Dear Professor Guillaume,

Thank you for considering my manuscript *Gene network robustness as a multivariate character* for recommendation in PCI Evol Biol. I am terribly late for this revision, please accept my apologies. This delay is partly (but of course not completely) justified by the new simulations needed for the revision.

Your and the reviewers' comments were very useful to prepare this revision. I have uploaded a new version of the manuscript including the improvements suggested in your decision letter, and I attach a pdf illustrating the changes. I tried to address as thoroughly as possible every idea; I hope I have managed to do it convincingly for most of them. Below are my responses to individual comments.

Yours sincerely,

Arnaud Le Rouzic

Decision letter

One salient point of the reviewers' comments is about the kind of pleiotropy at work in the gene network and how it affects the genetic correlation between the robustness traits. Is pleiotropy direct (as when one gene regulates multiple targets in the network) or implicit (as in affecting the emergent robustness traits without clear patterns of pleiotropic regulation)? Reviewer two suggests to estimate to mutation co-variance matrix (M-matrix) as a way to understand the genetic basis for the genetic correlation among robustness traits. I think it would be very informative, and innovative, to make the link between network structure and M-matrix structure.

Many thanks to you and to reviewer #2 for this suggestion. I computed the M matrix in the initial populations and compared the predicted evolvabilities in various bivariate directions of the multivariate robustness space to the realized evolvabilities (new fig 4). This required heavy simulations, because average evolutionary trajectories from 20 replicates were not smooth; they are now computed based on 100 replicates. As you will see, the predictions from the M matrices are very convincing: the bivariate evolvability of robustness seem constrained by the pleiotropy across robustness components in a way that is very similar to any quantitative character. This point strenghtens the conclusions of the paper, as it illustrates how robustness can behave as a multivariate quantitative character.

A similar question from both reviewers is about the estimation of the G-matrix and its evolution. As reviewer one points out, the structure of G depends also on the strength of correlational selection, not only on the underlying genetic correlation arising from the structure of the gene network. Is there a tendency to decrease/increase the genetic correlation b/n robustness traits due to selection? Can the strength of correlational selection be deduced from the evolution of the G-matrix and of the M-matrix? A link with previous evolutionary quantitative genetics is awaited and would anchor the paper in a well known theoretical framework.

Unfortunately, my attempts to compute G matrices from populations were not conclusive. The main reason is that computing G is a very intensive task, as the robustness needs to be assessed for every single genotype in the population, and the measurement needs to be precise enough to quantify differences in robustness within the population (i.e. among close genotypes). As 4 out of 5 robustness components are calculated by a stochastic procedure, the burden to get a single G matrix from a single replicate is substantial; multiplying the effort by the number of replicated simulations

seem out of reach, especially because the G matrix evolves through time (G cannot be estimated from generation 0 as I did with M, as there was no genetic variance in generation 0).

Therefore, I focused the revision on M, and applied on M evolvability indicators developed for G (in particular, conditional evolvability Mc and evolvability in the direction of the gradient, proposed in Hansen & Houle 2008). The only drawback for using M instead of G in such a context is that M provides unscaled estimates of the selection response (i.e. the direction of the response), while G would have also provided an estimate of the magnitude of the response. I hope that the new results proposed in fig3 and fig4 now connect convincingly the manuscript to traditional multivariate quantitative genetics.

It is not clear how the results of the 2-dim network generalizes to larger network. In particular, the 2-dim network shows large neutrality in regulation network for stable expression phenotype. How does this generalise to larger network?

I am not aware of any attempt at investigating neutrality in a quantitative gene expression network (which does not mean that it has never been done). As far as I know, in discrete networks where neutral regions can be formally defined, there is a consensus about the fact that neutral spaces are larger and more connected in large networks compared to small ones (e.g. Ciliberti, Martin & Wagner 2007 PloS Comp Biol). In the revision, I have extended equation (4) to any network size; in an n x n network, there are n(n-1) dimensions of "neutral" evolution. Therefore, the ratio of neutral dimensions / total dimensions increases with the network size. I now mention and comment this result, thank you for the suggestion.

In general, little is said about the constitutive levels of expression and their role in the evolution of robustness traits. This is a point that should be clarified.

