The evolution of bacterial cell differentiation and multicellular organization
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Chapter 9

Regulatory mechanisms link phenotypic plasticity to evolvability

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Organisms have a remarkable capacity to respond to environmental change. They can either respond directly, by means of phenotypic plasticity, or they can slowly adapt through evolution. Yet, how phenotypic plasticity links to evolutionary adaptability is largely unknown. Current studies of plasticity tend to adopt a phenomenological reaction norm (RN) approach, which neglects the mechanisms underlying plasticity. Focusing on a concrete question – the optimal timing of bacterial sporulation – we here also consider a mechanistic approach, the evolution of a gene regulatory network (GRN) underlying plasticity. The two approaches differ strikingly in their evolutionary outcome. Most importantly, the GRN model results in a much higher diversity of responsive strategies than the RN model. We show that each of the evolved strategies is pre-adapted to a unique set of unseen environmental conditions. The regulatory mechanisms that control plasticity therefore critically link phenotypic plasticity to the adaptive potential of biological populations.
Introduction

Life is adaptable. Organisms can slowly adapt by means of evolution or they can directly respond to changing environmental conditions (Botero et al. 2015). The capacity of an organism to express different phenotypes in response to the environment is referred to as phenotypic plasticity (DeWitt and Scheiner 2003). All organisms express plasticity. Yet, despite its prevalence, it often remains difficult to understand how plasticity evolved. First, one needs to have a good understanding of the possible response strategies. Which information can an individual obtain from the environment? What are the available response options? Second, one needs a firm understanding of how phenotypic changes are related to changes at the genetic level. How do mutations affect the plastic responsiveness of an individual? A question like this may be difficult to answer, since the genotype-to-phenotype mapping (i.e., the relation between an organism’s genotype and phenotype) is often very complex and largely unknown (Beldade et al. 2011; Ritchie et al. 2015). Third, individuals not only respond to the environment, they also shape their environment (Lewontin 2002). For example, many bacteria influence their environment by secreting products like antimicrobials, communicative signals and waste products (Davies et al. 1998; Hammer and Bassler 2003; Fajardo and Martinez 2008; Flemming and Wingender 2010). Moreover, in social interactions the phenotype of an individual typically influences the social environment to which it is exposed (McNamara and Weissing 2010; Berg and Weissing 2015). The dual role of the environment as both the cause and consequence of an individual’s phenotype makes it difficult to study the evolution of plasticity.

In this study, we examine the evolution of plasticity using a mechanistic model, in which we explicitly account for the genotype-to-phenotype mapping and the interaction between individuals and their environment, without making the model intractable. Our goal is to understand how a mechanistic implementation of plasticity affects the outcome of evolution. To this end, we focus on a specific system: bacterial sporulation. Bacterial sporulation is one of the best studied forms of plasticity (Fabret et al. 1999; Sonenshein 2000; Higgins and Dworkin 2012). Sporulation forms the ultimate survival strategy that is triggered when cells face harsh environmental conditions (Piggot and Hilbert 2004). Sporulation is costly, involving both time and energy, and results in the production of a metabolically inactive spore. Spores can survive long periods of environmental stress, such as starvation, desiccation and radiation. The sporulation process is controlled by a gene regulatory network that integrates multiple cues, such as starvation cues, communicative signals and physiological cues (Sonenshein 2000). Together these cues determine when and where a cell sporulates. This is particularly apparent in bacterial colonies, where cells – in response to their environment – trigger sporulation in specific regions of the colony (Veening et al. 2006; Vlamakis et al. 2008).

Using individual-based simulations, we study the evolution of sporulation. We compare two alternative implementations of the genotype-to-phenotype mapping: (1) a classical reaction norm approach and (2) a simple mechanistic implementation of a gene
regulatory network. Reaction norms give a phenomenological description of plasticity, by directly considering the response of an organism to its local environment. Reaction norms are a valuable tool to compare the plastic responses of different genotypes, but they largely ignore the regulatory mechanisms that underlie plasticity (Scheiner 1993; Schlichting and Pigliucci 1998; Murren et al. 2014). The question therefore arises whether, and to what extent, an explicit consideration of these mechanisms would affect predictions concerning the evolution of plasticity. To address this question, we compare a reaction norm approach to a more mechanistic gene regulatory network approach. In both model implementations, cells grow in colonies, where they interact with the environment by consuming nutrients and secreting products. Over time, colonies go through consecutive rounds of colony growth and dispersal. Spores are more likely to survive dispersal and thereby to found new colonies, yet spores cannot divide and therefore hamper colony growth. The fitness of a sporulation strategy is determined by the timing of sporulation: when a cell sporulates too early it forgoes cell division and when it sporulates too late it increases the chance to die when conditions are getting harsh. Cells can evolve the timing of sporulation by changing their responsiveness to the environment.

For each model implementation, we investigate if and how the cell’s responsiveness evolves by running 500 replicate simulations. In addition, we perform a detailed evaluation of the evolved responsive strategies, by examining their differences and by exposing each strategy to a set of novel environmental conditions.

**Structure of the model**

We model a population of cells that grow inside a colony. The colony is placed on a two-dimensional surface that contains nutrients (Figure 9.1A). Cells consume these nutrients, convert them to energy, and subsequently use this energy to divide. Hence, while colony growth progresses, nutrients get depleted. The colony grows for a fixed time period. At the end, cells can disperse and colonize a new nutrient surface, which forms the beginning of a new cycle of colony growth. We assume that cells only interact with their local environment. Thus, cells only consume nutrients from the grid element on which they are placed (see Material and Methods). These consumed nutrients are partly replenished by diffusion. We also assume that cells secrete a product into their local environment (Figure 9.1B). We do not specify the nature of this product, so it can be anything from a waste product to a quorum-sensing signal. Henceforth we will refer to this product as ‘signal’. During colony growth, cells can decide to allocate their energy to cell division or sporulation (Figure 9.1B). Sporulation requires time and energy and at end of sporulation a cell transforms into a spore. Spores are metabolically inactive and cannot divide, yet spores are ten times more likely to survive dispersal than cells. Thus, the fitness of a genotype is largely determined by the spores it produces by the end of colony growth, which – as explained above – depends on the timing of sporulation. In fact, spore production forms an accurate proxy of fitness (Figure S1), even though cell production also contributes weakly to the reproductive success of a colony.
Figure 9.1. Colony growth and cell behaviours. (A) Individuals divide and differentiate on a two-dimensional grid (blue, cells; red, spores). Cells sense the local environmental conditions, determined by the quadrant on which a cell is placed. Nutrients (green) and signal (not shown) diffuse in space. Colony growth occurs for a fixed number of time steps, after which all individuals can disperse. Spores are ten times more likely to survive dispersal than cells. (B) Cells can express three behaviours: (B1) cells can consume nutrients, which are converted to energy, and secrete molecular products in the local environment (called ‘signal’); (B2) cells with sufficient energy have a fixed probability to divide; (B3) cells can sporulate, which requires time and energy. Only cells that finish the sporulation process are more likely to survive dispersal than cells.

In both model implementations – the reaction norm (RN) model and the gene regulatory network (GRN) model – cells can trigger sporulation in response to three environmental cues (Figure 9.2): nutrient concentration (N), signal concentration (S) and energy level (E). Both the nutrient and signal concentration are sensed from the local environment of a cell, while the energy level is associated with the physiological state of a cell. In the RN model, these cues directly determine if a cell sporulates or not. Each cue is multiplied by a certain weighting factor and a cell sporulates when the sum of regulatory input exceeds the activation threshold (Figure 9.2A, see Material and Methods). The weighting factors (α) and activation threshold (θ) are heritable and subject to evolution. Every time a cell divides, these parameters are transmitted to the offspring, subject to rare mutations of small effect size. In the GRN model, sporulation is not triggered directly, but determined by the output of a gene regulatory network. The network consists of three layers: input layer, regulatory layer and output layer (Figure 9.2B, see Material and Methods). The cues are processed by the input layer of the GRN and can affect gene expression. We assume that gene expression is Boolean, so genes are either expressed or
Figure 9.2. The genotype-to-phenotype mapping. Two different implementations of the genotype-to-phenotype mapping are considered: (A) a reaction norm (RN) and (B) a gene regulatory network (GRN). (A) In the RN model the environmental cues directly determine if a cell sporulates or not, as shown by the inequality below the three-dimensional reaction norm. (B) In the GRN model the environmental cues affect gene expression. The GRN consists of three layers: input layer; regulatory layer and output layer. The input layer processes the three environmental cues. These cues subsequently affect the gene expression in the regulatory layer, which affect the expression of the gene in the output layer. Only when the ‘output’ gene is expressed a cell sporulates. A gene is expressed when the regulatory input exceeds the gene’s activation threshold.

not. The expression of a gene is determined by the regulatory input it receives and its activation threshold. The regulatory input depends on the connection weights in the GRN. When the sum of regulatory input exceeds the activation threshold a gene is expressed. When the gene in the output layer is expressed a cell sporulates. The connection weights and activation thresholds are heritable and subject to evolution. Every time a cell divides, they have a small probability to mutate.

