Review of the thesis by Javier Puy Gutiérrez: Transgenerational effects of plant biotic interactions

The thesis examines the existence and the role of transgenerational plasticity in plant ecology. It has a general introduction of 19 pages, four chapters in the journal contribution style and general conclusions & summary. The first paper is methodological and as such constitutes an important baseline on which other two (II and IV) papers can rest. Two papers determine the existence and magnitude of transgenerational plasticity in biotic interactions (competition and mycorrhiza) of a dandelion species, the last paper examines effects of transgenerational plasticity in artificial communities of Arabidopsis thaliana. One of the papers is published, the other three still at the manuscript stage (two of them seem to have been submitted).

Strong points of the thesis

Choice of the subject. While there have been quite a few papers addressing different aspects of transgenerational plasticity in plant ecology, the thesis brings a fresh look at the subject by dealing with its role in biotic interactions. I particularly like the idea behind the paper II, which has a great, and ecologically highly relevant, potential, but has never been addressed by the transgenerational plasticity research. The same case in the paper III.

Publication status. I appreciate that the author has most papers in the thesis in a sort of a final form, but is presenting them before final publication. The success in publication of the first paper shows that the authors is capable of getting his work published, but still the papers leave more space for a reviewer to discuss.

Good data analysis. As far as I am able to say, the papers are based on careful experimental design and good data analysis (with a small proviso on multivariate analysis below).

Weak points of the thesis

Slight overinterpretation of the findings. While most experiments were able to detect some signal of transgenerational plasticity, it was never very strong. I would appreciate qualifying statements about the magnitude (not significance) of the effects, and also of their potential role in the field. I would tend to say that the necessary next step for the TGP research it to seek for such effects in the field, and to demonstrate their importance relative to e.g. genetic or purely environmental effects.

(Some) questions for the discussion

Introduction (p. 11). The candidate correctly discusses relationship between trait/functional dissimilarity and coexistence (the limiting similarity concept), but the limiting similarity concept comes mainly from animal ecology. I would appreciate some good examples from the plant kingdom. How do plants differentiate their niches that would fall into the category of limiting similarity?

Paper I (e.g. p. 10): This experimental design inevitably confounds toxic effects of the azacytidine and its effects on demethylation. (I appreciate the spraying approach as it seems to minimize the former.) Could the candidate propose an experimental design that would permit true separation of these two effects, and discuss whether they can be separated in principle?

Paper II and III: I would appreciate some rationale why *Taraxacum brevicorniculatum* was selected as a model species. Any other reason than its potential as an apomict (i.e. presumably genetically homogeneous) and a rubber producer? BTW The genetic homogeneity was assumed or tested?

Paper II (li 54): I might have missed something important, but I do not understand why the offspring experiment did not use crossed treatment of competition and demethylation (If I understand it well, demethylated plants were kept in common environments, whereas the competition treatment did not involve demethylated plants.)

Paper II (li 62): response to competition by more conservative strategy is somewhat unexpected for me. Many plants tend respond by faster growth of cheaper tissues to counter competition for light (which, I presume, was the case here as well – or was it? higher root mass fraction would indicate otherwise). How would you explain why the plants used this strategy?

Paper III: This is a really nicely conceived experiment, but it is a pity that the second generation was not combined with a demethylation treatment. Here it is difficult to argue (as the author does, p 95) that the transgenerational plastic effects were not due to carryover by seeds. There are several lines of evidence that this may be the case: (i) The effects clearly tend to disappear in adult stages, (ii) seed stoichiometry differs between parental treatments, but (if I am getting it right) has not been accounted for. In general, I am afraid that the candidate overinterprets the findings a bit, as interactions between parental and offspring treatments were generally weak even at the juvenile stage.

Paper III (p. 86): wasn't there any increase in nutrient availability after gamma-irradiation? (This is a commonly found effect, due to release of nutrients from bodies of dead bacteria and fungi.) I assume Fig. S1 shows only total nitrogen, which is not that much relevant, and there is no info about phosphorus.

Paper III (p. 89): using seed mass as covariate removed the linear part of the overall maternal investment, but may not remove the effect of differential seed stoichiometry (e.g. P content – see Fig. S3). Or did you take seed stoichiometry also into account?

Paper III (p. 93 and Tables S2 and S3): Given the high number of different response variables, multivariate analysis may possibly be appropriate for assessing the effects of individual treatments. Was there any specific reason for running univariate analyses only?

Paper IV (p. 124): it is not clear to me how you separated complementarity and selection effects. Were you able to identify individuals of different origins in the second generation mixtures? (By position?)

General: Most TGP experiments are based on two-stage design: first conditioning the plants by some manipulated factor, and then exposing the second generation to the similar (or possibly even different) set of factors to show whether the plants responded to them differently according to the first-generation conditioning (and possibly whether the effect could be nullified by demethylation). Such experiments are typically based on fairly extreme and stable values of the factor used in the conditioning (e.g. waterlogging in the paper IV). I would appreciate some discussion how such effects could work in the field, where extreme values can occur, but often come and go, and the plant's progeny may establish in condition that are variable as well.

