Introduction

Many bacteria regulate gene expression by producing and sensing pheromones. Because these signals can accumulate as culture density increases, pheromone-mediated responses often depend on high cell densities, giving rise to the term “quorum sensing” [1]. However, in many systems pheromone signaling is not simply a function of cell density. Instead, both synthesis of pheromones and responsiveness to them are often context dependent. Environmentally responsive regulators control expression of many pheromone synthases and/or their cognate receptors, rendering such signaling dependent on other parameters in addition to cell density [2–27]. Moreover, pheromone signals often stimulate an increased rate of their own synthesis [28–39]. This positive feedback can mean that even at the same cell density, the concentration and synthesis of a pheromone are partly a function of whether the system has recently been in a stimulated state.

Combining context-dependent regulatory control over pheromone synthesis with pheromone-mediated positive feedback has profound functional implications. Positive feedback can amplify the effects of other regulatory inputs that modulate pheromone synthesis, and the relative strengths of a regulatory input and positive feedback will affect communication, particularly in a population that spans a heterogeneous environment. If positive feedback is strong, a subpopulation of bacteria experiencing an environment that favors pheromone production might elicit a population-wide response, even in cells that would otherwise remain uninduced given their distinct environmental context. If positive feedback is weak, local environmental context becomes a more defining determinant of whether a pheromone system is induced. Understanding how bacteria integrate pheromone sensing, environmentally responsive regulation, and positive feedback to coordinate group responses requires model systems with pheromone-mediated behaviors that are easily observable and induced in natural environments.

Vibrio fischeri is an attractive model for studying pheromone-mediated gene regulation and host-microbe symbiosis [40]. Bioluminescence in V. fischeri is regulated in part by the LuxR-LuxI pheromone system [28], and it is induced upon infecting the
squid *Euprymna scolopes* in a natural and experimentally tractable symbiosis [40–42]. The *luxICDABEG* operon (Fig. 1) underlies bioluminescence and encodes the LuxI pheromone synthase, which produces N(3-oxohexanoyl)-L-homoserine lactone (3OC6) [43]. When 3OC6 accumulates to a threshold concentration, it combines with LuxR to activate transcription of *luxICDABEG* [44–46]. Because the LuxI product 3OC6 induces *luxI* transcription, this “autoinducer” pheromone initiates a positive-feedback loop resulting in both increased 3OC6 production and bioluminescence.

The *V. fischeri lux* system also is regulated tightly in response to the environment, and such control is especially pronounced in isolates from *E. scolopes* such as strain ES114. ES114 is dim and produces little 3OC6 in culture, but in the host light organ it is ~1000-fold more luminescent and produces more 3OC6 than in culture, even at similar high cell densities [47–49]. Several regulators modulate Lux expression [27,38,50,51], perhaps none more impressively than the redox-responsive ArcA/ArcB two-component regulatory system [52]. ArcA is a direct repressor of *lux* and a *V. fischeri* arcA mutant is 100- to 1000-fold brighter than ES114 in culture, achieving nearly symbiotic luminescence levels [52].

In this study, we used *V. fischeri* and ArcA-mediated control of *lux* to examine the interplay between direct regulation by an environmentally-responsive regulator and the positive feedback inherent in pheromone production. We also explored the potential for intercellular signaling by distinct symbiotic *V. fischeri* populations. Our data illustrate important potential roles for 3OC6-mediated communication beyond sensing a quorum.

**Materials and Methods**

**Media and Growth Conditions**

*V. fischeri* was grown at 28°C or 24°C in one of three rich media depending on the application, as indicated below. The media used were LBS medium [53], ASWT medium [54], or SWTO medium [52]. *E. coli* strains were grown in either LB medium [55] or Brain Heart Infusion (Difco) at 37°C. Antibiotic selection for *V. fischeri* and *E. coli* strains was performed as described previously [56].

**Plasmid and Strain Construction**

Bacterial strains, plasmids, and oligonucleotides used in this study are presented in Table 1. Plasmids were maintained in *E. coli* strain DH5α [57] except for plasmids that contained only the R6Kγ origin of replication (oriV<sub>R6K</sub>), which were maintained in strain DH5αpir [56], or in strain CC118pir [58] in the case of plasmid pEVS104. Plasmids that were stably maintained in *V. fischeri* were derived from shuttle vectors that contain both oriV<sub>R6K</sub> and the replication origin from *V. fischeri* plasmid pES213 (ori<sub>Escherichia coli</sub>) [56,59]. These shuttle vectors were maintained in *E. coli* DH5αpir prior to introducing them into *V. fischeri*. Plasmids were mobilized from *E. coli* into *V. fischeri* by triparental mating using CC118pir pEVS104 as a conjugative helper and exploiting the RP4 origin of transfer (ori<sub>Rp4</sub>) as previously described [60].

To generate mutations in *V. fischeri*, mutant alleles were mobilized on unstable plasmids into recipients, and allelic exchange was screened using appropriate antibiotic resistance markers and PCR. To construct a Δ*lux* mutant, sequence upstream of *luxI* was PCR amplified using primers ASLUX1 and ASLUX2 and cloned into pEVS122 at the Smal site resulting in plasmid pAS2. pAS2 was fused at the NheI site to NheI-digested pEVS114<sub>8</sub> which contains sequence downstream of *luxI*, resulting in the Δ*luxI* deletion construct pAS3. The Δ*luxI* allele on pAS3 was exchanged into ES114 to generate strain ANS3. To make the Δ*arcA lux* double mutant strain ANS7, plasmid pAHi containing the Δ*arcA* allele was fused to pBluescript at their respective SpeI sites, resulting in plasmid pAS6, and the Δ*arcA* allele on pAS6 was exchanged into VCW2G7 (*luxI* point mutant). To construct the Δ*arcA::erm* Δ*luxICDABEG* strain JB33, the Δ*luxICDABEG* allele on plasmid pEVS153 was exchanged into the Δ*arcA::erm* strain AM1. To construct the *V. fischeri* ΔluxICDABEG mutant, pAS2 was fused at the NheI site to NheI-digested pEVS149<sub>8</sub>, which contains sequence downstream of *luxG*, resulting in the Δ*luxICDABEG* deletion allele on plasmid pAS4. This Δ*luxICDABEG* allele was exchanged into ES114, generating strain ANS5. To make the Δ*arcA::erm* Δ*luxICDABEG* strain ANS6, the Δ*arcA::erm* allele on plasmid pJB169 was exchanged into strain ANS5. To construct plasmid pAS104 with an inducible *arcI*, the *arcI* gene including 24 bp upstream of the ATG and 21 bp downstream of the TAA stop codon, was PCR amplified using primers ASInd_arcAF and ASInd_arcAR and directionally cloned into the SacI and XbaI sites of plasmid pAKD601B.

