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LETTER TO THE EDITOR

Superfeminization as an effect of bisphenol A in *Marisa cornuarietis*

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Recently, we had the opportunity to read two articles in volume 66 of *Ecotoxicology and Environmental Safety*. Forbes et al. (2007a, , 2007b) declare "to explore the reproducibility of prior work" showing that bisphenol A (BPA) induces superfeminization in the freshwater snail *Marisa cornuarietis* (Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2001). Based on the outcome of a toxicity test with the same species, the authors conclude that their results "do not support previous claims of enhanced reproduction in *M. cornuarietis* in response to exposure to BPA." We take issue with the declaration of exploring the reproducibility of our challenged work and the validity of the conclusions made by Forbes et al. (2007a, 2007b). Furthermore, we feel the toxicity test is flawed because its experimental design and the selected exposure conditions result in an irresponsiveness of test animals to the tested compound.

The studies by Forbes et al. (2007a, 2007b) are not suitable for investigating the reproducibility of our challenged studies because of substantial differences in design (e.g., pair-breeding vs. spawning groups, duration of experiment, considered endpoints, and lack of positive control (PC)), exposure conditions (e.g., flow through vs. semistatic exposure, water parameter, and selected temperature), and test animals. Specifically, the aspects of (1) potential strain and probably even species differences, (2) mate choice and pair-breeding design, (3) temperature selection, and (4) the lack of a PC in Forbes et al. (2007b) demand attention.

(1) Offspring of wild-caught snails from a lake in Puerto Rico were used for the experiments reported by Forbes et al. (2007a, 2007b). These snails exhibit an external sex dimorphism but no seasonal pattern in reproduction, whereas snails used for our studies are not externally sexual dimorphic and have shown a marked reproductive cycle since 1994 with a 2-month spawning season starting at the end of the year (Schulte-Oehlmann et al., 2001). These substantial dissimilarities point to the existence of two different strains if not even to two cryptic species as has been shown for a number of other gastropods (Wilke et al., 2002; Pfenninger et al., 2003). The importance of animal model selection for tests with endocrine disrupters, including BPA, has been intensively discussed by the NTP Low-Dose Peer Review Panel, also highlighting the importance of a PC (see also (3) below): "Because of clear species and strain differences in sensitivity, animal model selection should be based on responsiveness to endocrine active agents of concern (i.e., responsive to PCs), not on convenience and familiarity" (NTP, 2001).

(2) The pair-breeding design in Forbes et al. (2007a, 2007b) was chosen to investigate the contribution of differences among snail pairs to the total variance in egg production. This approach is based on the tacit assumption that mate selection in *M. cornuarietis* is arbitrary. However, the occurrence of pairs without any egg production (five out of 180; cf. Forbes et al., 2007b, p. 321) and the high variability between pairs (causing 94% and 97% of the total variance according to Forbes et al., 2007a, 2007b, respectively) can be signs of an active mate selection in *Marisa*. A spawning group design as in our studies accounts for potential mate selection and is therefore the appropriate approach if sexual selection in a species cannot be ruled out. This is also indicated by the low coefficient of variation (1.2–12%) for egg production among replicate spawning groups in Oehlmann et al. (2006). Most prosobranch snails reproduce in spawning groups also in the field (e.g., Fretter and Graham, 1962; Crothers, 1985). Furthermore, there is increasing evidence that sexual selection is widespread in molluscs and during the World Congress of Malacology in Antwerp this year, a 2-day session is dedicated to this issue.

(3) Forbes et al. (2007b) conducted their experiment at 25 °C, whereas the challenged experiments were performed at 22 °C (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2001) and at 20 and 27 °C in parallel (Oehlmann et al., 2006). The latter study showed that control snails produced 34% more eggs at the higher temperature ($p = 0.026$) and that the stimulating effect of BPA on egg production was masked at 27 °C due to an increased reproductive output in controls. An increase of 55% is also reported at 25 °C when compared with 22 °C in the study of Forbes et al. (2007a) ($p = 0.02$). Because of the obvious temperature dependence of effects, a direct comparison between studies performed at different temperatures is not possible. Claims that temperatures in our experiments are too low and inappropriate for *M. cornuarietis* are not justified. The sustainability of culture conditions is demonstrated by the fact that our laboratory stock has now been maintained for more than 13 years and provided several thousand snails per year for toxicity tests. Furthermore, *Marisa* is reported in the field at comparable temperatures, e.g., in the San Marcos Spring ecosystem (Florida), where the species is widely distributed. Along the river, hourly measurements of temperature were performed at seven stations between November 1994 and May 1997, with mean values ranging between 21.0 and 22.1 °C (n = 10,221–19,189 for each station) (Saunders et al., 2001).

