Is There a Causal Association between Genotoxicity and the Imposex Effect?

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There is a growing body of evidence that indicates common environmental pollutants are capable of disrupting reproductive and developmental processes by interfering with the actions of endogenous hormones. Many reports of endocrine disruption describe changes in the normal development of organs and tissues that are consistent with genetic damage, and recent studies confirm that many chemicals classified to have hormone-modulating effects also possess carcinogenic and mutagenic potential. To date, however, there have been no conclusive examples linking genetic damage with perturbation of endocrine function and adverse effects *in vivo*. Here, we provide the first evidence of DNA damage associated with the development of imposex (the masculinization of female gastropods considered to be the result of alterations to endocrine-mediated pathways) in the dog-whelk *Nucella lapillus*. Animals (n = 257) that displayed various stages of tributyltin (TBT)-induced imposex were collected from sites in southwest England, and their imposex status was determined by physical examination. Linear regression analysis revealed a very strong relationship (correlation coefficient of 0.935, $p < 0.0001$) between the degree of imposex and the extent of DNA damage (micronucleus formation) in hemocytes. Moreover, histological examination of a larger number of dog-whelks collected from sites throughout Europe confirmed the presence of hyperplastic growths, primarily on the vas deferens and penis in both TBT-exposed male snails and in females that exhibited imposex. A strong association was found between TBT body burden and the prevalence of abnormal growths, thereby providing compelling evidence to support the hypothesis that environmental chemicals that affect reproductive processes do so partly through DNA damage pathways. *Key words:* ecotoxicology, endocrine disruption, genotoxicity, imposex, micronucleus, *Nucella lapillus*, tributyltin. *Environ Health Perspect* 114(suppl 1):20–26 (2006). doi:10.1289/ehp.8048 available via *http://dx.doi.org/* [Online 21 October 2005]

In recent years there has been increasing concern that certain pollutants may disrupt normal endocrine functioning in exposed human and wildlife populations (Colborn 1996; Jobling et al. 2002; Tyler et al. 1998). Current opinion suggests that pollutants that interfere with steroid action/production may be of most concern to human reproductive health (Fisher 2004). Moreover, it is well documented that alterations in steroid synthesis and production, particularly at critical *in utero* and early postnatal stages of development, may lead to permanent alterations in structure and function of endocrine systems in adulthood. In wildlife species, as in humans, an array of abnormal physiological and morphological responses likely to compromise reproductive fitness have been noted. These hormonal disturbances include partial sex change (intersex) in riverine and estuarine fish and marine snails, reproductive failure in birds, and abnormalities in the reproductive organs of alligators and polar bears [see Depledge et al. (1999) for review].

Temporal increases in the incidence of certain hormone-dependent cancers and the relationship between reproductive health and cancer may be linked to rising levels of endocrine-disrupting contamination in the environment (Weir et al. 2000). Such a possibility poses the question as to whether these phenomena are part of the same syndrome. To support this suggestion, there is growing evidence from clinical and epidemiological studies that indicates a synchronized increase, a possible common cause, and a toxicological mechanism for a variety of abnormalities in testicular development and function. This condition, termed "testicular dysgenesis syndrome," comprises testicular cancer, lowered fertility and cryptorchidism, and hypospadias (Sharpe 2003; Sharpe and Skakkebaek 1993). Moreover, the origins of the endocrine disruptor hypothesis may be traced to reports on adolescent daughters born to women who had taken the highly potent synthetic estrogen diethylstilbestrol (DES) while pregnant. Many of these daughters developed reproductive tract abnormalities and clear cell adenocarcinoma of the vagina and cervix, thus illuminating the causal association between developmental exposure to DES and reproductive cancers (Greenwald et al. 1971).

Despite the biological plausibility of a link between diverse environmental pollutants, reproductive abnormalities, and cancers, gaps remain in our knowledge of potential mechanisms of action. Many endocrine-disrupting chemicals (EDCs) are reactive chemicals that exhibit multiple mechanisms of toxicity acting at different sites within the body. For example, altered immune, nervous, and thyroid function and damage to genetic material may each contribute toward the overall toxic impact (Choi et al. 2004; Colborn 2002; Galloway and Handy 2003). Systematic review of the toxicity profiles of a range of EDCs has highlighted the potential for those that exhibit steroid-modulating effects also to possess mutagenic and carcinogenic activity (Choi et al. 2004). This suggests that direct damage to DNA and altered gene expression may contribute to endocrine disruption and the adverse effects seen *in vivo*. Indeed, evidence is accumulating to support this hypothesis for a range of different environmental contaminants, including those that are endocrine-active in different ways, as estrogen, androgen, or thyroid hormone mimics or through manipulation of hormone synthesis, transport, and metabolism. In mammalian studies, estrogenic activity and cell cycle components, including tumor suppressor genes, have been implicated in the promotion and progression of reproductive diseases, including cancer (Foster et al. 2001; Liao et al. 2000). Estrogens can also damage DNA, either directly or after conversion to genotoxic catecholestrogens (Adjei and Weinshilboum 2002). Hiraku et al. (2001) showed that catecholestrogens induced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in MCF-7 breast cancer cells in culture, highlighting their potential to initiate carcinogenesis through oxidative DNA damage. In fish and invertebrates less is known about the complex interactions of biochemical pathways that could lead to contaminant-induced biological effects in this way (Depledge 1998; Rotchell and Ostrander 2003). However, a very recent study in catfish (*Ictalarus punctatus*) showed that exposure to the potent genotoxin benzopyrene induced the formation of 4-hydroxyestradiol, a potentially genotoxic estrogen, metabolized by the liver (but not by the gills or the gonad) (Butala et al. 2004). Unlike in vertebrates, it has been suggested that genetic damage in invertebrates is manifested as a suite of changes known as the genotoxic disease syndrome

