

Author's response to reviews

Title: Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in cd-1 mouse pups

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Author's response to reviews: see over

February 10th 2009

Philippe Grandjean and David Ozonoff
Editors-in-chief
Environmental Health

Dear Editors

Please find enclosed the revised manuscript “Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in CD-1 mouse pups.” by Laura Ricceri, Maria Luisa Scattoni, Gemma Calamandrei, and myself, that we would like you reconsider for publication to Environmental Health. In the following pages we detailed the changes made in the manuscript according to the three referees’ comments

Best regards,
Aldina Venerosi

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Reviewer's report

Reviewer: Theodore Slotkin

Reviewer's report:

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There are some minor revisions:

The authors have a number of findings that are individually “at the margin of significance” but are all suggestive of biologically significant changes (p. 17). The collective finding, though, does not appear to be likely as a random event. The authors may wish to group the results for a chi-square test of combined p-values, ($\chi^2 = -2 \ln(p)$, where $df=2 \times \#p\text{-values}$) so that they can make a global statement about the overall pattern rather than having to qualify the conclusions as they do. The reason this is important is that human exposures are likely to be lower, and if the global test is significant, it would point to the need to perform a test battery likewise in humans, rather than one or two individual tests. So if the global result is positive, this could be an important discussion point.

There are a number of typos in the reference list that look like database glitches (edited volume citations indicating ed^eds).

We thank this reviewer for his comments to our study. We agree that a global statement about the overall pattern would be more informative. As a matter of fact, it has to be considered that the “Total daily score” included in our study somewhat corresponds to a global measure of sensorimotor development for each animal (Page x line x of the revised manuscript).

The chi square test of combined p-values, suggested by the reviewer, requires a set of p-values obtained from independent studies testing the same null hypothesis (G. Singh: Conventional Approach Of Combining P-Values Using Chi-Square Test: An Essence of Meta Analysis. The Internet Journal of Medical Informatics. 2007. Volume 3 Number 1. Westfall P.A. Combining P values. Encyclopedia of Biostatistics, 2005). Unfortunately this is not our case, since the tests we performed were applied to data collected on the same group of subjects.

Length is misspelled as “lenght” in Table 1.

female CPF PN3 body length value is missing some digits (should it be 3.00?)

Manuscript has been corrected for typos and grammatical errors.

Reviewer: Christine V Portfors

Reviewer's report:

• Major Compulsory Revisions

1. This study evaluates the effects of low-level exposure of chlorpyrifos (CPF) on ultrasonic vocalizations and motor behavior in mouse pups. While rodent models seem to have benefits for understanding short and long term neurological effects of CPF exposure on human infants, the rationale for this particular study is not well laid out. The information presented in the Background section is not in a logical order that makes the reader understand what is known and not known about the pertinent issues and it does not clearly outline the purpose of the study. These issues need to be clarified. For example, the rationale for studying ultrasonic emission patterns in isolated mouse pups is not clear. Why would these change with CPF exposure? What are the expected results? What is the hypothesis?

We have partially rephrased the background section to clarify the rationale for studying patterns of ultrasonic vocalizations in mouse pups perinatally exposed to CPF (page 5, 2nd paragraph to the end of Background section).

We stressed the point that developmental CPF exposure consistently targets serotonergic pathways and recent data also show that hypothalamic neuropeptides (oxytocin, vasopressin) are selectively affected by developmental CPF treatment. Since these systems unequivocally

regulates social behavior in adult mice, but also mouse neonatal vocalizations we decided to include the ultrasonic vocalization analysis in the assessment of neonatal behavioral patterns. Furthermore, USV have proven to be an extremely sensitive endpoint to detect even subtle effects brought about by different xenobiotics, including drugs of abuse and environmental contaminants (Elsner et al, Neurotoxicology and Teratology 1990; De Marco et al, Toxicology 2005; Rankin and Manning, Behavioral Neural Bio logy 1993). We considered that together with evaluation of sensorimotor development and analysis of spontaneous behavior, USV recording could give a more exhaustive picture of the potential adverse effects of prenatal CPF exposure in the early neonatal phase.

