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RESEARCH ARTICLE

The regulatory role of the NO/cGMP signal transduction cascade during larval attachment and metamorphosis of the barnacle *Balanus* (=*Amphibalanus*) *amphitrite*

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SUMMARY

The barnacle *Balanus amphitrite* is among the most dominant fouling species on intertidal rocky shores in tropical and subtropical areas and is thus a target organism in antifouling research. After being released from adults, the swimming nauplius undertakes six molting cycles and then transforms into a cyprid. Using paired antennules, a competent cyprid actively explores and selects a suitable substratum for attachment and metamorphosis (collectively known as settlement). This selection process involves the reception of exogenous signals and subsequent endogenous signal transduction. To investigate the involvement of nitric oxide (NO) and cyclic GMP (cGMP) during larval settlement of *B. amphitrite*, we examined the effects of an NO donor and an NO scavenger, two nitric oxide synthase (NOS) inhibitors and a soluble guanylyl cyclase (sGC) inhibitor on settling cyprids. We found that the NO donor sodium nitroprusside (SNP) inhibited larval settlement in a dose-dependent manner. In contrast, both the NO scavenger carboxy-PTIO and the NOS inhibitors aminoguanidine hemisulfate (AGH) and *S*-methylisothiourea sulfate (SMIS) significantly accelerated larval settlement. Suppression of the downstream guanylyl cyclase (GC) activity using a GC-selective inhibitor ODQ could also significantly accelerate larval settlement. Interestingly, the settlement inhibition effects of SNP could be attenuated by ODQ at all concentrations tested. In the developmental expression profiling of NOS and sGC, the lowest expression of both genes was detected in the cyprid stage, a crucial stage for the larval decision to attach and metamorphose. In summary, we concluded that NO regulates larval settlement *via* mediating downstream cGMP signaling.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/21/3813/DC1

Key words: barnacle, *Balanus amphitrite*, larval attachment, metamorphosis, larval settlement, signal transduction, nitric oxide, guanylyl cyclase, cyclic GMP.

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INTRODUCTION

The barnacle *Balanus amphitrite* is among the most dominant fouling organisms in tropical and subtropical areas. Because of its importance in the functioning and structuring of marine benthic ecosystems worldwide, larval settlement of this species has been extensively studied in recent decades (e.g. Clare et al., 1992; Harder et al., 2001; Khandeparker and Anil, 2007). The interaction between different environmental/artificial factors and larval attachment and metamorphosis of this species has been amply documented (e.g. Pawlik, 1992; Okazaki and Shizuri, 2000a; Okazaki and Shizuri, 2000b). Previous studies indicated that initiation of larval attachment is regulated upon the detection of inductive signal cues, which is followed by the appearance of a sequence of settlement behaviors, including crawling, temporary attachment, secretion of a cement substance and eventually permanent attachment and metamorphosis (Yamamoto et al., 1998). As the recognition of the inducer is specific in many benthic invertebrates, it has been suggested that particular signal transduction systems are involved in the transmission and/or translation of exogenous signals to the endogenous effectors and that they control larval settlement (Holm et al., 2000).

Over the past 20 years, a considerable body of research has focused on the molecular mechanisms controlling larval attachment and metamorphosis of *B. amphitrite*. For example, in the studies of exogenous settlement cues, a high molecular mass glycoprotein named ‘settlement-inducing protein complex’ (SIPC), which was shown to be a cue for gregarious settlement, was purified from *B. amphitrite* adults (Matsumura et al., 1998b). Immunostaining results revealed that SIPC mainly localized at the attachment discs of the antennules and the ‘footprints’ of *B. amphitrite* cyprids could also be specifically stained (Matsumura et al., 1998c). These results indicated that SIPC may be involved in adult–larva and larva–larva interactions during settlement. More detailed studies on the spatial and ontogenetic expression of SIPC have confirmed the previous findings and suggested that cyprids might detect this cue through contact with cuticle of adult barnacle (Dreanno et al., 2006a; Dreanno et al., 2006b; Dreanno et al., 2006c). Nevertheless, the SIPC receptor in cyprids is still unknown.

For endogenous molecular signaling studies, adenylate cyclase activator and inhibitor were both used to show the involvement of cyclic AMP (cAMP) in the pheromonal modulation of barnacle settlement (Clare et al., 1995). In a consecutive series of publications, Yamamoto and colleagues concluded that the protein kinase C (PKC) pathway might play an important role during larval metamorphosis but not during attachment of *B. amphitrite* (Yamamoto et al., 1995; Yamamoto et al., 1997). The same group of scientists exposed the cyprid larvae to serotonin, serotonin uptake blocker and serotonin antagonists, and postulated that serotonin may be involved in regulating the overall settlement process, which

includes both attachment and metamorphosis (Yamamoto et al., 1996; Yamamoto et al., 1999). Overall, these studies have provided some insights into the molecular mechanisms controlling this biological process. However, there has been no comprehensive study elucidating the regulation of interconnected signaling networks during larval settlement of *B. amphitrite*.

Nitric oxide (NO) is one of the smallest and most diffusible gaseous signaling molecules found in practically all phyla of living organisms investigated so far (Moncada et al., 1991). Fig. 1 shows a simplified schematic diagram of the NO–guanylyl cyclase (GC)–cyclic GMP (cGMP) pathway. NO is biosynthesized endogenously by a family of NO synthases (NOS), which convert L-arginine to NO. Subsequently, NO activates GC, which catalyzes the conversion of GTP to cGMP and subsequently activates the downstream components and effectors (Lucas et al., 2000). This signaling molecule undertakes a wide spectrum of functions affecting crucial biological processes including neurotransmission, muscle relaxation and inflammation, and host defense in vertebrate systems (Bruckdorfer, 2005). Recently emerging evidence showed the involvement of this simple molecule in regulating a variety of biological processes in invertebrate systems as well (Palumbo, 2005). Nevertheless, the involvement of NO signaling and related pathways during larval settlement of the barnacle *B. amphitrite* has not yet been studied.

To test whether NO signaling may be involved in barnacle settlement, we took advantage of a high-throughput *B. amphitrite* transcriptome database generated in our laboratory (Chen et al., 2011). Our searches against the annotated transcriptome sequences revealed the presence of all major components of the NO signaling and related pathways, which include nitric oxide synthase (NOS), GC, phosphodiesterase (PDE) and cGMP-dependent protein kinase (PKG) (Table 1; supplementary material Table S1, Fig. S1), suggesting that further investigation of this pathway in barnacle settlement was warranted. In addition, based on the results of our previous proteomics study (Zhang et al., 2010), we found that arginine kinase (AK), which uses the same substrate as NOS, was dramatically up-regulated when *B. amphitrite* cyprids were aging and approaching settlement. This might lead to a change in the availability of the substrate and therefore to the reduction of NO production. These results prompted us to hypothesize the involvement of NO and the related signaling cascade in larval settlement of *B. amphitrite*.

