Review

Quorum sensing and the confusion about diffusion

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Two hypotheses, termed quorum sensing (QS) and diffusion sensing (DS), have been suggested as competing explanations for why bacterial cells use the local concentration of small molecules to regulate numerous extracellular behaviours. Here, we show that: (i) although there are important differences between QS and DS, they are not diametrically opposed; (ii) empirical attempts to distinguish between QS and DS are misguided and will lead to confusion; (iii) the fundamental distinction is not between QS and DS, but whether or not the trait being examined is social; (iv) empirical data are consistent with both social interactions and a role of diffusion; (v) alternate hypotheses, such as efficiency sensing (ES), are not required to unite QS and DS. More generally, work in this area illustrates how the use of jargon can obscure the underlying concepts and key questions.

Quorum sensing

It is frequently assumed that bacterial cells use a process that has been termed 'quorum sensing' (QS) to regulate behaviours in response to cell density. Bacteria produce and release small diffusible molecules, usually termed signals (see Glossary), which have two main consequences [1,2]. First, the uptake of these molecules into cells regulates (autoinduction) a whole variety of behaviours, including the production of a range of exofactors that are released from the cells to aid growth, motility, and/or biofilm formation (Table 1). Second, the uptake of these molecules also leads to an increase in the production of the signal molecule itself (autoregulation). The production of these signal or autoinducer molecules therefore leads to positive feedback at high cell densities, which results in a considerable increase in the production of signal and QS-controlled factors. The hypothesis here is that producing certain extracellular factors is most beneficial at high cell densities, and that QS provides a mechanism which allows cells to increase the production of extracellular factors at high cell density [3].

In recent years, there has been debate over the extent to which the QS hypothesis described above represents the function of producing and responding to autoinducer molecules. This hypothesis assumes that QS is a social trait that coordinates behaviours across cells [4–6]. In an important challenge to this accepted idea, Redfield [7] suggested that the adaptive role of autoinducer molecules is not social communication between cells (signalling) but rather to assess the rate at which secreted molecules move away from the producing cell. This diffusion sensing (DS) hypothesis therefore suggests that autoinducer molecules allow cells to assess when producing exofactors will be directly beneficial to the cell that produces them, in response to the rate of diffusion in the environment. QS and DS are regularly seen as competing hypotheses [7–12], and a third hypothesis, termed efficiency sensing (ES), has been suggested as an attempt to unify both QS and DS into a single theory [9].

However, it is not clear whether QS and DS are competing hypotheses, let alone the extent to which they require the third hypothesis of ES to unify them. In particular, most comparisons of these three different hypotheses have been verbal and relatively informal, such that the key underlying assumptions have not always been made explicit and sometimes based on misconceived ideas about how natural selection operates. This matters, because if QS and DS are not competing hypotheses, then the increasing

Glossary

Coercion: when the sender does something that manipulates the behaviour of the receiver to the benefit of the sender and the detriment of the receiver.
Cooperation: a behaviour which provides a benefit to another individual, and which is selected for because of its beneficial effect on the recipient.
Direct fitness: the reproductive success of an individual that can be attributed to their own actions; that is, the part of the individual's inclusive fitness that owes to their own reproductive success.
Fitness: in non-class structured populations, the number of offspring that an individual produces over their lifetime; more generally, their expected asymptotic contribution of genes to distant future generations.
Inclusive fitness: 'the effect of one individual's actions on everybody's numbers of offspring . . . weighted by the relatedness' [42]; the sum of direct and indirect fitness; the quantity maximised by Darwinian individuals.
Indirect fitness: the reproductive success of an individual's genetic relatives that owes to the individual's own actions, weighted by genetic relatedness; that is, the part of the individual's inclusive fitness that owes to the reproductive success of their genetic relatives.
Relatedness: a measure of genetic similarity of two individuals, relative to the average; formally, it is the statistical (least-squares) regression of the recipient's breeding value for a trait on the breeding value of the actor [25].
Signal: acts or structures produced by the sender that alter the behaviour of the receiver; they have evolved because of that effect and are effective because the receiver's response has evolved.
Social : behaviour or trait is social if it has fitness consequences for another individual or individuals.

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Table 1. Traits controlled by quorum sensing

