**RESEARCH LETTER**

**FNR-mediated regulation of bioluminescence and anaerobic respiration in the light-organ symbiont *Vibrio fischeri***

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**Abstract**

*Vibrio fischeri* induces both anaerobic respiration and bioluminescence during symbiotic infection. In many bacteria, the oxygen-sensitive regulator FNR activates anaerobic respiration, and a preliminary study using the light-generating lux genes from *V. fischeri* MJ1 cloned in *Escherichia coli* suggested that FNR stimulates bioluminescence. To test for FNR-mediated regulation of bioluminescence and anaerobic respiration in *V. fischeri*, we generated *fnr* mutants of *V. fischeri* strains MJ1 and ES114. In both strains, FNR was required for normal fumarate- and nitrate-dependent respiration. However, contrary to the report in transgenic *E. coli*, FNR mediated the repression of lux. ArcA represses bioluminescence, and *ParcA-lacZ* reporters showed reduced expression in *fnr* mutants, suggesting a possible indirect effect of FNR on bioluminescence via arcA. Finally, the *fnr* mutant of ES114 was not impaired in colonization of its host squid, *Euprymna scolopes*. This study extends the characterization of FNR to the Vibrionaceae and underscores the importance of studying lux regulation in its native background.

**Introduction**

*Vibrio fischeri* is a model for investigations of bioluminescence and mutualistic symbioses, two fields connected by the importance of oxygen. O2 is a substrate for the luminescence-producing enzyme luciferase, and luciferase may benefit *V. fischeri* by generating a more reduced environment in or near cells (Visick et al., 2000; Timmins et al., 2001). Reduction of O2 could be especially advantageous for this facultative anaerobe when it is colonizing animal tissue and may minimize the host’s ability to generate reactive oxygen species (Visick et al., 2000). Luminescence emanating from bacteria colonizing the symbiotic light organ of the host indicates that O2 is present; however, evidence suggests that luciferase is O2 limited in this environment (Boettcher et al., 1996) despite its high affinity (*Km* ~ 35 nM) for O2 (Bourgois et al., 2001). Moreover, anaerobic respiration is apparently induced in symbiotic *V. fischeri* (Proctor & Gunsalus, 2000), consistent with the idea that [O2] is low in the light organ.

One regulator that might control anaerobic respiration and luminescence in response to [O2] is FNR (so named for its role in fumarate and nitrate reduction). FNR regulates genes during the switch between aerobic and anaerobic growth in *Escherichia coli* and other bacteria, and it often activates genes responsible for anaerobic respiration (Browning et al., 2002; Reents et al., 2006; Fink et al., 2007). Although FNR is expressed during both aerobic and anaerobic growth, it is only functional under microaerobic or anaerobic conditions due to its dependence on an oxygen-labile 4Fe–4S center (Khoroshilova et al., 1995, 1997; Lazazzera et al., 1996; Kiley & Beinert, 1998). Under anaerobic conditions, [4Fe–4S]-FNR forms a functional dimer that binds DNA at a 5′-TTGAT(N4)ATCAA-3′ FNR-box sequence (Eiglemeier et al., 1989), and it activates or represses transcription depending on the location of binding relative to the promoter (Wing et al., 1995; Meng et al., 1997; Marshall et al., 2001).

FNR was reported to activate bioluminescence in transgenic *E. coli* carrying the *V. fischeri* MJ1 luxR-luxICDABEG region, which encodes the autoinducer-dependent lux activator LuxR, the autoinducer synthase LuxI, and the Lux proteins that produce bioluminescence (Muller-Breikreutz & Winkler, 1993). Although FNR-mediated regulation of luminescence is cited frequently (Meighen, 1994; Spiro, 1994; Sitnikov et al., 1995; Ulitzur & Dunlap, 1995; Stevens & Greenberg, 1999), these data were only presented in...
whether FNR contributes to symbiotic competence. We have examined fnr in two V. fischeri strains: ES114 and MJ1. ES114’s genome is sequenced, and its symbiosis with the squid Euprymna scolopes can be reconstituted in the laboratory (Ruby et al., 2005; Stabb, 2006); however, like most isolates from these animals, ES114 is not visibly luminescent in culture (Boettcher & Ruby, 1990). In contrast, MJ1 has bright luminescence typical of isolates from the pinecone fish Monocentris japonica, but this symbiosis is not yet experimentally tractable. The genes required for luminescence and autoinduction are similar in the two strains, with the luxICDABEG operon adjacent to and divergently transcribed from luxR (Gray & Greenberg, 1992). However, there are differences in the luxR-luxI intergenic region, and notably there is a putative FNR box in MJ1 that is absent in ES114. Our goals were to examine V. fischeri to assess FNR’s regulation of luminescence and anaerobic respiration, and to determine whether FNR contributes to symbiotic competence.

