Reduced sperm counts after xenobiotic exposure in guppies (*Poecilia reticulata*)

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There is increasing evidence that normal male reproductive function can be disrupted by exposure to pollutants in the environment which act hormonally. A number of studies have now shown that a variety of common synthetic compounds (often at very low concentrations) can exogenously mimic, antagonise or block sex hormone function. One possible consequence of exposure to these xenobiotics is disruption to spermatogenesis and this action could explain the reported decline in human sperm counts. Here, we show that short-term adult exposure to very low levels of the common xenobiotics Tributyltin and Bisphenol A directly cause a significant decline in sperm counts of male guppy fish.

Chemicals which mimic hormones and disrupt endocrine function have enormous potential for compromising reproductive health in humans and wildlife *(1, 2)*. Since 1992 *(3, 4)* there has been growing evidence that human sperm counts are declining, and there has been an increase in the incidence of reproductive developmental abnormalities in men *(1, 4)*. Animals

exposed to these xenobiotic pollutants in the wild have manifested a range of reproductive defects at the anatomical, behavioural and physiological levels from reduced fertility to aberrant courtship behaviour *(1, 5)*. This study experimentally examines whether low levels of potentially xenobiotic pollutants have a direct influence on spermatogenesis, the fundamental level of male reproductive competence and recently identified as a key male trait to measure in relation to reproductive toxicity *(1)*.

A number of studies have shown that many common synthetic compounds can mimic, anatagonise, or block sex hormone function and disrupt normal reproduction *(2,6)*. However, as yet no studies have experimentally shown that short-term exposure to low concentrations of xenobiotics directly cause a decline in sperm counts *(1)*. We use guppies (*Poecilia reticulata*) as ideal aquatic vertebrate models for examining exposure to xenobiotics: adult males show continuous spermatogenesis and, although they are aquatic, fertilization is internal and reproduction viviparous which relates more closely to terrestrial vertebrate reproduction. Both Tributyltin (TBT) and Bisphenol A (BPA) are common water pollutants throughout the world and the concentrations we use in this study are widely encountered by animals and humans *(7-9)*. BPA is a phenolic plasticiser used extensively in food storage, plastics and dental restoration; BPA is a potent endocrine disruptor *(2)* and it is likely that humans are regularly exposed to BPA which can leach out of polycarbonate plastics, particularly when heated *(2)*. TBT is an organotin compound most often used as a ship anti-fouling agent (but is also used in agrochemicals) and was one of the first recognised xenobiotics which induces imposex in molluscs *(10)*. TBT use is now regulated but it

remains a widespread *(7-9)* and potentially damaging pollutant and its effects on sperm counts are not yet known.

Our experiment exposed mature male guppies to five non-lethal water treatments; aquaria contained either 11.2*n*g or 22.3*n*g of TBT per litre, 274*m*g or 549*m*g of BPA per litre, and a clean water control. All fish were maintained identically apart from variation in the level of TBT or BPA in the water. After 21 days, all fish were sacrificed to determine immediate effects on reproductive function. Weight of the single bi-lobed testis and number of mature sperm in the common deferent canal were determined. The deferent canal in the testis of internally-fertilizing fish is where mature sperm are stored in spermatophores prior to ejaculation. Mature spermatophores and their sperm were dispersed and total sperm number counted (Fig. 1 legend). The total lengths of 20 individual sperm per male were measured by tracing along the long axis of the head and axoneme using video microscopy.

As in other species *(11, 12)*, guppy testis size is allometric with body size (R=0.61, P<0.0001, n=72), and sperm number production depends upon testis size (R=0.25, P=0.03, n=72); we therefore control for any between-male variance that could be confounded by a natural variance in body size by analysing residual testis size and sperm numbers. We found no effect of TBT or BPA treatment on residual testis size ($F_{(4,67)}$=0.82, P=0.52). We found that individual males varied significantly in the total length of their sperm ($F_{(74)}$=11.94, P<0.0001) but this significant variation was not related to the TBT or BPA treatment that males received ($F_{(4,70)}$=0.85, P=0.5), nor did the variance within a male's sperm length relate to his pollutant treatment ($F_{(4,70)}$=2.15, P=0.08). Our central and most important result was that males exposed to xenobiotics show significantly reduced sperm

counts (Fig. 1). The decline in sperm count is significant whether we control for body weight and analyse residual sperm number ($F_{(4,67)}$=12.8, P<0.0001) or compare absolute sperm count values ($F_{(4,70)}$=14.07, P<0.0001). This significant reduction in sperm number is particularly consequential since the xenobiotic levels that we exposed the male guppies to were relatively low and may be encountered by animals and humans at higher levels *(2, 7-9)*. For example, up to 950 *m*g of BPA (double the concentration of our highest treatment) can leach out of a dental sealant within one hour of treatment *(2)* and into saliva. In the wild, levels of TBT are encountered that exceed 22.3 *ng* in both coastal and inshore waters where TBT use is regulated *(7-9)*.

We observed a sperm count decline after only 21 days of BPA and TBT exposure. Other studies have recorded male reproductive disruption after chronic long-term exposure to xenobiotics or at particular periods in development when individuals may be especially sensitive to hormone titre *(1,13)*, however, we observe a decline after only three weeks of exposure which suggests disruption in the production process, rather than a decline in the number of active spermatogonia in the germ line. Our finding that testis size is unaffected supports this interpretation. We do not yet understand the mechanism of such short term effects on sperm production, however Sertoli cells are essential in spermatogenesis *(14)* (functions include nutrition and release of developing sperm, and phagocytosis of degenerating gametes) and these cells are directly sensitive to xenobiotics. The estrogenic alkylphenol 4-tert-octylphenol causes Sertoli cell apoptosis within only 24 hours of exposure *(15)*. This apoptosis could block the nutritional activity of Sertoli cells on maturing spermatids and arrest the release of gametes from the eferent ducts into the testicular canal

and into storage in the deferent ducts of the testes. Such direct and short-term Sertoli cell sensitivity to xenobiotic action could be the mechanism by which male exposure to low levels of BPA and TBT leads to a significant, but short-term, decline in the production of sperm numbers.