Trait	Example organisms	Type of QS signalling[a]
Aggregation	*Yersinia pseudotuberculosis*	AHL
	Rhodobacter sphaeroides	AHL
Antibiotics	*Chromobacterium violaceum*	AHL
	Erwinia carotovora	AHL
	Serratia spp. ATCC 39006	AHL
Bacteriocins, lantibiotics	*Lactococcus* spp.	AIP
	Lactobacillus spp.	AIP
	Carnobacterium piscicola	AIP
	Bacillus subtilis	AIP
	Enterococcus faecium	AIP
	Streptococcus thermophilus	AIP
	Streptococcus mutans	AIP
Biofilm formation and maturation	*Aeromonas hydrophila*	AHL
	Burkholderia cenocepacia	AHL
	Pseudomonas putida	AHL
	Serratia liquefaciens	AHL
	Enterococcus faecalis	AIP
	Staphylococcus aureus	AIP
	Vibrio cholerae	CAI-1, AI-2
Bioluminescence	*Vibrio fischeri*	AHL
	Vibrio harveyi	AHL, AI-2
Biosurfactant production (aids motility)	*Serratia liquefaciens*	AHL
	Serratia marcescens	AHL
	Pseudomonas aeruginosa	AHL
Competence	*Streptococcus pneumoniae*	CSP
Conjugation	*Enterococcus faecalis*	AIP
Exoproducts (products excreted from cells with a variety of functions)	*Erwinia carotovora*	AHL
	Erwinia chrysanthemi	AHL
	Burkholderia cenocepacia	AHL
	Pseudomonas aeruginosa	AHL, AHQ
	Aeromonas salmonicida	AHL
	Pseudomonas aureofaciens	AHL
Exopolysaccharides (polymers which protect from environmental stresses and can be a component of biofilms)	*Pantoea stewartii*	AHL
	Pseudomonas syringae	AHL
	Vibrio cholerae	CAI-1, AI-2
Membrane vesicles (packaging and trafficking of toxins and small molecules)	*Pseudomonas aeruginosa*	AHQ
Motility (e.g., swimming, twitching, or swarming)	*Yersinia pseudotuberculosis*	AHL
	Burkholderia cenocepacia	AHL
	Pseudomonas aeruginosa	AHL
	Pseudomonas syringae	AHL
	Staphylococcus aureus	AIP
	Yersinia enterocolitica	AHL
Nodulation/symbiosis (nitrogen fixation which benefits host plant)	*Rhizobium leguminosarum*	AHL
Sporulation	*Bacillus subtilis*	AIP
	Clostridium perfringens	AIP
	Clostridium botulinum	AIP
	Clostridium acetobutylicum	AIP
	Clostridium sporogenes	AIP
Virulence	*Burkholderia mallei*	AHL
	Burkholderia pseudomallei	AHL
	Pseudomonas aeruginosa	AHL, AHQ
	Staphylococcus aureus	AIP
	Agrobacterium tumefaciens	AHL
	Bacillus thuringiensis	AIP
	Bacillus anthracis	AIP
	Clostridium perfringens	AIP
	Clostridium botulinum	AIP
	Enterococcus faecalis	AIP
	Listeria monocytogenes	AIP

[a]Abbreviations: AHL, N-acylhomoserine lactone; AIP, autoinducing peptide (can be linear or cyclic); AI-2, autoinducer-2; AHQ, 2-alkyl-4-quinolone; CAI-1, (S)-3-hydroxytridecan-4-one; CSP, competence stimulating peptide [2,43].

number of attempts to distinguish between them empirically will lead to conflicting and confusing results, which will hinder progress. Here, we address this potential problem, by examining the relationship among the QS, DS, and ES hypotheses. Our specific aims are to determine: (i) the extent to which QS and DS are competing or overlapping hypotheses; (ii) the fundamental empirical problems; and (iii) how ES links to QS and DS.

Evolutionary theory and QS

In much of the earlier literature it was assumed, either explicitly or implicitly, that QS had been selected for because it optimises growth or survival at the population level [1,13]. However, this is not how natural selection works – individuals who behave for the good of the group or species can be exploited and outcompeted by more selfish individuals [14–16]. Instead, evolutionary theory predicts that natural selection will lead to organisms that appear as if they were designed to maximise their inclusive fitness (Box 1). This formalises how an individual can increase the frequency of their genes in future generations, either by increasing their own reproductive success (direct fitness) or else by increasing the reproductive success of their genetic relatives who carry copies of the same genes (indirect fitness) [17,18]. Consequently, theory predicts that individuals should behave in ways that benefit themselves and/or their relatives.

To clarify how natural selection acts on a specific trait, it is often useful to produce a mathematical model. The advantage of a mathematical model, as opposed to a verbal argument, is that it forces one to formalise an argument, and hence make clear the underlying assumptions. This emphasises that testing the assumptions of a model is as important as testing the predictions. In 2001, Brown and Johnstone [4] modelled the evolution of QS, considering how natural selection would influence both the production and the response to autoinducer molecules. Their model built upon and used methodology developed in the extensive theoretical and empirical literature on the evolution of cooperation and signalling [19–24]. We will examine this model in some detail, as it provides an excellent framework for the issues that we need to cover.

Assumptions and implications

Brown and Johnstone's [4] model made four key assumptions that are relevant here:

1. The production of autoinducer molecules is costly to the cells that produce them.
2. The production of exofactors is costly to the cells that produce them.
3. The production of exofactors provides a benefit to the local 'social group' of interacting cells. The social group includes the actor and, depending upon parameter values, can be of any size, from unity (just the actor on their own) to very large.
4. The benefit of producing exofactors can vary with the number of cells in the social group. Although this variation could in principle take any form, for simplicity it is assumed that the fitness benefit from exofactors increases with higher cell density. The number of cells in the social group is defined according to the local

Box 1. Natural selection and fitness

Darwin's [44] theory of natural selection provided an explanation for the empirical fact that organisms appear as if they were designed to fit the environments in which they live (adaptation). His theory can be summarised as follows:

1. Character variation: individuals within a population vary in characters such as morphology, physiology, and behaviour.
2. Fitness variation: variation in characters can lead to variation in the ability of individuals to survive and produce offspring.
3. Heritable variation: some of this variation in characters is heritable. For example, offspring tend to resemble their parents more than other members of the population.
4. Natural selection: as a result of points 1–3, individuals with certain characters will tend to have more offspring than others, and these characters will accumulate in the population.
5. Organism design: as a result of point 4, organisms will appear as if they have been designed to maximise the number of offspring successfully produced (reproductive success or 'fitness').