In both models, cells evolve their responsiveness to the environment, either by changes in the RN or GRN. This responsiveness in turn determines the timing of sporulation. All simulations were initiated by a population of cells that could not sporulate – i.e. all evolvable variables were set to zero. The population could subsequently evolve for 400 consecutive colony growth cycles, to which we refer as ‘generations’.

Results

The evolution of plasticity in the Reaction Norm model
We first investigated the evolution of plasticity in the RN model. Figure 9.3 shows the number of cells and spores in the 500 replicate simulations over the course of 400 generations. Since at the onset of evolution cells are unable to sporulate, the colonies in the
first generation do not produce spores, with the exception of some mutants. Over evolutionary time, sporulation evolves and the number of spores that are present at the end of colony growth increases (Figure 9.3). At the same time the colony size decreases, because energy that is allocated to sporulation cannot be used for cell division. Despite the smaller colonies, the evolved genotypes have a higher fitness than their non-sporulating ancestors, because spores are more likely to survive dispersal than cells. The colony at generation 200 is characterized by two radial zones: the center and the edge. Spores mostly occur in the colony center, where nutrients are depleted first, while dividing cells occur at the colony edge. From generation 200 onwards, the colonies of most replicate simulations produce a constant number of spores until the end of evolution. Interestingly, when examining the distribution of spore production among the 500 replicate simulations, one can discriminate three phenotypic groups: colonies that produce a low (~600 spores), intermediate (~800) and high (~1,200) number of spores. Since the colonies

![Image of graphs showing cell and spore counts over generations](image)

**Figure 9.3. Evolution of sporulation in the RN model.** (left) Number of cells (blue) and spores (red) in 500 replicate simulations over the course of 400 generations. At each generation, cell and spore counts are collected at the end of colony growth. The black lines show the average number of cells and spores. (middle) Distributions of the number of cells and spores over the 500 replicate simulations at the end of evolution. (right) Colonies of the most productive genotype at generation 1, 200 and 400.
belong to separate replicate simulations, we hypothesized that three phenotypic groups belong to separate evolutionary trajectories, each trajectory leading to another level of spore production.

**DIVERSITY**
To check this, we had a closer look at the evolved reaction norms in generation 400. We compared the reaction norms of the 500 replicate simulations in a pairwise fashion by determining the fraction of conditions (N, S and E) for which two reaction norms would take different decisions: one reaction norm would sporulate, while the other would not. Using a hierarchical cluster analysis (see Material and Methods), the pairwise differences were converted into a phenogram, which illustrates the diversity of reaction norms present at the end of evolution (Figure 9.4A). Each dot is associated with a single evolved genotype and its associated reaction norm (corresponding to one simulation) and the branch lengths correspond to the differences between the reaction norms. The colors of the dots correspond to the productivity of the given genotypes.

The phenogram consists of four main branches (Figure 9.4A; numbered 1-4). The most productive genotypes all occur in the same branch (see label F in branch 4). The reaction norms and colonies that correspond to the tip of the branches are shown below the phenogram (Figure 9.4B; label 1-4). Figure 9.4B also shows the reaction norm and colony of the most productive genotype (label F; see also Figure S2). Interestingly, only the reaction norm of branch 3 is sensitive to all cues, the rest only responds to two of the three cues. The reaction norms of branch 1, 2 and 4 are insensitive to respectively the nutrient concentration, energy level and signal concentration. The reaction norm of the most productive genotype is insensitive to the signal concentration and mostly affected by the nutrient concentration: at low nutrient concentrations sporulation is triggered and spores were confined to the colony center. The genotypes that are associated with the other four reaction norms produce a low number of spores, as is apparent from their colonies, which are either small or show low sporulation efficiencies. The branched structure of the phenogram explains why evolution resulted in three more-or-less discrete phenotypic groups (Figure 9.3). Once a genotype belongs to a certain branch, it is expected to climb the local fitness gradient that is present in this branch. This genotype is unlikely to switch between branches, because this requires the accumulation of multiple mutations, many of which may be deleterious. Thus, genotypes follow alternative evolutionary trajectories that lead to different levels of spore production.

**The evolution of plasticity in the Gene Regulatory Network model**
Next, we investigated the evolution of plasticity in the GRN model. As for the RN model, sporulation evolved quickly and results in colonies with spores in the center and dividing cells at the edge (Figure S3). However, in contrast to the RN model (Figure 9.3), there are no clear groups differing in spore production at the end of evolution (Figure S3): most of the evolved colonies produce between 900 and 1,200 spores. Moreover, on average, the evolved genotypes in the GRN model produce more spores (median = 1,060, interquartile
range = [968; 1,173]) than those from the RN model (median = 849, interquartile range = [788; 936]). This is also the case when only comparing the 20 most productive genotypes, which produce approximately 250 spores more in the GRN model (mean and standard deviation = 1,491 ±66) than in the RN model (1,229 ±43).

Figure 9.4. The diversity in evolved reaction norms in the RN model. (A) Phenogram based on the distance between reaction norms of the most frequent genotypes in the 500 replicate simulations at the end of evolution. The distance between two reaction norms is given by the fraction of conditions at which they prescribe a different response. Colours indicate spore production of genotypes: low (red), intermediate (blue) and high (green). (B) The reaction norms and corresponding colonies that are associated with the tips of the branches in the phenogram (numbered 1–4) and the most productive genotype (F).
**INFORMATION PROCESSING AND CELLULAR MEMORY**

In the GRN model, the environmental cues do not directly determine the phenotype of a cell, but are first processed by the GRN. We use the mutual information metric (see Material and Methods) to quantify the extent to which the output of a network depends on a given input: when the mutual information that is associated with a network input is high, the output of the network is to a large extent determined by this input. Figure 9.5A shows for each network input – N, S and E – how, on average, the mutual information value increase over evolutionary time, especially within the first 200 generations. Thus, on average, the output of an evolved GRN depends on all network inputs.

In addition to the three cues, the output of a GRN can also be affected by the expression state of genes in the regulatory layer. Gene expression in the regulatory layer is inherited from one time step to the next and thereby forms an additional input to the network, to which we refer as the ‘expression background’. The expression background can be important for a cell’s decision. For example, suppose that one of the genes in the

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**Figure 9.5. Mutual information between network input and output in the GRN model.** (A) Average mutual information between network input – (1) nutrients (green area), (2) signal (blue area), (3) energy (red area), (4) expression background (grey area) – and network output (i.e. sporulation). The gene regulatory network on the left shows the relation between network input and output. The mutual information values were calculated for the most frequent genotype in each replicate simulation and averaged over all 500 replicate simulations. (B) Fraction of environmental conditions for which cells sporulate when having the expression background of a sporulating or a non-sporulating cell. The black area indicates the difference in the fraction of sporulation conditions between the two expression backgrounds.
regulatory layer strongly stimulates its own expression. Once this gene is expressed, it will continue to be expressed, even if the initial conditions that triggered its expression are absent. In fact, feedback interactions can lead to the inheritance of gene expression between generations, a phenomenon known as epigenetic inheritance through self-sustaining loops (Jablonka and Raz 2009). Figure 9.5A shows that the mutual information between the expression background and the network’s output increased over time (grey area). Thus, the evolved GRNs depend on the nutrient concentration, signal concentration, energy level and expression background.

Even though the mutual information shows that the expression background affects a cell’s decision to sporulate or not, it does not show how this decision is affected. We therefore determined how the expression background affects the fraction of conditions – possible combinations of N, S and E – for which a cell sporulates. Each evolved GRN was exposed to a large set of environmental conditions using different expression backgrounds: the expression background of a sporulating and a non-sporulating cell. The typical expression background of a sporulating and non-sporulating cell was determined separately for each evolved GRN (see Material and Methods). When cells have the expression background of a sporulating cell, they sporulate for a larger fraction of conditions than when they have the expression background of a non-sporulating cell (Figure 9.5B). Thus, cells that sporulate change their sensitivity to the environment such that they are more likely to continue sporulation in the presence of small environmental perturbations. In this way, no time or energy is wasted on failed sporulation attempts.

