

Author's response to reviews

Title: Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression in vivo: an experimental study in mice

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Author's response to reviews: see over

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Dear Dr. Ozonoff:

Please accept our revised manuscript, "Embryonic exposure to the fungicide vinclozolin causes virilization of females and alteration of progesterone receptor expression *in vivo*: an experimental study in mice," for further consideration for publication in *Environmental Health: A Global Access Science Source*. We have carefully responded to each of the comments from the reviewers and made changes accordingly. The reviewers' comments and each response, with quoted material from the paper as applicable, are appended below. We appreciate the reviewers' helpful and insightful critiques.

Please let us know if you need further information from us, and we thank you for your time.

Best regards,

A handwritten signature in black ink, appearing to read "Emily Willingham". The signature is fluid and cursive, with a long, sweeping underline that extends to the right.

Reviewer #1: Dr. R. Thomas Zoeller, University of Massachusetts

Reviewer's report: General

This manuscript describes work focused on the effects of vinclozolin exposure on gonadal tubercle development in mice and on the expression of steroid receptors (AR, ER, PR) in these tissues. While these are potentially interesting observations, there are a number of concerns about the manuscript as described below.

The language in this manuscript is quite imprecise. For example, the authors claim that progesterone receptors are required for appropriate signaling through the androgen receptor. This concept as stated suggests a signaling pathway that is fully unsubstantiated by the references cited, which are behavioral studies indicating that the progesterone receptor may mediate effects of progesterone on behaviors that might be traditionally considered to be regulated by androgens.

We agree that the question of whether or not androgen receptor interacts with or requires progesterone receptor remains wide open. Because there is simply very little data on this potential, our language must necessarily be equivocal. We feel that the language we used did not claim that progesterone receptors are required for appropriate signaling through the androgen receptor, but that research pointed to some intriguing possibilities in that regard, and that our own results add to this small accumulation of data and enhance the interest of the question. In the introduction, we state that, “Vinclozolin is known to affect androgen-receptor-mediated endpoints [1, 4–8], but some research indicates that progesterone receptors are required for appropriate signaling through the androgen receptor [9, 10]. Vinclozolin has been shown to affect androgen-receptor-mediated behaviors in mammals [11] and birds [12]. In addition, research indicates an inability for vinclozolin to compete for androgen receptor in the fathead minnow [13], even though it disrupts male steroid profiles and alters female gonadal condition in adults [13].”

In the discussion, we say, “In addition, if progesterone receptor is in fact required for appropriate androgen receptor activity (e.g., [9]), the increase in progesterone receptor in females may provide more opportunity for the existing androgen receptor to masculinize.”

Four of the studies cited are behavioral studies; these are the only studies we were able to identify that most closely addressed the possibility that progesterone receptor might influence androgen-mediated pathways, and we have added in some phrases to the text to clarify that. But we feel that “some research indicates” or “if progesterone receptor is in fact required” reflect the current, completely unresolved state of the question. Our only intent with these citations was to underscore the fact that some other studies have found a potential interaction and that the possibility is intriguing, especially in light of our own results. Also, the lack of an androgen-receptor response in the fathead minnow even with the altered male steroid profiles etc., leaves open the interesting possibility that we also identified here: other steroid receptors may be responsive to vinclozolin.

There are many places in the manuscript with similar imprecise language.

We have attempted to make the language as clear as possible throughout the manuscript and have rewritten some areas that we think might have been construed as imprecise.

The authors do not specify whether they are referring to (or measuring) PRA or PRB. There is reasonably strong suggestions that PRB preferentially mediates progestin effects on behavior. Analogues that selectively bind to one or the other of these PRs could very easily have different effects on tissue development and behavior.

We used Applied Biosystem's Mm00435625 gene expression assay. It detects both PR-A and PR-B, so these results reflect expression of both isoforms. We have added a sentence clarifying this, as follows: "The gene expression assay used for progesterone receptor did not distinguish between the two isoforms; thus, quantification reflects expression of both PR-A and PR-B."

The dose of vinclozolin used is considerably higher (50 times) than the reported NOAEL. Why choose this dose? And, at this dose, there could easily be a broad spectrum of effects. How does this affect the conclusions.

