What’s in My Water Bottle? A Question of Environmental Estrogens

by

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Part I—The Problem

Susan packed up her laptop, stuffing all of the papers she had been reading into the case. She looked around her. The library was nearly empty—typical for a Saturday evening. Still, she was happy to have made so much progress on her paper, which was due the following week. She picked up her belongings and began walking toward the steps to the main floor of the library. As she descended the staircase, she saw her friend Steve sitting at his usual table. She paused a moment at the foot of the stairs. Steve looked up from his computer just as Susan walked over to his table.

“Hi, Steve, what are you working on?” she asked quietly as she sat down at his table.

“Oh, it’s that research paper we have to write for Seminar,” he replied, shaking his head. “There is just so much information to write about; I’m having trouble getting it organized. I think I’m getting tired, too.” He looked over at her. “What about you? Have you made much progress on yours?”

Susan smiled. “Yes, I think I have a pretty good draft, but I have to let it rest for a while. I’ll work on editing it tomorrow.”

“Yeah, I think it’s time for a break. Want to go check out the basketball game for a bit?” Steve asked.

“That sounds great! A basketball game is just what I need to clear my head.”

The two friends headed off to the field house to see the game. When they got there, the Biology Club students were at a table selling filled water bottles that were decorated with biological themes.

“Hey, Susan! Hey, Steve!” one of the students called out. “Where are you going? Don’t you want to support the club and buy a water bottle?”

Susan and Steve walked over to the table.

“We’re going to watch the basketball game,” Steve answered. “We’ve been working on our research papers at the library and needed a break.” Steve picked up two bottles and looked over at Susan. “Do you want one, Susan?” he asked her.

“You know, I really don’t,” Susan replied. “No offense, but I’ve been working on a paper about the chemicals used in making plastics, and some of them are really harmful!”

“What do you mean?” Steve asked. “Aren’t plastics inert? Surely the government wouldn’t approve putting food and drink in plastic containers if it isn’t safe. I have heard that it’s bad to heat food in plastic containers, but I thought that was just for people who are totally paranoid about their safety.”

"Well, what I've been learning, Steve," she replied, "is that plastics are not inert. I used to think that the harmful chemicals were limited to the making of plastics. But, if that's not bad enough, it turns out that harmful chemicals come out of the plastic into the water you're going to drink. They're there in the milk in plastic bottles, and everything else in plastic. I swear, I can't believe human beings are still alive and fertile, based on what I've been reading."

"I hope you're being a good scientist and looking at evidence rather than reading a bunch of crazy environmentalists' tracts," Steve responded.

Susan gave him a hard look. "Of course I am. You can read my paper when I've finished it. I've included several research articles that have good evidence showing that certain chemicals leach out of the plastic and into the water or whatever is being held in the plastic. It's really quite convincing."

"So, you're never going to drink out of a plastic bottle again?" Steve asked.

"I don't know," Susan replied slowly. "It's not easy to consider never using plastics, but I'm thinking about it. But you know, it's more than that. It seems to me like someone needs to get more people to understand the issue. We shouldn't just sit quietly by while we're being poisoned companies that we rely on for certain products."

Steve shook his head. "Well, let's go watch the basketball game."

Susan followed him into the arena, wondering what she would have to do to get people to understand the problem if she couldn't even get Steve to think about the issue.

Questions

1. How widespread is the use of plastics and how often do you think you come into contact with them? What are some less usual sources of plastic with which you come into daily contact?
2. What kind of information would you want, as a scientist, to begin to determine if there are health risks associated with plastic containers? For example, would you be interested in animal studies? If so, what kinds of animals? What kinds of controls would you need?
3. Design a simple experiment that could address the question about the safety of plastics.

Image credit: Water bottle photo ©Emin Ozkan/Fotolia.com

Part II—The Data

The following day Susan found Steve in the computer lab working on his research paper.

"Here's a copy of my paper," she announced as she walked up to him.

"Oh, cool," he replied, looking up from the computer. "I'm really ready for a break, so let's go get some coffee. No, first let me print out what I have so far. Will you read it and see what you think?"

"Of course, if you buy the coffee—and after you've had a look at mine!" Susan replied.