DIVERSITY

Above, we have focused on the average properties of the evolved GRNs; now, we explore the individual properties. To this end, the reaction norm corresponding to each evolved GRN was determined by exposing the GRN to all possible combinations of N, S and E (see Material and Methods). Figure 9.6 shows the phenogram based on these reaction norms, and the reaction norms that are associated with the twenty most productive GRNs at the end of evolution. In contrast to the RN model (Figure 9.4), the phenogram of the GRN model shows a much higher diversity in the evolved reaction norms. Even the twenty most productive genotypes, which produce approximately the same number of spores (Figure S4), differ considerably in their reaction norms: some genotypes only sporulate for a small set of conditions (e.g. genotype 15), while others sporulate for the majority of conditions (e.g. genotype 18). Moreover, while some genotypes are insensitive to a certain environmental cue (e.g. genotype 7 is insensitive to the amount of signal), others almost entirely base their decision to differentiate on that same cue (e.g. genotype 12 strongly depends on the amount of signal for its decision to sporulate).

Notice that the reaction norm of a GRN can only be determined for a given expression background. Figure S5 shows that the reaction norms of some evolved GRNs change substantially with a change in expression background. While the reaction norm of some genotypes is insensitive to the expression background (e.g. genotype 17), other genotypes are strongly affected (e.g. genotype 15). The diversity in GRNs is also apparent by
A Phenogram

Number of spores

LOW  HIGH

B Reaction norms of twenty most productive genotypes

1 Sporulation

Energy

Nutrients  Signal

2

3

4

5

6

7

8

9

10

11

12

13

14

15

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17

18

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20
comparing the mutual information values of individual GRNs (Text S1 and Figure S6). Thus, in conclusion, evolution in the GRN model results in a remarkably diverse set of responsive strategies. Even the twenty most productive genotypes are scattered throughout nearly the entire phenogram. This strongly contrasts the twenty fittest genotypes in the RN model, which cluster together in the phenogram and look nearly identical (Figure S2).

**INFORMATIONAL REDUNDANCY AND IRRELEVANT PARTS OF THE REACTION NORMS**

How can it be that GRNs produce similar colonies, with nearly the same number of spores, while having such different reaction norms? First, the GRNs might respond to different cues, but still sporulate at approximately the same time, thereby forming similar colonies. In other words, the same response pattern might be based on different sources of information. Second, reaction norms might mainly differ for conditions that are only rarely encountered during colony growth. The diversity in reaction norms might therefore result from the accumulation of mutations that are effectively neutral, since they only affect irrelevant parts of the reaction norm. Before now scrutinizing these potential causes of diversity one by one, we would like to stress that they are not mutually exclusive.

As illustrated in Figures S3 and S4, evolved colonies are characterized by two radial zones: the center (the ‘spore zone’) and the edge (the ‘dividing zone’). Evidently, cells in the dividing zone experience other conditions than cells in the spore zone (Figure 9.7A). Nutrients are abundant at the colony edge and gradually decrease in abundance towards the center. Signal is produced by dividing cells at the colony edge, but not by the spores. Consequently, there is a peak in the signal concentration that reaches its maximum in the transition between both zones. Cells in the dividing zone show a wide variety of energy levels, while the spores in the colony center are depleted of energy. Given their strong gradients, both the nutrient and the signal concentration can be used by cells to determine their position in the colony. In addition, there is a strong negative correlation between the nutrient and signal concentration in the dividing zone. Thus, the nutrient and signal concentration give redundant information about each other and the location of cells inside the colony. As a consequence, evolved GRNs can respond to different cues, but still express the same behavior: a GRN that is sensitive to the nutrient concentration can extract essentially the same environmental information as a GRN that is sensitive to the signal concentration.

**Figure 9.6.** (Left). Diversity in reaction norms in the GRN model. (A) Phenogram based on the distance between reaction norms of the most frequent genotypes in the 500 replicate simulations at the end of evolution. The distance between two reaction norms is given by the fraction of conditions at which they prescribe a different response. Colours indicate spore production of genotypes: low (red), intermediate (blue) and high (green). The twenty most productive genotypes are shown by larger dots. (B) The reaction norms associated with the twenty most productive genotypes ranked from the genotype that produces the largest number of spores (1) to the one that produces the smallest number of spores (20). For the twenty most productive reaction norms in the RN model see Figure S2.
The environmental gradients also make it evident that cells only experience a small subset of conditions. Figure 9.7B shows the conditions cells experience before (grey volume) and at (green volume) the onset of sporulation in the space of environmental conditions that is also used for the reaction norms in Figure 9.6. Cells clearly experience only a limited subset of conditions within the colony. Neutral mutations that alter the reaction norms for conditions that cells rarely or never encounter can increase the diversity of responsive strategies, without having an immediate effect on the phenotypes that are expressed. This, together with the informational redundancy between environmental cues, can explain the high diversity of responsive strategies that evolved among the twenty most productive genotypes in the GRN model (Figure 9.6).

**Figure 9.7. Environmental cues and informational redundancy in the GRN model.** The environmental conditions in ten replicate colonies of the most productive genotype (see Figures S3 and S4). (A) Environmental cues sensed by cells as a function of their distance to the colony center: N = nutrients (green), S = signal (blue), E = energy (red). Each dot corresponds to the conditions sensed by a single cell. The colonies are divided in two zones: spore zone and dividing zone. (B) Subset of conditions that cells sense before (grey volume) and at the onset of sporulation (green volume). The red dots correspond to the average conditions that cells experience at onset of sporulation in the ten replicate colonies.
Pre-adaptation to novel environments

Even though the diverse responsive strategies that evolved behave nearly identical in the environmental conditions that they encountered during evolution, they might behave differently under novel environmental conditions, which expose previously hidden parts of the reaction norms. To examine how the evolved genotypes respond to unseen environmental conditions, we exposed the twenty most productive genotypes from both the RN and GRN model to 250 randomly generated novel environments.

Before looking at the overall picture, we first will focus on five environments that were generated by changing one parameter only: the signal degradation rate. In contrast to nutrients and energy, signal is not required for the sporulation process, but the local signal concentration can be used by cells to time the onset of sporulation. Thus, by varying the signal degradation rate, the environment that cells perceive is altered, but the selection pressures on the timing of sporulation remain the same (Text S2 and Figure S7). Figure 9.8A shows colonies of the most productive genotype from the GRN model at different signal degradation rates. In comparison to the signal degradation rate at which genotypes evolved (δ = 0.1), the spore production of the twenty most productive genotypes of the GRN model varies strongly with the new environment (Figure 9.8BCD): whereas some genotypes robustly produce the same high number of spores under all signal degradation rates, others produce much fewer spores when encountering a new signal degradation rate. On average, genotypes tend to postpone sporulation at high signal degradation rates and advance sporulation at low signal degradation rates (e.g. Figure 9.8A). High signal degradation rates result in low signal concentrations, which cells associate with high nutrient concentrations (see the negative correlation between the signal and nutrient concentration in the dividing zone of Figure 9.7A). By the same token, low signal degradation rates result in high signal concentrations, which cells associate with low nutrient concentrations. Thus, by changing the signal degradation rate, cells get the ‘illusion’ that less or more nutrients are present in the environment and thereby falsely advance or postpone sporulation, which goes at the expense of spore production (Figure 9.8BCD; see also Text S3 and Figure S8). The most productive genotypes of the RN model are largely insensitive to the signal concentration (Figure S2). Accordingly, these genotypes are hardly affected by a change in the signal degradation rate (Figure 9.8EFG).