Darwin's theory was unified with Mendelian genetics by Fisher [17]. When Darwin formulated his idea he had no knowledge of the mechanism of heredity. Fisher showed how natural selection could be described by changes in gene frequencies. He showed that genes associated with greater individual fitness will increase in frequency and that this would lead to an increase in mean fitness, such that individuals would appear as if they had been designed to maximise their fitness. As Darwin had done before him, Fisher focused upon the number of direct descendants produced by individuals (reproductive success or reproductive value) as the measure of fitness.

Hamilton [18,45] extended Fisher's work to show that the general description of Darwinian fitness was what he termed inclusive fitness and not reproductive success. Fisher's [17] formalisation of natural selection focused upon how a gene influences their transmission to the next generation via the direct reproductive success of the individual in which they are in. Hamilton [18] realised that this did not provide a general description of natural selection, because genes can also alter their frequency in the next generation by influencing the reproductive success of other copies of that gene in other individuals. He showed that natural selection will lead to individuals that appear as if they were designed to maximise their inclusive fitness, which sums the consequences of their actions both for themselves (direct fitness) and for their relatives (indirect fitness), with the consequences for relatives being weighted by the extent to which they share genes in common with the actor (relatedness). To put it another way, if we were to ask 'what kind of organism would we expect natural selection acting on genes to produce?', then the answer is 'organisms that behave as if they are trying to maximise their inclusive fitness' [46,47]. The beauty of this is that it takes evolutionary theory based on gene dynamics and translates it into a theory about how individuals behave, which is what empirical workers can observe.

population density at the scale over which social interactions take place. Consequently, if cells do not interact, then the group size is one, whereas if cells do interact, then the group size is >1, and the trait being examined is social.

Three points should be noted from these assumptions. First, assumptions 2 and 3 imply that the production of exofactors could be a cooperative social behaviour, where the cost is paid by the individual (assumption 2), and the benefit is shared among the individuals in their social group (assumption 3). Second, assumption 3 makes clear that the modelled process can be either social or non-social. That is, QS could involve both direct and indirect fitness consequences (QS is social, with interactions between cells), or only direct fitness consequences (QS is non-social, and cells only interact with themselves). Put another way, if the size of the social group (assumption 4) is one cell, then trait is not social, and will evolve purely in response to the direct consequences.

Third, by varying the form of the benefit function in assumption 4, the fitness benefit from producing exofactors can be varied. This allows a range of biological complexities to be incorporated, such as non-linear returns from exofactors, diffusion, convection, decay, and degradation [9]. Note that the purpose here is not to hide the complexity of the environmental factors that may be important but rather to emphasise that from a functional perspective, we are interested in how they lump together. Indeed, an important issue is how the shape of the fitness benefit varies across environments or species, in response to the multitude of factors that could be important, and if/how cells respond to this.

It is important here to distinguish our discussion of this formal evolutionary model of QS, from the kind of verbal descriptions that are sometimes given in the literature. First, as discussed above, no assumption is made that the purpose of QS is to optimise growth at the population level – instead the population level consequences follow from an evolutionary model. Second, we do not assume that only cell density matters – instead, because they would influence the fitness return from producing exofactors, a variety of environmental factors could be important [9]. Third, QS does not have to be social – this depends upon whether cells can utilise the exofactors produced by other cells (i.e., is the social group size >1?).

Predictions

Brown and Johnstone [4] developed a general model that did not assume specific functions for the different relationships above, but then showed results for the simplest case. They considered the consequences of variation in two parameters: the average genetic relatedness between individuals in a social group (r) [25] and the size of the social group. Relatedness is a statistical concept, describing the genetic association between social partners – in the simplest case, if N unrelated lineages (clones) mix equally in an area, then average relatedness will be $r = 1/N$, which comes from the average of individuals being related by $r = 1$ to their clonemates, and $r = 0$ to the individuals in the other lineages [26].

Brown and Johnstone's aim was to predict the evolutionarily stable strategy (ESS) level of autoinducer and exofactor production, which cannot be beaten by any other strategy. They found three main results:

1. The ESS levels of autoinducer and exofactor production per cell both increased with larger social groups. This is because the benefit of producing exofactors was assumed to increase with the size of the social group (assumption 4).
2. The ESS level of exofactor production per cell increased with higher relatedness between interacting bacteria (Figure 1a). This is because greater levels of cooperation are expected between closer relatives.
3. The ESS level of autoinducer production per cell showed a domed relationship with relatedness (Figure 1b). At high relatedness, there is a shared