"Deal," Steve answered with a smile. They collected Steve's copy of his paper and went to the coffee shop next door.

When Steve got to their table with the two coffees, Susan had a pile of papers sitting in front of her.

"I hope I don't have to read all of those!" Steve said as he handed Susan her coffee.

"No," Susan said with a laugh, "this pile contains all of the information I have collected about plastics and whether they're safe for food use, and it's pretty convincing, I think. You can just read my paper to get the 'boiled down' version of the information." Susan picked up the top paper and started handing it to Steve.

"Wait," Steve said, holding his hand out so she couldn't put the paper in front of him. "I've been reading too much lately, so save my eyes, and just tell me what you found. My first question is, what is the evidence that things come out of the plastics and into the water, or whatever it is you have in the plastic?"

"OK, here is what I found on that." Susan showed him Table 1 (from Howdeshell, et al., 2003).

Table 1. Concentration of BPA in cage water samples.

	Amount of BPA leached per surface area (ng/cm^2)			Concentration of BPA per sample (µg/L)		
Cage description	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Glass dish						
1	< 0.07 LQ	—	< 0.13 LQ	< 0.1 LQ	—	< 0.2 LQ
2	< 0.07 LQ	—	< 0.13 LQ	< 0.1 LQ	—	< 0.2 LQ
Polypropylene cage						
1	< 0.03 LQ	—	< 0.12 LQ	< 0.05 LQ	—	< 0.2 LQ
2	< 0.03 LQ	—	< 0.12 LQ	< 0.05 LQ	—	< 0.2 LQ
Used polycarbonate cage						
1	62	10	10	110	18	18
2	29	23	12	51	41	22
3	—	—	90	—	—	280
4	—	—	160	—	—	310
New polycarbonate cage						
1	—	0.14	0.18	—	0.26	0.32
2	—	0.14	NQ	—	0.27	NQ
New polysulfone cage						
1	—	—	NQ	—	—	NQ
2	—	—	0.84	—	—	1.5

Abbreviations: LQ, less than method quantification limit; NQ, not quantifiable because ^{13}C-BPA surrogate not recovered.

"Whoa, Susan, you're supposed to be giving me the easy answer, not making me figure everything out on my own!"

"I'll explain it to you. What they did was to get used and new animal cages, either polycarbonate or polysulfone, to test. They used glass casserole dishes and also polypropylene cages as negative controls since neither is manufactured using bisphenol A, we just call it BPA …"

"Wait, what's this BPA stuff?"

"Oh, it's the monomer that's used to make polycarbonate and polysulfone, which are two common plastics," Susan replied. "Figure 3 from the same article shows the structure of BPA, and also how it is incorporated into the two kinds of plastics."