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(Kurelec 1993). These changes include impaired enzyme function and immune responses, cellular injuries, inhibited growth, a decrease in reproduction, and faster aging. Our previous studies have clearly shown that both estrogenic and androgenic contaminants are capable of inducing genetic damage in invertebrate species (Atienzar et al. 2002a; Hagger et al. 2002; Jha et al. 2000). Induction of chromosomal aberrations and developmental abnormalities by tributyltin (TBT) was demonstrated in early life stages of the marine worm *Platynereis dumerilii* (Hagger et al. 2002) and the mussel *Mytilus edulis* (Jha et al. 2000). Genetic damage (single-strand DNA breaks, micronucleus formation) was also induced in adult *M. edulis* exposed to TBT (Hagger et al. 2005). Atienzar et al. (2002a) reported changes at the genomic level detectable using the random amplified polymorphic DNA assay (Atienzar et al. 2002b) in developing barnacle larvae exposed to either nonylphenol or to the estrogen, 17β-estradiol. To date, however, there have been no conclusive examples in wildlife linking genetic damage with perturbation of endocrine function and adverse effects *in vivo*.

The induction of a pseudohermaphroditism in the gonochoristic marine gastropod *Nucella lapillus* remains the best-characterized example of endocrine disruption in wildlife and the only example where an unequivocal causal association with known chemicals has been proved. Smith (1971) first named the superimposition of male genitalia in female gastropods "imposex." Subsequent research has indisputably linked the condition in *N. lapillus* to exposure to the organotin compound TBT (Bryan and Gibbs 1991; Bryan et al. 1988; Gibbs and Byran 1986; Schulte-Oehlmann et al. 2000). However, exposure to testosterone as well as to methyltestosterone has been demonstrated to induce imposex in prosobranch mollusks (Bettin et al. 1996). In its most severe form, imposex restricts the reproductive capacity of affected females and leads to ecologically significant population declines (Bryan et al. 1986; Gibbs and Bryan 1986). The exact mechanism(s) by which TBT exerts its masculinizing effects on dog-whelks is poorly understood. Mechanisms have been proposed to bring about this effect. They include *a*) inhibition of aromatase and/or other steroid biosynthesis enzymes, *b*) modulation of testosterone conjugation and elimination pathways, *c*) induction of neurohormones that control genital development, *d*) APGW-amide (Ala-Pro-Gly-Trp-NH_2) involvement, and *e*) retinoid X receptor (RXR) activation (Bettin et al. 1996; Gibbs et al. 1991; Matthiessen and Gibbs 1998; Nishikawa et al. 2004; Oberdörster and McClellan-Green 2000). More recently, a combination of neuropeptide and aromatase involvement was proposed by Oberdörster and McClellan-Green (2002) and Oehlmann and Schulte-Oehlmann (2003).

The aim of this present study was to address the hypothesis that environmental chemicals that affect reproductive processes do so partly through DNA damage pathways, by examining dog-whelks with developmental and reproductive abnormalities induced by TBT for evidence of genotoxic damage.

Materials and Methods

N. lapillus is a carnivorous intertidal snail found on rocky shores in Europe and Northeast United States (Cooke 1915). For field evaluation for genetic damage, dog-whelks were collected from three locations in southwest England. Figure 1 shows the location of the three sites: *a*) Port Quin, North Cornwall; *b*) Whitsand Bay, South Cornwall; and *c*) Mansands, South Devon. Venous hemolymph was removed from the cephalopedal sinus at the junction where the proboscidal sinus (head) and the pedal sinus (foot) unite (Malek and Cheng 1974), after which the sex and imposex stage of the females were determined as described by Gibbs et al. (1987) and Oehlmann et al. (1991).

Comet assay. Blood cells were used for analysis of single-strand DNA breaks using the comet assay. The comet assay was performed essentially according to the procedure described by Singh et al. (1988), although it was modified for use with marine organisms (Lee and Steinert 2003). The hemolymph suspension was centrifuged at 2000 rpm at 4ºC for 2–3 min, after which the supernatant was removed and 85 µL of 1% low melting point agarose was pipetted onto the cell pellet. The cell suspension was then carefully pipetted onto high melting-point agarose covered frosted slides, sandwiching the cells between two layers of agarose gel. After the gel had set, the slides were placed into a freshly prepared lysing solution at 4°C for 1 hr [2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Na sarconisate (pH 10.0) 1% Triton X-100, 10% dimethyl sulfoxide (DMSO)]. The lysis and following steps of the comet assay protocol were carried out in the dark and at 4°C to prevent any additional damage to cell DNA.