**Luminescence Assays**

To assay luminescence, *V. fischeri* cultures were grown overnight in LBS medium and diluted 1:1000 into either 25 ml SWTO medium in 125 ml flasks or 50 ml SWTO medium in 250 ml flasks. Within each experiment, the same flask and medium volume combinations were used for all strains and treatments. Media was supplemented with 2 mM isopropyl beta-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO) or 50 nM 3OC6 (Sigma-Aldrich) where indicated. Cultures were incubated at 24°C with shaking at 200 rpm. At indicated time points, 0.5-ml samples were removed and the cell density was estimated by
Table 1. Strains, plasmids, and oligonucleotides used in this work.

| Strains or Plasmids | Relevant characteristics* | Source or Reference |
|---------------------|---------------------------|---------------------|
| **Strains**         |                           |                     |
| Escherichia coli    |                           |                     |
| DH5α                | F′/endA1 hsdR17 glnV44 thi-1 recA1 gyrA96 (Nal^R) relA1 | [57] |
| DH5αpir            | λpir derivative of DH5α | [56] |
| CC118JRpir         | Δ(lacZYA-argF)U169deoR1808lacIΔ(lacZYA-argF)M15 | [58] |
|                       |                           |                     |
| **Plasmids**        |                           |                     |
| pA14                | ΔarcA allele; oriV<sub>RE</sub>, oriT, Cm<sup>R</sup> | [52] |
| pA17                | ΔarcA::erm allele; oriV<sub>RE</sub>, oriT, Cm<sup>R</sup>, Em<sup>R</sup> | [52] |
| pAKD601B            | lac<sup>R</sup> and IPTG-inducible promoter; oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Kn<sup>R</sup> | [81] |
| pAKD702             | promoterless lacZ, oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Cm<sup>R</sup> | [82] |
| pAS2                | pEV5122 with lux upstream sequence; oriV<sub>RE</sub>, oriT, Em<sup>R</sup> | this study |
| pAS3                | Δluxl allele; oriV<sub>RE</sub>, oriV<sub>C<sub>RE</sub></sub>, oriT, Em<sup>R</sup>, Kn<sup>R</sup> | this study |
| pAS4                | ΔluxICDABEG allele; oriV<sub>RE</sub>, oriV<sub>C<sub>RE</sub></sub>, oriT, Em<sup>R</sup>, Kn<sup>R</sup> | this study |
| pAS5                | ΔarcA allele (pA14) fused to pBluescript; oriV<sub>RE</sub>, oriV<sub>C<sub>RE</sub></sub>, oriT, Cm<sup>R</sup>, Amp<sup>R</sup> | this study |
| pAS104              | pAKD601B-arcA (IPTG-inducible arcA), oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Kn<sup>R</sup> | this study |
| pBluescript         | oriV<sub>C<sub>RE</sub></sub>, Amp<sup>R</sup> | Stratagene |
| pEV5104             | conjugative helper, oriV<sub>RE</sub>, oriT, Kn<sup>R</sup> | [60] |
| pEV5122             | oriV<sub>RE</sub>, oriT, Em<sup>R</sup> | [58] |
| pEV5148k            | pCR-Bluntl-TOPO with lux downstream sequence, oriV<sub>C<sub>RE</sub></sub>, Kn<sup>R</sup> | [61] |
| pEV5149k            | pCR-Bluntl-TOPO with luxG downstream sequence, oriV<sub>C<sub>RE</sub></sub>, Kn<sup>R</sup> | [61] |
| pJLB169             | ΔarcA::erm allele (pA17) fused to pBluescript; oriV<sub>RE</sub>, oriV<sub>C<sub>RE</sub></sub>, oriT, Cm<sup>R</sup>, Em<sup>R</sup>, Amp<sup>R</sup> | this study |
| pJLB171             | pAKD702 containing the lux promoter region, oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Cm<sup>R</sup> | [82] |
| pJV5102             | gfp, oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Kn<sup>R</sup> | [59] |
| pJV5208             | rfp, oriV<sub>RE</sub>, oriV<sub>PE2123</sub>, oriT, Cm<sup>R</sup> | [59] |
| **Oligonucleotides** |                           |                     |
| ASInd<sub>arcAF</sub> | ATGAGGCTCTAACCACTAGTAGTTAGGTACC   | this study |
| ASInd<sub>arcAR</sub> | TATCTAGAAAGCTGATAGAAGAGATTCTTA | this study |
| ASLUX1             | CGGCTAGCCCTAGCACCCTCTCTCTATTATCATGATC | this study |
| ASLUX2             | ACCCTAGGTCGCGTGAGTTATTTAGGACGACACATG | this study |

*Kn<sup>R</sup>, Kanamycin resistance; Cm<sup>R</sup> and cat, Chloramphenicol resistance; Em<sup>R</sup> and erm, Erythromycin resistance; Amp<sup>R</sup>, Ampicillin resistance; Nal<sup>R</sup>, Nalidixic acid resistance; Sp<sup>R</sup>, Spectinomycin resistance; Cb<sup>R</sup>, Carbencillin resistance. Plasmid replication origins are designated ori<sub>RE</sub> with a subscript indicating the source, and ori<sub>T</sub> indicates the R4 origin of transfer.