Moreover, there is little (or incorrect) information on ultrasonic vocalizations in normal mouse pups. For example, it is stated on p. 5 that “USVs present clear ontogenetic profile...” but it is never explained what this is and how this may be expected to change with CPF exposure. Two pieces of information in this same section (p. 5) are incorrect. First, mouse pups emit vocalizations much higher than the 30-50 kHz stated (This is even shown in Fig 2).

We added a description (page 5, lines 5-11) of the ontogenetic profile: “USVs present a clear ontogenetic profile, peaking around day eight after birth and decreasing close to zero when pups are two weeks old (Ihnat et al. 1995; Noirot, 1969; Elwood, 1982)”, even if considerable differences have been found among mouse strains (Roubertoux et al., Behavioral Genetics 1996; Sewell, Nature 1970).

The range of mouse pup vocalizations was actually incorrect: it has now been replaced by 30-90 kHz.

Second, the statement that USVs are considered indicators of the emotional state of the animal is only true for rats. Mice emit USVs under different social conditions than rats and there is evidence suggesting that rodent pup vocalizations are artifacts of laryngeal braking rather than emotional “cries for help.” Thus, the interpretation that mouse pup USVs can be used to assess emotional state is not sound. This also potentially lessens the translational impact of the results in terms of relating levels of USV emissions in mouse pups to cries in human infants.

We are aware of the fact that rats emit different kind of vocalizations (22 and 50 KHz) that have never been detected in mice (Ultrasonic Communication by animals Eds: Sales G and Pye D, p 150. Chapman and Hall, London). However, a large body of evidence reported that USV are emitted in different social conditions throughout the entire life span in mice too, ranging from neonatal calling, including the so-called maternal potentiation response (Moles et al, Science 2004; Scattoni et al, Behavioral Brain Research.2008), to female-female interactions in adulthood (Rev. D'Amato).

As pointed out by this referee, there has been considerable debate (for both rat and mouse species) about the communicative value of ultrasonic vocalizations. It has been postulated that these sounds are the incidental by-product of a physiological response to a thermal challenge, e.g. the reflexive abdominal compression reaction that helps return venous blood to the heart (Blumberg and Alberts, Behavioral Neuroscience 1990; Blumberg and Sokoloff, Psychological Review 2001). However, it is a sound ethological evidence, not necessarily in contrast with the USV view of *artifacts of laryngeal braking*, that pup vocalizations elicit maternal approach and retrieval (Noirot, Developmental Psychobiology 1972; Smotherman et al, Behavioral Biology 1974; Cohen-Salmon et al, Physiology and Behavior 1985; Ehret and Bernecker, C., Animal Behaviour 1992) and reduce attacks or rough manipulation by the dam (Ihnat et al, Behavioral Processes, 1995; Noirot, Animal Behaviour, 1966). A dynamic relationship between maternal responsiveness and pup calling rate has been also confirmed in a study on maternal responsiveness to USVs, carried out in two different mouse strains (D'Amato et al., Behavioral Genetics 2005).

Moreover, a series of pharmacological studies have demonstrated that USV production in both rats and mice are modulated by several anxiolytic and antidepressive compounds (Hodgson et al,

Pharmacology Biochemistry and Behavior 2008; Cirulli et al. Developmental psychobiology 1994), thus supporting the hypothesis that ultrasound emission, whatever its physiological function, is an important component in the establishment of the mother-offspring bond.

2. Ultrasonic vocalizations (USVs) were recorded at day 4, 7 and 10. Significant differences in call rate, duration and frequency at maximum amplitude were found only at day 10. While this is interesting, it is unclear how real this effect is without further data collection. The differences for call duration and peak frequency are not great and could be due to individual variations that occur near the onset of hearing in mice. Additional subjects are necessary to adequately determine the importance of these differences. The significant decrease in number of calls/min at day 10 in the treatment group is very interesting but it is problematic that USVs were not recorded at subsequent days. If for some reason, day 10 is an anomaly and the differences are gone at day 11, 12 or later, then the significance of the results is lost. If the changes persist for additional days, then the interpretation that CPFs actually alter USV emissions is stronger and important. However, without additional evidence (i.e. more days of data collection), the data are not strong enough to justify the interpretations made in the study. Moreover, the authors provide no adequate explanation for why the differences would exist at day 10 and not at day 7.

