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Masculinization and reproductive effects in western mosquitofish (*Gambusia affinis*) after long-term exposure to androstenedione

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ABSTRACT

Androstenedione (AED) is a naturally occurring steroid hormone. It is metabolized to potent androgens, which may induce androgenic effects in fish. However, little is known whether and how the androgens interfere with the fish gonadal development and reproduction. This study aimed at demonstrating the effects of long-term AED exposure on reproduction and development in mosquitofish (*Gambusia affinis*). The growth, development and several morphological endpoints, including the segment number and length of anal fin, histological changes of gonads and liver, were evaluated in mosquitofish during development from fertilized embryo to adulthood (180 days) after exposure of AED at environmentally relevant concentrations. We found that the growth (length, body weight and condition factor) of fish was negatively correlated with AED concentration in females, but not in males. The significant elongation of the ray and increment of segment numbers in the anal fin, were detected in all mosquitofish after exposure. Moreover, AED exposure (0.4gμ/L) caused damages in gonads and reduced the number of pregnant females. These findings indicate that AED has adverse effects on the growth and development of the western mosquitofish after long-term exposure (180d). Long-term exposure (180d) to AED, including environmentally relevant concentration (0.4 μg/L and 4 μg/L), induced masculinization in female mosquitofish under the experimental conditions.

1. Introduction

Environmental contamination by chemicals may interrupt the endocrine systems of wildlife (Corcoran et al., 2010; Fent et al., 2006; Kloas et al., 2009). As such evaluations are crucial, yet most investigations have focused predominantly on effects from antiestrogens and estrogens (Flammarion et al., 2000; Van Aerle et al., 2001). In contrast, substances and mechanisms that generate androgenic actions remain vastly under-investigated (Ankley et al., 2003; Morthorst et al., 2010).

Various steroidal androgens have been widely detected in aquatic environments receiving wastewater discharge from pulp and paper mills, domestic sewage treatment plants, and livestock farms (Jenkins et al., 2004; Larsson and Frlin, 2002; Liu et al., 2012a, 2012b, 2012c). These substances may cause adverse reproductive effects and developmental toxicity in fish at low concentrations (Ankley et al., 2001). Laboratory and field evaluations have documented masculinizing changes in female mosquitofish (*Gambusia holbrooki*) living in waters contaminated by pulp mills (Ellis et al., 2003; Howell et al., 1980). Androstadienedione (ADD) and androstenedione (AED) have been suggested as causative agents for this effect (Denton et al., 1985; Jenkins et al., 2001; Parks et al., 2001); however, other researchers have refuted some of these findings based on methodology (Durhan et al., 2002). A recent study has indicated that AED may have the potential to cause masculinization of mosquitofish following six-week exposure (Stanko et al., 2007).

AED has been detected in the effluent of seven Chinese wastewater treatment plants in Beijing at concentrations of up to 270 ng/L (Chang et al., 2011), in two different types of wastewater treatment plants in Guangdong Province at concentrations of up to 1368 ± 283 ng/L (Liu et al., 2012a, 2012b, 2012c), and in natural rivers of Japan at concentrations of up to 480 ng/L (Chang et al., 2008). However, little research has been done to address the androgenic effect of AED in fish using in vivo assays. Although a single endpoint assay in fish after a simple exposure was reported (Stanko et al., 2007), more comprehensive assessment of growth and development, including multiple

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endpoints in a full life cycle from fry to sexual maturation in fish exposed to AED, is still lacking.

Mosquitofish (*Gambusia affinis*) have been extensively used previously for many types of environmental studies (Hou et al., 2011). Typical female mosquitofish have a standard, rounded anal fin, but this can be altered into a gonopodium structure (analogous that employed by males during internal fertilization) when they are exposed to androgenic chemicals in paper mill waste (Howell et al., 1980).

In our previous study, we reported an abnormally high concentrations of AED in Dengcun River waters contaminated with pulp mill effluent and masculinization of female mosquitofish from the river (Hou et al., 2011). The present study aimed at determining whether AED could induce abnormal morphological responses and alter reproduction and development in western mosquitofish after a long-term exposure.

2. Materials and methods

2.1. Test species and experimental design

Adult mosquitofish were collected from upstream areas of the Liuxi River, which is sheltered from contamination and is the source of drinking water in Guangzhou (Xie et al., 2010). Pregnant females were incubated overnight in a pregnancy box and fry were collected the following day (designated as Day 0). Day 0 fry were placed in 5 L glass tanks and kept at 26 ± 1 °C with a 14/10 h light/dark cycle. Larvae were provided with a nutrient solution 2 times per day up to the 20 days post-fertilization (dpf), and then the larvae were fed with freshly hatched baby brine shrimp (Shenggong Co., Shanghai, China). The fish were daily fed with commercial red worm flakes (Haisheng Co., Shanghai, China).