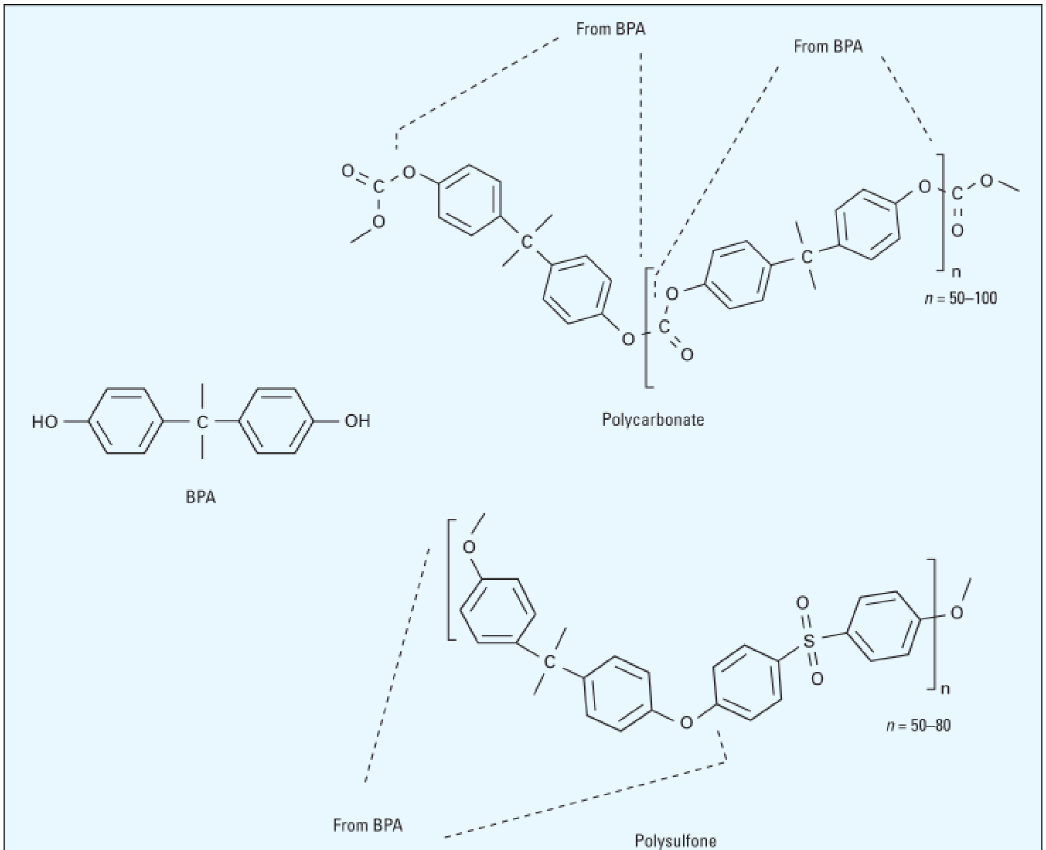

Figure 3. Structure of BPA and partial structure of the copolymers polycarbonate and polysulfone shown by monomeric chain units (n) within brackets. Both the rigidity of the aromatic rings and the inherent flexibility of the C—O, C—S, and C—C single bonds are depicted. Polycarbonate is joined by ester linkages (O—C=O—O) whereas polysulfone has ether linkages (C—O).

Susan looked at Steve. "OK, are you ready to go on and look at that table now?"

He nodded, intent on looking at the material in front of him.

"So," Susan said, going back to Table 1, "they had these two different kinds of plastic cages, new and used. They rinsed them all, first with just tap water and then with highly purified water (HPLC grade). Then they added 250ml of that HPLC grade water to each cage and let them sit for a week. They then collected the water and measured the amount of BPA found in the water from each type of cage. You can see that they repeated the experiment several times."

"Yes," Steve answered, "but what are all these dashes? I don't get that."

"Oh, well, I think they were making adjustments in the experiment as they did more replicates. You know, they learned something and applied it as they repeated the experiment."

"Then those aren't really replicates, are they?" Steve asked.

Susan ignored his comment and went on. "First, they just compared glass and the used cages. They saw that glass didn't leach out any BPA, so they didn't repeat it the second time. But that time they added new polypropylene cages to compare."

"I guess the third time they thought they'd do a more complete test," Steve added. "Did they add more used cages?"

"Yes," Susan replied. "I guess they wanted a larger sample number."

"But those released a lot more BPA," Steve observed. "Why? Do they explain that?"

"They said those cages looked more worn than the other ones—there were more marks on the plastic, like they had been damaged more during their use."

Questions

1. Are the replicates helpful? Why or why not?
2. What do you conclude from these data?
3. What other results would you like to see?
4. What are the strengths and weaknesses in these data?

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Part III—Is It So Bad?

"Well, I'm still skeptical," Steve said after looking at the data. "I mean, I think it's pretty convincing that this stuff is in the water, but so what? There could be lots of things in water. That doesn't mean it's bad, you know."

"OK, but look at this experiment," Susan responded, showing him Figure 1 from the Howdeshell, et al., 2003 paper.