The slides were then removed from the lysis solution and placed in a horizontal gel electrophoresis tank. The tank was filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM Na_2–EDTA), and the DNA was allowed to unwind for 40 min before electrophoresis, which was performed at 25 V for 30 min. After electrophoresis the slides were washed 3 times, for 5 min each time, in neutralizing buffer (0.4 M Tris, pH 7.5). The slides were stained with 20 µL of 5 µg/mL ethidium bromide solution, viewed under ultraviolet fluorescence light, and scored using the Komet software (version 5.0; Kinetic Imaging Ltd, Wirral, UK). Twenty-five cells were scored in duplicate per dog-whelk (50 in total).

Micronucleus test. Hemolymph was used for analysis of micronuclei formation, as described by Venier et al. (1997). Approximately 50–100 µL of hemolymph was smeared on slides that had been precoated in 10% poly-L-lysine solution. The slides were left to air dry and then fixed in methanol for 15 min, followed by staining in 5% Giemsa/buffer solution for 20 min, after which the slides were mounted with a coverslip. Slides were scored blind under ×40 magnification and micronuclei (Mn) validated under oil immersion. A total of 500 blood cells per dog-whelk were analyzed

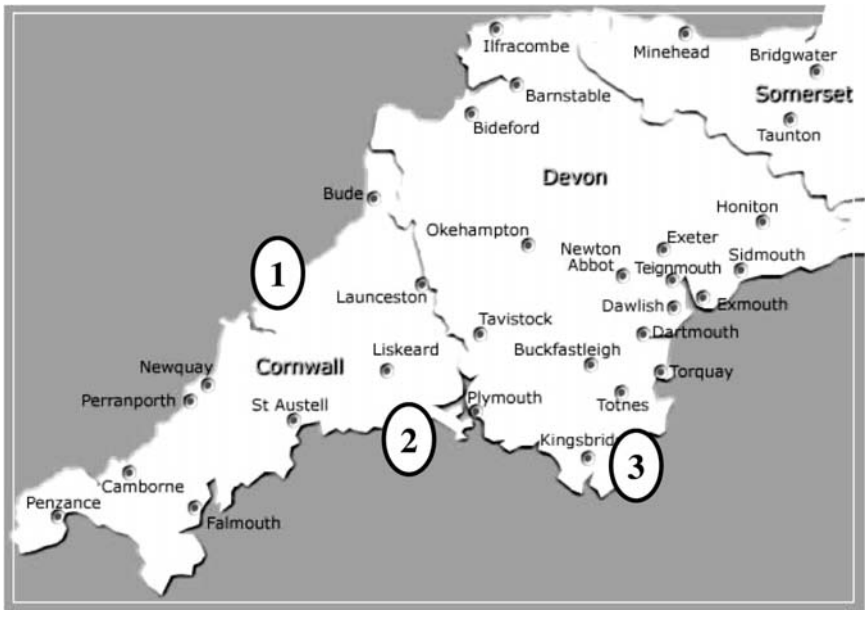

Figure 1. Map of southwest England indicating the three sites used for genotoxic analysis.

for the presence of Mn (Countryman and Heddle 1976).

Further field studies. In a separate series of field investigations, 18,697 specimens of *N. lapillus*, collected at more than 100 stations along the French and Irish Atlantic coast and at single stations in Great Britain, Germany, and Russia were analyzed between March 1988 and March 2004. Wherever possible, 30 or more sexually mature dog-whelks were sampled intertidally. Before further analyses, snails were narcotised using 7% magnesium chloride in distilled water. The individual imposex stage of each snail was determined according to Gibbs et al. (1987) and Oehlmann et al. (1991), and the vas deferens sequence index (VDSI) as the mean value of all imposex stages occurring in a sample was calculated. The occurrence of excrescences of proliferating tissue on genital organs such as the penis and vas deferens or on nongenital organs in the mantel cavity, primarily on gill and osphradium, the olfactory organ in prosobranch snails, was also assessed and the prevalences were calculated. For histological investigations, specimens were fixed with Bouin's fluid, preserved in 70% ethanol, and embedded in paraplast. Serial sections (7–10 µm) were stained with hemalun–chromotrope. Specimens for scanning electron microscopy were also fixed in Bouin's fluid, dehydrated via graded ethanol series, critical-point dried, coated with gold, and examined with a Hitachi S-530 scanning electron microscope (Hitachi Ltd, Tokyo, Japan).

TBT determination. The determination of TBT compounds in tissue was based on Stroben et al. (1992). Briefly, TBT and dibutyltin (DBT) compounds were extracted with hexane. DBT was eliminated by washing the hexane extract with sodium hydroxide followed by quantification using atomic absorption spectroscopy (Perkin-Elmer HGA-500 attached to a PerkinElmer 5000 AAS (PerkinElmer GmbH, Überlingen, Germany) with background correction; wave length 224.6 nm; slit 0.7 nm; injection volume 25 µL). Internal standardization (standard addition with spiked samples) was employed. Certified reference material (CRM: PACS-1, National Research Council of Canada) was also analyzed. Our results were within the standard deviation of the certified values for the CRM. The detection limit (3δ) in a single sample was 8.8 ng TBT-Sn. TBT tissue concentrations are given on a dry weight basis as Sn and can be converted to concentrations on TBT cation basis by multiplying by 2.44.