Fifty selected fry were exposed to AED at sublethal concentrations of 0.04, 0.4, 4, 40, and 120 μg/L for 180 days. Each exposure level was replicated three times with 50 fish per replicate. Experiments were performed in a semi-static system at 25 ± 1 °C under a 14/10 h light/dark rotation. Treatment water in the tanks was renewed daily. Conductivity (152–167 μS cm-1), pH (6.87–7.56), and oxygen saturation (≥ 76%) were documented every 5 days to ensure water quality. Fish were placed randomly in 5 L glass tanks with 1–5 L of filtered and aerated tap water (1 L from 0 to 40 dpf; 5 L from 40 to 60 dpf). At 60 dpf, the fish were put in the 30 L glass tanks with 20 L of filtered and aerated tap water. For a solvent-only control group, we added 0.001% (v/v) ethanol to water without AED. After exposure for 180 days, sex ratio was identified based on an external evaluation of the urogenital sinus (Noggle, 2005). Gender was determined by urogenital papilla examination, since the males cling to the papilla while copulating using terminal differentiation of the gonopodium. Gravid females were established by their Mongolian spots. Feces and uneaten food were removed from the bottom of the tank each morning prior to feeding

2.2. Test compounds

AED of > 99.9% purity was obtained from Sigma-Aldrich (St. Louis, MO, USA). A stock solution was prepared by diluting it with analytical-grade ethanol. Fresh working solutions were prepared daily by diluting the stock solutions with MilliQ water to the final concentrations.

2.3. Endpoint measurements

2.3.1. Body size and fin morphology

At 180 dpf, 50 fish were collected and sacrificed with excess anesthesia (MS-222, Sigma A5040), and their body normal length (± 0.1 mm) and mass (± 0.1 mg) were examined. The length of anal fin ray 3 and the number of segments on the identical ray were determined using a calibrated dissecting microscope (Olympus SZH-ILLK American, Melville, NY, USA) (Fig. 1) Then the tissues, including testis, ovary and liver were dissected and the propagules in the ovary were tallied. Developmental phases of the propagules were staged as described previously (Haynes, 1995) and the assessment of testicular mass (± 0.1 mg) were established. The viability of sperm was determined following the described previously (Toft and Guillette, 2005).

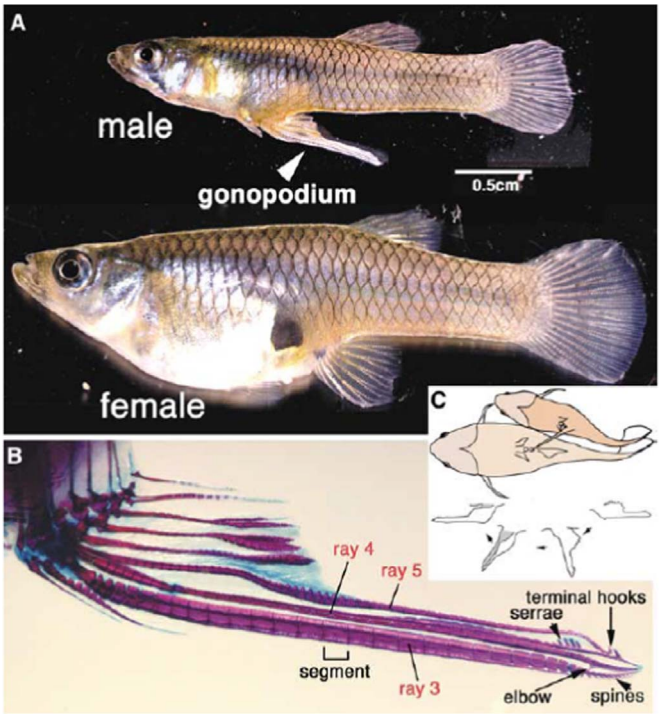

Fig. 1. Androgen-dependent sexual dimorphism in western mosquitofish (*Gambusia affinis*). (A) Mature male and female. (B) Bone staining of gonopodium (GP). The distal portion of the GP is composed of the 3rd, 4th, and 5th fin rays, and the distal tip is equipped with spines, serrae, an elbow, and hooks. The 3rd fin ray, as the axial center of rotation for the GP, is prominently thickened (33 mm TL, 1 year old, n = 4). (C) The GP swings forward for copulation, and sperm bundles (spermatozeugmata) are directly transported into the female urogenital sinus (Rosa-Molinar et al., 1996a, 1996b).

2.3.2. Histopathology

After recording their sex characteristics, we selected five adult males and five adult females from each glass tank for liver and gonadal histological evaluation. The gonads were dissected and fixed in Bouin's solution and prepared for histological sections by being embedded in paraffin. Nine longitudinal sections (2 μm) were cut from the ventral side of each fish stained with hematoxylin and eosin. Sections were photographed under a Leica DMRXE light microscope with an attached Leica DFC550 camera (Leica Microsystems, Switzerland). Image processing was carried out using Adobe Photoshop CS6 ver-sion 13.0 (Adobe Systems Inc.). We analyzed male mosquitofish for unusual connective tissue in the testes and female mosquitofish were examined for the development of sperm, regression of oocytes, and abnormal connective tissue in the ovaries.

2.4. Chemical analysis

Exposure water was renewed daily during the entire periods of exposure. Hence, the actual AED concentrations in the exposure water (500 mL) were only measured at 0 h and 24 h (just before water renewal) on the first day of exposure. The measurement of AED concentrations in the water samples followed the procedures reported in a previous study (Liu et al., 2011). Briefly, 500 mL of water was collected from each glass chamber. A method of solid phase extraction (SPE) was used to enrich the target compound in the water sample. This was loaded to a preconditioned CNWBOND LC-C18 SPE cartridge (200 mg