As stated above the number of USV emitted by neonatal mice follows a clear ontogenetic profile with a peak around day eight after birth followed by a rapid decrease afterwards thus reaching a value close to zero when pups are two weeks old. We agree with the reviewer that it would have been interesting to know if the CPF effect on USV observed on pnd 10 were confirmed at successive days. Indeed, in developing mouse studies the age range analyzed is usually the one used in this study. We know from our previous studies and the literature that in outbred CD-1 mice no USV are detectable after pnd 12 [pnd 2-9: Simeoni et al, Acta Neurobiologiae Experimentalis 2005; pnd 5 and 8: Branchi et al Behavioral Brain Research 2004; pnd 5-11 Ricceri et al Neuroscience Biobehavioral Review. 2003 (with less than 10 calls per min on pnd 11); pnd 3- pnd 11: Penner et al, Developmental Psychobiology 2001]. In planning our study, we did not expect to record a sufficient level of vocalizations on pnd 12. On the basis of the present findings, we are planning a new experiment aimed at assessing if levels of USV are still altered during the second week of life in CPF-exposed mice. A sentence has been added in Discussion in this respect (page 17, line 19).

The referee states that no adequate explanation for the existence of CPF effects on USV on pnd 10 and not on pnd 7 is provided. In the discussion, however we attempt to interpret these findings linking the particular time course of CPF effects to the expression of oxytocin receptors during development, also in light of the enhancement of oxytocin protein levels found in hypothalamus after the same dose of prenatal CPF used in the present study (Tait et al, Environmental Health Perspective 2009).

3. The Results section is dramatically disorganized. How this could not be caught by proof-reading is unknown to me. The first 4 sections (2 ½ pages) are repeated later on. The first figure referred to is Fig. 3 and this is not even the correct figure. The next figure referred to is Fig. 4 and there isn't even a Figure 4 in the Results! Obviously, the organization is very messed up and this did not make the paper any easier to read.

An error was done when formatting the manuscript for electronic submission that requires the different Sections to be separately copied in specific paragraphs. We apologize for this inconvenience. The Result section has now been reorganized.

• *Minor Essential Revisions*

1. *The entire manuscript needs to be edited and corrected for grammatical errors. There are many errors and sections that are not clearly written.*


Manuscript has been corrected for typos and grammatical errors.

• *Discretionary Revisions*

1. *It is unclear how the sex of mouse pups can be determined at birth. I did not think this was possible (at least no one in my lab can do it accurately until the pups are weaned). Explicitly how the sex of the animals was determined at day 1 should be included in the Methods.*

2. *I also find it surprising that each litter contained 6 males and 6 females. Is it common for this strain to have some many pups in a litter that 6 males and 6 females are in each one?*

1. At birth it is possible to determine the sex of mouse pups by the evaluation of anogenital distance. This methods is largely used in our and other laboratories. Details have been added in Methods section (pag 7, lines 10-11);

 2. The number of male and female pups which composed the litter at culling has been corrected in the Method section (pag 13, first paragraph). Indeed in the Results section we reported correctly the data relative to reproductive performance (mean number of pups : Veh: 11.5 ± 0.55 CPF: 10.18 ± 0.70). However we found 5 out of 18 litters with a number of both males and females ≥ 6 in the Veh group, and 4 out of 16 in the CPF group.

Reviewer's report

Title: Prenatal chlorpyrifos exposure alters motor behavior and ultrasonic vocalization in cd-1 mouse pups

Version: 1 **Date:** 30 December 2008

Reviewer: Francesca D'Amato

Reviewer's report:

This paper describes the effects of chlorpyrifos given to the mothers during late gestation, on the neurodevelopment of the offspring. The authors have already conducted several studies on chlorpyrifos exposure in mice but they present these earlier studies only in the discussion. I suggest to describe their previous results in the Introduction to better explain their aims, according to their already published data.

We have modified the Introduction following the reviewer's suggestion.

Some paragraphs in the results section have been erroneously repeated several times. I imagine that the last paragraph of page 12, page 13 and the first two paragraphs of page 14 should be deleted and the results section should start with: "On PND 0 data analysis on number..."

An error was done when formatting the manuscript for electronic submission that requires the different Sections to be separately copied in specific paragraphs. We apologize for this inconvenience. The Result section has now been reorganized.