Materials and methods

Bacteria and media

The bacterial strains used in this study are described in Table 1. Escherichia coli was grown in Luria–Bertani (Miller, 1992) or in M9 (Sambrook et al., 1989) supplemented with 1 mg mL⁻¹ casamino acids, 40 mM glycerol, and 40 mM of either sodium nitrate or sodium fumarate. Vibrio fischeri was grown in Luria broth plus salt (LBS) (Stabb et al., 2001), sea water tryptone (SWT) (Boettcher & Ruby, 1990), wherein seawater was replaced with Instant Ocean (Aquarium Systems, Mentor, OH), sea water tryptone at high osmolarity (SWTO) (Bose et al., 2007), or in a defined salts medium (Adin et al., 2009) with 40 mM glycerol as a carbon source, 1 mg mL⁻¹ casamino acids, and 50 mM of sodium nitrate or sodium fumarate. Agar (15 mg mL⁻¹) was added to solidify media for plating. Anaerobic growth on plates was assessed using the GasPak EZ Anaerobic Container System from Becton, Dickinson and Company (Sparks, MD). Antibiotics were added as described previously for selection (Stabb & Ruby, 2002), and N-3-oxo-hexanoyl homoserine lactone (3-oxo-C6-HSL) autoinducer was added to the media at 140 nM.

Genetic manipulations

Cloning was performed using standard procedures, with plasmids transformed in E. coli strain DH5α or DH5α×2pir, as described previously (Bose et al., 2008). Cloned PCR products were sequenced to ensure that unintended alterations were not incorporated. Sequencing was conducted at the University of Michigan DNA Sequencing Core Facility or at the University of Georgia Molecular Genetics Instrumentation Facility. Plasmids were mobilized into V. fischeri from E. coli by triparental mating using strain CC118pir with pEVS104 as a helper (Stabb & Ruby, 2002), and mutations were placed on the chromosome by allelic exchange. Parent strains and plasmids used for allelic exchange are listed in Table 1.

Key plasmids and oligonucleotides are described in Table 1, and an overview of allele construction follows. To mutate fnr, an ~3.3 kb region of the V. fischeri genome centered on fnr was PCR amplified with primers EVS97 and EVS98 using ES114 or MJ1 genomic DNA as a template, and the fragments were ultimately subcloned into pEVS136 and pJLB69, respectively (Table 1). We generated Δfnr::tmpR alleles by replacing the ClaI to AvrII fragment of fnr with the trimethoprim-resistance gene (tmpR) from pJLB1 (Dunn et al., 2005) on a BstBI to AvrII fragment, resulting in tmpR replacing an internal 255-bp fragment beginning in the middle of fnr, with tmpR in the same orientation as fnr. The ES114-derived Δfnr::tmpR allele was placed in pJLB5 and pJLB70, and the MJ1-derived Δfnr::tmpR allele was used in pCDW5. For complementation of E. coli with ES114 fnr, we ligated the fnr-containing BsrBI–PstI fragment from pEVS136 into Smal- and PstI-digested pDMA5, generating pJLB6. To place lacZ under control of the arcA promoter, we PCR amplified an ~3.1-kb fragment containing an engineered lacZ (Tomich et al., 1988) using pVSV3 (Dunn et al., 2006) as a template and primers JBLACZ1 and JBLACZ2 (Table 1). We cloned this product into Smal-digested pAJ4 and pJLB55 (Bose et al., 2007), which carry regions flanking arcA from ES114 and MJ1, respectively, with the sequence between the start and the stop codons of arcA replaced by a 6-bp Smal recognition site. The ParcA-lacZ alleles contain the arcA start codon, followed by a 5′-CCC-3′ proline codon, and then the lacZ reporter (Tomich et al., 1988) from its second codon onward. These ES114- and MJ1-derived alleles were subcloned into pAS31 and pJLB139, respectively.