As far as I can tell, our "version" of the Wagner model is the only one to introduce a constitutive expression term (actually there was one in Wagner 1994 but it has not been explored). Traditionally, constitutive expression is set implicitly to the mid-point between minimum and maximum expression, and is thus not considered as a variable. Because our model explicitly sets the constitutive expression, I could run simulations to check how the parameter a conditions the evolution of robustness (Appendix 7 column 3). Changing the parameter a from ~ 0 to 0.5 has a minor effect on most indicators, except robustness to early environmental noise and stability (both being very correlated throughout the simulations). Networks are more sensitive to changes in their initial state (and more prone to cyclic dynamics) when the constitutive expression was low. I am not sure whether this has a biological interpretation, or can simply be attributed to the way regulation is scaled – across simulations, mutations have the same effect on genes expressed at their constitutive expression; this means that mutations will have a different effect on genes expressed at the same level among simulations differing by the constitutive expression parameter. Yet, the fact that constitutive expression affects some, but not all, robustness components suggests that there is more than a simple scaling effect. The discussion has been updated accordingly: "F*inally, the sigmoid response function was made asymetrical by introducing a constitutive expression parameter (as in e.g. Rünneburger and Le Rouzic, 2016) in order to avoid the unrealistically high expression of unregulated genes (half the maximum expression) from the default setting. This constitutive expression was not evolvable in the model, but simulations (Appendix 7) show that two robustness components (ρ E and ρ S) were very sensitive to this parameter (larger constitutive expression was associated with more robust networks). It is thus not unlikely that real systems could evolve towards more robustness by increasing the constitutive expression of key genes, as already suggested (for dfferent reasons) by Draghi and Whitlock (2015). "*

I attach the manuscript with some additional comments and grammatical corrections (grammatical mistakes are highlighted in orange).

Edited manuscript:

*** Abstract: "whether or not robustness to various sources of perturbations is independent conditions ..."**

Changed → *"whether or not robustness is independent to various sources of perturbations conditions..."*.

*** Methods: "In addition, direct, directional selection on robustness indicators was performed ..."**

Changed → *"Directional selection on robustness indicators was also performed..."*

*** Results: "The simulated behavior of networks A to E are illustrated in Appendix 5"**

Changed→ *"In order to assess the variation of the robustness properties, five networks of contrasted robustness, labeled from A to E, were tracked more specifically"*

*** "This 2-gene network analysis thus confirms the results obtained for larger gene networks"**

Changed → *"This 2-gene network analysis thus confirms the results obtained for large random networks"*.

*** "due to stabilizing selection. (lower scores..."**,

Changed → *"due to stabilizing selection (lower scores..."*

*** "all homogeneous to a variance of gene expression"**

Changed → *"all homogeneous to a sum of squared difference in gene expression (i.e., the variance in gene expression induced by various disturbances)"*

*** "This could a be consequence from the canalizing selection"**

This paragraph has been deeply reformulated.

*** Figure 4: very hard to understand:**

Reviewer #2 also noticed this, and I agree. The figure has been removed and replaced by a more traditional representation (M matrices vs realized responses).

*** Discussion, "mchanisms"**

Changed → *"mechanisms"*.

*** "mutational effects were correlated in my simulations"**

I agree that the simulation setting (new allele drawn into a Gaussian centered on the former allelic value) looks pretty standard in quantitative genetics, and this would not deserved to be discussed in another context. I am not sure to know why most similar models draw allelic effects in a constant distribution (centered around 0), and I am unsure about the consequences, but the difference deserves to be mentioned. The sentence has been reformulated as: *"Unlike in Wagner (1996) and Siegal and Bergman (2002), mutations had cumulative effects (the value of the mutant allele was drawn in a Gaussian centered around the value of the parental allele), which allows for cumulative evolution."*

*** "Could you elaborate on effects of network sparcity and density on robustness and evolvability?"**

This is a very interesting question, which is also difficult to address here due to the limited size of simulated networks. From the evolutionary simulations in Appendix 7, larger networks are slightly more robust (only to genetic disturbances) at equilibrium, but network density has no effect. An additional set of simulations was run to test the effect of changing the network density in random networks (new fig 1), suggesting that more complex networks (larger and more connected) have their robustness components more correlated. The discussion now states:
"Computational constraints also limit the network size to a few dozen genes, which was not enough to generate realistic levels of sparcity – simulated gene networks were too dense to be realistic. Decreasing network density and smaller network sizes made robustness components slightly less correlated (Figure 1E and F), suggesting that the integration of robustness components increases with network complexity."

Next time, please submit a manuscript with line numbers to help with minor corrections.