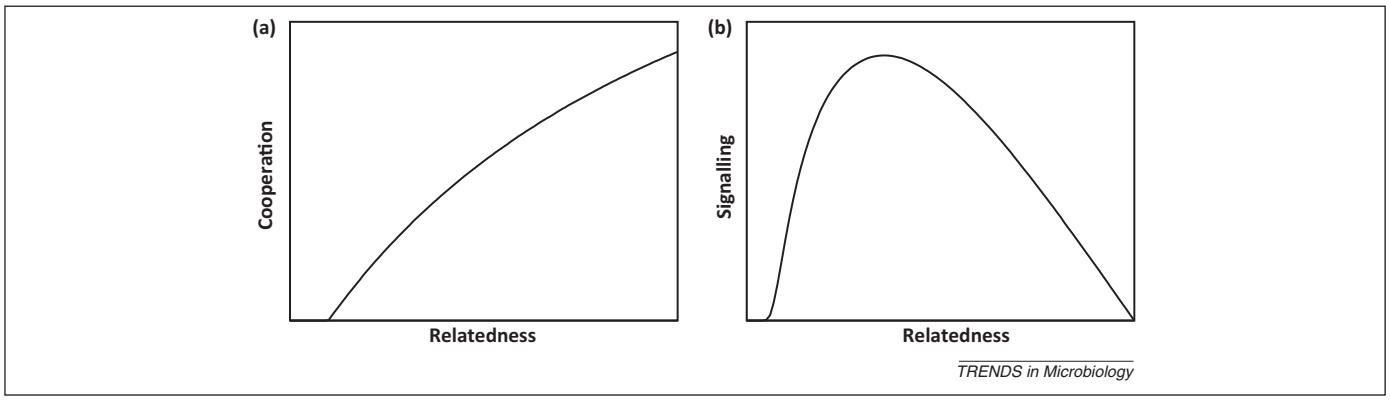

Figure 1. The predictions of Brown and Johnstone's theoretical model. **(a)** The evolutionarily stable strategy (ESS) level of exofactor production increases with increasing relatedness (*r*), because the inclusive fitness benefits of cooperation are maximal at high relatedness and minimal at low relatedness. **(b)** The ESS level of an autoinducer (signal) molecule shows a dome-shaped function with relatedness. At low relatedness there is little inclusive fitness benefit to the cooperative production of exofactors. At high relatedness there is little conflict, thus cheap signalling is favoured to coordinate cooperation, whereas at intermediate relatedness cooperation is worthwhile, yet there is also a selection to increase the cooperation of others. Note that relatedness here measures the relativeness to the social group, which includes the focal actor (termed 'whole group' relatedness) and not relatedness to the other members of the group (termed 'others only' relatedness) [48]. Consequently, for example, $r = 1$ could correspond to a cell interacting with either only clone-mates or only itself.

interest in the cooperative production of exofactors and cheap signalling to coordinate this. Specifically, the minimum level of signalling that is required to be detected will be favoured. At low relatedness, there is no selection for cooperative production of exofactors, and hence no selection for signalling to coordinate this. With intermediate relatedness, there can be selection to cooperatively produce exofactors, but it is in the individual's interest to produce lower levels of exofactor than the other local cells (because $r < 1$). This favours higher levels of autoinducer production, in an attempt to manipulate competitors to produce more exofactors, which in turn leads to the autoinducers being increasingly ignored. This is termed competitive devaluation of signal strength.

Three points should be noted from these results. First, the highest level of exofactor production is favoured when social groups are clonal, such that $r = 1$ (Figure 1a). In this extreme special case, cells should behave as if they are maximising the growth of their social group [27]. This is because when $r = 1$ an individual values the growth and reproduction of any other cell in the group as much as they value their own growth and reproduction. This means that all the cells in a social group will 'agree' on how best to maximise their inclusive fitness, and hence behave as if they were a single, multicellular organism with a unity of purpose. Because $r = 1$ will not always hold, it is incorrect to think of bacterial populations in general as multicellular organisms that maximise population survival.

Second, although a lower relatedness leads to lower production of exofactors, appreciable production of exofactors can still be favoured when relatedness is relatively low (i.e., $r < 1$; Figure 1b). Third, the production of autoinducers to coordinate exofactor production can also be favoured in non-clonal populations. Indeed, an intermediate relatedness can even favour a greater amount of costly signalling (Figure 1b).

Diffusion sensing as an alternative to QS

In 2002, Redfield [7] challenged the idea of QS and suggested an 'alternative' explanation of DS for why autoinducers would be used to control the production of exofactors, Redfield's argument was based around four points.

1. The acceptance of QS in the microbiological literature was based upon: an assumption that cells behave for the good of the group; and a lack of empirical evidence that the production and response to autoinducers is a social trait.
2. "[B]ecause bacterial populations are rarely clonal" (i.e., $r < 1$), 'cheats' who do not produce or respond to signal would invade [7]. Hence, using autoinducers to socially control the production of exofactors would not be evolutionarily stable.
3. The production of both autoinducers and exofactors could be for purely direct fitness benefits to the cell producing them.
4. A direct benefit of producing autoinducers could be to assess the rate at which secreted molecules would move away from the cell that produced them. This measurement of the diffusion rate would allow cells to avoid producing costly exofactors in situations where they would diffuse away before the benefit could be obtained.