Growth and luminescence

Overnight cultures in LBS were diluted 1:1000 into SWT and incubated at 24 °C with shaking (200 r.p.m.). Aerobic cultures contained 50 mL of SWT in 250-mL flasks. For anaerobic cultures, aerobically grown overnight cultures were diluted 1:10 in LBS before inoculation of 0.2 mL into 20 mL SWT in 165-mL sealed bottles with a headspace containing 5% CO₂, 10% H₂, and 85% N₂. Samples (500 μL each) were removed periodically and culture optical density (OD₅₉₅ₙ₅) was determined using a BioPhotometer (Brinkman Instruments, Westbury, NY) or a SmartSpec 3000 (BioRad Laboratories, Hercules, CA). After measuring OD₅₉₅ₙ₅ cuvettes were covered with parafilm and shaken vigorously for ~10 s to aerate the sample, followed by...
determination of luminescence using a GLOMAX 20/20 luminometer (Promega, Madison, WI).

**Quantitative reverse transcriptase (RT)-PCR**

Triplicate aerobic cultures of ES114 and JB1 were grown in LBS to an OD

removed, added to microcentrifuge tubes containing 1/5 volume 5% (v/v) phenol, pH 4.3, with 95% (v/v) ethanol, and placed on ice for 30 min. Samples were centrifuged and the pellets were stored at $-80^\circ$C overnight. Pellets were thawed, and RNA was isolated using Absolutely RNA Mini-preps (Stratagene, La Jolla, CA). RNA was treated using the Turbo DNA-free kit (Applied Biosystems, Foster City, CA),
and RNA quantity and purity were assessed using a Biotek Synergy 2 plate reader with Take3 Multi-Volume Plate and software (Winooski, VT). RNA was then stored at −80 °C. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA), and reactions were cleaned using a DNA Clean & Concentrator-5 kit (Zymo Research, Orange, CA). cDNA was quantified using the Synergy 2 plate reader. Real-time PCR was performed using the MyIQ Single-Color Real-Time PCR Detection System (BioRad Laboratories), and reactions were set up using the BioRad IQ SYBR Green Supermix. Primers AS1310RTF2 and AS1310RTR2 were used to determine the level of VF1310 cDNA. ES114 genomic DNA was used to generate a standard curve. Real-time PCR data were analyzed using BioRad IQ™5 software.

**lacZ reporter expression**

To determine P_{arcA-lacZ} reporter expression, strains were grown overnight in LBS and diluted 1:1000 in 20 mL SWT in 250-mL baffled flasks and grown at 24 °C with shaking to an OD of ~0.1. Four hundred microliters were inoculated into 20 mL SWT in anaerobic bottles. These were grown overnight in LBS and diluted 1:100 in 20 mL SWTO in 250-mL baffled flasks and incubated at 24 °C with shaking until peak luminescence was reached. Strains were also grown aerobically in 20-mL tubes at 28 °C with shaking to an OD of 0.3–1.0, and this was checked by plating the inocula on LBS. To study infection kinetics, the squid were placed in 5 mL of inoculum for up to 14 h before being rinsed in Instant Ocean. To study infection kinetics, the squid were placed in 5 mL of inoculum in scintillation vials, and the onset of luminescence was monitored using an LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). For mixed-strain competitions, hatchlings were exposed to an inoculum containing an ~1:1 ratio of wild type and mutant. At 48-h postinoculation, individual squid were homogenized and dilution plated on LBS. The resulting colonies were patched onto LBS with added trimethoprim to determine the ratio of strains in each animal. Inocula were similarly plated and patched to determine the starting ratio. The relative competitiveness index (RCI) was determined by dividing the mutant to wild-type ratio in each animal by the ratio of these strains in the inoculum. The mean RCI was calculated from log-transformed data.

