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May 18, 2015

Dear Prof. Higley,

Thank you, Prof. Queller and the two anonymous reviewers for your time and insight in reviewing our manuscript. We have make the alterations suggested. These are detailed below with reviewers comments numbered and italicised and our response in normal font. Line numbers refer to the lines in the tracked changes version of the manuscript. In the tracked changes version, new sections are in blue. We feel, and I hope you and the reviewers agree, this is a much improved manuscript.

Editor's comments

1. *From my perspective, the key issues are justifying your methods and establishing whether or not they actually support genetic imprinting.*
   As you say, there were two main criticisms. The first we answered by carrying out the DNA controls suggested by Prof. Queller's 5th comment. We also redid our analysis to include a measure of efficiency. After this both of our genes showed significant results (see our response to Prof. Queller's 5th comment).
   The second criticism was that we had not found genomic imprinting. This is a fair point. As we said in our original manuscript there is no way we could have confirmed genomic imprinting with our study. However, our original manuscript was dominated by discussion of genomic imprinting. We have extensively rewritten our introduction and discussion to focus on allele specific expression, making clear that our study can not differentiate between genetic and epigenetic causes. Genomic imprinting is now only mentioned in the second to last paragraph. Even here we only say our data is consistent with the genes being imprinted. Please see our response to Prof. Queller's fourth comment for details.

Reviewer 1 (Anonymous)

1. *Check order of citations (Weiner and Toth, 2012; Yan et al., 2014; Welch and Lister,*

2014; Glastad et al., 2011)) these may need to be reordered according to journal style.
Line 15 corrected

2. *Table 1. Remove the comma after Cardoen , Ecdysteroid regulated gene E93/Mblk-1 transcription factor Mushroom body large-type Kenyon cell specific protein 1 -like (LOC100645656) Upregulated in honeybee reproductive workers (Cardoen,et al., 2011)*
Corrected

3. *Here also:Ecdysone inducible gene L2/ ImpL2 Neural/ectodermal development factor IMP-L2-like (LOC100645498) Upregulated in honeybee nonreproductive workers (Cardoen,et al., 2011)*
Corrected

4. *Page 6 Space needed between exons and (Araujo et. al) "coding exons(Araujo et al., 2012; Lytle et al., 2007)"*
Line 181 corrected

5. *Page 7, Figure 3. wilcoxon is a proper name, it should be Wilcoxon and the type of test. Is it signed-ranks? matched-paired signed ranks? OR Wilcoxon Mann-Whitney test?*
This test is now not used.

Reviewer 2 (David Queller)

1. *The introduction is pretty sketchy in terms or providing context. Imprinting and epigenetics are said to be important for certain areas, but reasons arent given. Haigs theory is mentioned but an outside reader will have no idea what that is (and, the Patten review is cited on this, doesnt Haig deserve a citation or two for the particular ideas, not just for what imprinting is). In fact, Haigs theory provided special motivation for studying social insects before any indications of methylation in the social Hymenoptera. There are lots of taxa that have methylation but comparatively few that have a strong theoretical expectation for imprinting. Thats what makes the social insects particularly exciting in my view.*
We have extensively rewritten the introduction and discussion to focus on allele specific expression. Genomic imprinting is now only mentioned in the second last paragraph as a possible explanation (Line 201).

2. *Figure 3. Why no whiskers on the maternal plot? And can it really true that the 95% confidence interval for the Difference exactly matches the range?*
The boxplots have now changed, however some of them still have no whiskers. This is due to how R calculates outliers and whiskers. Less than Q1 - 1.5*IQR (inter quartile range) or greater than Q3 + 1.5*IQR then that point is classed as an "outlier". The whiskers goes to the first data point before the "1.5" cut-off. So plots without whiskers do not have extreme data points less than the 1.5 cut off.
The 95% confidence intervals for the second graphs have been removed as the graphs now show proportions as requested by reviewer three (comment 3).

3. *Im not very familiar with the required MIQE standards for quantitative PCR, but I dont see any mention of these in the article.*
We have reported what is required in MIQE standards. Where we differ from them is in our normalisation process. From the MIQE guidlines "Normalization involves reporting the ratios of the mRNA concentrations of the genes of interest to those of the reference genes. Reference gene mRNAs should be stably expressed, and their

abundances should show strong correlation with the total amounts of mRNA present in the samples." Clearly this would not be useful in allele specific qPCR as each sample is compared against itself. Rather we used the F3 primer sets as a reference. These amplify both of the alleles and provide the same information as a standard reference gene in normal qPCR.