Results

Imposex development. In *N. lapillus,* imposex development can be described by a scheme with six different stages representing increasing degrees of masculinization (Gibbs et al. 1987; Oehlmann et al. 1991). Within individual imposex stages, up to three different types or substages can be distinguished that have been observed mainly in continental European populations (Oehlmann et al. 1991). From stage 0, a normal female without any signs of imposex, to stage 4, the number and size of male sex organs increases gradually but without affecting the reproductive capability of snails. Imposex development starts in stage 1 with the formation of a penis oriment (stage 1a) or of an isolated vas deferens section (stages 1b, c). In stage 2a the penis oriment develops a penial duct, whereas in stages 2b and 2c a further vas deferens section and a penis oriment can be found, respectively. The vas deferens sections grow farther out over the bottom of the mantle cavity in stage 3 (Figure 2A), but it is not before stage 4 that both male formations are fully developed with the vas deferens expanding from the penis base up to the vaginal opening. In stage 5 the oviduct is blocked, either by proliferating vas deferens tissue (Figure 3B) or by the development of a prostate gland instead

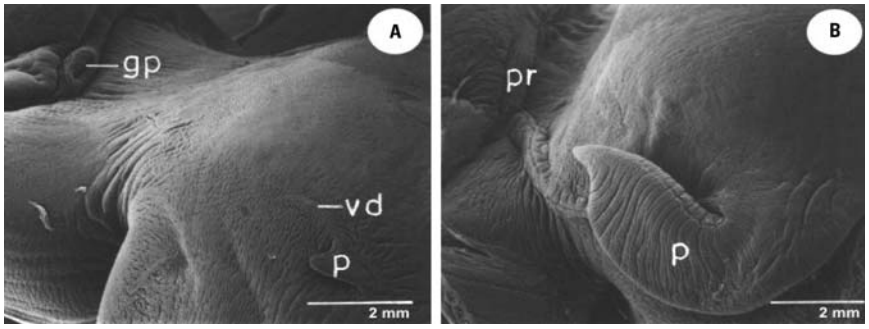

Figure 2. Scanning electron micrographs of *Nucella lapillus* depicting (*A*) imposex stage 3a with a penis and an anterior section of the vas deferens and (*B*) imposex stage 5a with a fully developed penis, vas deferens, and a prostate gland supplanting the vagina. Abbreviations: gp, genital papilla; p, penis; pr, prostate gland; vd, vas deferens.

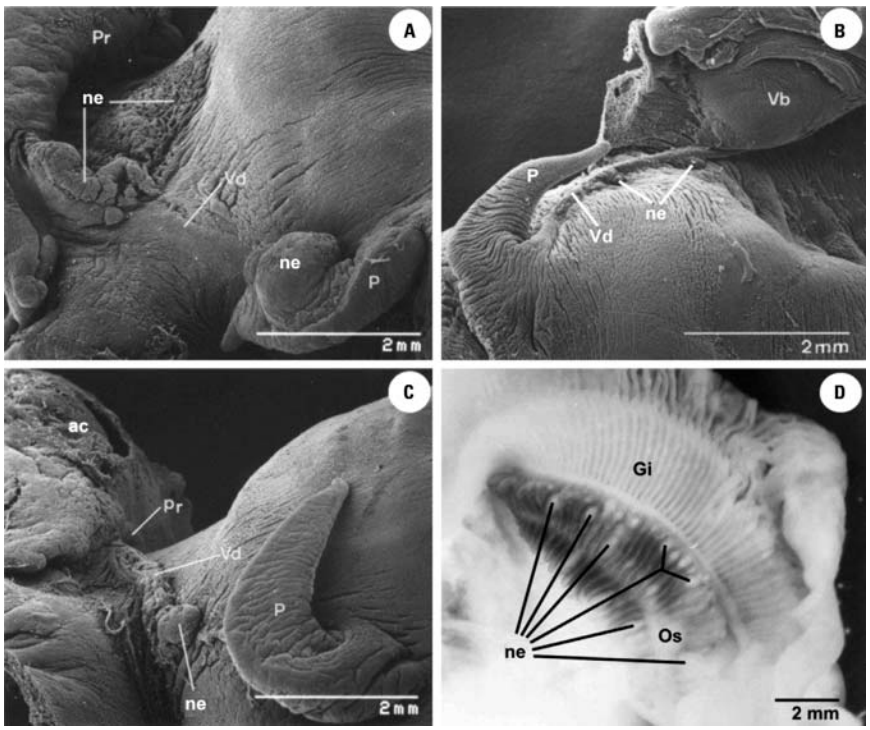

Figure 3. *Nucella lapillus.* Abbreviations: ac, abortive capsule mass penetrating the mantle wall; Gi, gill; ne, neoplasmic like growth; P, penis; Pr, prostate gland; Os, osphradium; Vb, blockage of vaginal opening; Vd, vas deferens. (*A–C*) Scanning electron micrographs of specimens with excrescences of proliferating tissue on male genital organs (penis and vas deferens). (*A*) Male with neoplasia like growth on penis, vas deferens, and the mantle bottom epithelium. (*B*) Imposex stage 5 with smaller excrescences on vas deferens. (*C*) Imposex stage 6 with growth on vas deferens. (*D*) Micrograph of specimen with numerous proliferations on the osphradium.

of a vagina (Figure 2B). This development causes reproductive failure and a functional sterility because egg capsules can no longer be released so that they accumulate in the oviduct and can be seen as an abortive black mass in imposex stage 6 through the walls of the capsule gland. Finally, the oviduct ruptures, and as a consequence of female sterility and increased mortality, populations decline and the sex ratio shifts in favor of males.