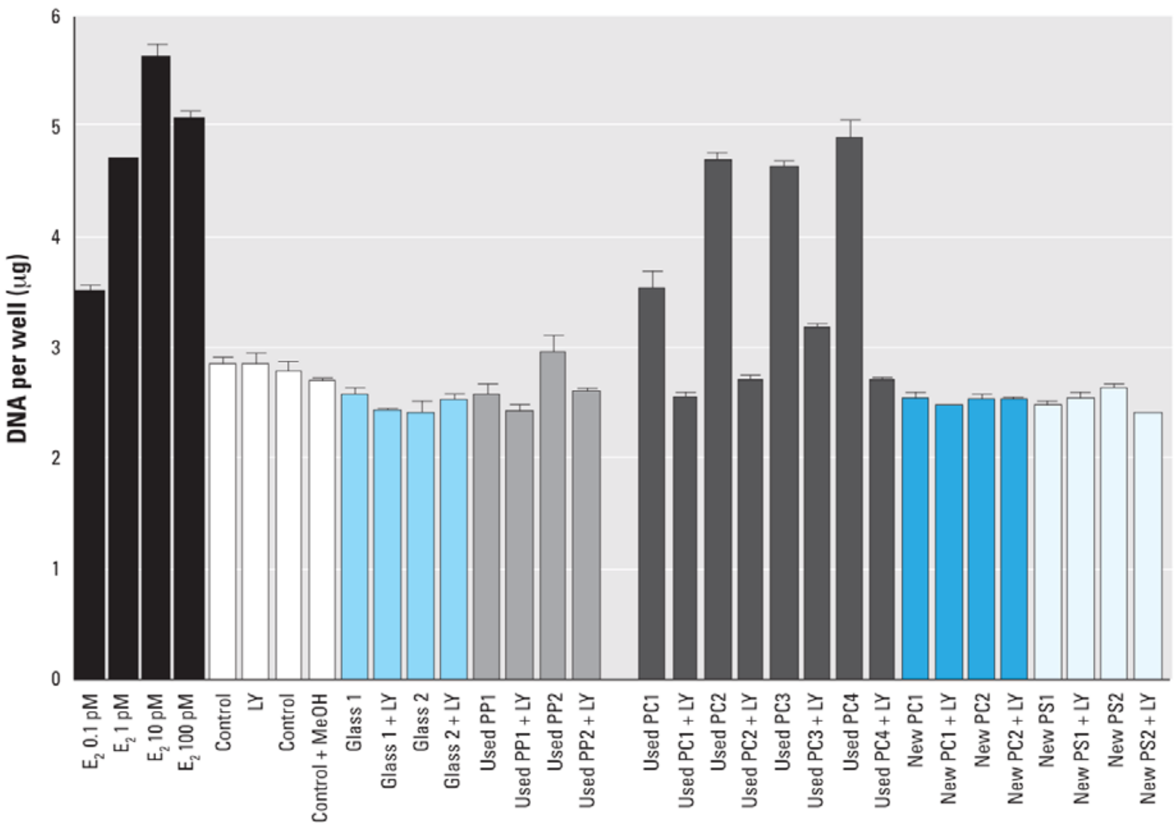

Figure 1. Cell proliferation in MCF-7 cells exposed to water samples of plastic animal cages. Abbreviations: Control, control estrogen-free media; Glass, glass dish; LY, an antiestrogen, also called keoxifene; MeOH, methanol; PC, polycarbonate cages; PP, polypropylene cages; PS, polysulfone cages. Cells grown in phenol red–free (estrogen-free) maintenance medium were treated for 4 days with the media containing the reconstituted cage and glass dish water samples, a range of 17β-estradiol (E_2; 0.1–100 pM), and/or 0.1 mM LY156758 with daily medium changes. Values are the mean ± SE; $n = 3$.

"Susan, I hope you explain your findings more clearly in your research paper. You know if you just show a bunch of tables you're going to get a bad grade."

"I know, I will. But don't you see what's going on in this figure?"

"Well, not immediately," Steve responded. "There are a lot of bars, and why are they measuring DNA?"

"They're measuring the amount of cell proliferation," Susan explained. "Obviously, the more cells there are, the more DNA there is."

"OK, I got that," Steve answered a bit annoyed at her tone.

"What are all of these different labels on the x axis, though?" he asked.

"Well, they wanted to test if the BPA is estrogenic or acting like estrogen," Susan explained. "Estrogen causes breast cancer cells to divide. They added estrogen, or water from the cages, to human breast cancer cells. See these first four bars?"

Steve nodded. "Those show that adding estrogen increases cell number because it increases DNA amount in the well holding the cells."

"OK," Steve nodded, "and this next group of bars shows the controls, right?"

