Reduced sperm counts in guppies (Poecilia reticulata) following exposure to low levels of tributyltin and bisphenol A

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There is increasing evidence that normal male reproductive function can be disrupted by exposure to pollutants in the environment that can exogenously mimic, antagonize or block sex-hormone function. One possible consequence of exposure to these xenobiotics is disruption to spermatogenesis, but results thus far provide only indirect and inconsistent evidence. In this study we exposed adult male guppies (Poeciliidae: 'tetraoi) to environmentally relevant levels of the common xenobiotics tributyltin (11.2–22.3 ng l⁻¹) and bisphenol A (274–549 μg l⁻¹) in experimental aquaria. After 21 days of exposure, we found significant declines (by 40–73%) in total sperm counts for male fishes exposed to tributyltin and bisphenol A compared with controls. This short-term decline in sperm count is unlikely to be the result of endocrine-mediated alteration of the germ line, and we found no change in testis size or sperm lengths between treatments. However, Sertoli cells, which facilitate the transport of maturing sperm into the testicular deferent duct (where they are stored prior to ejaculation), are directly sensitive to xenobiotic action and it is therefore possible that spermatogenesis was inhibited through in vivo interference with normal Sertoli-cell function.

Keywords: pollution; spermatogenesis; endocrine disruption; oestrogenic; Sertoli; xenobiotic

1. INTRODUCTION

Polluting chemicals that mimic or antagonize hormones and disrupt endocrine function have great potential for compromising reproductive health in humans and wildlife (reviewed in Crisp et al. 1998; Sonnenschein & Soto 1998; Risbrough 1999). However, this area of study remains controversial because environmental evidence may be based on atypically high levels of pollution, and experimental evidence may derive from environmentally irrelevant concentrations of pollutants (Juberg 2000). There is a clear need to conduct controlled experimental investigations of xenobiotic action on important male reproductive traits such as sperm count.

Animals exposed to potentially 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 (Crisp et al. 1998; Tyler et al. 1998). Fishes are particularly prone to direct pollutant exposure through their aquatic environment and an increasing range of synthetic compounds associated with sewage effluent, detergents, pesticides, agrochemicals, pulp mill effluents and domestic and industrial chemicals have been shown or suggested to act as endocrine disruptors (although it has proved difficult to identify the exact chemical(s) causing the oestrogenic effects) (Crisp et al. 1998; Sonnenschein & Soto 1998; Risbrough 1999). Fishes in the wild exposed to these compounds can exhibit a range of reproductive problems including constrained or disrupted testicular development (Johling et al. 1996), physiological and anatomical feminization of males coupled with elevated levels of the female phospholipidprotein vitellogenin (Harries et al. 1999) or masculinization of females (Howell et al. 1980), anomalous behaviour (Jones & Reynolds 1997) and compromised fertility (Leatherland 1992). These conditions require more detailed experimental investigation, but under xenobiotic treatments that are environmentally relevant (Saïdi et al. 1999).