Redfield's [7] paper led to the idea that DS is an alternative and competing hypothesis to QS. However, this perspective is incorrect and can lead to confusion. To illustrate this, it is useful to compare the formal QS theory described in the previous section with the points made by Redfield.

Redfield's [7] first point was correct at the time but not anymore. The wider microbial literature on QS had generally presumed that if a behaviour was in the best interests of the bacterial population, then it would be straightforwardly favoured by natural selection. However, although this was true in the verbal explanations of QS that had dominated the microbial literature, clonality is not required to favour QS, or signalling more generally, as illustrated by Brown and Johnstone's [4] model which had been published in the previous year. It was also true at the time, that the "quorum-sensing hypothesis rests on very weak foundations" [7], because there had been no empirical work testing whether QS was social. However,

other exofactors had been examined [28] and, since then, relevant empirical work has been carried out, which we discuss below.

Redfield's [7] second point is incorrect. As shown by Brown and Johnstone's [4] model, appreciable signalling and cooperation can still be favoured in mixed (non-clonal) populations where $r < 1$ (Figure 1). Indeed, higher levels of costly signal can actually be favoured at intermediate levels of relatedness (Figure 1b). This is further supported from numerous theoretical and empirical studies on animals, where cooperation and signalling is favoured between individuals that are not clone-mates [19,20,23]. Indeed, arguably the most extravagant animal signals are those involved in sexual selection, such as tails of peacocks, which are for signalling between unrelated individuals.

Related to this second point, Redfield [7] also argued that if the accepted explanation for QS was true, then selection would favour cheats, which did not produce or respond to signal, but that there was no empirical evidence for such cheats. However, since then, such cheats have been discovered in a both natural systems and laboratory cultures (Table 2).

Redfield's [7] third point is correct, in that the production of autoinducers and exofactors could have direct benefits, but this does not pose a problem for the QS hypothesis. Specifically, the fact that exofactors could have direct benefits was already explicitly assumed with QS. As made clear in assumption 3 of Brown and Johnstone's [4] model, the benefits of exofactor production are shared between all members of a social group, including the producing cell.

Instead, the key point with Redfield's [7] suggestion was that she was suggesting the extreme end point of the continuum of social group size, where the size of the social group is fixed at one cell, such that there are no indirect consequences for other cells. Although this scenario is allowed for by Brown and Johnstone's [4] model, both they and other authors were focusing on the scenario where the size of the social group is greater than one cell, and the benefit of producing exofactors increases with population density.

Redfield's [7] fourth point is correct, in that autoinducers can provide information about diffusion rates. However, it is incorrect to imply that this is purely a direct benefit, because diffusion will also have a strong influence on the social or indirect fitness consequences of producing exofactors. Specifically, the diffusion rate will influence the relationship between the amount of exofactors produced and the fitness benefit provided to the social group (assumption 3 of Brown and Johnstone's model) [29]. Furthermore, this relationship will be influenced by other environmental factors, and not just diffusion, such as convection, degradation, and decay [9].

QS versus DS?

The above discussion makes it clear that it is incorrect to see DS and QS as competing hypotheses. Primarily, DS and QS are not competing hypotheses because: (i) viewing DS and QS as competing hypotheses is based upon a mischaracterisation of what would be required for QS to be stable; and (ii) diffusion is likely to be important in many scenarios, irrespective of whether or not QS is a social trait. Indeed, the DS scenario envisaged by Redfield [7] is a special case of Brown and Johnstone's [4] model, where the social group comprises only one cell, and hence there are no social (indirect) consequences of producing autoinducers and exofactors.

Conceptualising DS and QS as competing hypotheses will impede research in at least three ways. First, because both density and diffusion could be important in the same system, attempts to carry out empirical work that distinguishes between QS and DS could be a fruitless waste of time or be forced into misleading conclusions. For example, a study on *Streptococcus pneumoniae* elegantly tested the assumptions of both QS and DS, finding that the benefits of exofactor production were shared socially between cells (assumption 3 of Brown and Johnstone's QS model, and in contradiction to Redfield's DS hypothesis), but also that diffusion rates mattered (the fourth point of Redfield's DS argument, but also consistent with QS) [12]. Although these results were taken as strong support for the DS hypothesis, and against the QS hypothesis, they actually show that both are at play, with a social trait where diffusion matters. A study on quorum size in *Pseudomonas syringae* on leaf surfaces also suggests that both the social environment (cell density) and diffusion rate (water availability) matter [10].