The present study was undertaken to comprehensively examine the regulatory role of NO, a simple but potentially crucial signaling molecule, during the pelagic-to-benthic transition in *B. amphitrite*. As many biological functions of NO are mediated *via* a downstream signaling molecule, namely cGMP (Warner et al., 1994; Seidel and Bicker, 2000), we also examined the involvement of this important second messenger. Specifically, the role of this signaling cascade was investigated at the transcriptional level by real-time PCR and at the functional level by specific inhibitor treatments. Furthermore, the interaction between the exogenous inductive cue (SIPC) and NO signaling was determined by *B. amphitrite* adult extracts and NO donor co-incubation assay.

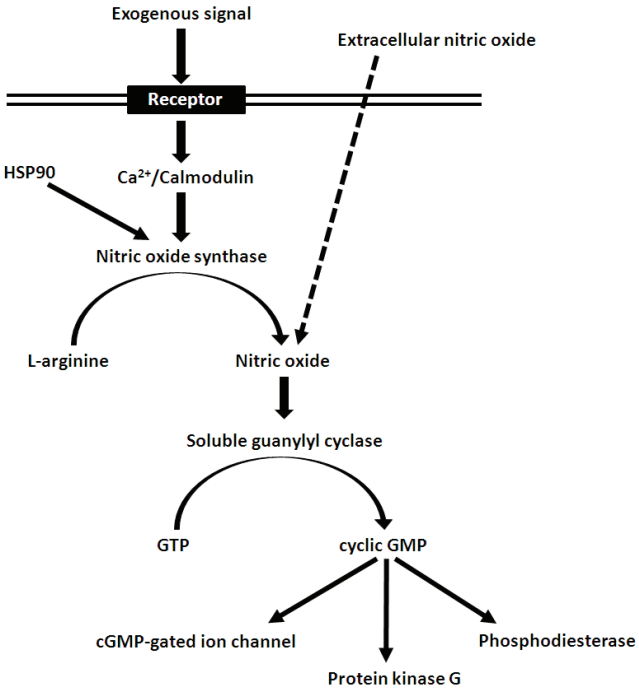

Fig. 1. A simplified schematic diagram of the nitric oxide (NO)–soluble guanylyl cyclase (sGC)–cyclic GMP (cGMP) pathway. Upon reception of exogenous stimuli (environmental and artificial), nitric oxide synthase (NOS) is activated, which leads to the enhanced biosynthesis of cGMP. Eventually, the downstream cGMP-gated ion channel, cGMP-dependent protein kinase G (PKG), and phosphodiesterase (PDE) are activated and the signal cascade transduces to cellular effectors. As NO is cell permeable, it can be generated by adjacent or even further away cells. It diffuses and passes through cell membranes of distant target cells.

MATERIALS AND METHODS

Sampling the larvae

Balanus amphitrite Darwin adults were collected from the concrete columns of the pier at Pak Sha Wan in Hong Kong (22°21′45″N, 114°15′35″E) and the released naupliar larvae were reared to the cyprid stage (see Thiyagarajan et al., 2002; Thiyagarajan et al., 2003). Briefly, *B. amphitrite* adults were induced to release nauplii using bright artificial light. Nauplii were collected within a 2 h period. When reared on a diet of *Chaetoceros gracilis* at 28°C with a 15 h:9 h light–dark cycle, the nauplii usually developed into cyprids on day 4. Because of different growth rates of each individual, the nauplii did not transform to the cyprid stage simultaneously. In order to eliminate the variance generated by cyprid aging, the cyprids were collected every 4 h to ensure that the collected cyprids had just transformed from the naupliar VI stage. The newly collected cyprids were transferred to a 24-well polystyrene plate (Nalge Nunc International, Rochester, NY, USA) for a series of pharmacological bioassays. Each well contained 10–15 larvae in 1 ml of filtered seawater (FSW) or an experimental solution in FSW. The same batch of cyprids was used for all replicates within the same experiment. For the analysis of differential gene expression during the development of *B. amphitrite*, stage IV nauplii (Nau4), stage VI nauplii (Nau6), cyprids, just metamorphosed juveniles and adults were collected. All of these samples were immediately frozen in liquid nitrogen until use.

Quantitative PCR

To investigate whether the expression of NO/cGMP-related genes changes during the process of settlement, quantitative real-time PCR (qRT-PCR) of two key components of this signaling pathway, namely NOS and soluble guanylyl cyclase (sGC), were performed following protocols described elsewhere (Qian et al., 2010). Briefly, total RNA was extracted from each developmental stage of *B. amphitrite* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA),

Table 1. NO signaling components present in 454 pyro-sequencing generated *B. amphitrite* transcriptome database

NO signaling component	Symbol	Function in NO signaling	No. hits
Nitric oxide synthase*	NOS	Synthesizes nitric oxide from L-arginine	1
Nitric oxide synthase interacting protein	NOSIP	Regulates distribution and activity of NOS	5
Nitric oxide synthase 1 (neuronal) adaptor protein	NOS1AP	Involved in NO synthesis regulation *via* its association with nNOS/NOS1	3
Soluble guanylyl cyclase/guanylyl cyclase*	sGC/GC	Synthesizes cGMP from GTP	1/14
cGMP-dependent protein kinase or protein kinase G	PKG	Phosphorylates downstream target proteins	10
Phosphodieterase	PDE	Hydrolyzes the second messengers cGMP, cAMP or both cGMP and cAMP	26

NO, nitric oxide.
Corresponding information for the two key components marked with an asterisk is detailed in supplementary material Table S1 and Fig. S1.

according to the manufacturer's instructions. Total RNA was then digested with TURBO DNA-free kit (Applied Biosystems, Carlsbad, CA, USA) to remove trace DNA contaminants. Total RNA concentration and purity were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) and its quality was evaluated by agarose gel electrophoresis. First-strand cDNA was synthesized from 2 μg of total RNA from each stage using M-MLV Reverse Transcriptase (USB Corporation, Cleveland, OH, USA) with $pd(N)_6$ random primer (Bio Basic Inc., Shanghai, China). The *cytochrome b* gene was chosen as the reference gene to normalize expression levels of selected genes (De Gregoris et al., 2009). Specific DNA primers of *NOS* and *sGC* were designed based on the *B. amphitrite* transcriptome (raw sequencing data NCBI accession number: SRA029164.1) (Chen et al., 2011) using the NCBI Primer-BLAST program (Table 2). Real-time PCR was carried out using a Kapa SYBR FAST qPCR master mix (Kapa Biosystems, Boston, MA, USA) and run on an ABI 7500 fast real-time PCR system (Applied Biosystems). All of the assays were performed in triplicate and repeated twice. The qRT-PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method (see Livak and Schmittgen, 2001).