"Right—they took the water from the glass containers and from the used polypropylene, which didn't produce any BPA. But see here," she moved her finger to the next set of bars, "the water from the used polycarbonate cages acted just like estrogen did."

"What's the 'LY' mean?" Steve asked.

"LY is an antiestrogen," Susan answered. "See what it does?"

Steve continued to look at the graph. "Yes, I do, that is interesting."

Susan turned the page to show Steve Figure 2 (see next page). "OK, here they used HPLC to separate out the different chemicals that could be in the water from the cages. Table A is water from a used polycarbonate cage, and Table B is water from a used polypropylene cage."

"Yes, look," Steve pointed out, "here is where the BPA is in the used polycarbonate cage, and there's that increase in cell number again. It really does cause those cells to divide. Do you think that means BPA can cause breast cancer?"

"I don't know," Susan answered thoughtfully, "they don't really address that. These were breast cancer cells in the first place."

"What does this next table show?" Steve asked, referring to Table 2 (see next page).

"Well, this was an *in vivo* study, so they could actually look at the whole animal and see if the BPA was having any negative effect."

"What would you test, though?" Steve asked.

"They tested how much the uterus grew in female mice that had not yet gone through puberty," Susan answered.

"It doesn't look like there's much of a difference."

"No, it doesn't, and they say in the paper that they don't think uterine weight like this is a good measure of estrogenic activity."

"Do you think they're just saying that because they're biased?" Steve asked. "Do they say what else they should test?"

"Yes, they say that the transcriptional activity of estrogen receptor is very sensitive, and so is the fetal male prostate."