(4) The importance of considering a PC in tests with endocrine active chemicals such as BPA was recently highlighted by vom Saal and Welshons (2006). This issue is particularly important if non-standard test organisms are used and for studies showing no effects, such as Forbes et al. (2007b), because the general responsiveness of the test organisms can be proven only if a PC is applied. Otherwise, negative results from an experiment cannot be interpreted and statements that a test chemical does not show an effect are inappropriate.

Forbes et al. (2007a) argue for a "lack of basic knowledge of the influence of husbandry conditions on the performance of *M. cornuarietis*" in our studies. This is unjustified. As already stated above, we have maintained a stable and healthy laboratory stock since 1994. This stock has been intensively characterized regarding reproductive output (including seasonality), growth, development (including sexual differentiation), sex ratio, and longevity. Given our knowledge, we had already predicted before the start of the experiment now published in Forbes et al. (2007b) that the selected exposure conditions would result in null effects (letter to the UK Environment Agency, October 11, 2005). Other claims are also unjustified, such as "no replication" in our experiments, of "inappropriate statistical techniques," and that we did not control "for differences in snail age within or among treatments." Exposure series II in Oehlmann et al. (2006) used replicates for all treatments. Furthermore, all experiments reported in the three challenged publications provided identical effects and even EC_{10} values, demonstrating the intra-laboratory reproducibility of our work.

The data in Forbes et al. (2007a) show that the reproductive output of *M. cornuarietis* declined in all three laboratories with time over a period of 13 months (by 50%, 42%, and 75% at ABC, BEL, and RUC, respectively). These data are interpreted by the authors as an effect of snail age and as an argument against seasonality because of the lack of "any signs of returning to starting egg production rates toward the end of the full-year trial." Not considering that a 13-month experiment is not suitable for analyzing seasonality under constant conditions with a free-running period, these results point to a rapid and early reproductive exhaustion of the snails under the chosen test conditions. In our laboratory, snails do not show any signs of reduced egg production over their reproductive life period, except at the end of the already described spawning period.

A direct comparison of fecundity data between the different experiments reported in Forbes et al. (2007a, 2007b) indicates a poor intra-laboratory reproducibility and a dramatic increase in egg numbers produced per female and week at ABC in the second compared with the first experiment: while Forbes et al. (2007a) report an average of 440 eggs per snail and month at ABC (p. 311), the number for the same laboratory increases by almost 40% to 600 eggs per female and month in the control group during the toxicity test (cf. in Forbes et al., 2007b, p. 322). This is comparable to the increase of between 45% and 69% under BPA exposure, reported by Oehlmann et al. (2006).

In summary, considering the differences in experimental design, exposure conditions, and test animals, the results of Forbes et al. (2007a, 2007b) cannot answer the question of reproducibility of the earlier challenged studies. The remaining question from the studies of Forbes et al. (2007a, 2007b) is how to benefit from a biotest performed under conditions, which were deliberately selected to maximize the reproductive output in the controls and thus to mask the previously reported BPA effects.

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Response from Forbes et al. to Oehlmann et al.

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To the Editor,

We thank the editor for giving us the opportunity to respond to Oehlmann et al.'s comments (Oehlmann et al., 2008) on our papers (Forbes et al., 2007a,b). In response to the criticisms of our studies we would first like to emphasize that it was not our aim to exactly repeat the experiments of Oehlmann and colleagues, but rather to produce robust and reproducible results that were statistically valid and that therefore could be used in the risk assessment of bisphenol A (BPA).

We are disturbed by the highly speculative suggestion by Oehlmann et al. that we may have studied a different 'cryptic' species of *Marisa cornuarietis*, as there is no evidence to support such suggestion. Furthermore, the snails used in our studies were collected from a documented pristine field site and identified to species by Dr. Sharon File-Emperador from University of Puerto Rico, a recognized snail expert. They were reared for a known number of generations under tightly controlled and documented conditions. In contrast, the snails used in Oehlmann et al.'s studies (e.g., Oehlmann et al., 2006) came from the Dusseldorf Zoo (original site of field collection not indicated) and were periodically supplemented with snails from Florida (location not indicated). There is likewise very little information in the published studies about the culture and husbandry conditions of Oehlmann et al.'s snails, whereas a major portion of our research aimed to identify appropriate husbandry and culture conditions for *M. cornuarietis*. It is on the basis of such detailed study that our toxicity tests with BPA were conducted.