Chemicals

The NOS inhibitors aminoguanidine hemisulfate (AGH) and *S*-methylisothiourea sulfate (SMIS), and the soluble guanylyl cyclase (sGC) inhibitor 1*H*-(1,2,4)oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) were purchased from Sigma-Aldrich (St Louis, MO, USA). The NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolineoxyl-1-oxyl-3-oxide (carboxy-PTIO) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NO donor sodium nitroprusside (SNP) was obtained from Beyotime Institute of Biotechnology (Shanghai, China). AGH and SMIS were prepared as 500 mmol l^{-1} stock in Milli-Q water. SNP and carboxy-PTIO were prepared as 1 mol l^{-1} stock in Milli-Q water. ODQ was prepared as 500 mmol l^{-1} stock in dimethyl sulfoxide (DMSO). All stock solutions were divided into aliquots and stored at –20°C until use. Table 3 summarizes information on the bioassays, including biochemical/physiological action, concentrations and exposure duration of these chemicals. A range of concentrations of each chemical used in this study was chosen based on previous reports using the same chemical, which showed significant effects on modulating the corresponding molecular target.

Modulation of the endogenous NO level

To examine how alteration of the endogenous NO level affects larval settlement, chemicals that enhance or reduce the formation of NO were used. To elevate the endogenous NO level, one of the most studied NO donors, namely SNP (Kowaluk et al., 1992), was utilized during the pharmacological incubation. A dilution series of SNP (125, 250, 500 and 1000 μmol l^{-1}) was applied to investigate the most effective concentration of the chemical. The percentages of larval settlement among treatments and control were scored at 4 days post-treatment. Experimental results for other chemicals were scored at 1, 2, 3 and 4 days post-treatment. To reduce the endogenous NO level, both chemical removal of NO using carboxy-PTIO and enzymatic inhibition of NOS using AGH and SMIS were used in this study (Maeda et al., 1994; Laszlo et al., 1995; Szabó et al., 1994). Carboxy-PTIO (100 μmol l^{-1}) was used in the treatment, while for NOS inhibitors a dilution series (100, 200, 400 and 800 μmol l^{-1}) of AGH and SMIS was utilized.

NO donor and adult extract co-incubation assay

To determine the relationship between the natural inductive cue and NO signaling, larvae were co-incubated with 1000 μmol l^{-1} of SNP and 40 μg ml^{-1} of *B. amphitrite* adult crude extract, which contains the gregarious settlement-inducing cue SIPC. The results of this experiment were scored at 12, 24, 48 and 72 h post-treatment. The adult crude extracts were prepared as detailed elsewhere (Matsumura et al., 1998a). Briefly, adult barnacles were homogenized in 50 mmol l^{-1} Tris-HCl, pH 7.5. The homogenates were filtered with gauze and then centrifuged at 40,000 ***g*** for 30 min. The supernatant was immediately stored at –20°C until use.

Table 2. *NOS* and *sGC* gene expression analysis during development and metamorphosis of *B. amphitrite*

Gene name	Isotig no.	Gene expression variation					Primer sequence (forward, reverse)
		Nau4	Nau6	Cyprid	Juvenile	Adult	
NOS	GBQDZ6L01A97M3_3	21.9	2.9	1	10.9	46.3	5′-GCCGGCCGTCTCCGGCATGA-3′ 5′-GTCACGCGCCGCCACCTCGG-3′
sGC	GBQDZ6L01AIVFU_6	4.2	2.7	1	3.9	27.9	5′-GGTGGGCGGGTGTCCCGAGGT-3′ 5′-TGGGCACCAGCACATGGCCCG-3′

Gene expression levels at the cyprid stage were taken as the baseline in both analyses.
Nau4, stage IV nauplii; Nau6, stage VI nauplii.

Table 3. Summary of chemicals used to modulate NO/cGMP signaling pathway in this study

Selective compounds	Symbol	Linear structure	Biochemical/physiological actions	Concentrations used (μmol l^{-1})	Exposure duration/ scored time	Related references on selected concentrations
Sodium nitroprusside dihydrate	SNP	$Na_2[Fe(CN)_5NO]\cdot 2H_2O$	NO donor/releaser and potent vasodilator, which releases NO *in vivo*	125, 250, 500, 1000	4 days/Day 4	Leise et al., 2001; Ebbesson et al., 2005; Krönström et al., 2007
Carboxy-PTIO	Carboxy-PTIO	$C_{14}H_{17}N_2O_4$	Stable radical compound used to scavenge and trap NO radicals,which reacts stoichiometrically with NO	100	4 days/Day 1, 2, 3 and 4	Pfeiffer et al., 1997; Froggett and Leise, 1999; Cao and Reith, 2002
Aminoguanidine hemisulfate salt	AGH	$CH_6N_4\cdot 1/2H_2SO_4$	NOS inhibitor, which inhibits both constitutive and inducible NO synthase	100, 200, 400, 80	4 days/Day 1, 2, 3 and 4	Han et al., 2007; Pechenik et al., 2007
S-methylisothiourea sulfate	SMIS	$C_4H_{14}N_4O_4S_3$	Potent and selective inhibitor of inducible NOS (iNOS)	100, 200, 400, 800	4 days/Day 1, 2, 3 and 4	Yet et al., 1997; Pechenik et al., 2007
1*H*-(1,2,4)oxadiazolo [4,3-a]quinoxalin-1-one	ODQ	$C_9H_5N_3O_2$	Selective and potent inhibitor of NO-sensitive guanylyl cyclase. ODQ reversibly inhibits cGMP generation in response to endogenous NO or exogenously added NO donors	2.5, 5, 10, 20	4 days/Day 1, 2, 3 and 4	Bishop and Brandhorst, 2001; Bishop and Brandhorst, 2007; Krönström et al., 2007; Pechenik, 2007
SNP and ODQ co-incubation	SNP + ODQ	As above	ODQ inhibits cGMP generation in response to exogenously added NO donors	SNP: 1000; ODQ: 2.5, 5, 10, 20	4 days/Day 1, 2, 3 and 4	This study

Name of the selective compounds, their symbol, linear structure, biochemical actions, concentrations, exposure duration and scored time, and related references are provided.