*Oligonucleotides are in the 5′ to 3′ orientation with introduced restriction sites underlined.

doi:10.1371/journal.pone.0049590.t001
measuring the optical density at 595 nm (OD\textsubscript{595}) using a BioPhotometer (Brinkman Instruments, Westbury, NY). The cuvette was then shaken to aerate the sample and luminescence was measured using a GLOMAX 20/20 luminometer (Promega, Madison, WI) with a 10 sec integration setting. Luminescence values were normalized to cell density (OD\textsubscript{595}) unless indicated otherwise.

\section*{3OC6 Bioassays}
Previous studies assayed 3OC6 by adding samples to \textit{V. fischeri} wild-type ES114, and endogenous 3OC6 production did not impede its utility in assaying exogenous 3OC6 [48,62]. However, it has more recently been found that ES114 produces \textit{N}-octanoyl homoserine lactone (C8) at much higher levels than 3OC6, and C8 can affect LuxR expression and activity [63-65]. For our bioassay we therefore used strain NL11 (\textit{Ains3; litR::kan}), which lacks both the C8 synthase (\textit{AinS}) and the \textit{LitR} regulator that modulates LuxR in response to C8 (or to the LuxS-generated AI-2).

To determine the level of 3OC6, \textit{V. fischeri} cultures were grown in SWTO medium in aerobic shake flasks to an OD\textsubscript{595} of 2.0 when cells were near peak luminescence. Cells were removed by centrifugation and the supernatant was extracted using equal parts supernatant and acidified ethyl acetate (1:1000 acetic acid in ethyl acetate). The ethyl acetate layer was removed and allowed to evaporate in sterile glass flasks. After evaporation the extracted HSL was resuspended in a volume of SWTO equal to the original culture volume, and this extract-amended SWTO was inoculated 1:1000 with \textit{V. fischeri} bioassay strain NL11. Cultures were incubated at 24°C with shaking at 200 rpm, and cell density and luminescence were measured over time. Luminescence values for each sample extract were compared to those for 3OC6 standards to quantify the pheromone levels. For standards, 3OC6 (Sigma-Aldrich) was added to culture supernatant from a \textit{V. fischeri} \textit{ainsS} mutant (CL21) to known concentrations (0, 1, 3, 10 nM) and these C8-amended supernatants were extracted and processed as described above. We used a Student’s t-test to determine whether strains had significantly different C8 accumulation.

\section*{Squid Colonization Assays}
\textit{V. fischeri} cultures were grown in ASWT to an OD\textsubscript{595} of 0.3–0.7 and diluted in Instant Ocean (United Pet Group Inc., Cincinnati, OH) to 600–3000 CFU ml\textsuperscript{-1}. Aposymbiotic squid were added to the inoculum water overnight. The next morning squid were transferred to \textit{V. fischeri}-free Instant Ocean. To measure the onset of luminescence in symbiotic animals, the luminescence per squid was measured using a LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). At designated time points, squid were anesthetized with MgCl\textsubscript{2}, dissected and imaged using a Nikon (Melville, NY) Eclipse E600 epifluorescence microscope with a Nikon 96157 red filter cube, a Nikon 41017 green filter cube, and a Nikon Coolpix 5000 camera. After imaging, squid were homogenized and plated to determine CFU per squid.

\section*{Results}

\subsection*{The arcA Mutant Produces High Levels of 3OC6 Pheromone}
Previous work in \textit{V. fischeri} showed \textit{arcA} and \textit{arcB} mutants have bright luminescence in culture relative to the parent strain [50,52]. Since lux\textsubscript{D}, the 3OC6 synthase gene, is encoded in the same operon as the genes directly involved in generating bioluminescence, we predicted that an \textit{arcA} mutant would also produce more 3OC6 pheromone than wild type. We found that at an OD\textsubscript{595} of 2.0 when cultures are near peak luminescence, \textit{ΔarcA} mutant cultures contained on average 55 nM 3OC6 pheromone while the wild-type, \textit{luxA} and \textit{ΔarcA luxA} mutant cultures were below the level of detection for the assay (<1 nM). Thus, as predicted, ArcA mediates repression of not only bioluminescence but also 3OC6 pheromone synthesis.

\subsection*{Bright Luminescence of the \textit{arcA} Mutant is Mostly due to 3OC6-mediated Positive Feedback}
The ~500-fold increase in luminescence in \textit{arcA} mutants (documented previously [52]) should be a combined effect of the loss of direct, \textit{ArcA}-dependent repression of the \textit{luc} operon together with the 3OC6-mediated positive feedback inherent in the LuxR-LuxI regulatory circuit. To test the relative importance of these two effects we first assayed luminescence in a \textit{ΔarcA} mutant strain with or without a functional \textit{luc} 3OC6 synthase gene. It is important to note that under these broth culture conditions, LuxR-mediated activation of the \textit{luc} operon in ES114 is stimulated primarily by C8 [63], which is the product of \textit{AinS} \textit{[68,69]}. C8 measurements ranged from 150 to 500 nM in different experiments but were never significantly different between ES114 and the \textit{ΔarcA} mutant (p>0.2). The \textit{arcA} mutant was >350-times more luminescent than wild type; however, in the \textit{luc} mutant background, the \textit{arcA} mutation had only a 2-fold effect on luminescence (Fig. 2A). Similarly, we saw a much greater effect of the \textit{ΔarcA} allele on a \textit{P\textsubscript{lux-lacZ}} reporter in the wild-type background (10-fold) than in a \textit{lucA} mutant (2-fold) (Fig. 2B). These results indicate that in the absence of \textit{ArcA}-dependent repression of \textit{luc}, bright bioluminescence is mediated primarily through the 3OC6-dependent positive feedback regulation of the \textit{luc} genes.

