Author’s response to reviews

Title: Urinary Mycoestrogens and Age and Height at Menarche in New Jersey Girls

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Response to Reviewers:

Reviewer #1
This very interesting study is quite suitable for publication in EH. It is well written, and the Discussion particularly is excellent.
We appreciate the reviewer interest in our study. We acknowledge the fact that we didn’t measure conjugated mycoestrogen concentrations as a limitation of our study. We have included a new sentence to address this more clearly (Lines 269-270, “However, we were not able to measure conjugated ZEN (or metabolites) which may explain the higher number of participant with undetectable levels of α-ZAL and other metabolites.”). Despite this limitation, given the paucity of research on this topic, we believe these results are important and will stimulate future work.

(1) There are a few questions and clarifications. First it is confusing, how the exposure biomarkers are handled, when most are <LOD.

We appreciate this point and the need for additional clarification in the text. Please refer to Question/Answer #8 for a complete discussion on this issue.

(2) Second also confusing are the mixed models and what variables are used, in particular whether they include multiple hts and wts from enrolment to menarche. The way it reads now, in Table 3 and the text, a single weight/height is the outcome. Indeed, the authors undertake an analysis that considers BMI in/out of the pathway, which they justify correctly. But there doesn't appear to be much BMI-mediation here. Regarding mediation and the causal pathway, our puberty studies also examined growth trajectory in relation to several exposures of this kind, with findings that are generally consistent with BMI mediation (phthalates, phenols, elemental lead).

Regarding mixed models, we appreciate that additional details were needed. Height and weight measurements were longitudinally collected within each follow-up questionnaire until girls reached menarche, thus mixed models are longitudinal models which accommodate repeated measures. We have specified this in the Methods section (Lines 160-162, “These models included repeated measures for height- and weight-z-scores, consequently models calculate estimates using longitudinal height- and weight-z-scores for each girl”). Please refer to Question/Answer #10 for further details on mixed models and changes in the manuscript.

(3) Third, the Z urinary concentrations could be put in context with exposures seen in other populations, if any. However, a serious limitation is that only unconjugated (free) Z metabolites were measured. Most studies measure total or some combination of free/bound, and most urinary metabolites of this type are mainly conjugated. The laboratory method did not include the deconjugation step that is done in such studies. This is addressed in the Discussion, but is clearly a major limitation, and accounts for so many of the levels being LOD. It is perhaps likely that the
free concentrations are highly correlated with the total, but this is my speculation, though it might be supported by the literature, experimental or human.

We recognized that only measuring free ZEA (and metabolites) is a limitation of our study and that future work should quantify both conjugated and unconjugated forms. Notably, since the primary function of conjugation is to detoxify xenobiotic molecules glucuronidation typically results in a reduction of biological activity (Ritter, Chem Biol Interact 2000 129: 171-193). Thus the unconjugated form measured here may more accurately reflect the bioactive portion. We have included a new sentence to address the lack of conjugated mycoestrogen measurements (Lines 269-270, “However, we were not able to measure conjugated ZEN (or metabolites) which may explain the higher number of participant with undetectable levels of α-ZAL and other metabolites”).

(4) Z’s are somewhat homologous to some of the other phytos, so maybe analogy can be used to extend this argument. Finally, it is really a stretch to interpret an effect from "(adjusted HR: 0.35; 95% CI: 0.06, 2.00)." The only thing that might be reasonable would be comparison of results to a similar analysis for B2 in the earlier paper, if the estimate is similar to that. Otherwise, perhaps the BMI effects may be responsible for the imprecise estimates in Table 2 for menarche. And the small numbers can flip-flop the models. Rather than overinterpret, rely on the BMI findings which are interesting and consistent with the literature. Either a larger cohort or better exposure measures might improve the models.

