggest that several interacting inhibitory as well as excitatory mechanisms are involved in the normal control of light production and are affected by the NO tonus in the preparation.

cGMP

In some crustacean tissues the effects of NO are mediated through stimulation of cGMP production. This is shown pharmacologically in preparations from the abdominal nervous system of the crayfish *Pacifastacus leniusculus* (Aonuma and Newland, 2002). Moreover, cGMP IR is elevated after NO stimulation of certain cells in the cardiac ganglion of the crab *Cancer productus*, and in the abdominal nervous system of the crayfish *Procambarus clarkii* (Aonuma, 2002). In contrast, NO does not affect luminescence through the cGMP pathway in fireflies and hatchetfish, two species in which NO has been demonstrated to be involved in the regulation of light production. The action of NO in these systems instead seems to be linked to mitochondrial respiration (Trimmer et al., 2001; Krönström et al., 2005). Similarly, in the present study, injections of a membrane-permeable analogue of cGMP did not mimic the effect of the NO donors, suggesting that the inhibitory effect of NO on the total light production does not involve cGMP formation. This is further supported by the lack of effect of the cGMP-antagonist ODQ. However, the effect of the analogue on RLP_{max} is contradictory, and again implies the involvement of several mechanisms. It also remains to be established as to what extent and how the light reaction in krill is dependent on cellular respiration.

In conclusion, the results from this study indicate that NO has a role, or several roles, in the control of bioluminescence in krill. NO could possibly restrict the supply of oxygen to the photocytes, or act directly on photocytes or photocyte-controlling nerves inside the photophore. The inhibitory effects of NO on krill photophores differ from the stimulatory effects of NO on firefly and the dual effects on hatchetfish light production. This highlights the diversity of bioluminescent systems as well as the multiplicity of physiological systems in which NO is involved.

Abbreviations

cGMP	cyclic guanosine monophosphate
eNOS	endothelial nitric oxide synthase
5-HT	5-hydroxytryptamine, Serotonin
iNOS	inducible nitric oxide synthase
ip	inflection point
IR	immunoreactivity
L_{tot}	summed total amount of light
L-NAME	N ^G -nitro-L-arginine methyl ester
Mq	megaquanta
NO	nitric oxide
NOS	nitric oxide synthase
NOS-LI IR	nitric oxide synthase-like immunoreactivity
nNOS	neuronal nitric oxide synthase
nNOS-LI IR	neuronal nitric oxide synthase-like immunoreactivity

ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
$q\ s^{-1}$	quanta per second
RLP_{max}	maximum rate of light response (growth rate of Gompertz graph)
SNAP	S-nitroso-N-acetylpenicillamine
SNP	sodium nitroprusside
$Tq\ s^{-1}$	tera quanta per second
t_{ip}	time to the inflection point
uNOS	universal nitric oxide synthase

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