Despite the wide recognition that xenobiotics can disrupt male reproductive function at the behavioural, anatomical and physiological levels, few studies have examined effects at the gametic level and whether exposure to low levels of potentially oestrogenic compounds affects the number of sperm produced. Sperm count is a fundamentally important predictor of male fertility and it is therefore important to assess the effects of xenobiotics so that risk assessments of endangered or commercially important species can be addressed. In humans, there is evidence that sperm counts are in decline, possibly as a result of exposure to xenobiotics (Carlsen et al. 1992; Sharpe & Skakkebæk 1993). Such longitudinal trends, however, can be confounded by spatio-temporal variation in data derivation and sampling, and similar studies have reported no decline in human sperm counts (e.g. Fisch et al. 1996; Saïdi et al. 1999). Accordingly, a controlled experimental approach is necessary that focuses on xenobiotic effects on sperm counts in an animal model, and the few experiments reported so far have generated mixed results. Reproductive and semen traits of male rats were not affected by octylphenol in direct doses as high as 2000 ppm and Cagen et al. (1999a,b) reported no effect of prenatal exposure to bisphenol A (BPA) (up to 10 ppm) on male offspring in rats (Cagen et al. 1999a) and mice (Cagen et al. 1999b). In contrast, male mice that were prenatally exposed (gestation days 11–17) to BPA showed a 20% reduction in sperm production per unit of testicular tissue (VomSaa et al. 1998). Similarly, lactational
exposure of male rat pups to nonylphenols resulted in a lower epididymal sperm count at adulthood (Lee et al. 1999). Japanese medaka males (Oryzias latipes) exposed to 20–230 ppb 4-tert-octylphenol showed reduced fertility and histological examinations revealed that spermatogenesis had been inhibited (Gronen et al. 1999). Nickel sulphate directly generated declines in sperm counts of adult male mice (Pandey et al. 1999) while an intraperitoneal dose (250 mg kg⁻¹ body weight) actually resulted in an increase in epididymal sperm count but a depletion in seminiferous tubules (Contreras & BustosObregon 1999). Results are therefore inconsistent.

Accordingly, the few studies that have experimentally explored xenobiotic actions on sperm counts generate mixed results, which depend upon the animal model, the chemical compound and the timing and level of exposure. In this study we extend the experimental research into xenobiotic effects on sperm counts by examining how the endocrine disruptors tributyltin (TBT) and BPA influence sperm production in adult male guppies (Poecilia reticulata). Guppies are aquatic vertebrates with internal fertilization and viviparous reproduction, and therefore make useful models for examining whether direct exposure to low levels of synthetic xenobiotic compounds influences sperm counts.

We explore the influences of TBT and BPA on sperm counts, gonad size and sperm length. Both TBT and BPA are common water pollutants throughout the world and the concentrations we use in this study are widely encountered by animals and humans (Fent & Humin 1995; Fent 1996; Shawky & Emons 1998). BPA is a phenolic plasticizer used extensively in food storage, plastics, resins and dental restoration. BPA shows endocrine-disrupting properties (Sommerstein & Soto 1998) and it is likely that humans are chronically exposed to BPA, which can leach out of polycarbonate plastics, particularly when heated (Sommerstein & Soto 1998). TBT is an organo-tin compound most often used as a ship anti-fouling agent (but also used in agrochemicals) and was one of the first recognized xenobiotics that induces imposex in molluscs (Gibbs & Bryan 1988). TBT use is now regulated but it remains a widespread pollutant in freshwater and marine aquatic environments (Fent & Humin 1995) and its effects on sperm counts and male reproduction are not yet known.

While there appear to be windows within development when males are particularly sensitive to xenobiotics, such as in utero or before puberty (VomSaal et al. 1998; Lee et al. 1999), little attention has been focused on the direct and proximate actions of xenobiotics on male reproductive function. In this experiment we exposed mature and reproactively active male guppies to two concentrations of TBT and BPA for a 21-day period prior to bioassay in order to measure the short-term effects of xenobiotics on sperm counts in adulthood. In addition, we examine whether xenobiotic exposure influences testis size and we measure sperm lengths to determine whether exposure influences the elongation and normal development of sperm length.

2. MATERIAL AND METHODS

Commercially supplied mature male guppies (n = 75) were randomly assigned to one of five treatment groups, which differed only in the concentrations of BPA or TBT in the aquarium water. All five treatment groups were replicated three times each with five individuals in each replicate (i.e. five treatments × three replicates × five fishes = 75 individuals) to control for tank effects. All fishes were maintained within guidelines for the use of animals in research at Gomelsbury University and we took all necessary steps for all fishes to ensure that any stress or suffering was minimized. Aquaria (110 cm × 30 cm × 50 cm) were maintained with 11.2 ng or 22.3 ng of TBT or 274 μg or 549 μg of BPA per litre of aquarium water with a clean-water control group. All fishes were sustained identically under standard tropical-fish regimes (24±1 °C and 14 L:10 D) apart from variation in the level of TBT or BPA in the water. TBT and BPA were solubilized in 5 ml of acetone prior to dilution in each aquarium. Acetone is a suitable solvent and does not disrupt development of zebra danio embryos or juveniles at concentrations up to 150 ng ml⁻¹ (Gonge & Nagel 1990) and acetone treatments were identical for all aquaria (including controls) at a dilution of 5 ml per 1000 ml of tank water. Water in each aquarium was continuously pumped through a porous Siporax filter (Schott Glas, Mainz, Germany) and the xenobiotic and water levels were replaced every 48 h in all tanks (including controls) to maintain treatment concentrations. Three males from the 11.2 ng l⁻¹ TBT treatment died prior to bio-assay from indeterminate causes and these were the only individuals omitted from analysis.