Let us now consider the performance of the twenty most productive RN and GRN genotypes when exposed to 250 novel environments, which were generated by randomly varying seven parameters of our model (Table S2). In each environment, we determined the relative and absolute spore production of a genotype by growing ten replicate colonies. To evaluate the responses of the evolved genotypes over the 250 environmental conditions, we performed a cluster analysis (see Material and Methods). Genotypes were clustered with respect to their relative spore production over the 250 environments and environments were clustered with respect to the absolute spore production of the 40 genotypes. Genotypes that appear close in the cluster analysis have approximately the same relative spore production – i.e. relative fitness with respect to the other genotypes –
Figure 9.8. Colonies and the signal degradation rate in the RN and GRN model. For each of the twenty most productive genotypes (see Figure 9.6B for their reaction norms), 10 replicate colonies are grown at five signal degradation rates (δ): 0.025, 0.05, 0.1, 0.2 and 0.4. Cells evolved at a signal degradation rate of 0.1. (A) Colonies of the most productive genotype in the GRN model at different signal degradation rate (blue = cells and red = spores). (B) Average spore production of twenty most productive genotypes over the different signal degradation rates. (C) Fraction of cells that failed to sporulate (i.e. having insufficient energy to sporulate). (D) Average nutrient concentration at which cells initiate sporulation. The lowest row of graphs show (E) the number of spores, (F) fraction of failed sporulation attempts and (G) average nutrient concentration at onset of sporulation for the twenty most productive genotypes of the RN model.
Figure 9.9. Spore production of RNs and GRNs in randomly-generated novel environments. The twenty most productive genotypes of the RN model (grey circles) and GRN model (black circles) were exposed to 245 randomly-generated novel environments (Table S2). For each environment ten replicate colonies were grown per genotype and the average number of spores at the end of colony growth was used for the cluster analysis (see Material and Methods). The colours indicate the relative spore production of genotypes in each novel environment: red = relative low spore production, green = relative high spore production. Histograms on the right show the distribution of relative spore production for the genotypes. Graph on the bottom shows range of absolute spore production in RN model (light grey area) and GRN model (dark grey area) over all 245 environments. The two bars at the bottom compare genotypes from the RN and GRN model directly, by showing where the most productive genotype comes from (grey = a genotype from RN model, black = a genotype from GRN model) and which set of genotypes produce most spores on average (grey = genotypes from RN model, black = genotypes from GRN model).
in all novel environments. Environments that cluster together have a similar effect on the absolute spore production of all genotypes. Figure 9.9 shows that the relative spore production of each genotype varies strongly between the novel environments (see histograms on the right of the cluster diagram in Figure 9.9). Moreover, the differences in spore production between genotypes from the GRN model are much more pronounced than the differences between genotypes from the RN model. Thus, the higher diversity of responsive strategies in the GRN model (Figures 9.4 and 9.6) is translated to a higher diversity in spore production among the tested novel environments (Figures 9.8 and 9.9). Even though there are many novel environments for which genotypes from the RN model on average perform better than the genotypes from the GRN model, the best performing genotypes nearly always come from the GRN model. In the GRN model, each genotype has a distinct profile along the 250 novel environments. In other words, each responsive strategy is pre-adapted to unique set of environmental conditions. Some genotypes perform relatively well under most novel environments, but never have the highest relative spore production (e.g. genotype 10 from the GRN model). Others perform very well in some environments, but badly in others (e.g. genotype 1 from the GRN model). Importantly, none of the genotypes performs best in all environmental conditions. The diversity in responsive strategy is therefore important for the capacity of a population to cope with many potential future environments.

**Discussion**

Plasticity is prominent in all forms of life, but it often remains hard to explain how the mechanisms underlying plasticity evolved. We examined the evolution of plasticity in the context of bacterial sporulation by comparing two alternative model implementations: a classical reaction norm (RN) model and a more mechanistic gene regulatory network (GRN) model. Even though the GRN is inspired on real signal transduction cascades, it still strongly simplifies the regulatory interactions that underlie sporulation (Sonenshein 2000). In both model implementations, cells rapidly evolved the capacity to sense, interpret and respond to the environment by triggering sporulation. The evolved GRNs typically produce more spores than the evolved RNs, in part because many RNs got stuck on local fitness peaks. In the RN model, three distinct evolutionary trajectories were apparent, each making use of different combinations of environmental cues. Apparently, switching from one trajectory to another one is unlikely, because gaining sensitivity to a new cue will typically perturb the current responsiveness of a cell. Such constraints play a minor role in the GRN model, where regulatory redundancy in the GRN allows cells to evolve sensitivity to novel environmental cues without compromising the already existing response to the environment. Each gene in the regulatory layer can process all environmental cues and is therefore fully redundant with respect to the others. Such functional redundancy allows cells to accumulate mutations that do not immediately affect their phenotype (i.e. neutral mutations), but can form a gateway to new adaptive
mutations. This form of mutational robustness has been suggested to facilitate the evolvability for a number of biological properties (e.g. RNA molecules, proteins, metabolic networks) (Wagner 2005; Wagner 2007; Ciliberti et al. 2007; Tokuriki and Tawfik 2009; Soskine and Tawfik 2010; Hayden et al. 2011).

The evolved RNs and GRNs not only differ in their capacity to process direct environmental information, they also differed in their capacity to transmit epigenetic information. Only in the GRN model positive feedback interactions could evolve that allow for the transmission of epigenetic information (Jablonska and Lamb 2005; Alon 2007; Jablonska and Raz 2009). This information is used by cells to adjust their sensitivity to the environment such that they are more likely to continue the sporulation process once initiated. Sporulation is a costly process; switching back and forth is therefore a waste of time and energy. In the absence of epigenetic feedback, small environmental perturbations during sporulation are sufficient to stop the sporulation process. The epigenetic switch that evolved in the GRN model makes the sporulation process largely irreversible and prevents cells from prematurely stopping sporulation (Kuchina et al. 2011). Similar feedback mechanisms also underlie other forms of terminal cell differentiation (Hemberger et al. 2009; Gilbert 2014). We hypothesize that, quite generally, epigenetic inheritance through self-sustaining loops will be an adaptive strategy when switching back and forth between states is costly: when organisms encounter environmental ‘borderline’ conditions, epigenetics can prevent them from responding in a too sensitive manner to environmental fluctuations, which could otherwise induce repeated switches between states.

A particularly striking difference between the RN and GRN model is the much higher diversity of responsive strategies in the GRN model. Two factors contributed the evolution of this diversity: (i) informational redundancy and (ii) the limited set of conditions cells experience in the colony. Two cues provide redundant information, when the value of one cue can be inferred from the other, which is the case when cues strongly correlate. In our model, the nutrient and signal concentration strongly co-varied in the dividing zone (see Figure 9.7). In the presence of informational redundancy, cues become interchangeable, which allows for the evolution of various responsive strategies that express the same adaptive phenotype, but respond to different environmental cues (Perkins and Swain 2009; Bowsher and Swain 2014). The second factor that underlies the diversity in response strategies is the small set of conditions cells encounter inside the colony. Since cells only sense a small number of conditions, only a part of their reaction norm gets exposed, which allows for the accumulation of neutral mutations elsewhere in the reaction norm (i.e. the so-called ‘hidden reaction norm’; Schlichting 2008). A large fraction of the diversity in responsive strategies remains hidden under the conditions in which organisms evolved. Yet, under novel conditions, this diversity gets exposed (Rutherford 2000; Ghalambor et al. 2007; Le Rouzic and Carlborg 2008; Schlichting 2008). We showed that each responsive strategies is pre-adapted to a specific set of potential future environments (Masel and Siegal 2009). The capacity of a population to cope with environmental change therefore strongly depends on the diversity of responsive strategies that is present in the population (Rutherford 2000; West-Eberhard 2003; Gibson and

In our analysis, we focused on the most frequent genotypes that evolved in different replicate simulations. We therefore neglected the stable coexistence of genotypes, while previous studies have shown that the coexistence of responsive strategies can be a likely outcome, especially when individuals engage in social interactions (Botero et al. 2010; Wolf and Weissing 2010; van Gestel et al. 2012). In our model, such coexistence is of marginal importance, since populations repeatedly experience severe bottlenecks during the dispersal phase. We suspect that, in model implementations with a larger effective population size, a variety of genotypes with similar fitness will coexist, in particular if their responsive strategies are somewhat complementary (Wolf and Weissing 2010). Under these conditions, we therefore expect a higher degree of within-population diversity in the GRN model than in the RN model, resulting in a higher adaptive capacity when encountering novel environmental challenges.

Material and Methods

Model

Colony growth
Cells are placed on a continuous two-dimensional surface with fixed boundaries, which is placed on top of a discrete grid (the model therefore combines continuous and discrete spacing; see Kreft et al. 1998). The grid consists of 200 by 200 equally-sized elements. The radius of a cell is smaller than that of one grid element (r_{cell} = 0.8). The grid element on which a cell is placed forms its local environment. The nutrients and signal that are associated with this grid element can be sensed by the cell. In addition, any exchange in the form of nutrient consumption or signal production occurs through the local environment. Cells consume the locally available nutrients with a fixed rate, V (see Table S1 for model parameters). Nutrients (N) are converted into energy (E); assuming that one unit of nutrients is converted in one unit of energy. When the energy level of a cell passes the threshold level E_d, the cell divides with a fixed probability P_d. If cell division occurs, the energy necessary for cell division is consumed (E_d) and left-over energy is divided equally among the two daughter cells. One of the daughter cells takes in the position of the mother cell and the other one is placed in a random direction next to it, thereby potentially pushing aside neighboring cells that were already there. Cell pushing is an iterative process, in which cells are randomly selected and examined for their overlap with neighboring cells. If a cell overlaps with its neighbor it is moved such that the overlap between cells disappears (for growth dynamics of colony see van Gestel et al. 2014).