DNA damage. There were no statistical differences in the percentage of DNA damage (as detected using the comet assay to detect the percentages of DNA in the tail of the cells' comet) between males and females with no imposex ($p = 0.3938$) and among females with different stages of imposex ($p = 0.8304$). Nevertheless, all females with imposex demonstrated a statistically significant increase in Mn formation in hemocytes when compared to the females without imposex and with males ($p = 0.0005$). Figure 4 shows the relationship between micronuclei formation and sex and imposex stage of females. There was a highly significant correlation between the induction of Mn and increasing imposex stage ($p = 0.001$; $R^2 = 0.9356$); hence, females that exhibited the highest degree of masculization (stage 6) also had hemocytes containing the highest numbers of Mn.

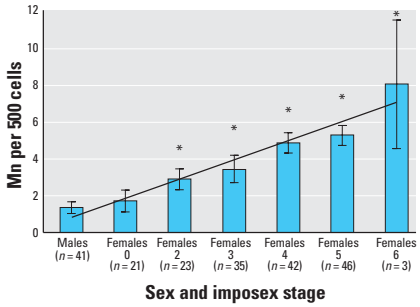

Figure 4. Induction of micronuclei formation in relation to the sex and imposex stage of female dog-whelks *Nucella lapillus* from three beaches in southwest England ($n = 257$). Error bars are 2× SE. $y = 1.0232x - 0.2124$. $R^2 = 0.9356$.
*Significantly different from females without imposex.

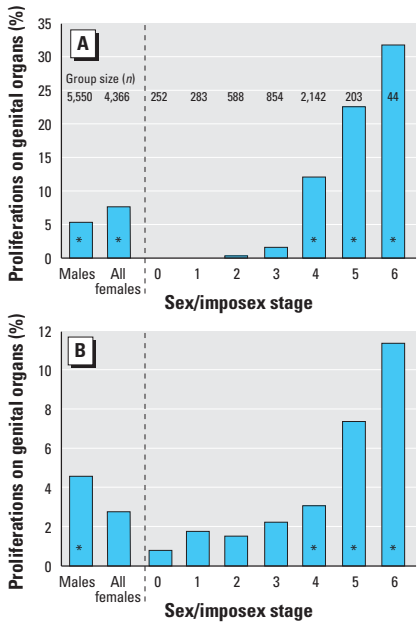

Figure 5. *Nucella lapillus*. Prevalence of proliferations on genital (*A*) and nongenital organs (*B*) in males, females, and different imposex stages. Dashed line separates all females from those with different imposex stages.
*Statistically significant differences compared with [imposex stage 0] females (χ^2 test, $p < 0.01$).

Further field studies. In additional field studies, we found examples of all different imposex stages [Figure 5A for the number of males, all females (imposex stages 0–6), and the different imposex stages]. Growths of proliferating epithelial tissue were also found in some of the snails sampled. The occurrence of growths of proliferating tissue was first visible as small nodules that developed into voluminous cauliflower-shaped structures (Figure 3). Macroscopically, they could be found almost exclusively on mantle cavity organs. Primarily affected were the vas deferens and the penis of males and imposex females, but the growths were also observed on the osphradium, the gill and, to a lesser extent, the bottom of the mantle cavity. Accordingly, genital and nongenital excrescences could be distinguished (Figure 3). All proliferations emanated from the epithelium, as indicated in Figure 6, with the example of a mantle epithelium excrescence. Neither the underlying connective tissue nor the musculature or other tissues contributed to these formations. The normal epithelium in all these organs comprised ciliary and various glandular cells (Figure 6B), whereas exclusively ciliary cells could be found in the altered epithelium (Figure 6D).

The incidence of proliferations on genital and nongenital organs was almost comparable in males, with 5.42 and 4.57% incidences, respectively. Females exhibited a significantly higher prevalence of genital growths (7.67%; $\chi^2 = 20.6$, $p < 0.01$) and a lower prevalence of nongenital excrescences (2.77%; $\chi^2 = 21.9$, $p < 0.01$) when compared with males (Figure 5). Moreover, the incidence of growths increased with the imposex stage of the females and attained peak incidences of 31.8 and 11.4% in stage 6 imposexed mollusks for genital and nongenital outgrowths, respectively. The achieved individual imposex stage of a female has been shown to be a function of the TBT exposure level (Bettin et al. 1996; Bryan and Gibbs 1991; Gibbs et al. 1991;) during the life span. Therefore, the data shown in Figure 6 provide some indication that TBT may be a cause for the observed neoplasms in dog-whelks.