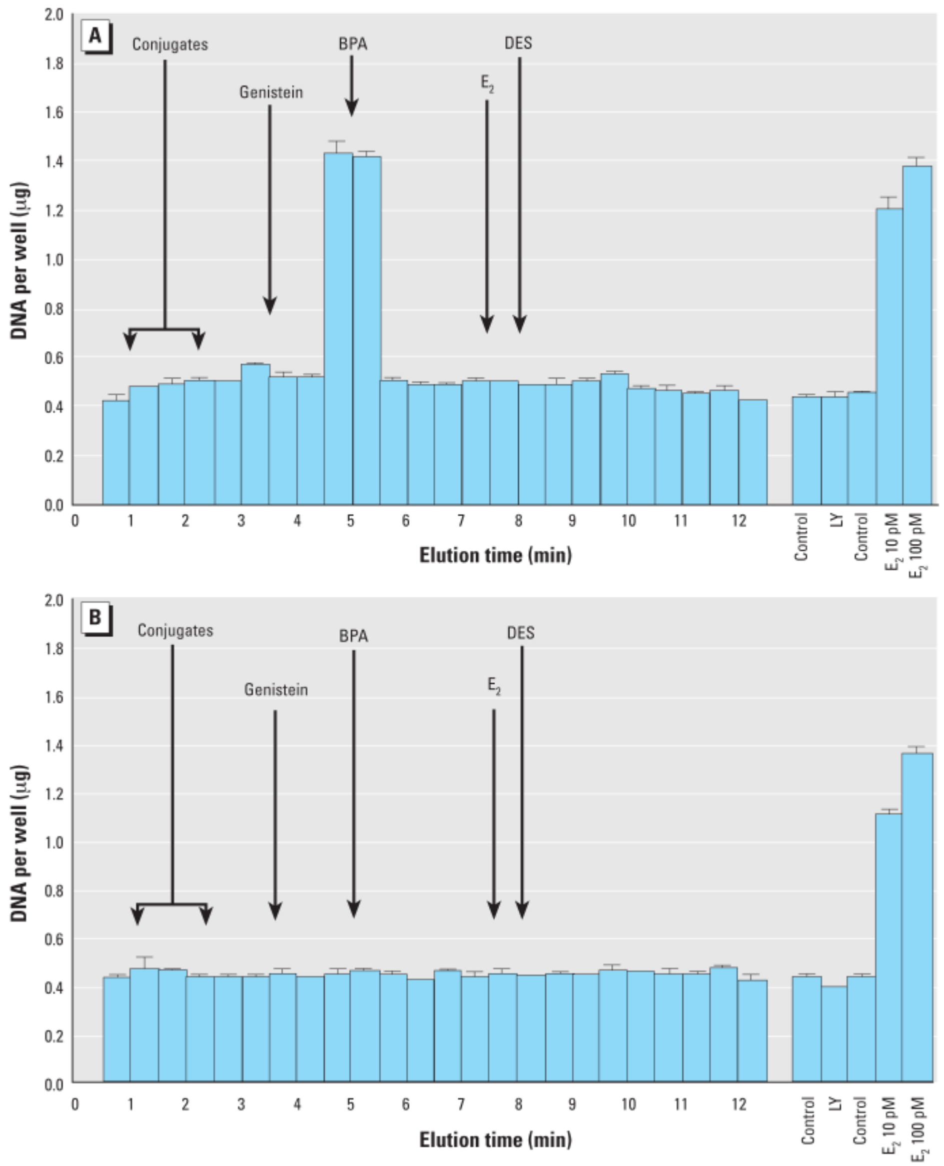

Figure 2. HPLC/bioassay separations of a water extract of a used polycarbonate cage (*A*) and a polypropylene cage (*B*), separated in 60:40 methanol:water as described in Grady et al. (1991). Abbreviations: Control, control estrogen-free medium; E_2, estradiol; LY, 100 nM antiestrogen LY156758. Fractions are shown from 0.5 through 12.5 min. Arrows indicate approximate elution times of standards, separated in separate HPLC runs. Estrogenic activity as increased proliferation is shown on the vertical axis. The bars to the right of the separation are controls. Values are the mean ± SE; $n = 3$.

Table 2. Prepubertal mouse uterotrophic assay assessing estrogenic influence of used polycarbonate animal cages versus polypropylene cages.

Housing condition	PND19 bw (g)	PND26 bw (g)	Uterine wt (mg)
PP cages with glass bottles ($n = 57$)	9.75 ± 0.16	16.30 ± 0.2	17.25 ± 0.70
Used PC cages with used PC bottles ($n = 57$)	10.11 ± 0.16	17.12 ± 0.24	20.56 ± 1.13
Statistical significance	$p = 0.17$	$p = 0.83$	$p = 0.31$

Abbreviations: bw, body weight; PC, polycarbonate; PND, postnatal day; PP, polypropylene; wt, weight. All data shown are mean ± SE; n = number of mice per housing condition.

"Really? That's interesting," Steve commented.

Questions

1. What were the positive and negative controls used for each of the figures discussed in this section? Why were these included, specifically?
2. Why do you think they included the data in Table 2?
3. Susan and Steve seem pretty convinced by these data. Are you? Defend what it is that either makes you agree or disagree.

Image credits: Figure 1, Figure 2 and Table 2 from Kembral L. Howdeshell et al., "Bisphenol A Is Released from Used Polycarbonate Animal Cages into Water at Room Temperature," *Environmental Health Perspectives*, 111:9, 2003. Reproduced with permission from *Environmental Health Perspectives*.

Part IV—Human Implications

"Well, I'm more convinced than before," Steve admitted after reviewing the data. "It certainly seems to be a problem for rats."

"Not just rats, Steve, those breast cancer cells were human, remember. Besides, they also explain that this could be a problem for animals that we keep in water in these cages, like fish and frogs. Here's what they say." Susan read aloud from the paper:

In guppies (Poecilia reticulata), *BPA exposure (274 µg/L for 21 days) in adulthood significantly decreased the number of mature sperm stored in deferent testes canals before ejaculation by 50%, relative to control males (Haubruge et al. 2000). A significant decrease in spermatozoa has also been reported for fathead minnows* (Pimephales promelas) *after exposure to 16 µg/L BPA in water for 164 days in adulthood (Sohoni et al. 2001).*

"Still, what about people? We're not really affected by this, are we? I mean, tests of cells alone, does that really say something about what we're drinking and eating?"

"Here, check out the last paragraph in this paper," Susan said as she handed it to him.

BPA migration into human serum has been reported with the use of polycarbonate and polysulfone plastic hemodialysis equipment (Yamasaki et al. 2001). Another potential route of BPA exposure in the laboratory is polyvinyl chloride (PVC) pipes used in the supply of tap water; BPA is added as a stabilizer in the production of PVC products.