To further explore the relative regulatory strengths of 3OC6-mediated positive feedback and direct repression of \textit{luc} by ArcA, we tested whether addition of 3OC6 at concentrations found in
cultures of the ∆arcA mutant could overcome ArcA-mediated repression of lux in wild-type cells. When 50 nM 3OC6 (the amount accumulated in arcA mutant cultures) was added to wild-type cultures, luminescence increased to levels comparable to that found in the ∆arcA mutant (Fig. 2C). Taken together, the results above suggested that ArcA-mediated repression of lux might be rendered inconsequential if cells have previously induced LuxI-mediated positive feedback or if a distinct population of neighboring cells lacks active ArcA-mediated repression of lux. Below we describe tests of both of these ideas.

3OC6-mediated Positive Feedback Results in Irreversible Luminescence Induction

Because an ∆arcA mutant produces high levels of 3OC6 and exogenous 3OC6 can overcome ArcA-mediated lux repression, we asked whether ArcA can repress luminescence once the LuxI-mediated positive feedback circuit is initiated. To test this, we constructed an IPTG-inducible arcA expression vector (pAS104) and moved it into an ∆arcA mutant to control when arcA is expressed. When the ∆arcA mutant carrying pAS104 was grown in aerobic shake flasks without IPTG, luminescence reached the same high level observed when the empty vector was present (data not shown), suggesting ArcA expression in the absence of IPTG, luminescence reached the same high level observed when the empty vector was present (data not shown), suggesting ArcA expression in the absence of IPTG was low enough to have little regulatory impact. When IPTG was present from the start of the experiment (T0), luminescence was repressed to a level similar to that of the wild-type control (Fig. 3). However, when IPTG was added to cultures after luminescence was induced (T1), arcA expression was no longer able to repress luminescence, resulting in cultures with bright luminescence similar to that in the ∆arcA mutant without IPTG (Fig. 3). These results suggest that once 3OC6-mediated positive feedback is initiated fully by the loss of Arc-dependent repression, expression of ArcA cannot reverse this effect.
3OC6 can Induce Luminescence in Neighboring Cells

We next asked whether 3OC6 from arcA mutant cells could induce luminescence in neighboring wild-type cells, overcoming ArcA-mediated repression of lux in the wild-type population. To examine this possibility we tested whether a dark (ΔluxCDABEG arcA) mutant could induce luminescence in wild-type cells in a 3OC6-dependent manner. When wild-type cells are co-cultured in the presence of dark (ΔluxCDABEG arcA) mutant cells in shake flasks, the specific luminescence of the wild-type cells increases nearly 500-fold (Fig. 4A), and this luminescence induction is dependent on luxI and 3OC6 pheromone production in the arcA mutant cells (Fig. 4B).

Additionally, we asked whether pheromone produced by the arcA mutant could be communicated across a distance when strains were spatially segregated. To test this we streaked wild-type and luxI mutant cultures onto an agar plate next to a spot of dark arcA mutant culture. After incubation, the wild-type streak showed high luminescence nearest the pheromone-producing arcA mutant, with elevated but diminishing luminescence in cells further from the pheromone source extending nearly 500-fold (Fig. 4A), and this luminescence induction is dependent on luxI and 3OC6 pheromone production in the arcA mutant cells (Fig. 4B).

3OC6 from arcA Mutant Cells can Induce Luminescence in Neighboring Cells in Culture

We next asked whether 3OC6 from arcA mutant cells could induce luminescence in neighboring wild-type cells, overcoming ArcA-mediated repression of lux in the wild-type population. To examine this possibility we tested whether a dark (ΔluxCDABEG arcA) mutant could induce luminescence in wild-type cells in a 3OC6-dependent manner. When wild-type cells are co-cultured in the presence of dark (ΔluxCDABEG arcA) mutant cells in shake flasks, the specific luminescence of the wild-type cells increases nearly 500-fold (Fig. 4A), and this luminescence induction is dependent on luxI and 3OC6 pheromone production in the arcA mutant cells (Fig. 4B).

Additionally, we asked whether pheromone produced by the arcA mutant could be communicated across a distance when strains were spatially segregated. To test this we streaked wild-type and luxI mutant cultures onto an agar plate next to a spot of dark arcA mutant culture. After incubation, the wild-type streak showed high luminescence nearest the pheromone-producing arcA mutant, with elevated but diminishing luminescence in cells further from the pheromone source extending nearly 500-fold (Fig. 4A), and this luminescence induction is dependent on luxI and 3OC6 pheromone production in the arcA mutant cells (Fig. 4B).

3OC6 from One Population of Symbiotic Cells can Induce Luminescence in 3OC6-deficient Cells in the Squid Light Organ

Theoretically, inter-strain induction of luminescence such as that shown in Figure 4 could reflect a similar phenomenon in the light organ, with a subpopulation inducing luminescence in a broader population through amplification and diffusion of pheromone originating from a distinct subgroup. Therefore, we were interested in testing whether a subpopulation of symbiotic cells can induce luminescence in the wider community in the squid light organ. While the intercellular signaling experiments described above used arcA mutants as a subpopulation of cells with altered regulation of pheromone synthesis, we did not use arcA mutants in the squid colonization experiment described below.
Previous studies showed wild-type *V. fischeri* cells produce high levels of pheromone in the light organ [48]. Moreover, ArcA does not repress luminescence in symbiotic cells [52], suggesting ArcA most likely does not repress pheromone synthesis in these cells as well. For these reasons, we used arcA* V. fischeri* strains to test for intercellular signaling in the squid light organ.