Modulation of the endogenous cGMP level

The biosynthesis of cGMP is catalyzed by GC, which is the principal receptor for NO and probably the most prevalent downstream effector of NO signaling (Lucas et al., 2000). To evaluate the involvement of cGMP in larval settlement of *B. amphitrite*, a dilution series (2.5, 5, 10 and 20 μmol l^{-1}) of the specific GC inhibitor ODQ was used to treat the cyprids (Brunner et al., 1995). To determine whether the biological function of NO during larval settlement is through the second messenger cGMP, the cyprid larvae were co-incubated with a dilution series (2.5, 5, 10 and 20 μmol l^{-1}) of ODQ and 1000 μmol l^{-1} SNP. If cGMP is a downstream effector of NO in regulating larval settlement, the effect of SNP against the settling larvae should be attenuated by ODQ rescue.

Data analysis

Each pharmacological treatment and control was replicated at least three times. Data were expressed as percentages of settlement and were arcsine transformed prior to statistical analysis. In experiments with a single concentration of chemical being tested, results were tested for statistical significance using Student's *t*-test. In experiments with a dilution series treatment, analysis was performed by simultaneous multiple comparisons of treatment means with a control using one-way ANOVA, followed by Dunnett's HSD test. For the co-incubation assay, significant effects on larval settlement were analyzed by simultaneous multiple comparisons of different means using one-way ANOVA, followed by Tukey's HSD test. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

NOS and sGC are differentially expressed during larval settlement

NOS and sGC are two key components of the NO/cGMP signaling pathway. In order to examine the involvement of this pathway in regulating larval settlement of the barnacle *B. amphitrite*, differential expression of genes encoding these two proteins during larval development was analyzed by qRT-PCR. Fig. 2 shows the results from stage IV nauplii, stage VI nauplii, cyprids, just metamorphosed juveniles and adults. Expression of the *NOS* gene significantly changed during larval development, which showed a continued decrease from Nau4 to Nau6 and from Nau6 to cyprid development (Fig. 2A). The lowest level of NOS expression was detected in cyprids, which is the critical stage for the larval decision to attach and metamorphose. It then significantly increased by 10.9-fold in juveniles and reached the maximal expression level (increased by 46.3-fold) in adults. Interestingly, the differential regulation of sGC at the transcriptional level was very similar to that of NOS, showing decreased expression during larval development and increased expression after attachment and metamorphosis (Fig. 2B). Similarly, cyprids exhibited the lowest, while adults displayed the highest expression level of sGC in the developmental stages examined. These results suggested that the reduction of NOS and sGC expression to an appropriate level in the cyprid stage is important for the initiation of larval attachment and metamorphosis in *B. amphitrite*.

Enhanced formation of endogenous NO inhibits larval settlement

The endogenous level of NO was manipulated using both chemical and enzymatic methods. SNP, one of the most widely used NO donors, was included in the pharmacological incubation to determine whether an increase in the endogenous NO level would affect larval settlement of *B. amphitrite*. Fig. 3 shows the response of cyprids to a dilution series of SNP. At 4 days post-treatment, the percentage settlement reached 93.0±5.7% in the control group. In the treatment groups, settlement was significantly inhibited by higher concentrations of SNP, with only 27.7±12.5% settlement in the

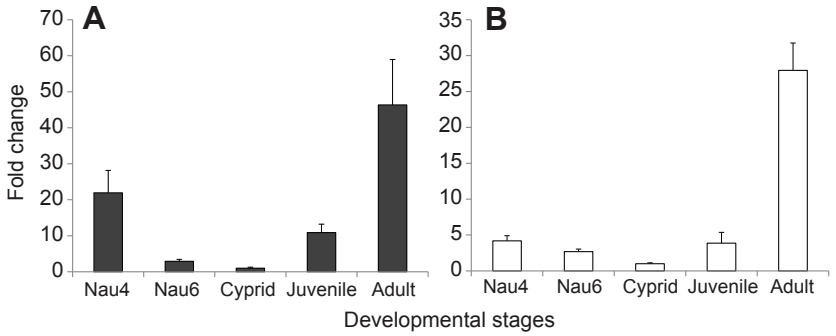

Fig. 2. Gene expression pattern of (A) *NOS* and (B) *sGC* during development and metamorphosis of *Balanus amphitrite*. Gene expression levels at the cyprid stage were taken as the baseline in both analyses. qRT-PCR assays for both genes were performed in triplicate. Each bar indicates mean and s.d. Nau4, stage IV nauplii; Nau6, stage VI nauplii.

500 μmol l^{-1} treatment (control *versus* treatment, P<0.001) and 5.9±9.4% settlement in the 1000 μmol l^{-1} treatment (control *versus* treatment, P<0.001). In contrast, the percentage settlement showed no significant difference at lower concentrations of SNP compared with the control group, with 88.3±6.5% settlement at 125 μmol l^{-1} and 80.8±9.0% settlement at 250 μmol l^{-1} SNP. Importantly, for most of the concentrations tested, the cyprids remained actively swimming compared with the control group, indicating that SNP is non-toxic to *B. amphitrite* at the concentrations tested. This is also suggested by the low mortality rate at the tested concentrations (control, 0%; 125 μmol l^{-1}, 2.6%; 250 μmol l^{-1}, 0%; 500 μmol l^{-1}, 2.4%; and 1000 μmol l^{-1}, 0%). Interestingly, cyprids in the SNP treatment seldom explored the substratum, suggesting that the inhibitory effects of SNP may be due to its interference in larval searching behaviors.