We are surprised to find that Oehlmann et al. believe our paired design to be flawed. They cite the high inter-pair variability in egg production as evidence for mate selection and imply that their spawning group design would have avoided such source of variability. Not only is the claim of mate selection highly speculative, but the fact is that there may just as well have been high inter-snail variability in the Oehlmann et al. studies. However, they had no way to quantify such source of variability when snails were reared in large groups in which individual snail performance could not be assessed. Oehlmann et al. cite low inter-tank variability in their studies as additional evidence for mate selection. We would like to point out that our studies have also shown very low inter-tank variability (e.g., only 2.6% of the total variance in egg production in Forbes et al. (2007b) was due to differences among replicate tanks). Additional evidence for mate selection is claimed to be the occurrence of pairs in our studies with no egg production. There were 5 of 180 pairs that did not produce eggs in Forbes et al. (2007b). This is equivalent to 2.8%, which is not a particularly high number. Non-reproducers could also have occurred in the Oehlmann et al. studies, but the group-spawning design used prevented identification of such non-reproductive individuals. Our work has also shown that snail density has a major negative effect on reproductive output (Aufderheide et al., 2006). The densities used in the Oehlmann et al. studies were in most cases greater than those that have been shown to reduce snail reproduction, and in all cases densities varied markedly during the course of the tests as snails were harvested for analysis or died. Finally we would like to point out that, if mate selection were a large source of error in our experiments, one would expect the average reproductive output in our studies to have been substantially lower than that observed in the Oehlmann et al. studies. In fact the opposite is the case.

Perhaps the most serious criticism of Oehlmann et al. is the accusation that we designed our studies to intentionally mask the effects of BPA. Our studies were carried out based on the balanced advice of an expert steering group that was set up by the rapporteur country of the EU risk assessment for BPA. Several years of preparatory study went into identifying husbandry conditions (i.e., food regime, animal density, photoperiod, temperature, water hardness, water turnover rate) in order to develop breeding and toxicity test conditions to ensure high fitness of snails. This is standard proce-

dure in the development of toxicity tests, since all other sources of stress (other than the chemical(s) to be tested) should be minimized. Aufderheide et al. (2006) found no difference in egg production rates between snails reared at 22 and 25°C. However, egg-hatching success was impaired at the lower temperature and rates of egg development and juvenile growth were also lower at the lower temperature. Selck et al. (2006) also found that juvenile snails grew more slowly at 22°C than at 25°C, and mortality was twice as high at the lower temperature. These results, together with the temperature range experienced by the snails in their native habitat (Lake Guajataca, Puerto Rico), indicated that fitness was reduced at 22°C compared to 25°C, and it was on this basis that we chose 25°C as the test temperature. However, to explicitly accommodate concerns of Jörg Oehlmann regarding the temperature effect, we performed an additional reproduction test with BPA at 22°C and found no evidence that snails were more sensitive at the lower temperature (Forbes et al., 2007c).

Oehlmann et al. criticize us for not using a positive control (e.g., estradiol) in our test with BPA. Two important reasons for not doing so are that the mode of action of BPA in *M. cornuarietis* is not known, and there are no reliable reference data for candidate substances. This issue was discussed in detail in the expert steering group, and our decision was based on the outcome of this discussion.

The published studies of Oehlmann and colleagues have been criticized elsewhere (Dietrich et al., 2006), and these criticisms will not be repeated here. However, in response to Oehlmann et al.'s claim that they observed "identical effects and even EC_{10} values" in their published studies, we would like to point out that in only one of two exposure series in Oehlmann et al. (2006) and in none of the earlier studies (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2001) were BPA treatments replicated. Therefore differences in the effects observed among their studies cannot be quantified and, with the exception of exposure series II in Oehlmann et al. (2006) in which two replicates per treatment were used, no EC_{10} values can be validly estimated from their data.

In summary, we have gone to great lengths to develop a robust and statistically valid test design for *M. cornuarietis* that is based on extensive study of the snail's husbandry requirements and an understanding of the sources of variability in its life-history characteristics. As our papers clearly demonstrate, we have succeeded in this goal.

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