Further field evidence for the causative role of TBT can be derived from the highly significant relationships between TBT body burden and the prevalence of genital excrescences in male and female dog-whelks (Figure 7). In populations with TBT tissue concentration < 50 µg as Sn/kg, generally no or only a few males and females with outgrowths on genital organs can be found. The incidences of such malformations increase to an average of > 30% in males and > 50% in females at TBT tissue concentrations > 500 µg as Sn/kg .

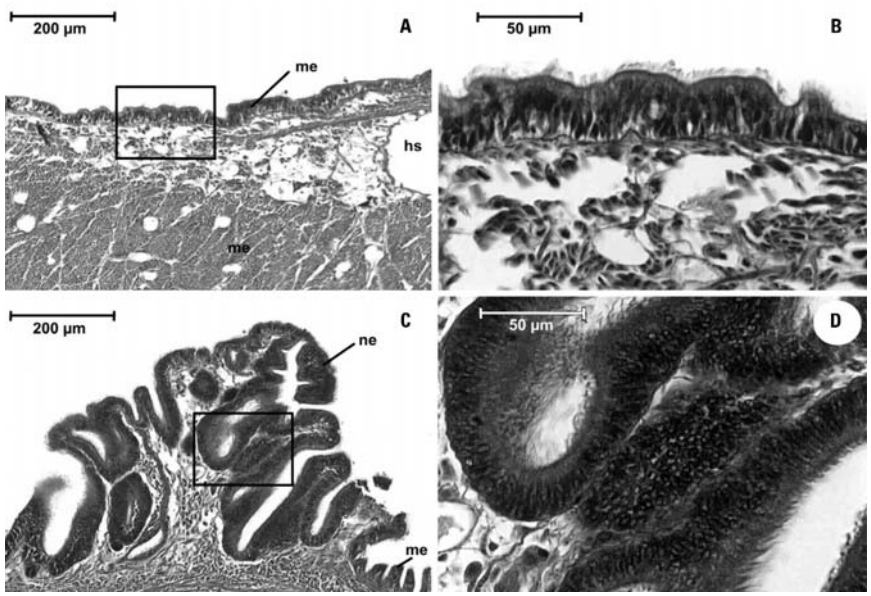

Figure 6. *Nucella lapillus*. Histological sections through the mantle epithelium. Abbreviations: hs, hemolymph sinus; me, mantle epithelium; mu, musculature; ne, neoplasmic like growth. Overview and detail of a normal epithelium in (*A*) and (*B*) and of an epithelium with growth in (*C*) and (*D*).

Discussion

The induction of pseudohermaphroditism in *N. lapillus* remains the best-characterized example of endocrine disruption in wildlife (Matthiessen and Gibbs 1998) and the only example where an unequivocal causal association has been proved with the organotin compound TBT. It is, therefore, possibly the best example of endocrine disruption (ED) in a wildlife species in which to study the association between ED and other mechanisms of toxicity. During this study we have clearly shown an association between DNA damage (micronucleus formation) and imposex in *N. lapillus*. Furthermore, we have shown that there are abnormal growths of epithelial tissue, primarily associated with the reproductive organs of these dog-whelks, and that (as with the micronucleus formation) the occurrence of these growths is positively correlated with the imposex stage and with the tissue concentration of TBT. There was no observed difference in single-strand DNA breaks using the comet assay, although this similarity may be a result of efficient repair mechanisms in the dog-whelk that may not be as successful for repairing large occurrences of DNA damage as represented by Mn formation.

Smith (1971) first named the superimposition of male genitalia in female gastropods as imposex, and subsequent research has indisputably linked the condition in *N. lapillus* to exposure to organotins (Bryan and Gibbs 1991; Schulte-Oehlmann et al. 2000). At water TBT concentrations of < 1 ng Sn/L, the female exhibits the initial stages of penis and sperm duct development, and these organs are fully formed at TBT levels around 1–2 ng Sn/L. At TBT levels > 2 ng/L, further proliferation of sperm duct tissue occurs, which invades the anterior oviduct, blocking the vulva and preventing the release of egg capsules from the capsule gland (Gibbs and Bryan 1986; Matthieson and Gibbs 1998). This blockage of the oviduct prevents the release of egg capsules, with a consequent demise of populations, because of poor female fecundity. The blockage also leads, on the individual level, to a distension of the oviduct and finally to its rupture, resulting in the female's death (Gibbs et al. 1987; Oehlmann et al. 1991). During the present study we often observed that the entire mantle cavity was filled with these proliferations, which may reduce the respiratory water stream as well as the ultimate survival of these snails. As stated above, it has been shown conclusively that genital proliferation in imposex-affected females exhibits a negative impact on both the individual and population level. Such outgrowths of vas deferens tissue may further occlude the vaginal opening, thereby leading to an accumulation of abortive egg capsules in the pallial section of the oviduct. Gibbs and Bryan (1986) also observed such proliferations of the pallial epithelium in imposexed dog-whelks that were classified to be hyperplasia. Although they concluded that because the proliferations were found to a lesser extent in males, such cell proliferation could not be regarded as a direct result of imposex but may be a further response to TBT contamination. During the present study, however, we have demonstrated a possible link between these growths and imposex because of significant correlation of their prevalence with the incidence of imposex as well as with TBT concentrations.