Table 2. Examples of naturally arising 'cheats'[a]

Organism	Isolated from	Mutated QS genes	Refs
Pseudomonas aeruginosa	Trachea, urinary tract, wound	*lasR*	[49]
	Urinary tract infection	*rhlR*	[50]
	Pneumonia, bacteremia, swimming pools	*lasR*	[51]
	Cornea (microbial keratitis)	*lasR, lasI, rhlR*	[52,53]
	Wound, urinary and respiratory tract	*lasR, rhlR*	[54]
	Mechanically ventilated patients	*lasR, rhlR*	[32,55]
	Cystic fibrosis lung	*lasR, lasI, rhlI, pqs*	[56–58]
	Evolution selection experiments in test tubes	*lasR, pqsR*	[31,38]
Staphylococcus aureus	Blood specimens, urine samples, wounds	*agrA, agrC, agrB*	[59]
	Nasal cavities	*agrA, agrC*	[60]
	MRSA from blood, wound, sputum, nasal cavities	*agrA, agrC*	[61]
	Bacteremia	*agr* (+ or –)	[62]
Vibrio cholerae	Epidemic and environmental strains	*hapR*	[63]
Bacillus cereus	Laboratory strain collections	*plcR*	[64]

[a]The majority of strains isolated are mutants that do not respond to signal (*lasR*, *rhlR*, *pqsR*, *agrA*, *agrC*, and *plcR*), rather than not produce signal (*lasI*, *rhlI*, and *agrB*) or post-transcriptionally regulated genes involved in a QS response (*hapR*).

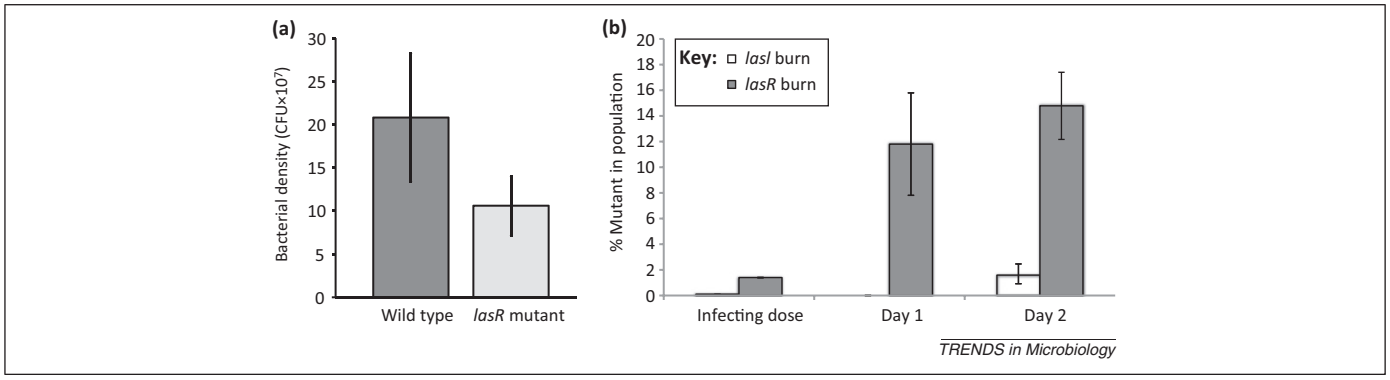

Figure 2. Testing whether quorum sensing (QS) is social in *Pseudomonas aeruginosa*, when infecting burn wounds of mice. **(a)** Infections initiated with the QS wild type grew to higher densities than those initiated with the *lasR* mutant that does not respond to a signal [39]. **(b)** When infections are initiated with a mixture of the QS wild type and either the *lasR* mutant that does not respond to signal, or the *lasI* mutant that does not produce signal, the mutant increases in frequency over time [33]. Taken together, these results show that QS provides a benefit at the group level, which can be exploited by cells that do not QS.

Second, data that support the DS hypothesis could naively be interpreted as posing a problem for the social QS hypothesis, and data supporting the QS hypothesis could naively be seen as posing a problem for the DS hypothesis. However, both of these conclusions would be incorrect: the existence of apples does not disprove the existence of oranges. For example, the observation that: (i) diffusion matters [10,12] does not mean that the production and response to autoinducers is not social; (ii) autoinducers control 'private goods' formation, such as nutrients from the internal metabolic breakdown of adenosine, and which provide purely direct (not social) benefits [3,11], does not mean that the exofactors produced in response to QS are not social; (iii) exofactors are social [12,30–33] or that population density matters [3] does not mean that diffusion does not matter. Overall, we suspect that it is highly likely that both the diffusion rate and the social environment influence the benefit of producing and responding to autoinducers (i.e., that both apples and oranges exist) [9,29,34,35].

Third, viewing DS and QS as competing hypotheses obscures the most important underlying questions. Specifically, rather than searching for predictions to differentiate between two potentially overlapping hypotheses, it is more useful to distinguish what the key questions are from a more general perspective.

A social trait?

From an evolutionary perspective, the most fundamental question regarding autoinducers and exofactors is whether they are social traits. Put simply, are the benefits of producing exofactors shared with other cells, or do they simply flow back to the cell that produced them? The traditional QS view rests upon the assumption that they are, whereas Redfield [7] pointed out that they need not be. Despite the fact that it is more than 10 years since Brown and Johnstone [4] clarified the key assumptions, and 10 years since Redfield [7] pointed out the need for these assumptions to be tested, there is still a shocking lack of empirical work addressing this question.

The first step in testing whether a microbial trait is social is to examine whether the relative costs and benefits of the trait vary with the social environment [26]. Specifically, if exofactors are shared socially between cells, then we predict that: (i) populations of cells that produce both autoinducers and exofactors (wild type) should grow better than populations of mutants that either do not produce or respond to autoinducers; (ii) when grown in mixed populations, mutants that either do not produce, or do not respond to autoinducers, should be able to exploit cells that do (i.e., they should be able to 'cheat'), and hence increase in frequency. If the benefit of exofactors flowed only to the cell that produced them, and was not social, we would make the first prediction, but not the second. Analogous predictions have been used to test whether exofactors not controlled by QS, such as siderophores, are social [36].