**Results**

**Identification of V. fischeri fnr**

BLAST searches (Altschul et al., 1990) of the V. fischeri ES114 genome revealed the similarity of ORFs VF1308 and VF1309 to the N and C termini of E. coli FNR, respectively (Fig. 1a). We suspected that a sequencing error had led to the misannotation of fur as two genes, and we therefore cloned and sequenced the region spanning VF1308 and VF1309. We found five errors in the genome database, leading to an erroneously predicted truncation of VF1308, which we corrected in GenBank (Mandel et al., 2008). In the revised sequence, VF1308 encodes a protein that is the same length as, and shares 84% identity with, E. coli FNR. This ES114 FNR is identical to the previously deposited V. fischeri M1 FNR (accession no. CAE47558). Importantly, the residues necessary for interactions with RNA polymerase (Williams et al., 1997; Lonetto et al., 1998; Blake et al., 2002; Lamberg et al., 2002), 4Fe–4S center assembly (Spiro & Guest, 1988; Kiley & Beinert, 1998), and DNA recognition (Spiro et al., 1990) in E. coli are conserved in V. fischeri FNR. Using TransTermHP (Kingsford et al., 2007), we also found a likely Rho-independent transcriptional terminator downstream of fur (Fig. 1a and b). Given the 142-bp spacing and strong putative terminator between fur and VF1310 (Fig. 1b), it seems likely that these are expressed on separate transcripts. Using quantitative RT-PCR, we found that the fur::tmpR allele in mutants described below did not affect the transcript levels for VF1310.

We next generated mutants disrupted in the putative fur in V. fischeri ES114 and MJ1. We did not observe any attenuation of these strains under aerobic growth conditions, consistent with the role of FNR in other bacteria. *Escherichia coli* fur mutants do not grow anaerobically with nitrate or fumarate as an electron acceptor (Lambden & Guest, 1976), and we found that *V. fischeri* fur mutants were similarly attenuated. Specifically, when grown with minimal medium under anaerobic conditions, ES114 and MJ1 displayed nitrate- or fumarate-dependent growth on a non-fermentable carbon source (glycerol) that was lacking in the fur mutants (e.g. Fig. 1c). Restoring fur by replacing the fur::tmpR allele with the wild-type allele by a crossover exchange back into these mutants recovered the ability to respire anaerobically. We restored the wild-type fur allele on the chromosome in this way (replacing fur::tmpR) rather than providing it in trans due to concerns that fur provided in multicopy can show uncharacteristic effects such as gene repression of arcA.
activation under aerobic conditions (Reyes-Ramirez & Sawers, 2006) and a narrowing of the difference between better and poorer FNR activation sites (Scott et al., 2003). However, because our V. fischeri-derived allele-replacement constructs were not appropriate (homologous) for exchange into E. coli, we provided the putative fnr of V. fischeri ES114 to E. coli in trans on plasmid pJLB6, which restored anaerobic respiration of E. coli fnr mutant PC2 on nitrate (Fig. 1d). Taken together, our results indicate that the putative V. fischeri FNR is similar in both sequence and function to E. coli FNR.

Repression of luminescence by FNR

We tested whether FNR regulates lux expression by monitoring the luminescence of strains grown aerobically or anaerobically (Fig. 2a and b). The luminescence of the fnr mutants was similar to that of their parent strains under aerobic conditions (Fig. 2a). FNR is inactivated by oxygen, and we therefore also assessed lux expression anaerobically. Luciferase uses oxygen as a substrate, and so anaerobic cultures do not luminesce; however, as with all luminescence measurements, samples removed from anaerobic bottles were shaken for ~10 s to saturate luciferase with oxygen before measuring luminescence. When grown anaerobically, luminescence was higher in fnr mutant EVS601 than in MJ1 (Fig. 2b). The magnitude of this difference varied between 1.5- and 20-fold, and averaged eightfold, in five experiments. The luminescence of ES114 and fnr mutant JB1 was below the background, appearing the same as a dark ΔluxCDABEG strain (data not shown), which raised the possibility that FNR regulates lux in ES114, but that the overall luminescence is below detection. To test this possibility, we added the luminescence-stimulating autoinducer 3-oxo-C6-HSL to anaerobic cultures of ES114 and its fnr mutant JB1. 3-oxo-C6-HSL stimulated the luminescence of ES114 and fnr mutant JB1, and under these conditions, JB1 was brighter than ES114 (Fig. 2c).