"So maybe we get some BPA from tap water—where's the evidence that it's bad for humans?" Steve asked.

"Actually, there have been some interesting findings that show that it is," Susan replied. "Let me see," she said as she rifled through her papers. "Ah, here are some of those findings. So, you know how girls seem to be going through puberty sooner than they used do?"

"Uh, well, I don't think I had noticed that."

"Well, I have. I thought maybe it was hormones in milk. And maybe it is. But this group of researchers (Howdeshell et al., 1999) suggests it has to do with BPA. They gave pregnant mice BPA fed in oil (or just oil as a control). They used a dose that is equivalent to what would be found in the environment. They took the babies and put them with foster mothers once they were born. Here's what they found. What do you think of that?"

"Well, this is kind of confusing," Steve commented as he stared at Figure 1. "I guess they were looking at reproductive

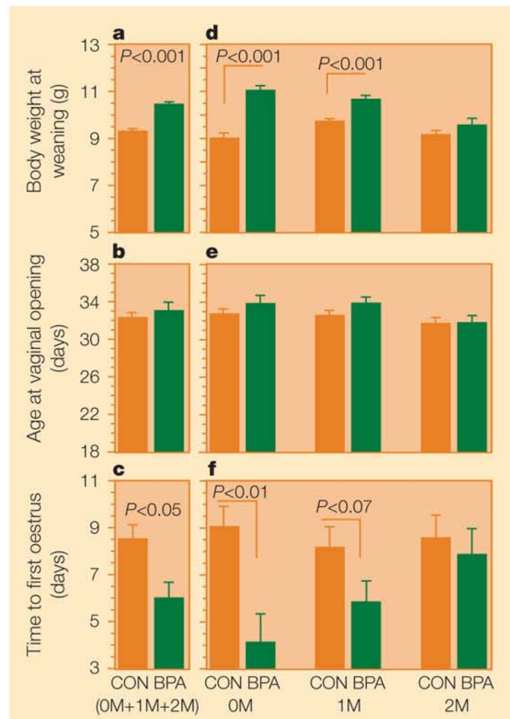

Figure 1 Mean (±s.e.m.) body weight at weaning, age at vaginal opening, and interval between vaginal opening and first vaginal oestrus. Data are for all females combined (**a–c**) and as a function of intrauterine position (**d–f**). Body weight at weaning includes data for all females surviving to weaning from control (CON, orange bars) and bisphenol A-exposed (BPA, green bars) litters. All data were adjusted for litter membership to control for maternal effects. Vaginal opening and interval data were also corrected by analysis of covariance for body weight at weaning. 2M, located between two males fetuses; 1M, located next to one male fetus; 0M, located next to female fetuses. Wean weight was calculated on 41 0M, 47 1M and 23 2M control females, and 20 0M, 43 1M and 12 2M bisphenol A-treated females. Vaginal opening and interval data were calculated on 19 0M, 20 1M and 19 2M control females, and on 19 0M, 21 1M and 11 2M bisphenol A-treated females. We attempted to include females from each intrauterine position from each litter, but some litters did not contain a 2M female.

maturity issues, but they talk about whether the mice were next to a male or not—that gets confusing to me!"

"You can ignore that, though it is interesting," Susan answered. "If you just look at panels a, b, and c, you can see that the weight of mice at weaning was larger for the mice whose mothers had been fed BPA. And they also are ready to mate earlier, by about two days."

"Is two days a lot?" Steve asked.

"Well, mice do mature a lot faster than we do, so I would think two days is meaningful. They do show that there is a significant difference."

"Now, look at the results of this study (Figure 2) by Masuno and colleagues," Susan continued. "They looked at the effects of BPA on obesity."

"Obesity?" Steve asked. "Why obesity? Though I do have to admit I've read in the newspapers that more and more people are becoming obese—some people are even calling it an epidemic."

"Well, what they explain is that estrogen has an effect on lipid metabolism, at least in rats. I wonder if that's why women gain weight easier than men," she added with a frown. "Anyway, these folks were studying whether BPA could change 3T3 fibroblasts into adipocytes. These are fibroblasts that do change into adipocytes in the presence of insulin, for example. As they change, they express more lipoprotein lipase (LPL) and less triacylglycerol (TG), and that's what they measured in this graph."