To determine if a subpopulation of cells can induce luminescence in the wider light organ community, aposymbiotic juvenile squid were co-infected with a 1:1 mixture of a gfp-labeled dark mutant (ΔluxCDABEG), that can still synthesize 3OC6, and a rfp-labeled lux mutant that cannot make 3OC6. Neither strain alone can induce symbiotic luminescence [63] (Fig. 5A). However, when these strains co-colonize the light organ, if sufficient pheromone from the gfp-labeled dark mutant can diffuse into the pheromone deficient rfp-labeled strain, luminescence should be observed. 24 hr after infection, luminescence values were recorded for each squid before it was dissected and imaged with an epifluorescence microscope to visualize the spatial distribution of the two strains within the light organ (Fig. 5B). We observed wild-type levels of luminescence in the rfp-labeled luxI mutant cells in the squid when co-infecting the light organ with a dark 3OC6-producing strain (Fig. 5C). Interestingly, even when the dark pheromone-donor strain (ΔluxCDABEG) comprised as little as 8% of the light organ population in a mixed infection with the 3OC6-deficient (luxI) strain, the latter achieved luminescence comparable to that of wild-type in a clonal infection, and this robust induction of the luxI mutant by the dark strain occurred despite significant segregation of the two strains in the light organ. These results indicate that 3OC6-mediated intercellular signaling can occur between distinct subpopulations of symbiotic cells and that high levels of 3OC6 synthesis in only a subset of the population are sufficient to induce luminescence fully in the remaining cells occupying the light organ.

**Discussion**

Bacterial pheromone signaling often is governed by environmental regulators and by pheromone-dependent positive feedback loops, both of which modulate the LuxR/LuxI pheromone signaling system that controls bioluminescence in *V. fischeri*. The relative strengths of environmentally responsive regulators and positive feedback have important implications for the biological functions of pheromone signaling in nature, and in this study we explored their contributions to lux regulation in the squid symbiotic strain ES114. We previously found that ArcA is a direct repressor of the luxICDABEG operon, and that arcI or arcB mutants are 100- to 1000-fold brighter than ES114 in culture [50,52]. In this study we were surprised to find that in the absence of LuxI-mediated positive feedback, ArcA is actually a very weak repressor of the luxI operon, repressing luminescence only about 2 fold. However, its influence over lux expression is amplified by LuxI-dependent positive feedback resulting in more than a hundred-fold additional increase in luminescence (Fig. 2).

Given the interest in mathematically modeling the lux circuit, we should note that our data do not necessarily indicate that a simple positive feedback circuit has such a large effect on lux operon transcription. Importantly for quantitative assessment of this circuit, luminescence output does not appear to correspond linearly with transcription from the luxI promoter. In studies either using gfp embedded in the lux operon or measuring transcripts by microarray analysis, conditions that induce luminescence 100- to 1000-fold only had a 10- to 20-fold effect on the GFP reporter or lux mRNA [52,70,71]. Similarly, in Figure 2 we show a ~10-fold effect on expression of a plasmid-borne PluxI-lacZ reporter under conditions where luminescence was affected by at least an additional order of magnitude. Using the strain with gfp added to the lux operon, Perez et al. noted the relationship between fluorescence and luminescence followed a power law, and speculated that this may be due to the association equilibrium of...
LuxA and LuxB, which must dimerize to form active luciferase [71]. Unpublished data also suggest the lux operon may be post-transcriptionally controlled by a regulator that can be titrated with sufficient mRNA, which would also lead to a non-linear response between lux transcription and luminescence.

Given the overriding strength of positive feedback, we speculated that ArcA-mediated repression of lux would be rendered insignificant after a culture has engaged LuxI- (3OC6-) mediated positive feedback, or if lux in neighboring cells was highly expressed, for example following deactivation of the Arc system. Figures 3 and 4 illustrate support for these two ideas. Taken together these results have significant implications for the symbiotic role of pheromone signaling in at least two ways.

The first functional implication of our results is that the data in Figure 3 indicate an environmental condition in the host necessary for induction of 3OC6 signaling need not be maintained over time, because once the lux system is induced it essentially becomes more difficult to turn off. In the case of the ArcA/ArcB two component system, ArcB renders ArcA more active under reducing conditions and less active under oxidizing conditions. Bose et al. previously proposed that initial V. fischeri colonists might experience an oxidizing environment, leading to inactivation of ArcA/ArcB and induction of the lux operon but that later in infection, cells would become more crowded in the light-organ crypts, consume $O_2$, and generate more reducing conditions that activate ArcA/ArcB [52]. They suggested that while ArcA might regulate other genes at this time later in infection, it would no longer effectively repress lux due to the increased synthesis of LuxI, accumulation of 3OC6, and positive feedback. More recently, studies by Williams et al. using transgenic E. coli strains also noted autoregulatory feedback by LuxR leading to more of this pheromone receptor in the cell and hysteresis [72], which may occur in strain ES114. The model proposed by Bose et al. reconciled the observations that ArcA did not repress lux in symbiotic cells but did appear to have a role in symbiont fitness later in infection [52]. The results of our study lend support for this model; however, our data also underscore the need to understand regulatory inputs during colonization of the host light organ. Given the discovery that direct regulation of the lux promoter by ArcA is quite weak, it is possible that the effect of another regulator is amplified by positive feedback and overpowers the repression by ArcA. The Arc activation state in symbiotic cells remains unknown, but as discussed below it is a priority for future research.