We considered the possibility that increased luminescence in V. fischeri fnr mutants could result from increased availability of luciferase’s substrates due to the physiological effects of this global regulator. To test this possibility, we disrupted fnr in a background where the luxCDABEG genes are under the control of LacIq and a non-native promoter. In this background, FNR had no significant effect (P > 0.05) on luminescence (Fig. 2c). Thus, the repressive effect of FNR on luminescence is dependent on the native lux promoter.

The luxCDABEG operon can be subject to positive feedback regulation, because the autoinducer synthase LuxI generates 3-oxo-C6-HSL, which, in combination with LuxR, stimulates luxCDABEG transcription. Given the amount of 3-oxo-C6-HSL added exogenously to the cultures (Fig. 2c), we predicted that endogenously produced autoinducer
would have no further stimulatory effect, and therefore the effect of FNR on luminescence in this experiment would not have a significant LuxI-mediated positive-feedback component. We examined luxI point mutant VCW2G7 and found that, as predicted, it achieved the same luminescence as the wild type under anaerobic conditions with added 3-oxo-C6-HSL (data not shown).

**Analysis of FNR boxes**

It was suggested that a putative FNR box upstream of luxR might underpin the FNR-mediated regulation of luminescence in MJ1 (Muller-Breikreutz & Winkler, 1993); however, attempts to define a footprint using FNR*, an E. coli FNR derivative that is active aerobically (Kiley & Reznikoff, 1991), failed to show binding to this site (A.M. Stevens, pers. commun.). To further explore how FNR might affect luminescence, we conducted a 'Virtual Footprint' analysis with the PRODORIC database (Munch et al., 2005), searching the V. fischeri genome for FNR boxes using a weighted consensus matrix based on data from E. coli. As expected, high Position Weight Matrix (PWM) scores (≥7.0) were skewed toward intergenic regions. Such putative FNR boxes numbered in the hundreds, consistent with FNR's global role in E. coli, and these included intergenic regions upstream of genes involved in anaerobic metabolism (e.g. upstream of nitrate and nitrite reductase genes). However, the best FNR box matches in the lux intergenic region of MJ1 and ES114 returned scores of 6.73 and only 5.88, respectively. To put this in perspective, > 25 000 sites with no skew toward intergenic regions returned scores ≥5.9. Although we cannot rule out the possibility that FNR directly binds to the lux intergenic region, we believe this model is unlikely, especially in strain ES114.

**FNR-mediated repression of arcA**

Virtual Footprinting did suggest a possible indirect effect of FNR on luminescence. The highest PWM score returned in this analysis (7.67) was found in six intergenic regions, one of which was upstream of arcA. In E. coli, FNR activates arcA (Compan & Touati, 1994), and in ES114, ArcA strongly represses the lux operon (Bose et al., 2007). If FNR activates arcA in V. fischeri, this might explain FNR's repressive effect.

**Fig. 2.** Luminescence per OD_{595 nm} of fnr mutants. (a, b) Specific luminescence is shown at different culture densities for *Vibrio fischeri* ES114 (solid diamonds), ES114 fnr mutant JB1 (empty diamonds), MJ1 (solid squares), MJ1 fnr mutant EV5601 (empty squares), and dark ΔluxCDABEG mutant EV5102 (solid triangles) grown in batch cultures that were (a) aerobic (50 mL medium in 250-mL flask) or (b) anaerobic (20 mL medium in 165-mL bottles with anaerobic headspace) at 24°C with shaking (200 r.p.m.). ES114, JB1, and EV5102 were excluded from (b), because luminescence was not detected above the background for these strains under these conditions. Bars in (b) indicate the SD from (a), because luminescence was not detected above the background for these strains under these conditions. Bars in (a) indicate the SD from (b), because luminescence was not detected above the background for these strains under these conditions. 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Discussion

biotic competence (Adin et al., 2009). The onset of symbiotic luminescence (Fig. 4a), colonization levels (Fig. 4b), and colonization competitiveness (Fig. 4c) were similar for ES114 and fnr mutant JB1 during the first 2 days of infection. The fnr mutant was also equally competitive up to 90 h after inoculation (data not shown). Furthermore, the fnr mutation did not appear to affect the symbiosis in a ΔarcA mutant background (data not shown). We conclude that FNR is not necessary for colonization during the first days of a symbiotic infection.

