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Determination of the androgenic potency of whole effluents using mosquitofish and trout bioassays

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Abstract

This study combined bioassay-derived and direct chemical analysis of steroidal compounds in pulp and paper and municipal sewage effluent in order to determine the cause of masculinization of female mosquitofish. The bioassays used in this study consisted of androgen and estrogen receptor binding, and aromatase inhibition using tissues from rainbow trout. This study observed no masculinization of female mosquitofish from a pulp and paper mill effluent that was previously observed to cause this effect. Mosquitofish sampled from the receiving environment of the same mill verified that masculinization was not occurring in the wild. The municipal sewage effluent also had no masculinizing effect. In vitro bioassays indicated significant sources of both androgens and estrogens in the effluents tested with sewage effluent having both the highest estradiol (41 ng/L) and testosterone (182 ng/L) equivalent concentration. These results could not be attributed to any particular compounds measured in the effluents. Two compounds implicated in the masculinization of mosquitofish by pulp and paper effluent, androstenedione and androstadienedione required relatively large (10–100 μg/L) waterborne concentrations to elicit a masculinizing effect and neither of these compounds are likely to occur at levels this high in the natural environment. The potent aromatase inhibitor, 4-hydroxyandrostenedione also did not cause masculinization at 100 μg/L indicating that masculinization did not occur through this mechanism. The mammalian anti-androgen, cyproterone acetate was only partially effective in mosquitofish and reduced the severity of masculinization in the presence of methyl testosterone. While neither effluent masculinized mosquitofish, there was a significant reduction of in vitro ovarian steroid production with the most severe effects observed with the sewage effluent. Overall, this study found the disappearance of a masculinizing effect that had been previously observed; concluded that based on 21 days aqueous exposures androstenedione and androstadienedione are not likely candidates for mosquitofish masculinization, and showed that masculinization and in vitro steroid production are unrelated biological endpoints.

Keywords: Endocrine disrupter; Androgen; Bioassay; Mosquitofish

1. Introduction

The androgenic effects of pulp and paper mill effluent to fishes have been documented in a number of receiving environments throughout the world. Scandinavian studies demonstrated increased male sex ratios in the progeny of eelpout (*Zoarces viviparous*) that reproduce near a pulp and paper mill (Larsson et al., 2000; Larsson and Förlin, 2002) and caused male sexual characteristics in female guppies (*Poecilia reticulata*; Larsson et al., 2002). Perhaps the best known of the androgenic impacts is development of a male sexual appendage, the gonopodium, on the anal fin of female mosquitofish. This has long been observed in a number of pulp mills in the USA (Howell et al., 1980; Bortone and Cody, 1999) and has recently been observed in a New Zealand effluent (Ellis et al., 2003). One of the first observations of an androgen-mediated effect at a Canadian pulp mill was a strong male sex bias in fathead minnow (*Pimephales promelas*) life cycle tests (Kovacs et al., 1995). There has also been evidence presented that female white sucker (*Catostomus commersoni*) in the wild showed an increased level of nuptial

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tubercles, a male secondary sex characteristic, after the installation of secondary treatment (Munkittrick et al., 1997). Effluents from a number of Canadian pulp mills, with varying pulping and bleaching processes, have been observed to contain chemicals that bind to goldfish (*Carassius auratus*) androgen receptors (Hewitt et al., 2000, 2003, 2005) and those compounds are observed to bioaccumulate in the tissue of exposed fish.

The compounds androstenedione and androstadienedione, presumably derived from the biotransformation of sterols, have been implicated as being the steroids responsible for mosquitofish masculinization (Denton et al., 1985; Howell and Denton, 1989). Recent studies have isolated and measured those compounds in water and sediment from the Fenholloway River, USA where mosquitofish masculinization still occurs (Jenkins et al., 2001, 2004). Some of those findings have been refuted by other authors on the basis of methodology (Durhan et al., 2002). One data gap in associating those steroids with the observed effect of masculinization is the lack of any dose–response data. Androstenedione or androstadienedione were observed at very low concentrations in Fenholloway River samples (~40 ng/L) and it is not know if those concentrations were sufficient to cause masculinization.

Most work on municipal sewage effluents has focused on the impacts of estrogens (Sumpter and Johnson, 2005). Municipal sewage effluents can also contain substantial quantities of androgenic chemicals (Leusch et al., 2006a). While the potential of sewage effluent to elicit androgenic responses has not been widely studied, municipal sewage effluent has also been observed to masculinize female mosquitofish (Leusch et al., 2006b).