I apologize for the missing line numbers. Fixing it in the preprint will be difficult: the manuscript is hosted in arXiv (which is in the list of recommended preprint servers https://evolbiol.peercommunityin.org/help/help_practical#To%20prepare%20a%20preprint), and arXiv does not accept preprints with line numbers (they rejected my first submission). Perhaps something to discuss with the PCI staff. I will attach a line-numbered version together with the resubmission.

Reviewer 1

Robustness is often treated as the generic property resistance to perturbations, with little differentiation as to how different perturbations can require different kinds of robustness. This manuscript uses simulations to investigate how direct selection for different types of robustness in gene networks lead to different outcomes. By explicitly defining several kinds of perturbations, the manuscript shows that, while the different measures of robustness are correlated, not all types of robustness are equivalent, and observing robustness to several kinds of perturbations in organisms can potentially indicate different adaptations to separate selection pressures. This observed scenario is contrasted with the possibility that robustness to several kinds of perturbations shares the same underlying basis or that different kinds of robustness are completely different.
This is a simple and well-motivated investigation, the manuscript is well written and direct. The explicit description of the many axes of what we may convincingly call robustness is a worthwhile contribution and brings some much-needed precision to the discussion of the evolution of robustness.

I warmy thank the reviewer for constructive feedback.

General comments:
The inclusion of a measure of stability is an interesting necessity for there to be a stable state to which the system can return after a perturbation, but I'm not sure it would traditionally be included under robustness to perturbations. Network C in the two-gene network illustrates this, as it is somewhat robust but not stable. In any event, the two-dimensional results illustrate nicely the relative independence of the different kinds of robustness.

I totally agree with the reviewer that stability is an outlier compared to other robustness measurements. The only reason why I considered stability among robustness indexes is that it was historically one of the first network feature correlated with mutational robustness in theoretical gene networks by Siegal & Bergman (2002), who showed that selection on stability could induce an indirect response of mutational robustness. I was thus curious to include stability in this study. It turns out that stability is largely correlated to other robustness measurements (especially to environmental perturbations), which confirms Siegal & Bergman's results. It is not completely clear to me why stability and robustness are so tightly associated (unstable networks tend to overcompensate disturbances?), nor whether this result depends on the model hypothesis ("Wagner" networks tend to be very unstable, Pinho, R., E. Borenstein, and M. W. Feldman (2012)).

I updated the introduction to make it clearer that stability is an outlier: *"Four robustness-related measurements were considered, two of them corresponding to environmental robustness (early vs. late disturbances), two corresponding to genetic robustness (early — inherited — or late — acquired — mutations). Gene expression instability was also included in the set of robustness-related traits, as it is related to the intrinsic stability of the expression phenotype."*

pg 9, 2nd paragraph: "Selection on all robustness components also lead to an indirect response of all other components, which confirms a general genetic correlation." - Not necessarily due to genetic correlations, selection could also be correlated due to phenotypic correlations.

I am not sure to follow the reviewer's argument here, perhaps simulation details were missing and/or we have different definition of correlated selection. Due to genetic correlations, unselected traits could be affected by some selection differential, and thus respond indirectly to selection, but there was no gradient (by construction) on unselected traits. My reasoning is that, based on the Lande equation $R = G \beta$, if $R_i \neq 0$ for a trait i for which $\beta_i = 0$, this has to be due to one (or several) genes j for which $\beta_j \neq 0$ and $G_{ij} \neq 0$. If there is a response R_i for all genes i whatever which $\beta_j \neq 0$, this means that all $G_{ij} \neq 0$ (which is what I meant with "a general genetic correlation").

Note that there was no correlational selection in simulations (the total fitness was the product of marginal fitnesses computed on each trait separately).

I thus kept the sentence unchanged so far, but I would happily update it if I misunderstood the reviewer's argument.

In the next paragraph and figure 4, it's not clear that the correlations themselves are not evolving, thus partially removing the genetic constraint. It would be interesting to measure the correlations over time and investigate if this is a factor in the response (or lack thereof) to selection, both direct and indirect. Could some of these robustness axes be decoupled and others not?

Tracking the evolution of the genetic correlations is a very interesting suggestion. I added a new figure (Figure 5) showing how mutational correlations evolve in the simulations. Interestingly, correlations evolved for some trait combinations (but not all), and the direction of evolution

depends on whether robustnesses were selected positively or negatively. Yet, this was not consistent among robustness traits (i.e., selected more robust systems ended up with more or less correlated traits depending on the pair of traits). This illustrates the complexity of the links between robustness components. The text was updated in the results and the discussion to describe this new figure.