After 21 days of treatment exposure, all fishes were sacrificed to determine immediate effects on reproductive function. Body weight was recorded and the single bladed testis was dissected out and weighed. Mature spermatozoa are stored prior to ejaculation in spermatozeugmata in the deferent canal of the testis in internally fertilizing poeciliid fishes (Billard 1986). All mature spermatozeugmata in the common deferent canal were carefully flushed out onto a cavity slide; the evacuated spermatozeugmata

![Figure 1. Total numbers of mature sperm stored in the deferent testis canals of male guppies after 21-day treatments of tributyltin, bisphenol A and a control. Sample sizes are 15 males in each group (apart from 11.2 ng tributyltin where n = 12). Treatment doses are per litre of aquarium water and were replaced every 48 h. Multiple comparisons (Tukey maximum p = 0.026) reveal that sperm counts decline significantly compared to the control group in both tributyltin and bisphenol A treatments. This result for total sperm count does not change if variation in body size is controlled for and residual sperm numbers are analysed (see §3).](http://rspb.royalsocietypublishing.org/Downloaded from)
were then recovered under magnification using a micropipette and diluted in 5 ml of 0.1 M (pH 7) phosphate buffer for even dispersal. Numbers of spermatozeugmata were counted in five 50 μl subsamples per male and the total spermatozeugmata for each male was calculated by multiplying the mean count by the dilution factor. Mature sperm were dispersed from three spermatozeugmata per male using a 100 mM−1 NaCl solution with 10% cosin; evenly homogenized dispersal was achieved with an orbital agitator. Three 20 μl subsamples from each male’s sperm diluent were air-dried on glass slides and the total number of sperm in each of these smears was counted using phase-contrast microscopy. The total number of mature sperm per male was then determined by multiplying the mean sperm count of the 20 μl subsamples by the dilution factor and multiplying this value by the calculated total number of spermatozeugmata per male. Spermatozeugmata counts were significantly repeatable across 72 males (variance between males is greater than the repeated measures within individual males: repeated-measures ANOVA, $F_t=1.88, p=0.12$) as were sperm counts (repeated-measures ANOVA, maximum $F_2=1.18, p=0.31$). The total lengths of 20 individual and undamaged sperm per male were measured in these dried smears (where sperm present a two-dimensional image for measurement) by tracing along the long axis of the head and axoneme using video phase-contrast microscopy at ×400 magnification. This technique also shows significant repeatability (Morrow & Gage 2000).

3. RESULTS

As in other species (Harcourt et al. 1981; Gage 1994), guppy testis size is allometric with body size ($r=0.55, p<0.0001, n=72$), and sperm counts are associated with testis size ($r=0.33, p=0.005, n=72$), remains significant after single Bonferroni correction (critical $\alpha=0.052$) due to duplicate analysis of testis size. We therefore control for any between-male variance that could be confounded by a natural variation in body size between individuals by analysing both absolute and residual testis size and sperm numbers.