Methodological/minor comments

The introductory part of the thesis reads well, but it has very broad scope, reviewing everything from plant community structure and species coexistence to methodological issues of DNA methylation. This is understandable given the subject of the thesis, but it necessarily means that discussion of many issues remains a bit shallow. (The introductory part is also full of typing errors which is annoying for the reader.)

p. 2: I find the usage of the term "mean field approach" a bit odd. Mean field approach typically refers to the fact that some state variables can be considered constant over some domain (typically spatial domain). Traits of individuals are not state variables in the common sense.

p. 9 and the Paper I (e.g. p. 31): I appreciate the methodological approach taken by the author in testing demethylation effects. However, effects of demethylation are bound to differ strongly depending on the specific position in the methylated cytosine in the genome. I understand that this is not that easy to obtain, but I would have appreciated some discussion of this critical issue.

p. 2: this is a decade-long discussion of traits vs. functions. I still would appreciate if the authors is more specific about their differences. (Traits do not underlie coexistence; functions do.)

p. 29 bottom: the author states that the analysis was using binomial distribution of the response variable for the analysis of percentage methylation. How was the percentage expressed? I would assume that – given the large number of potentially methylated sites – the percentage is almost a continuous variable, making use of binomial errors problematic (or at least not necessary), but I possibly overlooked something.

p. 73: I spent quite some time trying to understand structure of the table – the legend might be more informative.

p. 63 first para: a correct test of this would be testing interaction between demethylation and competition.

Conclusion

In summary, the thesis convincingly demonstrates ability of the candidate to perform good research in plant ecology. It is based on a solid amount of field, laboratory and analytical work, it shows a good understanding of the subject and the ability to present results in the form of scientific papers. I am fully convinced that the candidate deserves a PhD. title and wish him success with further work.

I am happy to recommend the thesis for defence.

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Evaluation of PhD Thesis submitted by candidate Javier Puy Gutiérrez

Overall this is a very thorough and impressive thesis with several complimentary and quite ambitious investigations into the transgenerational response to abiotic and biotic challenges. The candidate has done an admirable job of assembling quite complex data collected from solid experimental design and analyses. The projects are well researched and well motivated and the discussion of the results in all chapters is thoughtful. The scope of the work from developing methods to testing questions in response to competition as well as microbial symbiosis and effects on ecosystem processes is impressive. This work will add a nice body of knowledge to the nascent field of ecological epigenetics.

The general introduction does a nice job covering the landscape of concepts that are important for the thesis including the importance of intraspecific phenotypic variation, and how biotic interactions affect species interactions, biodiversity and ecosystem processes.

Some points of concern that are first referenced in this introductory chapter but also resurface throughout the thesis are related to definitions and use of terms. Perhaps most important is the definition of epigenetics which in the introduction relies entirely on changes in "DNA expression caused without modifying the underlying sequence". This definition is problematic for many reasons, particularly 1) its not clear what is "DNA expression", 2) many changes in e.g. DNA methylation will not affect gene expression or genomic function, but should still be considered epigenetic and 3) many of the claims in the thesis cannot isolate these types of epigenetic effects from non-genetic effects more generally. Epigenetics is a difficult term to define and the community of scientists who work in this field have debated it heavily. However, it is important to be more clear about what you mean. In Banta and Richards (2018 Heredity) we agreed on "chemical modifications of chromatin or transcribed DNA that can influence gene activity and expression without changes in DNA sequence"- which is more accurate because this is what we care about in ecology and evolution but also this definition doesn't exclude all of the epigenetic changes that don't have an obvious function (similar to changes in sequence variation that have no obvious function but are still considered "genetic"). Further, DNA methylation may be the best studied epigenetic mechanism so far (particularly in ecology and evolution), however there is not such good evidence to support that it is "the most

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significant one", in fact you could argue that it doesn't occur in isolation of other more important mechanisms when it does matter.

Similarly, defining all of phenotypic variation as due to only either genetic or epigenetic (or both; page 5) confounds other sources of "non-genetic" mechanisms with those that are specifically epigenetic. This is related to the concept that plasticity and epigenetic effects are not completely interchangeable which we addressed in our 2010 commentary in Bioscience (doi:10.1525/bio.2010.60.3.9) among other papers. In our 2017 review, we emphasize the DNA methylation can **mediate** plasticity but also that the capacity to adjust epigenetic modifications, and the potential for inheritance of these modifications might have different adaptive benefits. We also maintain that this is a property of the genotype and that genotype-specificity in epigenetic or transgenerational effects may be common (e.g. Herman & Sultan 2016). Clearly, the property of phenotypic plasticity is also heritable and a genotype specific property so it is critical to differentiate this concept from the so-called "transgenerational plasticity" which really refers to the inheritance of an induced response, not just the ability to respond.