Some general questions, not crucial to the results but perhaps good avenues for further investigation:Could the coupling between axes of robustness be reduced in higher dimensional networks? Are the correlations weaker in the n=6 simulations than in the n=2 simulations?

I added new panels in figure 1 showing how the loading of PC1 was affected by various parameters, including the network size and network density. Robustness components in large and dense networks actually tend to be more correlated, although the effect is rather minor. Thanks for the suggestion.

Related to the previous point: would using larger networks allow for the inclusion of measures of network architecture? For example sparsity, modularity (...), and their relation to the correlations between robustness measurements.

The effect of network density is now explored in appendix 7 (it seems rather negligible). In the model, the strength of regulation is quantitative, it is thus not easy to determine whether a connection exists or not (it is never exactly 0, except for connections that where canceled on purpose from the beginning of the simulation to manipulate network density). It is possible that quantitative topological indicators may exist in graph theory, but this goes beyond my competences.

Is the constitutive expression parameter relevant for the dynamics? How does setting different constitutive expression values alter the results? Could (should?) it be altered by mutations?

Thanks for the suggestion, which has been highlighted by the editor (see my comment above). Constitutive expression indeed changes the robustness level for some (but not all) robustness components. In the model, constitutive expression is not evolvable, as it provides the yardstick for the meaning of the size of a change in the regulation strength (a given mutation has always the same effect on gene expression around the constitutive expression). Adding a genetic basis for constitutive expression would thus require a different scaling function, and was thus beyond this study. Nevertheless, I added a discussion sentence (see my response to the editor above) about this interesting observation – note that the evolution of constitutive expression as a response to selection for more robustness has already been proposed by Draghi & Whitlock (2015).

Could the difference in the evolvability in the different robustness measurements be due to the choice of mutation? i.e., Could a different, larger-mutation scheme, alter the evolvability?

The model can be parameterized with two mutational effects: the mutation effect size for robustness tests, and the mutation effect size for the simulations. In the previous version of the manuscript, the influence of the size of mutations on the calculation of genetic robustness parameters was assessed (in a figure that is now in Appendix 1), but the influence of the mutation size during the simulations was not tested. Yet, it is relevant, because it is susceptible to have an effect on the robustness to early mutations, one of the only robustness components that had to be under (indirect) selection in all simulations (as an unavoidable consequence of stabilizing selection). It is now one of the parameters included in Appendix 7 (mutation size, column 2). It happens that the size of mutations has an effect on the evolution of robustness, in a way that is similar to the rate of mutations: more and larger mutations improves substantially the robustness of genetic architectures to mutations. The fact that correlated robustness components are also affected is by itself interesting, because it illustrates concretely how robustness can evolve as a correlated trait.

Reviewer #2 (Charles Mullon)

In gene network robustness as a multivariate character, the author uses computer simulations to investigate the evolution of various measures of robustness of gene expression. A gene regulatory network (based on the "Wagner" model) is evolved where the fitness of an individual depends on the capacity of the regulatory network to maintain a stable level of expression in the face of mutational or environmental perturbations. It is shown that selection for one type of robustness (e.g. against germline mutations) does not necessarily lead to a correlated response in another type of robustness (e.g. against late in life environmental perturbations). In fact, selection on different types of robustness can interfere with one another, slowing down or even preventing adaptation. Nevertheless, certain pairs of robustness types can be selected in different directions. This supports the notion that certain types of robustness are more evolvable than others (i.e. more independent from other traits than others).
This is an interesting manuscript, that is well written and easy to follow. The methods are particularly clear.

Thank you for the accurate summary and the suggestions.

In my opinion however there are a few points that would need to be addressed before recommendation.

1. **I think that the concepts of pleiotropy and genetic constraints could be further investigated with a multivariate quantitative genetics framework (see the seminal paper by Lande in Evolution 1979 33(1)). In particular, estimating the G matrix of genetic variance-covariance for the different types of robustness would characterize genetic constraints in a more traditional and quantitative manner than it currently is.**

This point was also commented above by the PCI recommender. Computing G was complicated due to several reasons, and I rather replaced G by the mutational covariance matrix M (see below).