Regarding the imprecise hazard ratios estimates, we agree is a stretch to provide a solid conclusion. We have reported the result and provided the factors that may affect them (unmeasured conjugated mycoestrogens, small sample size). In addition to that, we have deleted the sentence related to that in the abstract. Rather than trying to read into this result further, we have elected to discuss the potential impact of ZEA on menarche within the larger context of pubertal outcomes and endocrine disruptors. We still believe our manuscript provide important data worth publishing given the little knowledge on the impact of mycoestrogens on health.

Other comments:

1. Abstr should give some indication of urinary concs or %detect.

We have modified the first sentence of the Results section within the abstract to reflect the % of girls with detectable levels: “Mycoestrogens were detectable in urine in 78.5% of the girls” Abstract).
2. HR for menarche - mediation?

As mentioned above, we have deleted the sentence related to menarche hazard ratios from the abstract. In the results section, we edited a sentence to specify that BMI adjustment do not cause a change in estimate (Lines 201-202, In adjusted models, ZEN and total mycoestrogen results were largely null suggesting no effect by BMI).

3. Li 88: girls with negative levels - revise "negative"

We have revised this sentence to read “undetectable” instead of “negative.”

4. Li 119 The number of follow-up questionnaires completed ranged from 2-7 with most girls having 4-5 provide a number or % for 'most'.

We have added the % of girls with 4-5 completed follow up questionnaires (Line 124-125, “The number of follow-up questionnaires completed ranged from 2-7 with 83% of girls having 4-5 measurements within the follow-up period”).

4. Li 121 - give N for cohort.

To clarify sample size, we have added sample size for girls with mycoestrogen measures included in the main analyses (n=163) in the “population section” within Methods (Line 112-113). We have also specified sample size for the early menarche analysis (n=139) in the logistic regression models (Line 160-161).

5. Li 122 -Note these were included as right-censored.

Correct. We have made that clear in the Statistical section. “Loss to follow-up girls and girls with no menarche yet were right censored” (Line 151).

6. Li 133 - Note if external standards only, i.e. no internal standards used.

Noted, no internal standards used.

7. li 136 - edit? Grouped = summed concentrations or quantile values?
We have revised this sentence to read: “Secondly, we summed all of the analytes (ZEA, zearanol, and their related metabolites, i.e., α-zearalenol, β-zearalenol, β-zearalanol, and zearalanol) into a composite measure (“total mycoestrogens”).” (Line 138).

8. Li 139: generally if levels are <50% or 60% or 70% <LOD (depending on study), continuous concentrations are not used (Lubin and others). This would affect medians in Table 1, where most investigators do not report univariate data for LODs, except as ‘LOD’. LOD should be provided in a note. A table of urinary Zearalonine etc levels should be given.

We understand the Reviewer’s concerns on this point and it is one we have considered extensively. . Our first publication on this study (Bandera et al. Sci Total Environ 2011 409:5221-5227), presents details of study design and mycoestrogen analytical protocol. During that analysis, we evaluated various approaches to managing values below the LOD including dropping data points to different imputation methods. In the current analysis, we assigned samples below the LOD a value of the limit of detection divided by the squared root of two. This method has been shown to provide accurate estimation of the median and standard deviation and recommended when the data is highly skewed (Hornung & Reed, App Occup Environ Hyg, 1990 5:46-51). This allowed us to increase the power of the analysis. Nevertheless, in Question #10, we present an analysis where we categorized the exposure, <LOD and >LOD. We decided to present medians to be consistent with our previous publication were medians were presented. We have added a footnote on Table 1 clarifying the medians that were <LOD.

Additionally, Bandera et al. 2011 includes a table (Table 1, below) with all metabolites concentrations including individual metabolites not presented in the current publication. Therefore, we did not include them in the current study (see table below).

[See SUPPLEMENTAL MATERIAL FOR RESPONSE TO REVIEWERS to see the table including mycoestrogen concentrations from Bandera et al 2011. The EH submission system did not allow us to include it in this section]

9. Li 147 - note how time-to-menarche was treated for right-censored obs.

Thanks for this suggestion. We have added a new sentence to specify this (Line 154, “Loss to follow-up girls and girls with no menarche yet were right censored”).