At the onset of colony growth 100 cells are placed in the spatial center of the surface. Each grid element on the surface is supplied with the same nutrient concentration, N_{init}.
During colony growth, cells consume these nutrients, resulting in a gradient with a high nutrient concentration at the colony edge and a low nutrients concentration in the colony center. Nutrients diffuse with a given rate, $D_N$. Hence, the nutrient gradient results in a net flux of nutrients from the colony edge to the center. Like nutrients, signal produced by the cells can also diffuse ($D_S = D_N$, with the exception of Figure 9.9). Signal is produced by cells before the onset of sporulation and is secreted in the local environment (Figure 9.1). In addition, signal is degraded with a given degradation rate, $\delta$.

A colony can grow for a fixed number of time steps ($t_{colony}$). After this, cells and spores migrate. We assume that spores are ten times more likely to survive migration than cells. From the individuals that survive migration 100 individuals are randomly chosen for the onset of the next growth cycle.

**Sporulation**

We assume that sporulation requires time ($t_{spore}$) and energy. At every time step during sporulation, cells consume a fixed amount of energy ($E_\delta$). When cells have insufficient energy, the sporulation process is stopped. Sporulation can also be prematurely stopped when a cell reverses its sporulation decision, which could follow from a change in the environmental conditions. There are no consequences of such a premature stop, aside from the time and energy that already has been wasted on sporulation. During sporulation a cell continues to consume nutrients, so a part of the energy that is consumed during sporulation gets immediately replenished. When sporulation finishes, a cell is transformed into a mature spore. Mature spores do not consume nutrients and cannot divide, but they have a higher probability to survive dispersal than cells. In addition, we assume that spores do not respond to the environment. At the onset of a new growth cycles, spores are assumed to germinate and transform back to dividing cells. Thus, all cells are phenotypically identical at the onset of colony growth.

**REACTION NORM MODEL**

In the RN model, the cues – nutrient concentration (N), signal concentration (S) and energy level (E) – directly determine whether or not a cell sporulates (Figure 9.2A). A cell sporulates when $\alpha_N \cdot N + \alpha_S \cdot S + \alpha_E \cdot E > \theta$, where $\theta$ is the cell’s activation threshold while $\alpha_N$, $\alpha_S$, $\alpha_E$ are the cell’s weighing factors for the corresponding cues. A weighing factor can take on positive and negative values: in case of a positive value, the corresponding cue stimulates sporulation, while it inhibits sporulation in case of a negative value. When the sum of regulatory inputs exceeds the activation threshold, $\theta$, a cell triggers or continues sporulation. The three weighing factors and the activation threshold are encoded by four heritable loci, which evolve under the influence of mutation and selection. At the onset of evolution, we assume that all weighting factors and the activation threshold are 0, hence there is no sporulation. At every cell division, each locus can mutate with probability $\mu$. When a mutation occurs, a value is added to the connection weight or activation threshold; this mutational step size is drawn from a normal distribution with mean 0 and standard deviation $\sigma$. 


GENE REGULATORY NETWORK MODEL
We consider a GRN that consists of three layers (see Figure 9.2B): (i) input layer, (ii) regulatory layer, (iii) output layer. The input layer processes the three cues that cell can sense: N, S and E. The regulatory layer consists of three genes that encode for products that can affect one or several genes in the regulatory or output layer. The output layer consists of one effector gene that determines whether a cell sporulates or not. Genes have a Boolean expression: a gene is either expressed or not, \( G \in \{0,1\} \) (for alternative implementations of the GRN model see Text S3). A gene is expressed \( (G = 1) \) when the sum of inputs it receives is higher than the gene’s activation threshold \( (\theta) \). A gene in the regulatory layer can receive inputs from both the input and regulatory layer, while the gene in the output layer can only receive inputs from the regulatory layer. The input a gene receives is weighted by the connection weights, \( w_i \). For example, in case a gene is affected by the nutrient concentration (connection weight \( w_1 \)) and the expression of a gene in the regulatory layer (connection weight \( w_2 \)), the input towards the gene is determined by: \( w_1 \cdot N + w_2 \cdot G \), where \( N \) is the nutrient concentration and \( G \) is the expression state of a regulatory gene. This gene would therefore be expressed if \( w_1 \cdot N + w_2 \cdot G > \theta \).

Connection weights and activation thresholds are real-numbered values that can be negative or positive. When a connection weight is negative (positive) gene expression is inhibited (stimulated). When the activation threshold of a gene is negative, a gene is expressed by default and its expression can only be prevented through inhibition. In total, the network contains 21 connection weights (9 from the input to the regulatory layer; 9 between genes in the regulatory layer and 3 from the regulatory to the output layer) and 4 activation thresholds (one for each gene). All the connection weights and activation thresholds can mutate, therefore a genotype consists of 25 evolvable loci. At the onset of evolution, we assume that all connection weights and activation thresholds are 0. At every cell division, each locus can mutate with probability \( \mu \). When a mutation occurs, a value is added to the connection weight or activation threshold that is taken from a normal distribution with mean 0 and standard deviation \( \sigma \).

Analysis

Mutual information
Different response strategies could evolve in both the RN and GRN model. In the RN model the response strategies can easily be determined by examining the weighing factors and activation threshold. In contrast, for the GRNs it is very difficult to determine the response strategy by solely examining the connection weights and activation thresholds. Instead, one can make use of the concept of ‘mutual information’ [24] to understand how a GRN processes environmental information (Figures 9.5 and S6). Consider (i) a probability distribution \( p \) over the set of input states \( I \), where \( p_i \) is the probability of input \( i \); (ii) a probability distribution \( q \) over the set of output states \( O \), where \( q_j \) is the probability of output \( j \); and (iii) the joint distribution \( P \) describing the probability \( P_{ij} \) that
output \( j \) co-occurs with input \( i \). If inputs and outputs were statistically independent \( (P_{ij} = p_{i}q_{j}) \), knowing the input would not provide any information about the output and vice versa. If \( P_{ij} \neq p_{i}q_{j} \), knowing the input does provide information on the output. Mutual information, which is defined as

\[
M = \sum_{i \in I} \sum_{j \in O} P_{ij} \log_2 \left( \frac{P_{ij}}{p_{i}q_{j}} \right),
\]

measures the information that inputs and output share, that is, it measures how much knowing the input (resp. the output) reduces uncertainty about the output (resp. the input).

In order to calculate the mutual information for our GRNs, we first discretized the network inputs by dividing the range of possible input values into 50 equally-sized intervals, yielding the input set \( I = \{1,2,\ldots,50\} \). The output set is already discrete: \( O = \{0,1\} \) (i.e. sporulation or not). Since the input set is larger than the output set, the maximum information content of inputs (\( \log_2 (50) = 5.64 \) bits) is larger than the information content of outputs (\( \log_2 (2) = 1 \) bit). For that reason, the mutual information values are more likely to be constrained by the output of a GRN than by the network’s inputs. Besides the three cues that are processed by the input layer of a GRN (N, S and E), the genetic background of a GRN could also be viewed as an input. There are three regulatory genes, allowing for 8 possible expression backgrounds. The information content of the expression background (\( \log_2 (8) = 3 \) bits) is therefore lower than that of the three cues.

**Expression background**

For Figures 9.5 and S5, we evaluate the responses of GRNs with different expression backgrounds. We discriminated between the expression background of a sporulating and non-sporulating cell. These backgrounds were acquired for each genotype separately, by evaluating the gene expression of cells at the end of colony growth. The expression background (i.e. gene expression in the regulatory layer) of the majority of dividing cells at the end of colony growth was considered to be the typical expression background of a non-sporulating cell. By the same token, the expression background of sporulating cells at the end of colony growth was considered the typical expression background of a sporulating cell. These expression backgrounds were subsequently used to evaluate the performance of a given GRN. In Figure 9.5 we evaluated how the fraction of conditions at which a GRN would sporulate depends on the expression background. In Figure S5, we evaluated how the reaction norm generated by a GRN depends on the expression background.