We selected the 50-mg dose because of the work of Earl Grey, which showed that at this dose, rats exposed *in utero* to vinclozolin exhibited hypospadias. We tried this dose with the mice in preliminary studies and found that it resulted in a percentage of hypospadias that was high enough for us to collect pooled tissue for a hypospadias group for RT-PCR; for this reason, we used this dose in this study. We have added this information to the materials and methods section as follows: "We selected the dose of vinclozolin based on results published by Grey et al.⁴ showing that this was the lowest dose that elicited frank hypospadias in male rats exposed *in utero*; our goal was to elicit hypospadias for molecular studies of the pathway. We also selected it based on preliminary investigations of our own indicating that this dose would produce hypospadias at a frequency that would allow us to compare tissue from hypospadiac and nonhypospadiac animals at the molecular level."

As for the fact that this dose is higher than the NOAEL, we feel that this information does not affect our conclusions. This dose elicited an effect (hypospadias in males; virilization in females) that we examined at the molecular level. Because our goal was to induce the effect and look at the molecular underpinnings of it, the dose itself was not a major factor. Grey reported that lower doses—as low as 3.125 mg/kg—elicited changes in AGD and in androgen-dependent tissues, but did not elicit hypospadias. In our studies examining steroid receptor expression with this model, we consistently choose doses of compounds that elicit a high enough percentage of hypospadiac males to provide tissues for our molecular analyses. Indeed, other published studies, including those of Grey, have used considerably higher doses (200 mg/kg) in rats; e.g., Shono et al., *J Pediatr Surg.* 2004 Feb;39(2):213-6.

Reviewer #2: Duncan Wilcox, U. of Texas

Reviewer's report: General

This is an interesting paper looking at the effect of a fungicide on genital tubercle development. The study appears well designed and the authors discussion addresses the complicated issue of why the same compound both virulises females and undervirulises males.

1. Page 5 NOAEL should be spelt out completely the first time it is used.
We have made this change.

2. Page 7. RT-PCR Methods. I am assuming that control animals were also used, this appears to be the case in the results. If so it should be mentioned here more clearly. If not then the authors need to explain why they did not use a control.

Yes, we did include a control group and neglected to say so. We have corrected this in the materials and methods section as follows: "Mice were gavaged once daily from GD 13 through 17 with 50 mg/kg vinclozolin or with corn oil alone as the vehicle control."

3. Page 9. RT-PCR is used as a title on this page it does not seem to fit in, I suspect this is a typographical error and it should be removed.

We have changed this header to read "*RT-PCR results*" so that it can be more clearly construed as a subhead. The paragraphs that follow describe first the frequency of hypospadias identified in the mice used for RT-PCR, then describe separately the PCR results for each steroid receptor assessed.

Also the paragraph starting "When the treated males..." is not very clear and I think it needs to be expanded on.

We have changed this sentence to read, "When we separated vinclozolin-treated males into two groups, those with and those without hypospadias, we found significant differences between each group compared to controls."

Page 10. I am concerned that the outlier has been removed especially as there was only 6 Dams in each group. How does this effect the statistics. I would like to know what a statistician thinks of this.

Prior to performing the outlier analysis, we consulted with statistics experts on the validity of doing so. We have provided below the original data for this group, which was the vehicle-control males group in the estrogen receptor analysis.

4.84
2.39
17.73
4.95
3.71
3.94
4.6
4.26
4.43
5.07

The outlier that was removed was the 17.73 for the pooled tissue. There were eight dams in this group, and each data point from the group represents pooled tissue of 4–5 male pups. We had a total of 10 data points; removal of the outlier left us with nine for this group. We believe that this outlier meets the criterion of having emanated from an error in sampling or other experimental analysis error, given the tightness of the other datapoints. In addition, this group did not exhibit this extreme differential in value for other receptors, again implying an experimental error for this specific procedure.

The critical Z value for a datapoint from a group of this size (10 datapoints) to be considered an outlier at a $p < 0.05$ is 2.29 (two-sided). The Z-value for this particular outlier was 2.8, which would meet the Z value cutoff for a sample of 24 datapoints; this was an extreme outlier. With the outlier present, the mean of the data is 5.59 and the SD is 4.33; without the outlier, the mean is 4.24 and the SD is 0.83.

5. Table 1. There is a typographical error I do not think that 46 was meant to be repeated.
This was a typographical error and has been fixed.