FNR is not necessary for host colonization

We tested whether FNR was important for symbiotic colonization by ES114 using established measures of symbiotic competence (Adin et al., 2009). The onset of symbiotic luminescence (Fig. 4a), colonization levels (Fig. 4b), and colonization competitiveness (Fig. 4c) were similar for ES114 and fnr mutant JB1 during the first 2 days of infection. The fnr mutant was also equally competitive up to 90 h after inoculation (data not shown). Furthermore, the fnr mutation did not appear to affect the symbiosis in a ΔarcA mutant background (data not shown). We conclude that FNR is not necessary for colonization during the first days of a symbiotic infection.

Discussion

In this study, we investigated the oxygen-sensitive regulator FNR in V. fischeri. Vibrio fischeri fnr complemented an E. coli fnr mutant, and like fnr in E. coli, it is required for fumarate- and nitrate-dependent anaerobic respiration. Moreover, our data and another recent bioinformatic analysis (Ravcheev et al., 2007) suggest that the FNR-box recognition site is conserved in V. fischeri. For example, we observed fnr-mediated regulation of reporters for arcA (Fig. 3), dmsA (Dunn & Stabb, 2008), torE (Dunn & Stabb, 2008), and yfID (data not show), which have predicted FNR boxes upstream. Taken together, FNR’s function in V. fischeri appears to be similar to that in its fellow gammaproteobacterium E. coli. As the first experimental examination of FNR in the Vibrionaceae, this study should underpin future efforts to understand FNR-mediated regulation in this important bacterial family.

We initiated this study largely because FNR is cited as an activator of luminescence in V. fischeri (e.g. see Meighen, 1994; Spiro, 1994; Sitnikov et al., 1995; Ulitzur & Dunlap, 1995; Stevens & Greenberg, 1999). However, that paradigm was based on a preliminary study that used the MJ1 lux genes cloned in E. coli (Muller-Breikreutz & Winkler, 1993). Our results appear to contradict that report, showing instead that FNR mediates repression of the luminescence-generating lux system in V. fischeri under anaerobic conditions (Fig. 2). It is perhaps not surprising that lux regulation should be different in transgenic E. coli than in V. fischeri. For example, LitR, which activates luxR transcription, is absent in E. coli (Fidopiastis et al., 2002). It is also possible that FNR does activate luminescence in V. fischeri under conditions different from those tested here, and that the discrepancy between our study and previous work simply reflects methodological differences.

Repression of the lux genes anaerobically may minimize the production of luciferase when its O2 substrate is unavailable. This is consistent with the finding that luminescence is repressed by the ArcAB two-component regulatory system, which is more active under relatively reduced conditions (Bose et al., 2007). The observation that arcA::lacZ reporters showed a lower expression in the absence of fnr (Fig. 3) suggests that the effect of FNR on bioluminescence may at least in part be indirect and mediated by FNR’s stimulation of arcA. Consistent with this idea, fnr did not exert much influence on luminescence in arcA mutant backgrounds, although arcA fnr double mutants were noticeably attenuated in anaerobic growth (data not shown). We speculate that FNR may amplify the
The repressive effect of ArcA on luminescence under reduced conditions. Although we cannot rule out the possibility that FNR exerts a direct effect by binding the lux region, as described above, we believe this model is unlikely. In either case, FNR apparently contributes to regulation that effectively turns off expression of the lux genes under ES114 under anaerobic conditions, which is easily rationalized, given that luciferase requires O2 to generate light.

Given the suggestion that anaerobic respiration is important for symbiotic V. fischeri (Proctor & Gunsalus, 2000), and the fact that FNR can contribute to virulence factor production and/or colonization by pathogens (Baltes et al., 2005; Bartolini et al., 2006; Fink et al., 2007; Zigha et al., 2007), we hypothesized that fnr would play a role in the symbiotic light organ. However, the fnr mutant had no discernable attenuation in colonizing E. scolopes during the first 90 h of infection. Vibrio fischeri, like other members of the Vibrionaceae family, is a cosmopolitan member of marine communities that is found in fish gut tracts and sediments where [O2] is low. Future studies may show the ecological relevance of FNR for V. fischeri in such environments outside E. scolopes.

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Authors' contribution

J.L.B. and A.N.S. contributed equally to this work.

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