**Reaction norms and phenograms**

In order to assess the diversity of reaction norms that evolved in the 500 replicate simulations, we constructed phenograms. This was done for both the RN model (Figure 9.4) and the GNR model (Figure 9.6). The phenograms were based on the reaction norms that were associated with the most frequent genotypes present in the 500 replicate simu-
tions at the end of evolution. In case of the GRN model, the reaction norm was determined by exposing each evolved GRN to all possible combinations of network inputs – nutrient concentration \(N = [0, 10]\), signal concentration \(S = [0, 10]\) and energy level \(E = [0, 15]\) – and determining if the GRN would sporulate or not. Since feedback interactions in the regulatory layer of a GRN can result in cyclic changes in gene expression, it would be misleading to evaluate the GRNs at one specific time point. Instead, GRNs were updated 2 to 5 times when evaluating their response to the environment. Finally, one should note that is not possible to associate a unique reaction norm to each GRN, because the GRN’s response to the environment also depends on the expression background of a cell (i.e. gene expression in the regulatory layer). For the reaction norms evaluated in Figure 9.6, we simply assume that none of the regulatory genes were expressed before exposure to the environment.

For each pairwise combination of reaction norms, we determined the fraction of conditions at which their associated reaction norms would give a different outcome. This distance measure ranges from 0 (i.e. identical reaction norms) to 1 (i.e. opposite reaction norms). All pairwise differences were combined into a distance matrix, a symmetric matrix with zeros on its diagonal. A neighbor-joining algorithm was used to convert the distance matrix into a phenogram: a tree-like diagram that visualizes the phenotypic differences between the evolved genotypes (using the ‘ape’ library in R version 3.1.1).

**Cluster analysis**

The cluster analysis of Figure 9.9 is based on a different distance measure than the phenograms shown in Figures 9.4 and 9.6. The distance between genotypes was measured in terms of relative spore production among the 250 novel environments. The relative spore production of the genotypes was determined for each environment separately (e.g. does a genotype produces the most or the fewest spores in a certain environment?) and compared between the genotypes over all novel environments. The distance between environments was measured in terms of absolute spore production of the 40 genotypes. The distance matrix of both the genotypes and environments were converted in cluster diagrams using a standard hierarchical clustering algorithm (using the ‘ape’ library in R version 3.1.1).

**Acknowledgements**

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SUPPLEMENTARY INFORMATION

Text S1. Mutual information of evolved GRNs

In Figure 9.5 of the main text, we showed that the evolution of sporulation is associated with an increase in the mutual information values between the network inputs and output. The mutual information values were *averaged* over the most frequent genotypes from the 500 replicate simulations. However, when comparing the reaction norms that were associated with the most frequent (phenogram in Figure 9.6A) and most productive genotypes (reaction norms shown in Figure 9.6B) at the end of evolution, a high diversity of responsive strategies was apparent. One might therefore expect that the same degree of diversity is probably also present among the mutual information values that are associated with the *individual* GRNs. In this section, we therefore evaluated the mutual information values of all individual GRNs. We first collected the most frequent genotypes in the 500 replicate simulations at intervals of 5 generations over the entire course of evolution (from generation 1 till 400); therefore including one genotype per replicate simulation per examined generation. For every genotype we determined the mutual information values associated with the four network inputs: nutrient concentration, signal concentration, energy level and expression background. This data was subsequently used for a principal component analysis.

Before examining the outcome of our principal component analysis, there are a few expectations that we can outline in advance. First, we know that the space of possible mutual information values is constrained. The mutual information can never be higher than the amount of information (i.e. entropy) in a network’s output (see Material and Methods). Low mutual information values could indicate that a GRN sporulates for a small minority or large majority of conditions and high mutual information values indicate that a GRN sporulates for an intermediate number of conditions. Second, we know that there are tradeoffs between the mutual information values of different inputs: a genotype cannot have high mutual information values for two environmental cues simultaneously. When a network’s output depends on multiple cues, it will decrease the mutual information values that are associated with each one of them. Thus, low mutual information values can indicate that the evolved GRN is insensitive to the environment or that it is sensitive to many independent cues. In contrast, high mutual information values indicate that a GRN predominantly responses to one cue only.

Figure S6 shows the outcome of the PCA. Each data point corresponds to a single genotype and the colours indicate the productivity of the genotypes in terms of spore production. Figure S6A shows the most frequent genotypes at generation 1, 100, 200, 300 and 400 in the PCA plot. The arrows indicate how the mutual information values are projected on the first two principal components of the PCA. At the onset of evolution (generation = 1), none of the most frequent GRNs sporulates, therefore all having mutual information values of zero. Afterwards, some GRNs evolve the capacity to sporulate and occur predominantly occur at the edges of the PCA plot, indicating that there are high
mutual information values between the network inputs and output in these GRNs. Towards the end of evolution, more and more GRNs evolve towards the center of the PCA plot, which suggests that the GRNs respond to multiple environmental cues or sporulate for a small fraction of conditions. When examining all genotypes analyzed in the PCA (Figure S6B), it becomes apparent that genotypes that produce an intermediate number of spores predominantly occur at the edge of the PCA and genotypes that produce either many or a few spores occur closer to the center of the PCA analysis (Figure S6C). This confirms the above trend (Figure S6A) that over the course of evolution, genotypes first evolve a strong dependency towards one cue for triggering sporulation (resulting in a high mutual information value), but over time integrate information from multiple cues. In addition, the low mutual information values of the most productive genotypes indicate that GRNs decrease the number of conditions for which they sporulate relative to other sporulating genotypes. Figure S6D indeed confirms this view, by showing that the genotypes with an intermediate fitness sporulate for the largest fraction of conditions (this fraction is determined from their associated reaction norms, in which each genotype is evaluated with respect to all possible environmental conditions).

Overall, two important conclusions can be drawn based on the PCA on mutual information values. First, as indicated by the reaction norms (Figure 9.6), evolved genotypes strongly differ in their sensitivity to environmental cues. Even when having approximately the same spore production, like the twenty most productive genotypes, the sensitivity to a given environmental cue can vary strongly. Second, even though the average mutual information values increase over evolutionary time (Figure 9.5A), the most productive genotypes actually show reduced levels of mutual information (Figure S6). This results from the smaller number of conditions at which the most productive genotypes sporulate and their sensitivity to multiple environmental cues.

**Text S2. Timing of sporulation**

Sporulation requires both time and energy. In order to time the onset of sporulation, cells have to account for both the energy level and nutrient concentration. When cells have insufficient energy the sporulation process is stopped. The amount of energy that cells need to store before the onset of sporulation depends on the nutrient concentration. Cells continue to consume nutrients during the sporulation process. A fraction of the energy that is required for sporulation is therefore directly provided by the environment during sporulation. The signal concentration has no influence on the optimal timing of sporulation, because signal is not required nor consumed during the sporulation process.

Figure S7 shows the average energy level and nutrient concentration at the onset of sporulation for the most-abundant genotypes in 500 replicate simulations. The colors indicate the spore production of the genotypes: a low (red), intermediate (blue) and high (green) spore production. The onset of sporulation is delineated by two zones: (1) a minimal nutrient concentration below which cells should not divide, since otherwise
their daughter cells have too little energy to finish sporulation; (2) a nutrient-dependent level of minimal energy, below which cells have insufficient energy to sporulate. The most productive genotypes (green) sporulate at low energy levels and low nutrient concentrations, thereby maximizing the amount of nutrients that are allocated to cell division, while maintaining an efficient sporulation program. Genotypes that produce intermediate numbers of spores (blue) sporulate at either high nutrient concentrations or high energy levels. Genotypes that sporulate at high nutrient concentrations, sporulate relatively early and therefore leave many nutrients in the environment that could have been used for cell division. Genotypes that sporulate at high energy levels, consume a lot of nutrients for nothing, because they accumulate more energy than needed for sporulation. The consumed nutrients cannot be used by other cells for either sporulation or cell division. Thus, overall, the most productive genotypes postpone sporulation as long as possible, by sporulating at low nutrient concentrations, but they initiate sporulation as soon as there is no further potential for cell division.

Text S3. Alternative modelling implementations of GRN

Different model variants
There are many ways to implement a GRN in a model (Spirov and Holloway 2013). In this section, we compare five alternative implementations. One key property of the GRN is gene expression. For the model implementation in the main text, we assumed a Boolean gene expression: a gene is either expressed or not. This assumption greatly simplifies the analysis of evolved networks, because there is a limited set of possible gene expression patterns. In reality, gene expression is typically continuous. Therefore, we examine the robustness of our conclusions in case genes have a continuous gene expression. We assumed that the expression of a gene is described by the function:

\[ G(x) = \frac{1}{1 + e^{b(\theta-x)}}. \]

\(x\) is the sum of regulatory input towards a gene, \(\theta\) determines inflection point of the sigmoidal curve (corresponding to a gene’s activation threshold in the Boolean implementation of the GRN) and \(b\) is proportional to the slope of the sigmoidal curve at the inflection point. \(\theta\) and \(b\) form heritable loci that are subject to evolution. Thus, the number of evolvable parameters differs between the Boolean (i.e. connection weights and activation thresholds) and continuous implementation of the GRN (i.e. connection weights, inflection points and slopes).