10. Li 157 - "matrix were used to assess height and weight z-scores in relation to…” It is not clear if this is a single ht/wt measure or multiple longitudinal measures; perhaps specify the
model exactly \((y=x)\). Also, where this is mentioned at li 197, not clear what variables are in the mixed effect models.

Regarding dichotomous Zea, as it is 55% detected, an alternate categorization would be LOD (A), lod-midpoint of detected (B), and \(>(B)=(C)\), to provide a little more power and an indication of linearity. Or perhaps as a sensitivity analysis, as (C) might be similar to the small \(>LOD\) zeranol group. Alternatively, if "machine" values are available for the LODs, those can be used. There are a number of refs to justify this approach, and it would be further supported by the categorical exposure models.

We understand the need for clarification in our mixed model application and we have added details for clarity (Line 161-163). Height and weight measurements were longitudinally collected within each follow-up questionnaire until girls reached menarche (Lines 121-123) and included in the mixed models as repeated measures. The mixed models accommodate repeated measures and not only model the means but it accommodate their variance and covariance as well. These models were adjusted for family income and mid-parental height (Lines 180-181, 205). Levels below detection were assigned a value of the limit of detection divided by the squared root of two to below. In sensitivity analysis, only presented here, we dichotomized the exposure, \(<LOD\) and \(>LOD\) detection values as explained in question. Results are comparable to the main analysis presented in the manuscript. However, we believe that analyzing the data as continuous with no missing values gives us more power.

[See SUPPLEMENTAL MATERIAL FOR RESPONSE TO REVIEWERS to see the tables including the sensitivity analysis. The EH submission system did not allow us to include it in this section]

11. Li 198: does average weight and height z-scores = average weight- and height- z-scores

Correct. We have taken the Reviewer’s suggestion and have changed the terms as weight- and height- z-scores through the manuscript.

12. Li 199: Zeranol levels - note if this is detect vs non-detect.

Correct. We have added “\(>LOD\)” to the sentence. Thanks.

13. Disc li 209 This may mean to say zeranol, rather than ZEA? ("Girls with detectable ZEA concentrations were also less likely to have early menarche, but results were 210 not statistically significant")
Correct. We have made the correction. Thanks for bringing it to our attention.

14. li 281, regarding single urine sample for biomarker: in other studies some but not all biomarkers of this kind have been found to be consistent over wks to years (i.e. ICC>.5), so if the Z's come from common, usual exposures a single sample may be expected to be fairly reliable.

We agree with the Reviewer that this may be a possibility. However, because human biomonitoring of mycoestrogens is an understudied area we want to be cautious. Thanks for the note.

15. Table 1. see notes on handling of LOD values

Please see answer for Question #8. Additionally, we have added a note on our LOD approach as part of the Table 1 footnotes.

Table 2. Indicate concentrations of LOD/<LOD

Added, thanks.

Table 3. Indicate exposure concentrations. If these are ng/ml, then the difficulty of imputing <LOD makes this model problematic. If it is <>LOD, then note if exposure is coded 0,1. Not clear what the note means (Note: n=163 girls, observations used in mixed models= 2,021 (from 2,507).) Are there multiple ht/wt in these models? See also same question above. Describe the model more detail.

Please see answers for Question #8 and #10. The model includes multiple height and weight measurements and we have described the model in more detailed as suggested.

Reviewer #2:

Specific remarks and questions
It is unclear why the authors have not made efforts to collect additional urine samples from study participants to assess their exposure to zearalenone, zeranol and their metabolites for several reasons.

We appreciate the reviewer’s interest in our study. We would have liked to collect additional urine samples as suggested, however, due to funding constraints, our only face-to-face visit was at baseline with all additional follow up conducted by phone or mail.