Tributyltin, in common with many EDCs, is a reactive chemical that exhibits multiple mechanisms of toxicity that act at different sites within the body. Although TBT was not originally classified as a genotoxic agent (Davis et al. 1987), recent results have highlighted the potential for TBT to cause DNA damage. Fragmentation of DNA was induced by TBT in both human cells and gill cells of the mussel *Mytilus galloprovincialis* (Micic et al. 2002). Ferraro et al. (2004) demonstrated that TBT produced mutagenic effects with an increase in DNA damage in the fish *Hoplias malabaricus* measured as single-strand DNA breaks, the induction of Mn, and an increased incidence of chromosomal aberrations. Previous work in our laboratory showed that exposure of early life stages of the marine mollusc *M. edulis* and the polychaete worm *P. dumerilii* to TBT caused DNA damage via the production of both chromosomal aberrations and sister chromatid exchanges (Hagger et al. 2002; Jha et al. 2000). A postulated mechanism for this effect is through thiol-mediated inhibition of Ca^{2+}-ATPases, leading to increased intracellular Ca^{2+} influx and activation of Ca^{2+}-dependent degradative enzymes including endonucleases and in turn to DNA fragmentation (Collins et al. 1996; Mattioli et al. 2003; Orrenius et al. 1992). In addition, the immunotoxic effects of TBT may contribute to reduced immunosurveillance and persistence of transformed cells (Galloway and Depledge 2001). Direct oxidative damage to DNA may also occur. These observations raise the possibility that damage to DNA and altered gene expression may contribute to the development of imposex and the adverse effects seen *in vivo*. Indeed, evidence is accumulating to support the hypothesis of a link between genotoxic damage and endocrine disruption for a range of different environmental contaminants. Such contaminants include compounds that are endocrine-active in different ways, such as estrogen, androgen, or thyroid hormone mimics or those that act through manipulation of hormone synthesis, transport, and metabolism (Choi et al. 2004; Rotchell and Ostrander 2003; Roy and Liehr 1999).

The occurrence of visible excrescences, primarily on the reproductive tissues of the dog-whelks in association with imposex, also supports the contention that a genotoxic mechanism may contribute to the development of imposex and the adverse effects seen *in vivo*. Excresences in mollusks have also been reported as a consequence of infestations with ciliates. Laruelle et al. (1999) reported that the host-specific parasitic ciliate *Sphenophrya dreissenae* (Rhynchodida: Sphenophryidae) induced hyperplasia, cell hypertrophy, and vacuolization of gill and other mantle cavity epithelia in the zebra mussel *Dreissena polymorpha*. Chesapeake Bay populations of the softshell clam *Mya arenaria* and the razor clam *Tagelus plebeius* were infected by *Perkinsus* sp. protozoans at prevalences ranging from 30–100% (Dungan et al. 2002). In the present study, however, it seems highly unlikely that ciliates were the causative agents for the excresences, as the occurrences of epizootic protozoans in the dog-whelks with and without neoplasia were at a comparable infestation rate (< 5% for both groups).

Chemical induction of neoplasia and hyperplasia in mollusks is also described in the literature. Harris et al. (1999) reported that an exposure of juvenile greenlip abalone, *Haliotis laevigata,* to a pH of 7.16 for 50–68 days resulted in an increase in gill hyperplasia and abnormalities. Gardner et al. (1991) found neoplastic disorders in the oyster *Crassostrea*

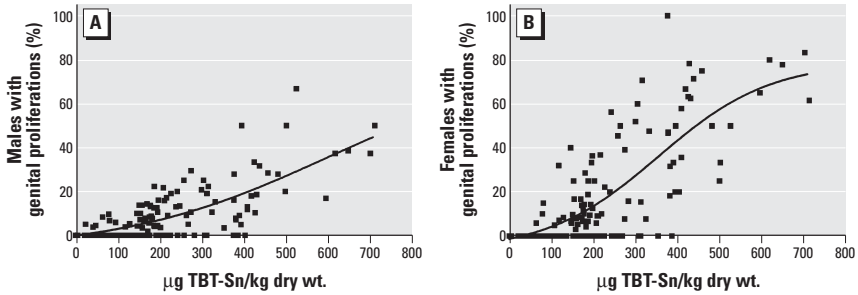

Figure 7. *Nucella lapillus*. Relationship between TBT body burden and prevalence of proliferations on genital organs such as penis and vas deferens in males (*A*) and females (*B*). Values for 149 samples analyzed with 2,945 males and 2,321 females are shown, including calculated regressions: (*A*) $y = 6.82 + (77.7 + 6.82) / (1 + 10^{(601 - x) \times 0.0018})$; $r = 0.745$; $p < 0.0005$. (*B*) $y = 6.85 + (79.0 + 6.85) / (1 + 10^{(335 - x) \times 0.0033})$; $r = 0.788$; $p < 0.0005$.

virginica when it was experimentally exposed both in the laboratory and field to chemically contaminated sediment from Black Rock Harbor in Bridgeport, Connecticut. Neoplasia were observed in oysters after 30 and 60 days of continuous exposure in a laboratory flow-through system to a 20 mg/L suspension of harbor sediment plus post exposure periods of 0, 30, or 60 days. Neoplasia incidence was 13.6%, with the occurrence being highest in the renal excretory epithelium, followed in order by gill, gonad, gastrointestinal, heart, and embryonic neural tissue. Regression of experimental neoplasia was not observed when the stimulus was discontinued. In field experiments, gill neoplasms developed in oysters deployed in cages for 30 days at Black Rock Harbor. Although the authors did not consider organotin compounds in the accompanying analytical program, it is evident that TBT and other organotins are present in such harbor sediments.