Besides the implementation of gene expression – Boolean or continuous – we also varied the number of genes in the regulatory layer of the network, the number of parameters that can evolve and the initial conditions of the network. Altogether this resulted in five different model variants.
Model variants
The upper row of graphs in Figure S8A shows the response curves of genes in each of the model variants: the regulatory input to a gene is shown on the x-axis and its response is shown on the y-axis. The response curves correspond to those at the onset of evolution. On top of the graphs we listed the number of evolvable parameters in the network. Here, a short description of each model variant:

- **Default implementation** – This model variant corresponds to the one we have in the main text. We assume Boolean gene expression and both the connection weights and activation thresholds are subject to evolution.

- **Model variant A** – In this model variant we also assume Boolean gene expression. However, in contrast to the default implementation, we assume that only the connection weights can evolve. In this way, we can examine how the degrees of freedom by which a network can change affect the results.

- **Model variant B** – In this model variant we also assume Boolean gene expression. However, in contrast to the default implementation, we assume that there are four genes in the regulatory layer of the GRN. This is another way to examine how the degrees of freedom affect the evolution of a GRN.

- **Model variant C** – In this model variant we assume continuous gene expression. The connection weights, inflection point (θ) and slope (b) can evolve. In addition, we assume the initial response curves of genes to resemble that of genes with Boolean gene expression (Model variant A and B).

- **Model variant D** – In this model variant we assume continuous gene expression. The connection weights, inflection point (θ) and slope (b) can evolve. However, in contrast to model variant C, we assume that at the onset of evolution genes show a more gradual response to the regulatory input (Model variant A and B).

Results
For each model variant, we ran 100 replicate simulations for 500 generations. At the end of evolution, we selected the 10 most productive genotypes. These 10 genotypes were grown as mono-clonal colonies at different signal degradation rates. Based on the results in the main text (Figure 8), we had the following expectations: (i) the variation between the genotypes in terms of spore production is lowest at the signal degradation rate at which cells evolved and higher at alternative signal degradation rates; (ii) the fraction of failed sporulation events is lowest at the signal degradation rate at which cells evolved and higher at alternative signal degradation rates; (iii) at high signal degradation rates cells postpone sporulation (i.e. lower nutrient concentration at onset of sporulation) and at low signal degradation rates cells advance sporulation (i.e. higher nutrient concentration at the onset of sporulation). Model variants A, B and C all satisfied the above expectations (Figure S8B). Only model variant E produced different results. In this model variant, genotypes did express a higher diversity in spore production and failed sporulation attempts at alternative signal degradation rates, but they did not postpone (advance) sporulation at high (low) signal degradation rates. How can these results be explained?
Model variant E differs from the other model variants in the initial response curve of genes (Figure S8A). In contrast to the other model variants, genes only weakly change their expression in response to changes in their regulatory input. As a consequence, it is difficult to evolve positive feedback interactions in the regulatory layer. Like explained in the main text, positive feedback interactions are necessary to ensure that cells continue the sporulation process in the presence of small environmental perturbations. As such, they are also necessary for cells that rely on the signal concentration for triggering sporulation. Cells stop producing signal after initiating sporulation. Sporulating cells will therefore experience a drop in the signal concentration, which can trigger cells to stop sporulating. If signal-responsive cells want to continue the sporulation process after its initiation, they need positive feedback interactions in the regulatory layer. Since these positive feedback interactions are difficult to evolve in model variant E, cells cannot evolve a dependency on the signal concentration, which explains why the evolved genotypes do not change the timing of sporulation when changing the signal degradation rate in model variant E.

Supplementary Tables:

Table S1. Parameter settings of model.

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<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
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<td>$N_{\text{init}}$</td>
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<tr>
<td>$r_{\text{cell}}$</td>
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</tr>
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<td>$D_N$</td>
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<tr>
<td>$D_s$</td>
<td>Diffusion rate of signal</td>
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</tr>
<tr>
<td>$\delta$</td>
<td>Signal degradation rate</td>
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</tr>
<tr>
<td>$V$</td>
<td>Nutrient consumption rate</td>
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<td>Duration of colony growth$^1$</td>
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<tr>
<td>$\sigma$</td>
<td>Standard deviation of mutational step size</td>
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</tr>
</tbody>
</table>

$^1$ The time duration is chosen such that colony does not exceed the surface boundaries.
Table S2. Parameter ranges used for generating novel environments in Figure 9.9. Parameter conditions are drawn from a uniform distribution between the minimum and maximum value.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Default value</th>
<th>Minimum value</th>
<th>Maximum value</th>
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</tr>
<tr>
<td>$D_S$</td>
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<td>0.15</td>
</tr>
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<td>0.15</td>
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</tr>
<tr>
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</table>

Supplementary Figures:

Figure S1. Spore production and fitness. The relationship between fitness and spore production for the 500 replicate simulations in the RN model at the end of evolution (generation = 400). Fitness is given by the number of spores plus a 10% fraction of the cell count, because cells have a relative chance of 10% to survive dispersal.
**Figure S2. Diversity of reaction norms in the RN model.** (A) Phenogram based on the distance between reaction norms of the most frequent genotypes in the 500 replicate simulations at the end of evolution. The distance between two reaction norms is given by the fraction of conditions at which they prescribe a different response. Colours indicate spore production of genotypes: low (red), intermediate (blue) and high (green). The twenty most productive genotypes are shown by larger dots. (B) The reaction norms associated with the twenty most productive genotypes ranked from the genotype that produces the largest number of spores (1) to the one that produces the smallest number of spores (20).
Figure S3. Evolution of sporulation in the GRN model. (left) Number of cells (blue) and spores (red) in 500 replicate simulations over the course of 400 generations. At each generation, cell and spore counts are collected at the end of colony growth. The black lines show the average number of cells and spores. (middle) Distributions of the number of cells and spores over the 500 replicate simulations at the end of evolution. (right) Colonies of the most productive genotype at generation 1, 200 and 400.
Colonies present at the end of the last growth cycle

Figure S4. Colonies of the twenty most productive genotypes in the GRN model. Colonies at the end of colony growth associated with the twenty most productive genotypes, showing cells (blue) and spores (red). The colonies correspond to the last cycle of the evolutionary process and therefore may contain multiple genotypes.
Figure S5. Diversity and expression background in the GRN model. Figure 9.6B shows the reaction norms that are generated by the twenty most productive genotypes, assuming that none of the genes in the regulatory layer are expressed before cells are evaluated with respect to all combination of N, S and E (i.e. expression background of a non-sporulating cell). In this figure, the same GRNs are evaluated with the expression background of a sporulating cell (see Material and Methods). The new reaction norms are shown as red meshes on top of the original green reaction norms. In most cases, cells sporulate for a larger number of conditions when having the expression background of a sporulating cell. The percentage on top of each reaction norms indicates the relative volume of the red reaction norm (i.e. expression background of sporulating cell) with respect to the green one (i.e. expression background of non-sporulating cell).
Figure S6. Diversity in mutual information values in evolved GRNs. In Figure 9.5A we evaluated the average mutual information values of the evolved GRNs over the course of 400 generations. Here, we analyse the individual mutual information values that are associated with the evolved GRNs using a principal component analysis (PCA). The principal component analysis is based on all GRNs evaluated in Figure 9.5A, which includes the most frequent genotypes in the 500 replicate simulations, collected at intervals of 5 generations over the entire course of evolution. The inner axes of the PCA show the relation between the mutual information values and the first two principal components: N = nutrients, S = signal, E = energy, B = gene expression background. Each genotype forms a single data entry (i.e. data point) to the PCA and is associated with four mutual information values (see Text S1 for details). The colour indicates the relative spore production of a genotype. (A) Four PCA plots showing the 500 most frequent genotypes at respectively generation 1, 100, 200, 300 and 400. (B) PCA plot showing all data entries. The twenty most productive genotypes at the end of evolution are highlighted by the larger data points. (C) The relationship between a genotype’s spore production and the Euclidean distance (mean ± SD) of its data point in the PCA from the centre of the PCA (see Text S1 for details). (D) The relationship between a genotype’s spore production and the fraction (mean ± SD) of environmental conditions (evaluated using the associated reaction norms, e.g. Figure 9.6) at which the associated GRN sporulates.
Figure S7. Environmental conditions at the onset of sporulation in the GRN model. The average nutrient concentration and energy level at which the most frequent genotypes initiate sporulation at the end of evolution. Colours indicate the relative spore production of the associated GRNs. The range of conditions at which sporulation occurs is delineated by two zones: (1) a minimal nutrient concentration below which cells should not divide, since otherwise their daughter cells have too little energy to finish sporulation; (2) a nutrient-dependent level of minimal energy, below which cells have insufficient energy to sporulate. Dotted line shows the average energy level of daughter cells after cell division (see Text S2 for details).
**Figure S8. Model variants and the accumulation of hidden diversity in GRNs.** The exposure of hidden variation in response to changes in signal degradation rate is discussed in the main text for one implementation of the GRN (i.e. default implementation; Figure 9.8): Boolean gene expression in which both the connection weights and activation thresholds can evolve. Here we show the results for 4 alternative implementations of the GRN: model variant A-D. (A) The initial response curves of genes at the onset of evolution for the different model implementations (model variant A and B have Boolean gene expression and model variant C and D have continuous gene expression, but with different initial conditions). (B) The relationship between the signal degradation rate and the (i) spore production, (ii) fraction of failed sporulation attempts and (iii) average nutrient concentration at onset of sporulation for the ten most productive genotypes in the different model variants (see also Figure 9.8). The ten most productive genotypes were collected at the end of evolution among 100 replicate simulations (for details see Text S3).
Plasticity: not by single genes alone