Chapter 1 describes an improved method for the application of 5-azacytidine which allows for experimental reduction of DNA methylation and tests of the role of this epigenetic mechanism. The candidate performed two experiments that demonstrate that the apparent toxicity of 5-azacytidine can be alleviated by spraying the chemical on established seedlings instead of germination of seeds. The study nicely shows that the differences in offspring traits were no longer significant after the sprayed application of the treatment, but also that the sprayed treatment did not seem to have the detrimental effects that the chemical had when applied at germination.

Although the results provide compelling evidence that the sprayed application alleviated the detrimental morphological effects of germination in azacytidine, one thing missing from this chapter is a discussion of the reported actual DNA damage that has been found from the use of this chemical. See Liu et al (DOI: https://doi.org/10.1105/tpc.114.135467) who claim extensive DNA single-strand breakage, in contrast with zebularine treatment where no large amount of DNA strand breaks could be detected. This effect may not have been relevant in the study presented in chapter 1, but could reveal itself under different environmental contexts and would only be revealed by detailed molecular study of the plant material.

Chapter 2 describes a complicated study of the inheritance of induced effects and the potential for epigenetic effects to contribute to this phenomenon. The study is interesting because it assesses response to different competitive environments with a very ambitious design and creative use of a "relative interaction intensity" index. The characterization of what is actually competitive is quite compelling. In addition, this study offers some of the first examinations of the importance of DNA methylation on an ecosystem function with studies of decomposition.

Some points of concern are related to how the concepts are presented as mentioned above. For example, its not clear what is "epigenetic plasticity" (page 51), but this is not a commonly used term and should be more accurately described. Again, its important to emphasize that epigenetic mechanisms can contribute to plasticity by eg modulating expression but plasticity and epigenetic are not interchangeable (we do not define these terms as such in Bossdorf et al 2008; Richards et al 2017 which are provided as support). We know that there are clearly plastic responses that are not epigenetic (including provisioning which the authors consider, but also eg biochemical function which is temperature sensitive and can constrain phenotype). Plasticity does not "drive" responses (page 51)- the term describes a response. Further, the use of the term "transgenerational" is confusing as mentioned above. Please carefully consider the difference between this term and the genotype property of plasticity which should be inherited anyway. It could be easier to say "inheritance of induced response", or use something like the description in the introduction of chapter 4 which is much clearer.

Some follow up questions: How can you be sure that the offspring are genetically identical throughout the studies? Aren't there examples of single nucleotide mutations that impact genome wide methylation and traits (eg FWA allele)? Similarly, epigenetic effects are not necessarily equalized in common garden. Without detailed molecular studies, these statements are overly bold.

Chapter 3 has a combination of several exciting ideas with tests for the inheritance of the effects of mycorrhizal symbionts and how the effects are context (water stress) dependent. The role of mycorrhizae in tolerating stress is already an interesting and current line of inquiry, but the study is even more compelling in the context of inheritance and non-genetic effects.

While these are very exciting questions, the design is quite complex and the results are somewhat confusing. There are clearly effects of drought stress and mycorrhizae, and an interaction, but its surprising that eg the total biomass is greatest under benign water conditions with mycorrhizae (instead of without). Could it indicate that somehow the benign conditions are not so benign? Somehow there is still a benefit of mycorrhizae there regardless of parental treatment. Or the drought stress was so severe that the mycorrhizae do not overcome the limitation? Further, as mentioned above, the claim that this is an epigenetic effect is unfounded since there could be other unmeasured non-genetic effects. More cautious language should be used. Isn't there more than just the possibility of seed provisioning or hormones?

Chapter 4 describes two more experiments that attempt to address the importance of genetic and epigenetic sources of biodiversity at a population level. The first experiment uses three different Arabidopsis accessions (Gue-0, Mer-6, Vav-0) to assess genetic diversity and Col-0 from different parental treatments to assess the effects of epigenetic diversity. These plants are exposed to 3 different conditions (control, waterlogging, fertilization) and application of 5-azacytidine. The results suggest an important effect of treatment (particularly a negative effect of waterlogging) and that epigenetic diversity seemed to have a more negative effect than genetic.

Again, these are interesting ideas, but the design is quite complex and the results are somewhat confusing. In particular, it is difficult to actually compare the genetic and epigenetic diversity findings since really the comparison is also between Col-0 and the other 3 accessions. It could be important to include Col-0 in the relevant 'Genetic diversity monocultures' comparisons to understand the differences between these genotypes. Also, in this current version of the thesis, I could not decipher how the two components of biodiversity (Fig 4. Complimentarity vs Selection) were actually calculated, nor exactly what they might mean.

The General Discussion is a fine summary of an ambitious thesis that involved a lot of experiments and a lot of work. It is a remarkable achievement. However, the writing still reflects the problem of confounding epigenetic inheritance with all of non-genetic inheritance. For this work, which largely relies on phenotypic response, and does not measure actual molecular level mechanism, "non-genetic" is more appropriate.

Sincerely,

Christina Richards

Christina Richards
Associate professor