A second key implication of our results is that not all symbiotic cells would have to experience a stimulatory environment in the host for there to be a population-wide induction of luminescence. As noted above, high cell density alone will not induce luminescence in ES114, but the combination of a quorum and some aspect of the host environment foster induction of lux and bioluminescence. In Figure 4 we show that in the absence of ArcA-dependent repression of lux, 3OC6 can diffuse into nearby cells and induce luminescence despite ArcA actively repressing the lux operon in the cells receiving 3OC6. Superficially, the experiment shown in Figure 4C resembles many others wherein added pheromone or a pheromone-producing strain stimulates a response in cells lacking pheromone production, with the signal diffusing over millimeter distances [73,74]. However, the key difference is that in Figure 4C an environmentally responsive regulator is what distinguishes pheromone levels in the adjacent strains. In short, Figure 4 shows that in a heterogeneous population, either mixed or spatially segregated, derepression of pheromone synthesis and the ensuing positive feedback override direct repression. These results imply that a group response can be coordinated based on physiological conditions that are not necessarily being experienced by the entire population.

Figure 6 further illustrates how we interpret the data shown in Figure 4C, and how this result could reflect luminescence induction in the symbiosis. In our model, some environmental
cue in a host microenvironment is found in a gradient. For example, if oxygen, reactive oxygen species or another good electron acceptor were provided by the host but also consumed by the bacteria (e.g. oxygen consumed by luciferase), there could be a relatively steep gradient of this cue. Perhaps symbionts nearer the host epithelium are exposed to a sufficiently oxidative environment to turn off Arc while others deeper in the crypt lumen experience more reduced conditions wherein Arc is active. The first group, with inactive Arc, might then initiate LuxX-mediated pheromone synthesis, producing a signal that spreads through the population. Quite the opposite of the original environmental cue, the pheromone signal would not be destroyed by other symbiont cells but rather it would be amplified by positive feedback, leading to a much more shallow gradient. Put together, this scenario could allow cell-cell signaling to induce a broader population response based on an environment only experienced by a subpopulation.

Our model in Figure 6B in part invokes a scenario wherein 3OC6 signal from a subpopulation in the light organ initiates a population-wide response, and while this seemed plausible to us it had never been tested. In Figure 5, we observed pheromone-mediated intercellular communication in the squid light organ, where dark 3OC6-producing cells induced luminescence in co-colonizing, 3OC6-deficient cells. This inter-strain signaling in the light organ was particularly remarkable in that only a relatively small and segregated portion of the population needed to be producing 3OC6 in order to induce luminescence fully in the remaining cells. Moreover, it is worth noting that only the 3OC6-producing cells retain positive feedback regulation of pheromone synthesis, and therefore such positive feedback was not required for the subpopulation receiving the signal to induce luminescence fully. These data are proof in principle that symbiotic luminescence induction could originate from a regulatory response by a subpopulation. We have previously shown that symbiotic lux induction is spatially heterogeneous, indicating that subpopulations in different light organ microenvironments experience different regulatory cues [59]. The regulators and environmental conditions underlying this heterogeneity, and whether in fact subpopulations are responsible for the ultimate population-wide induction of luminescence, are fertile areas for future research.

Pheromone production is required for luminescence induction in V. fischeri cells colonizing the squid; however, as noted above, the environmental cues and regulators responsible for activating symbiotic pheromone synthesis and luminescence remain uncertain. Previous work showed that arcA mutant cells are brighter than wild type in culture but not in the squid [52], consistent with a model in which inactivation of ArcA/arcB in the host may be important for luminescence derepression in symbiotic cells. Future work will focus on directly testing the role of ArcA/arcB in symbiotic luminescence induction in V. fischeri and on elucidating the physiological conditions that control the activity of this two-component system in V. fischeri. Studies of E. coli have indicated that oxygen itself is not perceived by ArcA/ArcB [73], and varied reports have suggested that ArcB may sense and respond to fermentation acids [76,77] and/or the redox state of the quinone pool [78,79]. The predicted ArcA regulon in V. fischeri [80] appears to be very similar to that of E. coli [90], and the V. fischeri arcI gene complements an E. coli arcI mutant [52], suggesting functional conservation of this two-component system across these two bacterial species. However, the mechanism underlying ArcB sensing of redox state in V. fischeri remains uncertain. Future work in this area will elucidate both how Arc functions and how it is integrated with pheromone signaling in a natural infection.

Our results are also consistent with a different regulatory input overpowering ArcA-mediated lux repression. Figure 2C directly illustrates that 3OC6 can induce luminescence and render ArcA-mediated lux repression insignificant. This result shows that if another regulator induces 3OC6-mediated positive feedback in symbiotic cells, whether or not Arc is repressing lux may not matter. We are actively investigating the Arc status of symbiotic V. fischeri cells, but we are also elucidating regulators other than Arc that could play key roles in symbiotic pheromone signaling. A recent mutant screen identified transposon-insertion mutants in thirteen loci other than arc that led to increased luminescence of ES114 in culture [50]. These and other regulatory inputs should also be considered with respect to lux induction in the symbiotic environment.

The lux pheromone system in V. fischeri is controlled by environmental regulators, and we have shown that such inputs can be powerfully amplified by the positive feedback inherent in the lux circuitry. Because bacterial pheromones are often regulated in response to environmental conditions and also subject to positive feedback, we expect that these results may reflect signaling systems in many other host-associated bacteria. Although pheromone signaling may require a sufficiently high cell density for effective communication, the pheromones cannot be considered simply as census-taking molecules. Scenarios similar to the model presented in Figure 6B may explain the combination of environmental regulation and positive feedback in the pheromone signaling systems of host-associated bacteria, and this possibility should be considered as we seek to understand the biological roles of these systems.

Acknowledgments

We would like to thank Jeffery L. Bose for use of pJLB169 and JB33, Lawrence Shimmekts for helpful discussions, and anonymous reviewers for insightful comments.

Author Contributions

Conceived and designed the experiments: ANS EVS. Performed the experiments: ANS. Analyzed the data: ANS EVS. Contributed reagents/materials/analysis tools: EVS. Wrote the paper: ANS EVS.