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Many organisms exhibit phenotypic plasticity, that is, they express different phenotypes under different environmental conditions. Whether phenotypic plasticity generally facilitates or hampers adaptive evolution is a matter of much debate. A recent study of Ghalambor et al. (2015) might shed light on this issue. Ghalambor et al. transplanted Trinidadian guppies from a habitat with predators to two predatory-free habitats. After a few generations, fishes from all three locations were reared in the lab to examine the degree of evolutionary divergence and the plastic responses of fishes towards the presence and absence of predators. Based on the comparison of gene expression data, Ghalambor et al. suggest that ‘adaptive’ plasticity hampers adaptive evolution, while ‘non-adaptive’ plasticity facilitates adaptive evolution (Merilä 2015). Here we argue that this conclusion is premature. First, Ghalambor et al. assign a direction of plasticity to each individual gene. Yet, genes act in concert to shape the phenotype, and very different expression patterns at the gene level may represent the same pattern of plasticity at the phenotypic level. Second, the expression of different genes is often not independent but reflects general regulatory processes. As we will demonstrate by a simple model, a small change in a single regulator may explain the change in expression pattern of the whole suite of 135 genes reported by Ghalambor et al. Hence, no statistical conclusions can be drawn concerning the relationship between plasticity and the speed of adaptive evolution.

Ghalambor et al. do not directly measure organismal plasticity or evolutionary adaptation at the organism level (Merilä 2015). Rather, they infer the evolutionary changes in plasticity from transcriptomic data (gene expression profiles). Ghalambor et al. conclude that there is a strong correlation between the degree of evolutionary divergence and the level of non-adaptive plasticity by analysing their data gene by gene, thereby implicitly assuming that selection optimizes the expression of each gene separately and that each gene represents an independent realization of plasticity. These assumptions are unrealistic, especially in a context where phenotypes are modulated by the environment (Beldade et al. 2011). Genes do not act in isolation to shape a particular aspect of the phenotype, but they are part of regulatory networks (Segrè et al. 2005; Davidson 2010). A change in the expression of one gene can propagate through the entire network, thereby affecting the expression of many down-stream regulatory genes. The analysis of the most well-studied gene regulatory networks have revealed that diverse regulatory patterns can produce the same phenotypic response, and that the relationship between the action of individual genes in the network and the resulting phenotype is often very weak (Wagner 2007, 2011; Beukeboom and Perrin 2015). Thus, one cannot expect that selection for the same organismal phenotype in two derived guppy populations is associated with convergent changes at the gene expression level (Whitehead and Crawford 2006). To illustrate this, we constructed an evolutionary model, in which a simple gene regulatory network could evolve to launch an adequate phenotypic response when sensing the absence or presence of predators. The optimal response readily evolved, but the network structures mediating this response differed substantially across replicate simulation runs (Figure 1). Hence, even in this simple network there is no convergent
evolution on the level of gene expression patterns. Accordingly, it does not make sense to classify the way how the expression patterns of single genes change with a change in environment as ‘adaptive’ or ‘non-adaptive’.

Ghalambor et al. assume that many of the expression changes between the ancestral and derived fish populations resulted from adaptive evolution. Yet, given the small number of generations, it is unlikely that evolution could have independently tuned the expression of tens or hundreds of genes. If many loci were involved, the speed of adaptive evolution would have been very low, in particular since the experimental populations are small (‘Haldane’s dilemma’; Nunney 2003). It is therefore more likely that the changes in gene expression found by Ghalambor et al. reflect a few regulatory changes, which affect the expression of many genes. We will show by a simple model that a small regulatory change could indeed explain the results of Ghalambor et al. To motivate this model, we need to have a closer look at their categorization of adaptive and non-adaptive plasticity.
Figure 2. Effect of small hormonal differences on gene expression patterns. In an ancestral and a derived population, the hormone-modulated expression pattern of 500 genes was assumed to follow the model $G_i = B_i + w_i \cdot H(E) + \varepsilon$ (see text). All parameters were the same in both populations, with the exception of hormone level in a predator-free environment (ancestral population: $H = 1.6$; derived population: $H = 1.3$). Conducting the same analysis as in Ghalambor et al.’s Figure 2 (A) and Figure 3 (B) produces very similar patterns (of high statistical significance) as those reported in Ghalambor et al. (2015). Parameters: baseline levels (which do not affect the results) $B_i = 0.0$; $H = 0.0$ in the presence of predators; $\varepsilon$ normally distributed with mean zero and sd 0.1.
Gene expression differences with respect to the ancestral population (living in the presence of predators) are considered ‘adaptive’ when they were consistently observed in three guppy populations (two of which are the introduced guppy populations) from predator-free environments. Genes in the ancestral population are assumed to express ‘adaptive plasticity’ when – in a predator-free environment – they exhibit an up- or down-regulation in the direction of the adapted genes of the derived populations in this environment, but only if the degree of regulation is weaker in the ancestral population. Thus, plastic responses that occur in the same direction in both ancestral and derived populations but have a smaller effect size in the derived fishes, are considered to be ‘non-adaptive’. According to this definition, any factor that weakens the change in gene expression in the derived populations would be a source of non-adaptive plasticity. Let us assume, for example, that a large set of genes is under (partial) control of a regulatory mechanism, which affects the expression of these genes in response to different environments. To be concrete, one might think of a hormone, like cortisol, which in guppies typically has higher levels in the presence than in the absence of predators (Fischer et al. 2014). Cortisol can have a major influence on gene expression (Aluru and Vijayan 2009) and it affects organismal phenotypes in a variety of ways (Becker et al. 2002). The simplest model for the action of a hormone on many genes posits that the expression level \( G_i \) of gene \( i \) is given by \( G_i = B_i + w_i \cdot H(E) + \varepsilon \), where \( B_i \) is the baseline expression level of this gene, \( H(E) \) is the environment-specific level of the hormone, and \( \varepsilon \) subsumes all stochastic effects. The effect \( w_i \) of the hormone on gene \( i \) can be positive or negative and large or small (in our simulation, the \( w_i \) are drawn from a standard normal distribution). If we now assume that fishes from a derived population have a slightly lower hormone level when reared in the absence of a predator than fishes from the ancestral population, this simple model reproduces the main results of Ghalambor et al. (compare their Figures 2 and 3 to our Figure 2A and 2B). Hence, a single regulatory change provides a much more parsimonious explanation for the observed transcription patterns than the large-scale evolutionary changes postulated by Ghalambor et al. Our alternative explanation does not refer to adaptive or non-adaptive plasticity, and it does not require evolution of gene regulation patterns (the remain constant). It does not even require adaptive evolution of the hormone level, since the lowered hormone level of the derived populations in a predator-free environment might just reflect a random change, for example due to a founder effect (Bonier et al. 2009).

Ghalambor et al. present an intriguing experiment to investigate the role of phenotypic plasticity on evolutionary adaptation. We agree that non-adaptive plasticity could in principle potentiate evolutionary change, but caution is required when inferring evolutionary changes in plasticity from transcriptomic data, especially when the underlying regulatory mechanisms are unknown. The complex relation between an organism’s genotype and phenotype make it often impossible to understand adaptive evolution by focusing on single genes alone.