There was no relationship between a male’s residual testis size and the xenobiotic treatment to which he was exposed ($F_t=2.834, p=0.1$; no effect of replicate, $F_t=2.253, p=0.17$). However, we found that males exposed to TBT or BPA show significantly reduced sperm counts (figure 1). The decline in sperm count is significant whether we control for body weight and analyse residual sperm number ($F_t=5.56, p=0.029$; no effect of replicate, $F_t=0.39, p=0.69$) or compare absolute sperm-count values ($F_t=10.13, p=0.003$; no effect of replicate, $F_t=1.09, p=0.38$). Sperm counts decline significantly compared to the control group in both TBT and BPA treatments (Tukey multiple comparisons, maximum $p=0.026$). There was 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.

We found that there was significant variation between individual males in the total length of their sperm ($F_t=11.94, p<0.0001$; figure 2) but this significant variation was not related to the TBT or BPA treatment that males received ($F_t=0.83, p=0.51$) nor did the variance of an individual male’s sperm lengths relate significantly to his pollutant treatment ($F_t=2.22, p=0.08$).
4. DISCUSSION

Our primary finding is that sperm count in adult male guppies declines by 40–75% after 21 days of exposure to 11.2–22.3 ng of TBT or 274–549 μg of BPA per litre of aquarium water (figure 1). We therefore show that exposure to low levels of these xenobiotics disrupts male spermatogenesis, and this study is clear evidence that these endocrine disruptors can directly generate a decline in sperm count. This reduction in sperm count is particularly important since the xenobiotics that the male guppies experienced may be frequently encountered by animals and humans at higher levels (e.g. Fent & Hunn 1995; Fent 1996; Sonnenschein & Soto 1998) and our experimental treatments were therefore environmentally relevant. It is probable that humans are chronically exposed to BPA, which can leach out of plastics and resins, especially when heated (Krishnan et al. 1993). For comparison, up to 950 μg of BPA (double the concentration of our highest treatment, which caused a 75% decline in sperm count) can leach out of a dental sealant and into saliva within 1 h of treatment (Sonnenschein & Soto 1998). In the wild, levels of TBT are encountered that exceed 22.3 ng·l−1 in both coastal and inshore waters where TBT use is regulated (Shawky & Emons 1988; Fent & Hunn 1995; Fent 1996). Our results suggest that male fishes in these waters may be reproducitively compromised by exposure to TBT (and BPA), but we are not yet aware of the fertility consequences of reduced sperm counts in guppies and whether this could affect population stability of guppies in polluted natural waters or whether these results have implications for other fishes and aquatic species.

We observed a sperm-count decline after only 21 days of BPA and TBT exposure. Other studies have recorded male reproductive disruption after chronic exposure to xenobiotics or exposure at particular periods in development when individuals may be especially sensitive to hormone titre (Crisp et al. 1998; VomSaal et al. 1998; Lee et al. 1999). 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 generated from the germ line. Our findings that testis size and normal spermatosal elongation are unaffected support this interpretation. Male Japanese medaka (O. latipes) show compromised fertility and elevated levels of vitellogenin after a similar short exposure period of only 21 days to the xenoestrogenic alkyphenol 4-tert-octylphenol (Gronen et al. 1999) but it is not known whether sperm numbers were compromised.

We do not yet understand the mechanism of such short-term effects on sperm production and it is unlikely that disruption in the germ line could lead to such a significant decline within 21 days. However, Sertoli cells are essential in spermatogenesis (Jegou 1992) (functions include nutrition and release of developing sperm, and phagocytosis of degenerating gametes) and these cells are directly sensitive to xenobiotics. Recent research shows that octylphenol (a related xenobiotic to BPA) causes Sertoli-cell apoptosis within only 24 h of exposure (Raychoudhury et al. 1999). This apoptosis could block the nutritional activity of Sertoli cells on maturing spermatids and arrest the release of gametes from the efferent ducts into the testicular canal and into storage in the deferent ducts of the testes (Billard 1986). This interference with release would lead to a decline in sperm numbers rather than disruption to sperm morphology and we find no effect of xenobiotic exposure on the development of sperm length. Such direct and short-term Sertoli-cell sensitivity to xenobiotic action could be the mechanism by which exposure to low levels of BPA and TBT leads to a significant, short-term, decline in the sperm counts of male guppies.

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