References

1. Fuqua WC, Winans SC, Greenberg EP (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176: 269–275.
2. Schuster M, Hawkins AC, Harwood CS, Greenberg EP (2004) The Pseudomonas aeruginosa RpoS regulon and its relationship to quorum sensing. Mol Microbiol 51: 973–985.
3. Van Delden C, Comte R, Bally M (2001) Stringent response activates quorum sensing and modulates cell density-dependent gene expression in Pseudomonas aeruginosa. J Bacteriol 183: 5376–5384.
4. Moris M, Braeken K, Schoeters E, Verreth C, Beullens S, et al. (2005) Effective communication between Rhizobium etli and Pseudomonas vulgaris requires the alarmone ppGpp. J Bacteriol 187: 5460–5469.
5. Chattree J, Miyamoto CM, Zouzoulas A, Lang BF, Skours N, et al. (2002) MetR and CRP bind to the Vibrio harveyi lux promoters and regulate luminescence. Mol Microbiol 46: 101–111.
6. Liang W, Pascual-Montano A, Silva AJ, Benitez JA (2007) The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in Vibrio cholerae. Microbiology 153: 2961–2973.
7. Liang W, Sultan SZ, Silva AJ, Benitez JA (2008) Cyclic AMP post-translationally regulates the biosynthesis of a major bacterial autorepressor to modulate the cell density required to activate quorum sensing. FEMS Lett 582: 3744–3750.
8. Wang I, Hashimoto Y, Tsao CY, Valdes JJ, Bentley WE (2005) Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autorepressor 2 in Escherichia coli. J Bacteriol 187: 2066–2076.
9. AbuA MS, Pesce EC, Runyen-Janecky LJ, West SE, Igleswki BH (1997) Vir controls quorum sensing in Pseudomonas aeruginosa. J Bacteriol 179: 3928–3935.

10. Reverchon S, Bouillant ML, Salmond G, Nauer V (1998) Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in Erwinia chrysanthemi. Mol Microbiol 29: 1407–1418.

11. Lenz DH, Mok KC, Lilley BN, Kulkarni RV, Wingreen NS, et al. (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in Vibrio harveyi and Vibrio cholerae. Cell 118: 1127–1138.

12. Bollinger N, Hassett DJ, Igleswki BH, Costerton JW, McDermott TR (2001) Gene expression in Pseudomonas aeruginosa: evidence of constitutive overexpression of genes on the small RNA transcribers. J Bacteriol 183: 1190–1996.

13. Kim EJ, Wang W, Deckwer WD, Zeng AP (2005) Expression of the quorum-sensing regulatory protein LasR is strongly affected by iron and oxygen concentrations in cultures of Pseudomonas aeruginosa irrespective of cell density. Microbiology 151: 1:7–17.

14. Loh J, Carlsson RW, York WS, Stacey G (2002) Bradyoxerin, a unique chemical signal involved in symbiotic gene regulation. Proc Natl Acad Sci USA 99: 14466–14471.

15. Makinen EC, Hastings JW (1982) Iron repression bioluminescence and affects catabolite repression of luminescence in Vibrio harveyi. Curr Microbiol 5: 181–186.

16. Krol E, Becker A (2004) Global transcriptional analysis of the phosphate starvation response in Staphylococcus meliloti strains 1021 and 2011. Mol Gen Genomics 272: 1–17.

17. Gristwood T, Finneran PC, Eversen L, Williamson NR, Salmond GP (2009) The PhoBR two-component system regulates biofilm biosynthesis in Seriata in response to phosphate deprivation. BMC Microbiol 9: 100.

18. Jensen V, Loua D, Zoua C, Redfern BC, Mieusser A, et al. (2006) PhoR expression in Pseudomonas aeruginosa is modulated by the PhoP/Pseudomonas quinolone signal via PhoB-dependent and independent pathways. J Bacteriol 188: 8601–8606.

19. Guina T, Wu M, Miller SI, Purvine SO, Yi EC, et al. (2003) Proteomic analysis of Pseudomonas aeruginosa grown under magnesium limitation. J Am Soc Mass Spectrom 14: 742–751.

20. Chacsey ST, Wood DW, Person LS, 3rd (1999) Two-component transcriptional regulation of N-acetylhomoserine lactone production in Pseudomonas aeruginosa. Appl Environ Microbiol 65: 2294–2299.

21. Cui Y, Chatterjee A, Liu Y, Dumont CK, Chatterjee AK (1995) Identification of a global repressor gene, ronl, of Erwinia carotovora subsp. carotovora that controls extracellular enzymes, Nα3-acetylhomoserine-L-homocatetate lactone, and pathogenicity in soft-rotting Erwinia spp. J Bacteriol 177: 5108–1115.

22. Lenz DH, Miller MB, Zhu J, Kalkurni RV, Basler BL (2005) CRa and three redundant small RNAs regulate quorum sensing in Vibrio cholerae. Mol Microbiol 57: 1186–1192.

23. Pessi G, Williams F, Hindle Z, Heuriker K, Holden M, et al. (2001) Global posttranscriptional regulator RsmA modulates production of virulence determinants N-acetylhomoserine lactones in Pseudomonas aeruginosa. J Bacteriol 183: 6676–6683.

24. Yakihin H, Baker CS, Berezin IA, Evangelista MA, Rassan A, et al. (2011) CRa repression translates of rsmC, which encodes the N-acetylhomoserine-L-lactone receptor of Escherichia coli, by blocking exclusively within the coding region of rsmC mRNA. J Bacteriol 193: 6162–6170.

25. Yan Q, Wu XG, Wei HL, Wang HM, Zhang LQ (2009) Differential control of the PhoP/PhoR quorum-sensing system in Pseudomonas fluorescens 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system. Microb Res 164: 95–26.