Reduction of the intracellular NO level accelerates larval settlement

Carboxy-PTIO is a stable radical compound used to scavenge NO radicals and widely used as an endogenous NO remover (Maeda et al., 1994). Fig. 4 shows the effects of 100 μmol l^{-1} carboxy-PTIO on the swimming cyprids. This drug significantly potentiated larval settlement starting from the beginning of the experiment, which is shown by the 3.2-, 1.6- and 1.3-fold increases in the mean percentage of larval settlement between the control and treatment on days 1, 2 and 3 of incubation, respectively. Treatment with AGH, an NOS inhibitor, is an enzymatic method that prevents endogenous biosynthesis of NO. This treatment has been widely used in pharmacological research (Laszlo et al., 1995). As shown in Fig. 5, although the larval settlement-promoting effect is not as strong as with carboxy-PTIO, application of AGH at 100 and 200 μmol l^{-1} accelerated larval settlement during the first 2 days of pharmacological treatment. On the first day post-treatment, pharmacological incubation with 100 and 200 μmol l^{-1} of AGH led to 2.2- and 1.6-fold increases in the mean percentage settlement, respectively. On the second day, percentage settlement was 87.5±6.3% with 100 μmol l^{-1} AGH (P<0.001) and 80.4±7.5% with 200 μmol l^{-1} AGH (P<0.01) compared with 55.6±4.8% in the control group. However, 400 μmol l^{-1} AGH did not promote larval settlement, while 800 μmol l^{-1} AGH significantly inhibited larval settlement during the second day of the experiment, suggesting possible side-effects of AGH at higher concentrations. Incubation with another NOS inhibitor, SMIS, showed a similar effect in accelerating larval settlement (Fig. 6). On the first day post-treatment, only larvae treated with 400 μmol l^{-1} SMIS showed a significant difference from the control in terms of percentage settlement, while on the second day the settlement-accelerating effects were observed in the 100–400 μmol l^{-1} SMIS treatments. Overall, our results

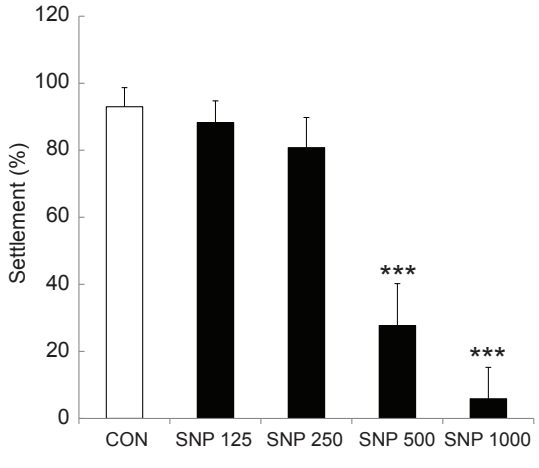

Fig. 3. Inhibitory effects of the NO donor sodium nitroprusside (SNP) on larval settlement of *B. amphitrite*. Cyprids were treated with a dilution series of SNP (125, 250, 500 and 1000 μmol l^{-1}). Data presented are percentages of larvae that both attached and metamorphosed at 4 days post-treatment. Each bar indicates mean and s.d. Multiple comparisons were made between the filtered seawater control and treatments. Data in this figure were assessed by one-way ANOVA and Dunnett's HSD test after arcsine transformation. ***P<0.001. N=3 replicate treatments.

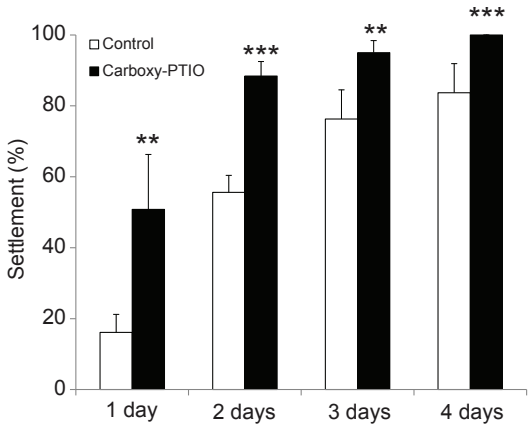

Fig. 4. Inductive effects of the NO scavenger carboxy-PTIO on larval settlement of *B. amphitrite*. Cyprids were treated with 100 μmol l^{-1} carboxy-PTIO. Data presented are percentages of larvae that both attached and metamorphosed 1, 2, 3 and 4 days post-treatment. Each bar indicates mean and s.d. Student's *t*-test comparisons were made between the filtered seawater control and treatments. **P<0.01, ***P<0.001. N=3 replicate treatments.

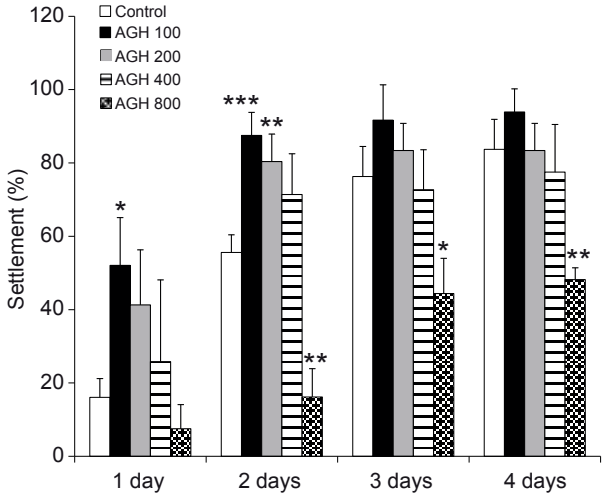

Fig. 5. Inductive effects of NOS inhibition (aminoguanidine hemisulfate salt, AGH) on larval settlement of *B. amphitrite*. Cyprids were treated with a dilution series of AGH (100, 200, 400 and 800 μmol l^{-1}). Data presented are percentages of larvae that both attached and metamorphosed 1, 2, 3 and 4 days post-treatment. Each bar indicates mean and s.d. Multiple comparisons were made between the filtered seawater control and treatments. Data in this figure were assessed by one-way ANOVA and Dunnett's HSD test after arcsine transformation. **P*<0.05, ***P*<0.01, ****P*<0.001. *N*=3 replicate treatments.

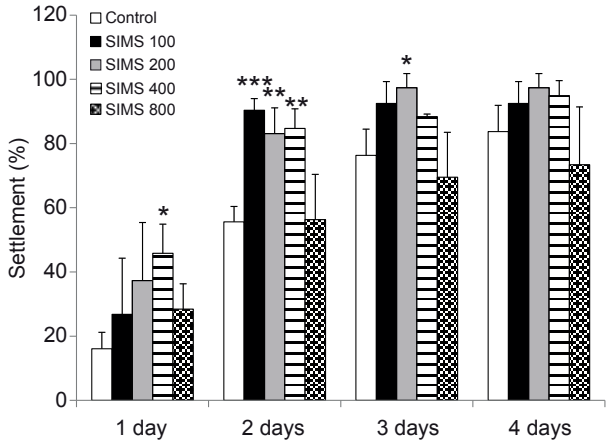

Fig. 6. Inductive effects of NOS inhibition (*S*-methylisothiourea sulfate, SMIS) on larval settlement of *B. amphitrite*. Cyprids were treated with a dilution series of SMIS (100, 200, 400 and 800 μmol l^{-1}). Data presented are percentages of larvae that both attached and metamorphosed 1, 2, 3 and 4 days post-treatment. Each bar indicates mean and s.d. Multiple comparisons were made between the filtered seawater control and treatments. Data in this figure were assessed by one-way ANOVA and Dunnett's HSD test after arcsine transformation. **P*<0.05, ***P*<0.01, ****P*<0.001. *N*=3 replicate treatments.

indicated that both non-enzymatic removal of NO and enzymatic inhibition of NO biosynthesis led to cyprid settlement in *B. amphitrite*.