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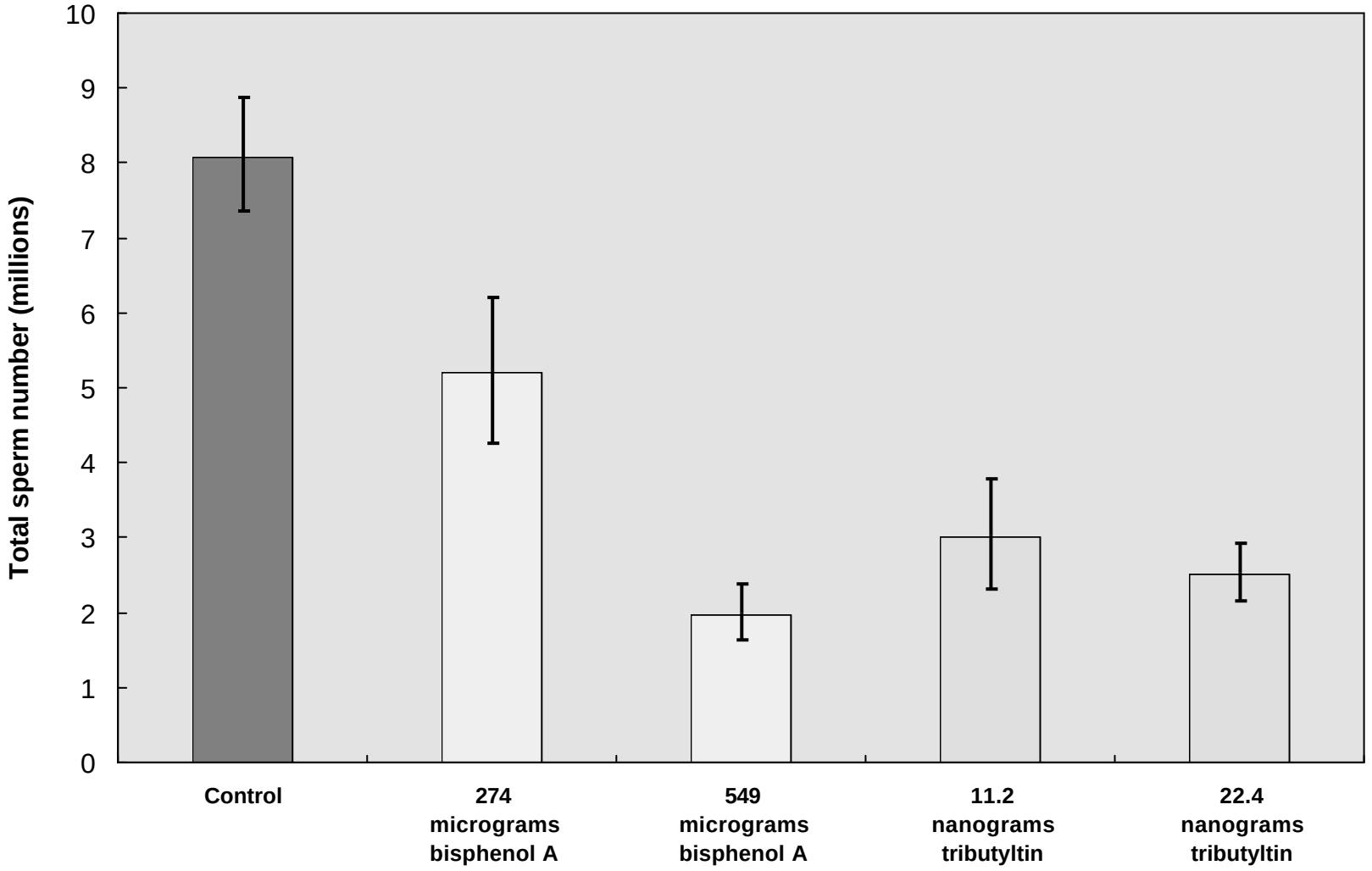
Fig. 1. Total numbers of mature sperm stored in the deferent testis canals of male guppies after different 21-day xenobiotic treatments. Mature spermatozoa packaged within each spermatozeugmata were mechanically dispersed and diluted in 0.1 *M* NaCl buffer with 10% eosin; total sperm count per male was determined by counting sperm in sub-samples of this diluent. Sample sizes are 15 males in each group apart from 11.2*n*g/L TBT where $n=12$. Sperm counts decline significantly from the control in both Tributyltin (TBT) and Bisphenol A treatments (Tukey multiple comparisons: maximum $P=0.026$). This result for total sperm numbers does not change if natural variation in body size is controlled for and declining residual sperm numbers are analysed (see text results). There is a dose response for BPA with increasing xenobiotic concentrations (Tukey multiple comparisons: maximum $P=0.026$), but not for TBT concentrations where a significant and equal decline is observed for both treatment doses.


Total sperm number (millions)
10
9
8
7
6
5
4
3
2
1
0
Control
274 micrograms bisphenol A
549 micrograms bisphenol A
11.2 nanograms tributyltin
22.4 nanograms tributyltin