These predictions have been tested in two bacterial species: the opportunistic pathogen *Pseudomonas aeruginosa*, and the usually commensal, but sometimes pathogenic, *S. pneumoniae*. Data on these species have supported the QS hypothesis by showing that the production of autoinducers and exofactors are indeed social traits in test tubes [12,31,37,38] and, in *P. aeruginosa*, also during acute and chronic infections of mice [33,39] and in the lungs of mechanically ventilated patients [32,40]. In each case, QS mutants (cheats) were worse at growing in monoculture, but were able to exploit QS cooperators, and hence increase in frequency when grown in mixed cultures (Figure 2). In Box 2, we discuss some of the ways that this empirical work needs to be extended, especially to more natural systems.

QS versus DS versus ES?

Hense *et al.* [9] regarded DS and QS as "competing evolutionary hypotheses" and developed another hypothesis, which they termed efficiency sensing (ES), in an effort to provide unification. Hense *et al.* [9] made two arguments that are relevant here. First, that sensing occurs in a complex environment, where a multitude of factors could be important, such as density, diffusion, convection, decay, and degradation. We agree completely. From a semantic perspective, terms such as QS and DS emphasise single factors and can hence underplay the complexity of the real world. However, although the term ES might better represent this complexity, we think that the term QS is already in such widespread use that it is better to acknowledge the underlying complexity, rather than replace QS with ES [41].

Box 2. Extending the tests of sociality of QS

First, studies are required on a greater range of species. To date, empirical tests of whether the autoinducers and exofactors are social have been restricted to only two species, *P. aeruginosa* and *S. pneumoniae*, limiting the generality of any conclusions.

Second, there are several finer level predictions from social evolution and signalling theory that can be made and tested. For example: (i) the production of exofactors should provide a greater benefit at higher cell densities [3,28,65]; (ii) the production of exofactors should provide a lower (direct and indirect) benefit at higher rates of diffusion, convection, decay, degradation, etc. [7,9,12,66,67]; (iii) the relative fitness of 'cheats', compared with 'cooperators' who produce and respond to autoinducers, should be higher when the cooperators are more common [12,33,37,68]; (iv) cheats should have a lower relative fitness at lower cell densities [69], or in more viscous media [66], or when populations show greater spatial aggregation/clustering [9]; (v) Brown and Johnstone's [4] predictions could be tested with an experimental evolution approach, by varying any of the parameters involved in their four key assumptions, and testing how this influenced the relative fitness of mutants [37,39]; (vi) if mutants could be created with a more variable response, then we would predict that the extent of response would correlate with their growth rate in monoculture, relative fitness in mixed cultures (with other strains), and the growth of the other strain in mixed cultures [70]; (vii) can the distribution of natural mutants, that either do not produce or do not respond to autoinducers (Table 2), be explained by variation in the social environment?

Third, empirical studies are required in environments in which the species has evolved. Laboratory liquid cultures could lead to artificially high population densities that would give misleading results, and although mice and human lungs represent more natural environments than test tubes, they are not the primary environment for *P. aeruginosa*. As well as measuring the costs and benefits of traits in more natural environments, it is necessary to determine the structure of natural populations, such as relatedness, density, and degree of clustering [9,71].

A related issue here is that different methodologies have different uses. For example, controlled experiments in test tubes or 'engineered landscapes' are useful because they facilitate precise experimental control [3,72]. This can be especially useful for investigating mechanistic questions. By contrast, if we are interested in investigating the selective forces that have favoured traits, then it is key to carry out the experiments in as natural an environment as possible, even though this may reduce the possibility for experimental manipulation. Often the best approach will be to combine methodologies.

Fourth, much previous work is based on intentionally simplistic descriptions of the underlying biology. Both theoretical models and empirical studies need to incorporate the biological complexities of different systems (Box 3).

Hense *et al.'s* [9] second argument was that QS was dependent upon "group fitness benefits" and that DS depended only upon "individual fitness benefits". However, it can be misleading to dichotomise benefits at the levels of the group versus the individual, because natural selection does not dichotomise between these two extremes. The general result from evolutionary theory is that natural selection will lead to organisms that behave as if they are trying to maximise their inclusive fitness, which includes both direct consequences to the individual and indirect consequences to other members of the group, weighted by genetic relatedness (Box 1). This result holds for both social and non-social traits.

By contrast, we would only expect individuals to maximise individual (personal) or group fitness in extreme cases. Specifically, at one extreme, if relatedness equals zero ($r = 0$), then individuals do not value the fitness of the other members in their group because they are completely unrelated to them, and so would be selected to maximise their personal fitness [18]. Alternatively, at the other extreme, if relatedness (r) equals 1, then individuals are genetically identical, thus value equally their own fitness and that of the other members in their group, and are selected to maximise group fitness (as, e.g., is the case with the different cells in complex multicellular organisms such as animals) [27]. These two extreme cases do not contradict inclusive fitness theory – instead, it is that maximisation of inclusive fitness also leads to maximisation of personal fitness when $r = 0$, and maximisation of group fitness when $r = 1$ (Figure 3).