"So," Steve said thoughtfully, "exposure to BPA could actually make people fatter? So maybe it's not video games or too much sugar that's responsible for people gaining so much weight?"

"That's right!" Susan replied. "Who would think that you could get fat from drinking out of plastic containers?"

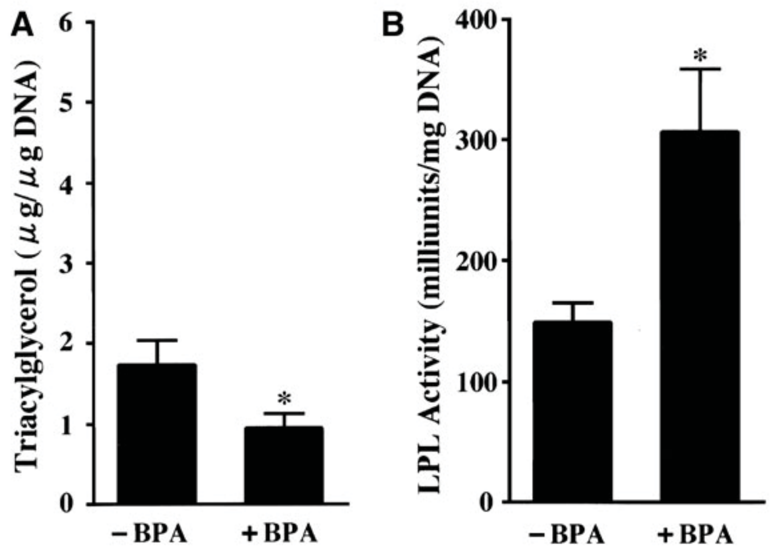

Fig. 2. Effect of BPA on triacylglycerol (TG) content and LPL activity. The confluent cultures of 3T3-L1 fibroblasts were treated for 11 days without or with 20 μg/ml BPA. The cells were harvested and sonicated briefly at 0°C. A: An aliquot of the homogenate was used to measure TG content. B: Another aliquot was used to make an acetone/ether powder and LPL activity in aqueous extracts of the powders was measured. The DNA content of the cultures treated without BPA was 39.1 ± 2.3 μg/plate and that of the cultures treated with BPA was 22.1 ± 1.6 μg/plate. Values given are the mean ± SD for four plates. *$P < 0.01$ (compared with the value obtained from the cultures treated in the absence of BPA throughout 11 days).

Questions

1. In the quotes that Susan shows, the authors state that levels of BPA can affect sperm production in fish. Would fish raised in used polycarbonate cages be subjected to these levels? Explain.
2. In the experiment where pregnant mice were fed BPA, the baby mice were raised with foster mothers. Why did they do that?
3. What is the evidence that BPA causes obesity? Explain what is or is not convincing about it.

Final Assignment

Write an essay that is at least one page long, double-spaced and typed, that addresses the following paragraph:

BPA has been found in the linings of metal cans as well as dental sealants in addition to water from polycarbonate containers. Overall, comment on how valid you think the data are that were presented in this case study and explain the reasoning behind your evaluation. Discuss how serious you think this problem is. Then consider your place in society as a scientifically educated person. What should you do to help solve the problem or perhaps eliminate unnecessary concern?

References

Howdeshell, K.L., A.K. Hotchkiss, K.A. Thayer, J.G. Vandenbergh, and F.S. vom Saal. 1999. Environmental toxins: Exposure to bisphenol A advances puberty. *Nature* 401:763–764.

Howdeshell, K.L., P.H. Peterman, B.M. Judy, J.A. Taylor, C.E. Orazio, R.L. Ruhlen, F.S. vom Saal, and W.V. Welsons. 2003. Bisphenol A is released from used polycarbonate animal cages into water at room temperature. *Environmental Health Perspectives* 111:1180–1187.

Masuno, H., T. Kidani, K. Sekiya, K. Sakayama, T. Shiosaka, H. Yamamoto, and K. Honda. 2002. Bisphenol A in combination with insulin can accelerate the conversion of 3T3-L1 fibroblasts to adipocytes. *Journal of Lipid Research* 43:676–684. **http://www.jlr.org/cgi/content/full/43/5/676**