While the estrogenic effects of sewage effluent are widely known, pulp and paper effluent has also been observed to be estrogenic in nature (Tremblay and Van Der Kraak, 1999). There are also a plethora of other biological effects of pulp and paper effluents, none of which has been definitively linked to a steroid receptor-mediated mechanism. One such widespread impact of pulp and paper mill effluents has been the reduction of circulating sex steroid hormones as primarily documented in the white sucker (Munkittrick et al., 1992, 1994). A short term bioassay utilizing in vitro steroid hormone production in white sucker was adapted to study this observation (Van Der Kraak et al., 1992). Studies have documented a number of North American effluents that caused reduced in vitro steroid production in both white sucker (McMaster et al., 1996) and in mummichog (*Fundulus heteroclitus*; Dube and MacLatchy, 2001). In the particular case of pulp and paper effluents, breakdown products of lignin were implicated in the reduction of in vitro steroid production in mummichog (Hewitt et al., 2002). Such effects have also been linked to phytosterols (Leusch and MacLatchy, 2003) and were caused by exposure to steroids as well (Sharpe et al., 2004). Aside from those clear cut cases of androgenic effects such as mosquitofish masculinization, there has been little or no study to determine whether other reproductive effects are related to an androgenic mechanism.

The purpose of this study was to contrast the potential for pulp and paper and municipal effluents to act in an androgenic fashion at two levels of biological organization, in vivo, through masculinization of female mosquitofish, and in vitro, through steroid receptor binding and aromatase inhibition. We also sought to compare the mosquitofish masculinization endpoint to another endpoint commonly used in the study of reproductive impacts, gonadal steroid production. Chemical analysis was used to examine for specific androgenic steroids in order to determine if the bioassay-derived androgenic activity could be accounted for by those compounds. To determine if some of the steroidal compounds were likely candidates for causative agents of the androgenic effects of whole effluents, the waterborne dose–response of androstenedione, androstadienedione and methyl testosterone was examined. The mechanism of mosquitofish masculinization was further studied by determining whether masculinization could be elicited by an aromatase inhibitor, or whether the masculinization effect could be reduced or eliminated by and anti-androgen, indicating an androgen-receptor based mechanism. Collectively, these experiments were designed to increase our knowledge of the causes and mechanisms of chemically-induced reproductive dysfunction in fishes by sequentially determining: (1) what in vivo effects are occurring that may or may not be mediated through androgen receptors? (2) are those reproductive effects mechanistically related to one another? (3) what are the mechanisms leading to those impacts? and (4) what are the identities of the compounds involved?

2. Materials and methods

2.1. Description of mills and collection sites

The New Zealand pulp mill (hereafter referred to as PM1) from which effluent was sourced for this study was an integrated bleached kraft and thermo mechanical (TMP) pulp and paper mill that primarily used softwood (*Pinus radiata*) furnish. The bleaching process used sodium hypochlorite (HH) and/or 100% chlorine dioxide (DEopDnD or DeopPD). A moving bed biofilm reactor pre-treated the TMP wastewater before it is combined with the bleached kraft mill effluent. Secondary treatment consisted of a three-pond aerated stabilization basin with effluent retention time of 5–6 days. The effluent was discharged from the secondary treatment system into the Tarawera River at a mean volume of 180,000 m^3/day. The effluent dilution in the Tarawera River ranged between 5 and 12%. Tarawera River samples used for upstream reference (R1) were collected well upstream of the Tasman outfall and at least 500 m upstream from other discharge points of effluent.

The Canadian bleached kraft pulp mill (hereafter referred to as PM2) (2500 t/day) was also a combined bleached kraft/TMP mill. Mill furnish consisted of jackpine (*Pinus banksiana*; 60–70%) and spruce (*Picea*) species (30–40%). Chlorine dioxide (100% substitution) was used in the bleaching process in three sequential bleaching stages (DWEopDEopD). Effluents from the TMP and kraft processes were settled in primary clarifiers before they were combined and sent to an activated-sludge stirred-tank reactor. Retention time in the activated sludge reactor was approximately 3 h and typically had a mixed-liquor suspended solids value of 4500 mg/L. Reference water (R2)