Increase of endogenous NO attenuates settlement-inducting effects of adult crude extracts

SIPC is known as a cue for gregarious settlement of *B. amphitrite* (Dreanno et al., 2006c). To investigate whether the induction of larval settlement through SIPC involves NO signaling, cyprids were co-incubated with the NO donor SNP and the crude extracts of *B. amphitrite* adults, which presumably contain SIPC. If NO signaling acts downstream of SIPC signal reception, the inductive effect of SIPC on barnacle settlement should be diminished or at least attenuated by modulation of NO level *via* SNP addition. As shown in Fig. 7, upon the addition of 40 μg ml^{-1} of adult crude extract, larval settlement was profoundly accelerated during the first 2 days of the experiment compared with the control. The mean percentage of larval settlement increased 3.2-, 2.1- and 1.3-fold at 12, 24 and 48 h of incubation, respectively, in comparison with the control. In contrast, in the adult extract + SNP co-incubation group, SNP diminished the settlement-inducing effect of the adult extracts, which is suggested by the significant difference between the co-incubation groups and the adult extract treatment group starting from the first day of incubation (P<0.001 at all time points). This result suggests that SIPC-induced larval settlement in *B. amphitrite* can be interfered with by NO modulation, and that NO signaling might be downstream of SIPC perception in regulating this process.

cGMP is involved in larval settlement

sGC has long been considered as the most prevalent downstream effector of NO signaling. Generally, the up-regulation of NO will lead to the activation of GC, which subsequently promotes the formation of cGMP (Lucas et al., 2000). To evaluate whether cGMP is involved in regulating *B. amphitrite* larval settlement, cyprids were incubated with a potent and specific sGC inhibitor, ODQ (Brunner et al., 1995). If the inhibitive effect of NO in larval settlement is *via* the elevation of cGMP, suppression of cGMP-producing enzyme (sGC) activity should initiate larval settlement. As shown in Fig. 8, ODQ promoted larval settlement in a dose-dependent manner. Compared with the control, larvae incubated with 10 and 20 μmol l^{-1} ODQ had significantly higher percentages of settlement during the 4 days of the experiment. At 10 μmol l^{-1} treatment, the mean percentage of larval settlement increased 3.8-fold (P=0.005), 1.9-fold (P=0.012) and 1.5-fold (P=0.012) on the first, second and third day of incubation, respectively, in comparison with the control. At lower concentrations (2.5 and 5 μmol l^{-1}), the difference was not significant during the first 2 days of treatment. In contrast, larval settlement was promoted on the third and fourth day of treatment with low concentrations of ODQ.

cGMP regulates larval settlement *via* NO/cGMP signaling

To exclude the possibility that the induction of ODQ is through a non-specific effect, and to further validate the involvement of cGMP as a downstream mediator of NO, we incubated the settling larvae with a dilution series of ODQ, a selective sGC inhibitor, when SNP was present. If the settlement inhibition effect of the NO donor is through elevation of the downstream cGMP level, the inhibition of cGMP biosynthesis should diminish the effect of the NO donor. As shown in Fig. 9, in the 1000 μmol l^{-1} SNP treatment group, larval settlement was strongly inhibited during the whole experiment. In contrast, in most of the tested concentrations from 2.5 to 20 μmol l^{-1}, ODQ significantly attenuated the inhibition of settlement by SNP, which is suggested by the significant difference between the co-incubation groups and SNP inhibition groups and the similar percentages of settlement in all of the co-incubation groups to the control group. At 1 day post-treatment, percentage settlement in most of the co-incubation treatments (2.5 μmol l^{-1} ODQ+1000 μmol l^{-1} SNP, 40.6±0.9%; 5 μmol l^{-1} ODQ+1000 μmol l^{-1} SNP, 40±6.2%;

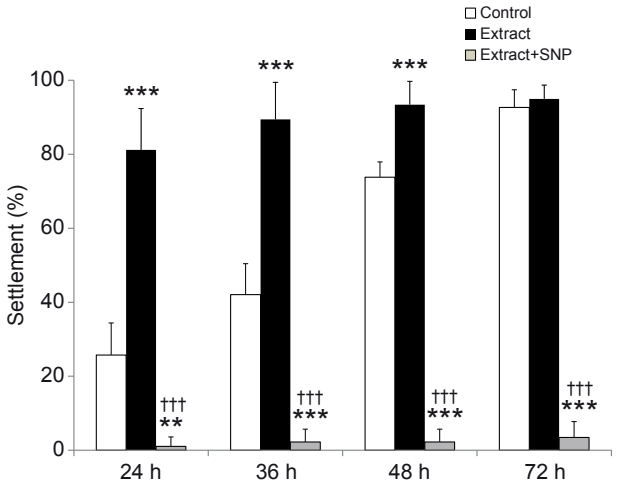

Fig. 7. SNP attenuates larval settlement-inducing effect of adult extract on *B. amphitrite*. Cyprids were co-incubated with 40 μg ml^{-1} of adult crude extract and 1000 μmol l^{-1} SNP. Data presented are percentages of larvae that both attached and metamorphosed at 24, 36, 48 and 72 h post-treatment. Each bar indicates mean and s.d. Data were analyzed by simultaneous multiple comparisons of different means using one-way ANOVA, followed by Tukey's HSD test. †Significant differences between treatments (including adult extract treatment alone and SNP + adult extract co-incubation). *Significant differences between treatments and control. A *P*-value of less than 0.05 was considered statistically significant. ***P*<0.01, †††,****P*<0.001. *N*=3 replicate treatments.