4. *The main problem with this study is mentioned by the authors in their discussion and it is a serious one. What they find is differential expression, but there are possible causes of this other than imprinting. One is that the two alleles simply differ in promoter sites, so they get expressed differently in a way that is independent of which parent they came from. The core problem of this study is that the differential expression data come from a single family. Even if the maternal allele and the paternal allele in this family have different expression, there is no way to tell if parentage is the cause. You would need many families to show this, with maternal and paternal alleles consistently showing the same direction of effect in each. Or, as some studies of imprinting do, you study reciprocal crosses to ensure that the same allele is studied both when it is inherited from the father and when it is inherited from the mother. The authors suggest that their result is still interesting because the differential expression is found in exactly the kind of gene predicted by the kin conflict theory. I see the point, but they only examined genes of that type, so any result showing differential expression (for whatever cause) would have matched a gene predicted by the kin conflict theory. If differential expression were really rare in general, then finding it in one of 12 candidate genes might be notable. But given that differential expression is quite common for a variety of reasons other than imprinting (see Palacios reference) then it would not be surprising to see an example of differential expression in a set of candidate genes, even if imprinting never occurs in this species. In the end, I dont think the study can claim to show anything definitive about imprinting.*

   We agree and have now rewritten the paper to focus on allele specific expression. We also make clear, as you suggest, that we can not with our study tell the difference between genetic and epigenetic causes of this allele specific expression.

   In our reanalysis, suggested by you, our results are now more interesting. Ecdysone 20 expression is consistent with patrigene expression, as before. Added to this, IMP-L2-like expression is now consistent with matrigenic expression. We couldn't help but note this is exactly as predicted by your 2003 BMC Evol. Biol. paper. We have added the following to line 201 onwards:

   "Given this, it is still interesting to note that the expression patterns of both ecdysone 20-monooxygenase-like and IMP-L2-like are consistent with those predicted for genomic imprinted genes involved in worker reproduction in a singly mated social insect colony (Queller 2003). Queller (2003) used Haig's kinship theory for the evolution of genomic imprinting (Haig 2000) to predict the imprinting patterns of genes involved in various functions under various social contexts in the social insects. He predicted that genes that are associated with the initiation of worker reproduction (e.g. ecdysone 20-monooxygenase-like) should be paternally expressed in social insect species such as *B. terrestris* with singly-mated, monogynous (one queen), queen-right (queen still alive) colonies. Ecdysone 20-monooxygenase-like's expression is consistent with increased paternal expression. Reciprocally we would expect a gene that inhibits worker reproduction (e.g. IMP-L2-like) to be maternally expressed. IMP-L2-like's expression is consistent with increased

maternal expression. Fascinating as this is, it must be tempered with the proviso that, as previously stated, this work was carried out on a single genetic line so cannot differentiate epigenetic from genetic causes."

5. *An important issue, about which I am not completely certain, concerns whether differential expression was actually shown, even in the single family studied. Quantitative pcr is used for this, with the key comparison being between pcr product specific to the maternal allele and one specific to the paternal allele (both subtracted from a reference product). I can accept that the measures of relative amount of product are accurate, but do they actually reflect the relative amounts of RNA template for the two alleles? I dont see why. The forward primers are different for the two, both in their terminal base (to match the snp) and in their length. So couldnt those amplify differently even if the templates were in the same initial concentration? An obvious control is to use the method on genomic DNA, which contains 50% of each allele, but this wasnt done.*
   We have carried out the DNA control as suggested. The ecdysone 20 primers do not amplify differently, but the IMP-L2-like do. To control for this we reanalysed all our qPCR data to include the efficiency of the qPCR reaction. In the original manuscript we used a modification of the $\Delta\Delta Ct$ method. This normally raises 2 to the power of some version of Ct. The idea being that the amount of DNA doubles each PCR cycle. Other well established methods use actual efficiency of the qPCR reaction in place of 2. This is what we now use, see line 127 onwards.
   This is a much better analysis. It does not affect the pattern of the ecdysone result (as would be expected) but now shows the IMP-L2-like result to be significant.

Reviewer 3 (Anonymous)

1. *That said, the authors do a decent job of discussing the limitations of their current work, though perhaps these drawbacks could do with a bit more emphasis. For example, there should be some more specific discussion on the other factors that can drive allele specific expression rather than just stating these other factors exist.*
   We have refocussed the whole paper on allele specific expression.

2. *First, this study tested for allele specific expression in a single Bombus colony. In order to find evidence for genomic imprinting, these tests need to be conducted on reciprocal crosses that can be used to uncouple allelic effects from parental effects. Only if expression is indeed dependent on parent of origin can it be concluded that there is evidence for genomic imprinting. At the very least, multiple alleles inherited from the mother or the father should be tested. Unfortunately in this study there were no additional screened colonies where a polymorphism was identified.*
   Please see our answer to Prof. Queller's fourth comment.

3. *Second, I think the allele-specific expression results could be more clearly represented in Figure 3. What is the scale for Difference? What proportion of the expression is of the maternal copy vs. the paternal copy? If this is the difference in relative abundance, its rather hard to interpret. It would be more useful to discuss this in terms of proportions. Are we talking about a difference that looks like 90:10 maternal:paternal or more like 60:40? Imprinting is most often defined as complete silencing of one of the parental alleles. In this case, there appear to be much subtler effects on gene expression, and this should be quantified and described more clearly.*
   We have recalculated the relationships as proportions as suggested.

Sincerely,

Eamonn Mallon