Thiriot-Quievreux and Wolowicz (2001) analyzed the spatial variation the prevalences of gill neoplasia in the bivalve *Macoma balthica* at different sites in the Gulf of Gdansk, a southern inlet of the Baltic Sea. This area is known to be highly contaminated with organotin compounds, including TBT (Kannan and Falandysz 1997; Senthilkumar et al. 1999). The prevalence of gill neoplasia, individually identified by the occurrence of abnormal metaphases with higher chromosome numbers than normal metaphases ($2n = 38$), ranges from 0–94% of individuals, according to the site studied. Sites near dockyards and other harbor installations were the most affected and showed the highest incidences of gill neoplasia. Laboratory experiments with the dog-whelk *N. lapillus* and triphenyltin (TPT), an organotin compound closely related to TBT, showed that the test compound induced a highly significant and concentration-independent increase in the incidence of excrescences on gills, osphradia, and other organs in the mantle cavity. This finding indicates a carcinogenic potential of TPT in this species (Schulte-Oehlmann et al. 2000). Tillmann et al. (2001) report that a co-exposure of dog-whelks to the antiandrogenic model compound cyproterone acetate and to potent androgens such as TBT and TPT resulted in partial or even total suppression of imposex proliferations in dog-whelks. This result suggests that the observed neoplasia under sole TBT or TPT exposure are androgen-mediated. Tumors, both kidney and enteric, were induced in eastern oysters (*C. virginica*) by a mixture of chemicals including the endocrine-disrupting compounds benzo[*a*]-pyrene, chlordane, Aroclors 1242 and 1254, cadmium, and lead (Gardner et al. 1992). The pathologies of such growths in invertebrates can closely parallel those in vertebrates. Soft shell clams, *M. arenaria*, have been shown to develop a proliferative disease in the hemolymph that has previously been called hematopietic neoplasia and whose pathogenesis is similar to that of leukemia in vertebrates (Miosky et al. 1989). Furthermore, Harring et al. (2003) used differential display PCR to perform gene expression analysis after toxicant exposure in the marine soft shell clam, *M. arenaria*, and identified an increase in a putative E3 ubiquitin ligase, which in humans degrades the tumor suppressor *p53* gene. In mammalian studies, estrogenic activity and cell cycle components, including tumor suppressor genes, have been implicated in the promotion and progression of reproductive diseases, including cancer (Foster et al. 2001; Liao et al. 2000). Estrogens can also damage DNA, either directly or after conversion to genotoxic catecholestrogens (Adjei and Weinshilboum 2002). Hiraku et al. (2001) showed that catecholestrogens induced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine in MCF-7 breast cancer cells in culture, highlighting their potential to initiate carcinogenesis through oxidative DNA damage. The estrogenic compound bisphenol A has been shown to cause genotoxicity in mammals (Metzler et al. 1998), and evidence has emerged for an association between environmental exposure to phthalates and DNA damage in human sperm (Duty et al. 2003). Taken together, this evidence strongly supports the notion that exposure to chemicals classified as endocrine disruptors can also cause damage to DNA, but to date there have been no examples of genetic damage occurring in organisms with recognizable alterations to their endocrine systems *in vivo*.

Numerous human epidemiological and experimental studies have been conducted to determine whether environmental pollutants may contribute to an increased risk of reproductive cancers. While both genetic and environmental factors are implicated in their etiology, the extent of the contribution of environmental factors to human reproductive abnormalities and cancers remains controversial. One of the problems has been the difficulty in establishing causality. Limited evidence suggests an association between an increased risk of breast cancer and elevated tissue levels of, or exposures to, polychlorinated biphenyls, organochlorines, for example, DDE [2,2-bis-(*p*-chlorophenyl)ethylene], and polyaromatic hydrocarbons (Davis et al. 1993, Laden et al. 1999). There is as yet no conclusive evidence of a causal association between EDCs and human cancer (International Programme on Chemical Safety 2002). Steroid receptor-mediated mechanisms have received the most attention, but other mechanisms, including hormone synthesis, storage/release, transport and clearance, and disruption of downstream signaling mechanisms, have been documented (Crisp et al. 1998; Goldman et al. 2000).

In summary, in dog-whelks exposed to TBT, DNA damage and endocrine disruption occurred simultaneously as did hyperplasmic growths. In mammals, DNA damage and cellular proliferation sometimes represent a precancerous state. For obvious ethical reasons, it is neither possible to perform controlled laboratory studies with human subjects nor to accurately probe the early developmental exposure to chemicals implicated in the disruption of endocrine processes. Therefore, by using *N. lapillus* as an alternative *in vivo* model, we can study the complex interactions between environmental exposure, genetic response, and reproductive abnormalities.

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