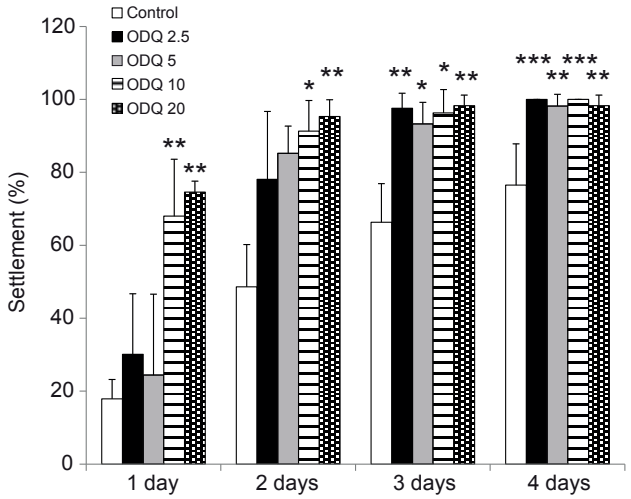

Fig. 8. Inductive effects of guanylyl cyclase (GC) inhibition (ODQ) on larval settlement of *B. amphitrite*. Cyprids were treated with a dilution series of ODQ (2.5, 5, 10 and 20 μmol l^{-1}). Data presented are percentages of larvae that both attached and metamorphosed 1, 2, 3 and 4 days post-treatment. Each bar indicates mean and s.d. Multiple comparisons were made between the filtered seawater control and treatments. Data in this figure were assessed by one-way ANOVA and Dunnett's HSD test after arcsine transformation. **P*<0.05, ***P*<0.01, ****P*<0.001. *N*=3 replicate treatments.

10 μmol l^{-1} ODQ+1000 μmol l^{-1} SNP, 44.4±13.6%) was higher than in the control group (16.1±5.1%) (Fig. 8). This result suggests that the regulatory role of NO in the larval settlement of *B. amphitrite* is *via* mediating the downstream cGMP level. It should be noted that, in most of the co-incubation groups, larvae actively explored the substratum for attachment, which was dramatically different from the behavior of the larvae in the SNP treatment group as described above. Furthermore, the resultant juveniles from all of the co-incubation experiments developed without any difference in terms of their morphology compared with the control group.

DISCUSSION

The selective settlement of *B. amphitrite* cypris larvae in response to different biotic factors has been substantially documented. In particular, the involvement of pheromones, hormones, neurotransmitters and proteins in regulating larval settlement of *B. amphitrite* has been intensively studied (e.g. Clare et al., 1995; Yamamoto et al., 1995; Yamamoto et al., 1997; Matsumura et al., 1998b; Matsumura et al., 1998c; Yamamoto et al., 1999; Delort et al., 2000; Endo et al., 2009; Gallus et al., 2010). Based on these results, it has been suggested that signaling transduction systems are likely involved in transmitting and/or translating these exogenous signals to endogenous effectors, resulting in the initiation of larval settlement (Clare et al., 1995; Yamamoto et al., 1997; Yamamoto et al., 1998). However, little is known about the signal transduction systems responsible for the regulation of cyprid settlement of *B. amphitrite*. NO signaling is one of the most conserved signaling systems found across different phyla of living organisms (Moncada et al., 1991). Although the biological function of NO signaling has been well studied using mammalian systems, invertebrates have received relatively little attention. For instance, Robertson and colleagues reported that NO regulates tactile learning (Robertson et al., 1994) and visual learning (Robertson et al., 1996) processes in *Octopus vulgaris*. It was also found that NO is involved in regulating neural transmissions to intestinal muscles of the common snail *Helix lucorum* (Röszer et al., 2004) and in the fine olfactory discrimination of the terrestrial slug *Limax valentianus* (Sakura et al., 2004), and, importantly, it was found that NO plays a crucial role in regulating metamorphosis of the marine snail *Ilyanssa obsolete* (Leise et al., 2004). In the present study, the involvement of NO and the associated cGMP signaling pathway during larval settlement of *B. amphitrite* were investigated.

The results of the present study suggest an inhibitory role of NO in larval settlement of *B. amphitrite*, which is supported by the following lines of evidence: (1) elevation of the endogenous NO level by the application of an NO donor, SNP, inhibited larval settlement in a dose-dependent manner; (2) removal of the intracellular NO using an NO scavenger, carboxy-PTIO, significantly accelerated larval settlement; (3) suppression of endogenous NO biosynthesis by pharmacological treatment with two NOS inhibitors, AGH and SMIS, promoted larval settlement; and (4) expression of NOS drastically decreased at the transcriptional level during larval development and reached its lowest value at cyprid stage, a stage at which the larva is competent to attach and metamorphose. In summary, our results indicate the inhibitive nature of NO as an endogenous regulator of larval settlement in *B. amphitrite*. In addition, based on the promoting effects of the NO scavenger and NOS inhibitors on larval settlement, together with the reduction of NOS expression at the cyprid stage, we suggest that the reduction of the endogenous NO level is important for the initiation of larval attachment and the subsequent metamorphosis of *B. amphitrite*. These results are consistent with previous reports, which indicated that the induction of larval metamorphosis could be counteracted by non-enzymatic and enzymatic enhancement of NO formation in several marine invertebrates, including the gastropods *I. obsolete* (Leise et al., 2001) and *Phestilla sibogae*

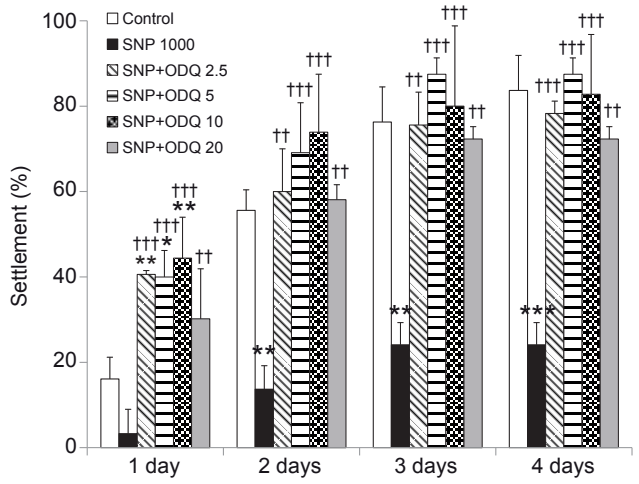

Fig. 9. GC inhibitor (ODQ) attenuates settlement inhibition effects of nitric oxide donor (SNP) in *B. amphitrite*. Cyprids were co-incubated with a dilution series of ODQ (2.5, 5, 10 and 20 μmol l^{-1}) and 1000 μmol l^{-1} SNP. Data presented are percentages of larvae that both attached and metamorphosed 1, 2, 3 and 4 days post-treatment. Each bar indicates mean and s.d. Data were analyzed by simultaneous multiple comparisons of different means using one-way ANOVA, followed by Tukey's HSD test. †Significant differences between treatments (including SNP treatment alone and adult extract + SNP co-incubation). *Significant differences between treatments and control. A *P*-value of less than 0.05 was considered statistically significant. *P<0.05, ††,**P<0.01, †††,***P<0.001. N=3 replicate treatments.