I greatly appreciate the detailed methodological protocols. I only suggest the authors to consider the following points

- *Delay in body length in CPF males: according to the statistics (no significant reported interaction effect of day x sex x treatment), these CPF pups seem to be shorter rather than showing a delay in body length.*

We changed in discussion (page x lines xx) the paragraph describing body length data according to the referee suggestion as follows:

"As for somatic development, CPF failed to affect body weight but reduced body length in male pups. This effect, that is suggestive of a subtle growth delay, is in partial agreement with what reported by one epidemiological study....."

- *The authors interpret the reduced level of USV on PD 10 as a "CPF depressive effect on pups". As USV tend to decrease as pups grow up, it is also possible to suggest that this decrease in vocalizations observed on PD 10 is due to an acceleration of development, as the first two sampled days (PD4 and PD7) showed no CPF effect.*

Although we considered the possibility that the effect seen on PND 10 might be due to an acceleration of development, however the absence of effect on PND 4 and 7 does not support such view. CPF pups presented a peak of emission at PND7 as expected in this strain. Thus CPF did not appear to alter the ontogenetic profile of USV, but rather a specific point of that profile, that could be temporally related to a crucial phase of activation of neuroendocrine functions linked to social competencies. We are aware that this is a still speculative hypothesis deserving further investigation. However, in the discussion, we based such hypothesis on the following evidences: the ontogenetic peak of expression of oxytocin receptors in the second week after birth, the rat data on oxytocin modulation of huddling response on PND 10 but not on PND 7 and the enhancement of oxytocin protein levels in hypothalamus after the same dose of prenatal CPF used in the present study.

- *As for the delay in body length, have you any justification for the observed gender effect?*

Previous data on developmental CPF neurotoxicity indicated, in rat studies, different effects in the two sexes possibly depending on windows of exposure (Garcia et al, Environmental Health Perspectives 2003). Our previous data in the mouse species similarly evidenced CPF effects on sex dimorphic behavioral items, thus indicating that CPF might interfere with mechanisms related to sex differentiation of the brain. It is possible that a greater vulnerability of the male sex to the somatic effects of CPF is attributable to a more general interference with endocrine regulation of growth, but any conclusion in this respect is highly speculative.

• *Page 20: In the sentence: Further investigations on neuropeptidergic CNS levels in pups developmentally exposed to CPF are needed to assess if the present reported changes in OT and AVP observed in adults exposed . delete the term “present” and add the citation*

We changed the sentence as indicated by the reviewer.

• *The differences observed in maternal behavior recorded only on PD 4 and only at reunion with the removed pup suggest, as stated by the authors, an effect of the treatment on emotionality, rather than on maternal motivation. However I ask the authors to be very caution in interpreting this result as it is based on only two minutes of observation x animal. In addition, even if a normalization between the two observational sessions (before and after) has been conducted, the experimental conditions are different (before: 8 min of observation after pup removal; after: two min of observation after pup reintroduction into the home cage) and I don't think that the comparison between the two sessions in CPF and control mice is relevant.*

We agree that the “Before” and “After” sessions refer to two different experimental conditions and that comparison between the two is not fully correct. In the revised version we decided to perform two separate analyses, where baseline levels of maternal responses after separation and reintroduction of the whole litter were scored for 8 minutes (Session 1) and responses to restitution of a single male pups were scored for two minutes (Session 2). In Figure 1, only data concerning Session 2 are now represented. (Result section, page 13)

• *The increase in grooming pup observed at reunion remind Meaney and collaborators data, showing that high levels of grooming resulted in lower emotionality at adulthood. Lower USV at PD10 could result from changes in maternal behavior, rather than direct changes of the OP on pup's SNC.*

We agree with the reviewer comment. At the present stage of the work, the possibility that CPF effects on the offspring could be in part mediated by CPF-induced changes in maternal behavior cannot be discounted and it is currently under study in our laboratory. However, on the basis of present data, it is very difficult to attribute the lower USV level recorded on PND 10 in CPF pups to the increase of maternal licking on PND 4. The increase in pup licking was indeed observed in both treatment groups and only after pup removal from the cage and its subsequent reintroduction. No data were recorded on the overall level of spontaneous licking and maternal behavior in the home cage following repeated observations at different days during the first two weeks after birth. We thus consider our data on maternal behavior very preliminary but suggestive of subtle changes in maternal responsiveness that need to be more systematically investigated (see revised Discussion, last paragraph).