2. **Pleiotropy could also be quantified by computing the mutation matrix (e.g. Jones et al. Evolution 2007 61(4)). Doing so would also allow to connect the notions of robustness and evolvability more clearly. From reading the title and introduction, these quantitative genetics analyses are in fact the types of analyses that I expected to see.**

I warmly thank the reviewer for this suggestion. As detailed above, this direction is now explored in the revised version. Former figure 4, proposing the analysis of the evolution of robustness under directional selection, has been replaced by an analysis based on how robustness responded compared to the M matrix. This analysis was performed using the geometrical tools from multivariate quantitative genetics (including measurements of evolvability and conditional evolvability from Houle & Hansen 2013, translated to M matrices instead of G).

3. **I thought the description of results were terse and rather vague at times (e.g. p. 9-10, see sentences with "possible outcome ...", "most cases ..."). Particularly disappointing is how little discussion there is about why different types of robustness are positively or negatively associated with one another. I understand this would be speculative without any further analyses, but it would nevertheless offer readers a message that goes beyond "it depends". Perhaps the quantitative genetics approach suggested above allows for a more thorough description and interpretation of the associations between different types of robustness.**

I tried to reformulate the text to provide more conclusive explanations. I also added a paragraph about the relationship between the interaction matrix and robustness estimates. I am afraid that this will not completely address the reviewers' legitimate concerns, because the problem is hard (at least, it looks hard to me). As far as I can tell from the results, robustness behaves as an emergent property of the gene network: it is obviously "encoded" in the matrix of interactions among genes, but it is a complex function of many interactions. In fig 2, illustrating the simplest case (a 2-gene network), the link between individual transcriptional intertactions and robustness components already appears quite complex, non-monotonous in every direction, and responding to different rules for different robustness components.

Here is my current understanding of the mechanisms, which are in my opinion too sketchy to deserve being published. Robustness components (as arbitrarily defined in the manuscript) can be classified in two clusters: Cluster I is Early environmental + stability, and cluster II are three other ones (late environmental, early genetic, and late genetic). Mathematically, cluster I is related to a measurement of the size, shape, and numbers of basins of attraction around equilibrium gene expressions. The underlying mathematics look tricky; solutions may perhaps be expressed in terms of the Lambert W function (this goes beyond my current mathematical skills). The stability of the equilibria depends on the eigenvalues of the matrix of second derivatives of the dynamic system around each equilibria (n-order polynomials of the 2nd-order derivatives (which are themselves in f(exp(x))^2 / f(exp(x))^3))). Cluster II reflects how the network responds to immediate disturbance, which is probably close to the first derivative of the dynamic system. This looks more tractable mathematically whenever the equilibrium point is known. However, figure 2 illustrates that this remains a naive interpretation, as two robustness components that happen to seem well-correlated in some areas of the "robustness space" can get uncorrelated in other areas, and I am not sure how relevant such conjectures might be.

I added a discussion paragraph about robustness as an emergent property of the network, justifying why the causes of correlations are not discussed more mechanically:

"Throughout this work, robustness was thus treated as an emergent property of the underlying network, which could not be easily deduced from a reductionnist approach. Yet, it remains possible to interpret the correlation patterns in terms of network dynamics. Two of the most correlated components are the robustness to early environmental variation ρ E and network stability ρ S , which both measure the ability of the network to converge to a given gene expression equilibrium. Conversely, the correlation between late mutational ρ m and environmental ρ e robustnesses can be attributed to the consequences of such disturbances over a single time step: for a single target gene, decreasing the concentration of a transcription factor and decreasing the sensitivity of the promoter to the same transcription factor may have very similar immediate consequences on gene expression. Yet, even if these measurements happen to be correlated by construction in the network model, their partial evolutionary independence highlights their potential for independent evolvability in real gene networks, which are substantially more complex and subtle than our mathematical approximation."

Minor comments / Typos:
Legend of figure 3: "robusnetss" -> robustness
Appendix 4 : "produced" -> produce

Thanks for the list of typos, all fixed in the revison.

Legend of figure 4: "univariare" -> univariate. In fact I did not understand what the thick horizontal lines corresponded to exactly.

Former fig 4 has been removed from the manuscript. I hope that the new figure is more intuitive.

Appendix 5: There is something wrong here. I am guessing the rows and columns have been inverted in the legend. The column for stability also seems to be missing. Please check the figure legend thoroughly.

Sorry about this, there was indeed a last-minute inversion of x and y axes, and I forgot to update the caption accordingly. I hope that the revised version is devoid of such inaccuracies.