(Bishop et al., 2008), and the sea urchin *Lytechinus pictus* (Bishop and Brandhorst, 2001). Furthermore, our observations are also supported by some previous studies indicating that the reduction of NOS activity accelerated larval metamorphosis in the ascidian *Ciona intestinalis* (Comes et al., 2007) and the gastropod *Crepidula fornicate* (Pechenik et al., 2007). In contrast to previous findings regarding the role of PKC during *B. amphitrite* settlement (Yamamoto et al., 1995), we did not observe metamorphosis of any cyprid without attachment for all of NO level manipulations, suggesting NO signaling may control the overall settlement process in this species.

Another important finding of the current study is that the regulatory role of NO during *B. amphitrite* larval settlement is mainly mediated *via* GC and cGMP. This was demonstrated by the following results. (1) Application of the GC inhibitor ODQ, which presumably suppresses biosynthesis of cGMP through inhibition of GC activity, accelerated larval attachment and metamorphosis. (2) Combined application of ODQ and the NO donor SNP greatly attenuated the settlement-inhibitive effect as a result of the increased NO level. (3) The expression level of sGC concomitantly decreased with the reduction of NOS expression when larvae were approaching attachment and metamorphosis. As sGC is the most prominent downstream effector of NO signaling, the selective sGC inhibitor ODQ has been widely used as a tool to study the interaction among NO, GC and cGMP (Brunner et al., 1995). Recently, using ODQ, different researchers have suggested the involvement of cGMP in larval metamorphosis of several marine invertebrates (e.g. Comes et al., 2007; Pechenik et al., 2007). However, direct experimental evidence showing how the interaction of NO and cGMP regulates larval settlement has not been demonstrated clearly. In this study, the rescue effect of ODQ in the presence of SNP has provided clear evidence demonstrating that the regulatory effect of NO during larval settlement is through the downstream effector cGMP. Interestingly, the inhibitory effects of SNP on larval exploration behavior are attenuated by the addition of ODQ, further supporting the proposal that cGMP acts on the downstream signaling cascade of NO in the larval settlement response of *B. amphitrite*. Additional supportive evidence is from the marine snail, *P. sibogae*, which showed that ODQ can attenuate the metamorphosis inhibitive effect of L-arginine (Bishop et al., 2008), the precursor of NO and a substrate of NOS, which presumably promotes endogenous NO biosynthesis.

Using the marine gastropod *I. obsolete* as a model species, Leise and colleagues found that an important neurotransmitter serotonin (5-HT) can induce metamorphosis (Couper and Leise, 1996), while exogenous NO can significantly reduce rates of 5-HT-induced metamorphosis (Leise et al., 2001; Leise et al., 2006). The authors suspected that the metamorphic induction effects of 5-HT may result from its inhibition of endogenous NOS activity and the subsequently decreased production of NO. Their hypothesis was later proved by real-time PCR results showing that the induction of larval metamorphosis by 5-HT leads to the reduction of neuronal *NOS* gene expression in this species (Hens et al., 2006). Similarly, in the barnacle *B. amphitrite*, Yamamoto and colleagues reported the inductive effect of serotonin in larval settlement (Yamamoto et al., 1996). Their later study indicated that 5-HT agonists promoted larval attachment and metamorphosis, while most of the 5-HT antagonists tested inhibited both processes. Based on their results, they concluded that serotonergic neurotransmission regulates the overall settlement process (Yamamoto et al., 1999). In the present study, treatment with NO donor also inhibited both larval attachment and metamorphosis, while both removal of endogenous NO and inhibition of NO production induced larval settlement of *B. amphitrite*. Our results are therefore in agreement with previous studies and indicate a possible interaction between 5-HT and NO in regulating larval settlement. The interaction between 5-HT and NO has also been demonstrated to control a variety of important physiological functions in different biological systems (Marcoli et al., 1997; Shimpo et al., 1997; Miller et al., 1997; Krönström et al., 2007). Importantly, it is suggested that the regulation of NO by 5-HT is at least partly *via* PKC signaling, which has also been shown to be involved in the larval settlement of *B. amphitrite*.

SIPC is a cuticle glycoprotein involved in gregarious settlement behavior of *B. amphitrite* (Dreanno et al., 2006a; Dreanno et al., 2006b). On basis of its localization at cuticles, the authors suggested that besides functioning as a contact pheromone, SIPC may also act as a waterborne cue, possibly released through cuticle regeneration and bacterial degradation (Dreanno et al., 2006c). More recently, through analysis of the *N*-glycan moiety of SIPC, Pagett and colleagues suggested that a mannose-type sugar chain may contribute to the settlement of cyprids (Pagett et al., 2012). These results are in agreement with the findings of a previous study, which suggested that a lentil lectin-binding sugar chain is involved in regulating this process (Matsumura et al., 1998a). However, we still know little about how this exogenous cue is transmitted or translated into endogenous signaling. In the present study, we have demonstrated, for the first time, that the settlement-inducing effect of *B. amphitrite* adult crude extracts (containing SIPC) can be attenuated by exogenous NO. This result suggests that NO may be a signal mediator located further downstream in the SIPC-initiated signaling cascade during larval settlement.

In conclusion, we have provided novel evidence showing that NO is a crucial endogenous regulator of larval settlement in *B. amphitrite*. Our results revealed the inhibitory nature of NO in

regulating larval settlement of this species. Importantly, we found that the regulatory function of NO during larval settlement of *B. amphitrite* is mediated *via* the downstream GC and cGMP. Overall, our findings provide a basis for more in-depth signal transduction studies of the larval settlement of *B. amphitrite*. Further studies on the functional link between exogenous cues (e.g. SIPC, etc.) and the endogenous signal transduction pathway during larval settlement will enable a more thorough understanding of the mechanism controlling this complex biological process. More detailed study of these interconnected signaling networks may aid in the development of antifouling technologies.

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Fig. S1. (**A**) Most similar match of isotig GBQDZ6L01A97M3_3. (**B**) Most similar match of isotig GBQDZ6L01AIVFU_6.

Table S1. Matching information of NOS and sGC

Isotig no.	EMBL DB: ID	Source	Query/subject match length	Score	Identity and positives	E-value
GBQDZ6L01A97M3_3	EM_INV: AY444337	*Acheta domesticus* nitric oxide synthase mRNA, partial cds.	327/631	867	74.0	6.1E–31
GBQDZ6L01AIVFU_6	EM_INV: AB204559	*Apis mellifera* AmsGC β3 mRNA for soluble guanylyl cyclase β3, complete cds.	225/3153	301	70.0	1.8E–4

Both transcripts were matched against the EMBL database.