26. Whitter CA, Ruby EG (2003) GaC regulates sybiontin symbiotic colonization traits of Vibrio fischeri and facilitates a beneficial association with an animal host. J Bacteriol 185: 7202–7212.

27. Engelbrecht J, Nealon K, Silverman M (1983) Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio ficheri. Cell 32: 773–781.

28. Fuqua WC, Winans SC (1994) A LuxR-LuxI type regulatory system activates Agrobacterium Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J Bacteriol 176: 2796–2806.

29. Sede PC, Paulevic M, Igleswki BH (1995) Activation of the Pseudomonas aeruginosa lasI gene by LasR and the Pseudomonas autoinducer PAI - an autoinduction regulatory hierarchy. J Bacteriol 177: 654–659.

30. Latifi A, Foglino M, Tanaka K, Williams P, Lardrinski A (1996) A hierarchical quorum-sensing cascade in Pseudomonas aeruginosa links the transcriptional activators LasR and RhlR (YamR) to expression of the stationary-phase sigma factor RpoS. Mol Microbiol 21: 1137–1146.

31. Niu C, Clemmer KM, Bonomo RA, Ruther PN (2008) Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii. J Bacteriol 190: 5336–5392.

32. Stauff DL, Basler BL (2011) Quorum sensing in Chromobacterium violaceum: DNA recognition and gene regulation by the CviIR receptor. J Bacteriol 193: 3071–3079.

33. Ji G, Beavis RC, Novick RP (1995) Cell density control of staphylococcal virulence mediated by an octapeptide phenol. Proc Natl Acad Sci USA 92: 12053–12059.
66. Schaefer AL, Hanzelka BL, Parsek MR, Greenberg EP (2000) Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of \textit{Vibrio fischeri} luminescence and other related molecules. Methods Enzymol 305: 289–301.

67. Flavier AB, Ganova-Raeva LM, Schell MA, Denny TP (1997) Hierarchical autoinduction in \textit{Ralstonia solanacearum}: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. J Bacteriol 179: 7089–7097.

68. Gilson L, Kuo A, Dunlap PV (1995) AinS and a new family of autoinducer synthesis proteins. J Bacteriol 177: 6946–6951.

69. Kuo A, Blough NV, Dunlap PV (1994) Multiple \textit{N}-acyl-L-homoserine lactone autoinducers of luminescence in the marine symbiotic bacterium \textit{Vibrio fischeri}. J Bacteriol 176: 7558–7565.

70. Antunes LC, Schaefer AL, Ferreira RB, Qin N, Stevens AM, et al. (2007) Transcriptome analysis of the \textit{Vibrio fischeri} LuxR-LuxI regulon. J Bacteriol 189: 8387–8391.

71. Perez PD, Weiss JT, Hagen SJ (2011) Noise and crosstalk in two quorum-sensing inputs of \textit{Vibrio fischeri}. BMC Syst Biol 5: 153.

72. Williams JW, Cui XH, Levchenko A, Stevens AM (2008) Robust and sensitive control of a quorum-sensing circuit by two interlocked feedback loops. Mol Syst Biol 4: 254.

73. Dilanji GE, Langebrake JB, De Leenheer P, Hagen SJ (2012) Quorum activation at a distance: spatiotemporal patterns of gene regulation from diffusion of an autoinducer signal. J Am Chem Soc 134: 5618–5626.

74. Flickinger ST, Copeland MF, Dosses EM, Braasch AT, Tuovu HH, et al. (2011) Quorum sensing between \textit{Pseudomonas aeruginosa} biofilms accelerates cell growth. J Am Chem Soc 133: 5966–5975.

75. Iuchi S, Chepuri V, Fu HA, Gennis RB, Lin ECC (1990) Requirement for terminal cytochromes in generation of the aerobic signal for the Arc regulatory system in \textit{Escherichia coli} - Study utilizing deletions and lac fusions of \textit{cyo} and \textit{cyd}. J Bacteriol 172: 6020–6025.

76. Iuchi S (1998) Phosphorylation/dephosphorylation of the receiver module at the conserved aspartate residue controls transphosphorylation activity of histidine kinase in sensor protein ArcB of \textit{Escherichia coli}. J Biol Chem 268: 23972–23980.

77. Rolfe MD, Ter Beck A, Graham AI, Trotter EW, Asif HM, et al. (2011) Transcript profiling and inference of \textit{Escherichia coli} K-12 ArcA activity across the range of physiologically relevant oxygen concentrations. J Biol Chem 286:10147–10154.

78. Malpica R, Franco B, Rodriguez C, Kwon O, Georgellis D (2004) Identification of a quinone-sensitive redox switch in the ArcB sensor kinase. Proc Natl Acad Sci U S A 101: 13318–13323.

79. Georgellis D, Kwon O, Lin EC (2003) Quinones as the redox signal for the arc two-component system of bacteria. Science 299: 2314–2316.

80. Ravcheev DA, Gerasimova AV, Mironov AA, Gelfand MS (2007) Comparative genomic analysis of regulation of anaerobic respiration in ten genomes from three families of gamma-proteobacteria (Enterobacteriaceae, Pasteurellaceae, Vibrionaceae). BMC Genomics 8: 54.

81. Dunn AK, Karr EA, Wang Y, Batton AR, Raby EG, et al. (2010) The alternative oxidase (AOX) gene in \textit{Vibrio fischeri} is controlled by NsrR and upregulated in response to nitric oxide. Mol Microbiol 77: 44–55.

82. Beur JL, Wollenberg MS, Cotton DM, Mandel MJ, Seger AN, et al. (2011) Contribution of rapid evolution of the \textit{luxR-luxI} intergenic region to the diverse bioluminescence outputs of \textit{Vibrio fischeri} strains isolated from different environments. Appl Environ Microbiol 77: 2445–2457.