(1) In the previous urine analysis the authors have only analyzed the unconjugated forms, omitting enzymatic hydrolysis of conjugates. But, these are known to comprise a large fraction of the excreted mycoestrogen and its reduced forms (see Mally et al. 2016; Metzler et al. 2011 and references cited in these reviews).

We recognized that only measuring free ZEA (and metabolites) is a limitation of our study and that future work should quantify both conjugated and unconjugated forms. Notably, since the primary function of conjugation is to detoxify xenobiotic molecules glucuronidation typically results in a reduction of biological activity (Ritter, Chem Biol Interact 2000 129: 171-193). Thus the unconjugated form measured here may more accurately reflect the bioactive portion. We have included a new sentence to address the lack of conjugated mycoestrogen measurements (Lines 269-270, “However, we were not able to measure conjugated ZEN (or metabolites) which may explain the higher number of participant with undetectable levels of α-ZAL and other metabolites”.

(2) Moreover, recent studies on the analyte pattern in human urines from several cohorts by state-of-the art methods vary considerably from the pattern found in the New Jersey girls: Whilst zearalenone was the predominant analyte in the latter, other studies found that urine levels of alpha-zearalenone exceeded those of the parent mycoestrogen (Shepard et al. 2013; Solfrizzo et al. 2014; Fleck et al. 2016; Ali & Degen, 2018; Sarkanj et al. 2018). These studies (not yet included) are of interest and the apparent discrepancy in analyte pattern requires a comment, also in light of the strong differences in estrogenic potency for ZEN, alpha- and beta-ZEL.

In addition, we have added Ali & Degen, 2018, Sarkanj et al. 2018 to the Discussion section. Thank you for the suggestion. “Lines, 263-265, Additionally, biomonitoring studies have reported that some of the metabolites levels in urine are comparable or exceeded those of ZEN.” Lines 269-270, “However, we were not able to measure conjugated ZEN (or metabolites) which may explain the higher number of participant with undetectable levels of α-ZAL and other metabolites.”
(3) Human biomonitoring studies in spot urines shed a light on exposure at a given point in time, but should be repeated if possible to gain also insight on variability as dietary exposure may change within a given period (e.g. Ali & Degen, 2018). In the present study this would have been particularly important in light of the controversial findings on the impact of mycoestrogen exposure on puberty in girls (Asci et al. 2014; Massart et al. 2008; Szuets et al. 1997).

We very much appreciate this input and recognize the limitation. Nevertheless, given the lack of research in this area, particularly in non-clinical populations, we feel that this work is novel and will stimulate future work that includes variation in concentrations over time, as the reviewer recommends.

Several other aspects in the present manuscript require amendments:

(4) In the Methods section, several references are mentioned by author and year whilst numbering is used in other sections.

Thank you for pointing this out, it has been corrected.

(5) In addition to the EFSA opinion mentioned (of 2011) there is a more recent one (EFSA 2016) which deals with the rational for setting a group value for ZEN and its reduced forms.

Both References are now included in the manuscript, Line 83 and 247.

(6) The analytical method described a bit more detailed in the previous paper (Bandera et al. 2011) may have been fit for purpose, but chromatograms of a real sample (and standards) would be useful, perhaps as supplementary information.

Currently, we are working on a manuscript on our analytical approach to ZEN (and its metabolites) detection. Although that level of analytic detail is beyond the scope of the current epidemiologic manuscript, the methods paper in preparation will include chromatograms.

(7) Table 1 contains (in the first column, characteristics) also the category 'missing'; this should be explained in a footnote.

We have explained the “missing” row in table footnote.
The acronyms used here for zearalenone, zeranol (alpha-zearalanol) and its congeners are outdated and should follow better nomenclature (Metzler M, 2011; Mycotoxin Res. 27:1-3).

We have updated the manuscript to reflect suggested nomenclature.