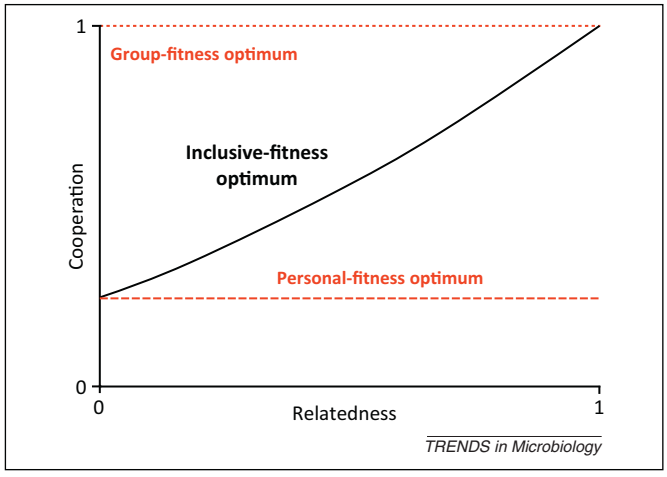

Figure 3. The individual and the group. Shown is the level of a cooperative trait, such as the amount of an exofactor produced, plotted against the relatedness (r) between interacting cells. The different lines show the optimal strategy from the perspective of an individual's personal fitness (broken red line), inclusive fitness (unbroken black line), and group fitness (dotted red line). Natural selection will lead to the evolutionarily stable (unbeatable) strategy being that which maximises inclusive fitness (i.e., the unbroken black line). We would only expect natural selection to lead to maximisation of personal or group fitness in extreme cases where $r = 0$ or 1, respectively.

The point here is that the general theory of adaptation is that individuals will be selected to maximise their inclusive fitness (Box 1), and that maximisation of personal or group fitness will only occur in extreme cases which cannot be expected to be generally applicable. Consequently, the distinction between QS and DS, and their unification with QS, does not link to how natural selection operates. Another way of looking at this is that QS theory makes no specific assumption about relatedness, which could therefore be anything from zero to unity or, in the absence of social interactions, undefined (equivalent to $r = 0$). Put simply, there is no gap between DS and QS to be occupied by hypotheses like ES. Furthermore, their verbal model of ES [9] corresponds to the QS scenario as previously modelled mathematically by Brown and Johnstone [4]. Consequently, the ES hypothesis could be considered a red herring.

Concluding remarks

To conclude, our main aim has been to show that treating QS and DS as competing hypotheses can lead to confusion and hinder progress. A major reason for this is that, in many cases, we would expect interactions between cells to

Box 3. Outstanding questions

- How can we explain the variation across species in the QS system? For example, why are different and/or multiple signals used in different species, and why do signals vary in factors such as diffusion rate, degradation rate, and production cost [2,29,43]?
- What are the evolutionary consequences of autoinducer molecules having multiple functions (such as immune modulators, iron scavenging molecules, or antibiotics) or being linked to other molecules via excretion in membrane vesicles [73]?
- What are the consequences of QS regulating a variety of traits, other than just simple exofactors, such as bioluminescence, conjugation, competence, sporulation, swarming, fruiting body formation, and intracellular traits [2,43]? How do the relative importance of the social environment and diffusion, or direct and indirect selection, vary across these traits? How would this alter the predictions given in Figure 1 in main text? Why does QS negatively control some traits, such as exopolysaccharide synthesis in the plant pathogen *Pantoea stewartii* subsp. stewartii [74]? Although there need not be one single explanation that applies to all traits controlled by autoinduction [34,41], is it possible to classify the uses of autoinduction into similar 'classes', such as enabling cells to 'produce public goods when this will be most efficient' or 'turn off virulence factors when they would cause too much damage', etc.?
- What are the consequences of cells being confined in small spaces for all or part of their life cycle, such as when *Staphylococcus aureus* is internalised within host endosomes, or when *P. syringae* is initiating aggregations on leaves [10,75]? Do interactions become social as soon as there is >1 cell within an area? Are exofactors shared more or less when cells are confined in small spaces? Do cells need to respond to variation in the extent of sociality within their life cycle? Does being in a biofilm change things [76]?
- Do interactions across species via autoinducer molecules represent cooperative signalling between species, that benefits individuals of both species, or individuals of one species exploiting the other [6]?

be social, but that diffusion would also matter. Consequently, to treat QS and DS as competing hypotheses would be incorrect, and hence force workers into conceptual errors. Empirical data supports the importance of both social interactions and diffusion rates, although this work is still in its infancy (Boxes 2 and 3). Furthermore, as well as DS and ES, many other hypotheses have been suggested to compete or unite with QS, ranging from positional sensing to cumulative gradient sensing [41]. These other hypotheses generally emphasise a single selective factor, that can be important in social interactions, and thus just as with DS, it is misleading to think of them as diametrically opposed competing hypotheses. Instead, it is more useful to emphasise that multiple factors are potentially at play, especially when interactions are social.

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