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Treatment of Fish with Hormones: Solubilization and Direct Administration of Steroids into Aquaria Water Using Acetone as a Carrier Solvent

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A variety of methods have been employed in attempts to administer steroid compounds to fishes. Such methods are summarized in a survey of the anabolic effects of steroid hormones in fishes by Donaldson et al. (1979). Perhaps the most commonly used methods involve the direct addition of steroids into the aquaria water or their incorporation into food sources. Such methods, however, are often inadequate because of their lack of precision with respect to solubility and even-dispersion throughout the aquaria water. Solubilization of steroids in oil-based carrier vehicles is also problematic to the researcher for the reasons stated above, and also because the oils tend to spread across the water surface. The latter effect is undesirable because such oil deposits often support fungal growth (personal observation). Some researchers have injected fish, either i.p. or i.m., with steroids using oil as a vehicle. This, of course, may cause considerable trauma to the fish and is time-consuming.

Ideally then, a method for delivery of steroids into aquaria water should ensure precise addition of small amounts of the compound, provide even and rapid dispersion of the compound throughout the aquaria water, cause minimal physical stress to the fish, and be relatively non-toxic to the fish. In our laboratory, we have achieved these criteria by using acetone to first solubilize steroids and then to serve as a carrier vehicle for their delivery into the aquaria water.

The purpose of this report is to illustrate the method, describe several experimental conditions in which it has been utilized, and show the acute lethality profile for acetone in a widely-used experimental aquatic model, the mosquitofish, Gambusia affinis.

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MATERIALS AND METHODS

All steroids used in this study were obtained from Sigma Chemical Co., St. Louis, Missouri. Reagent grade acetone was purchased from Sargent-Welch Chemical Co., Skokie, Illinois. In order to determine the solubilities of the steroids in acetone, 10 mg of each steroid were individually placed into 15-mL glass vials. Acetone was slowly added to each vial in 10 μL increments. The vials were then gently swirled after the addition of each acetone increment, until the sample was completely dissolved. The final concentration of each steroid was then calculated in mg per mL.

All fish used in this study were adult (32 - 34 mm, standard length), female mosquitofish, Gambusia affinis affinis, which were collected from several streams in Jefferson County, Alabama. Before experimental use, fish were allowed to acclimate to holding tank conditions for at least 1 wk. An additional 24-h period was allowed for acclimation to experimental conditions (dechlorinated water; pH = 7.4; temperature = 22-23°C; conductivity = 200 μOhms/cm; total alkalinity = 60 mg/L), prior to testing.

Stock solutions of the various steroids were prepared in acetone. Aliquots from these stock solutions were added directly to the aquaria water and thoroughly mixed using a glass stirring rod. For masculinization studies, 5-mL aliquots of androstenedione (ADD) in acetone were added to 12-L glass aquaria containing 10 fish each in 10 L of water. The final ADD concentration in each aquarium was 10 mg/L. Five mL of acetone were added to the water in a control tank in order to test for possible effects of the carrier solvent. The fish were examined daily using a dissecting scope to determine the stage of gonopodial development within the anal fin (see Howell and Denton, 1989, for a full description of this masculinization phenomenon).

For ADD LC_{50} determinations, ten female fish per treatment group were placed into glass beakers (2-L capacity) containing 2-L of water. Based upon preliminary studies, various concentrations of ADD, solubilized in 5 mL of acetone, were added to the beakers and thoroughly mixed with a glass stirring rod. Final concentrations of ADD used were 6-,8-,10-,12-,14-,18-, and 20-mg/L. Lethality in each beaker was recorded for up to 96 h, and dead fish were immediately removed to prevent fouling of the water. LC_{50} values and 95% CI were calculated using the method of Litchfield and Wilcoxon (1949).

For acetone LC_{50} determinations, female fish were placed

into 12-L aquaria containing 10-L of water (10 fish per aquaria). Acetone was added to the various aquaria in the following volumes: 80-,100-,120-,140-, and 160-mL. Aquaria were monitored, dead fish were removed, and LC_{50} values were determined as previously described.

RESULTS AND DISCUSSION

Table 1 represents the solubilities of three selected steroid compounds in acetone, as determined in our laboratory. As can be seen, significant amounts of methyltestosterone, androstenedione, and androstanol may be solubilized in acetone.

Table 1. Acetone Solubilities of Selected Steroids

Steroid	Approximate Solubility in Acetone (mg/mL)
Methyltestosterone	**76.9**
Androstenedione	**67.3**
Androstanol	**25.0**

Typically in our work, 2-L treatment beakers were readily prepared by first solubilizing the steroids in 1 mL of acetone. From these stock solutions, 0.1-mL aliquots were pipetted into the beakers of water. Initially, when the acetone-solubilized steroids were added to the water, a cloudy precipitant was seen. However, upon stirring with a glass rod, they rapidly cleared and easily went into solution. We have also prepared steroid treatment solutions for larger fish tanks (up to 10-L) by increasing the stock concentration of steroid and using larger volumes of acetone (i.e., 5 to 50 mL).

In 1980, Howell et al., reported that a population of female mosquitofish, Gambusia affinis holbrooki, living in a stream receiving effluent wastes from a papermill, was extensively masculinized. The masculinization involved a modification of the female anal fin into a gonopodium, a structure normally seen only in male mosquitofish. For a full description of the stages of gonopodial development, see Howell and Denton (1989). Other studies showed that phytosterols, which were present in pine-tree pulp, could be degraded by microorganisms into androgenic steroids (Charney and Herzog 1967; Denton et al. 1985; Howell and Denton 1989; Marsheck et al. 1972). Analytical work has shown that androstenedione is one of several metabolites found in

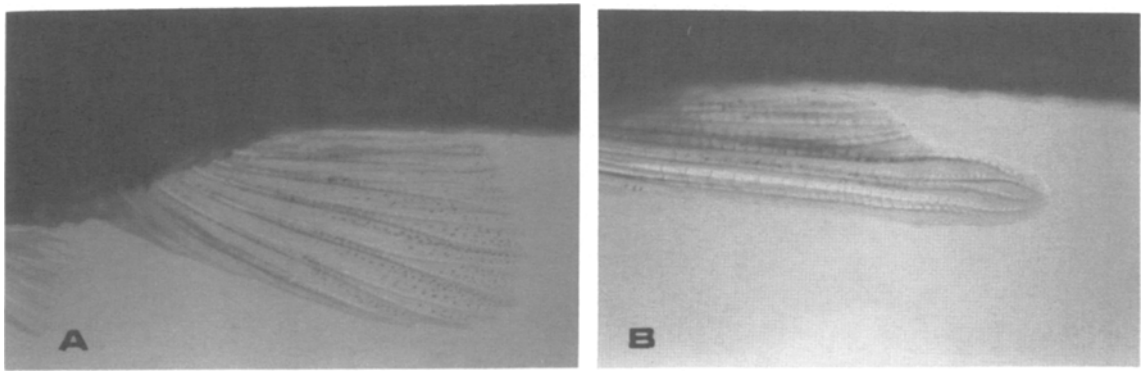

Figure 1. Anal fins in female mosquitofish: A - normal, unmodified fin; B - experimentally modified fin after treatment with androstenedione. This fin structure is similar to a gonopodium, normally seen only in the anal fin of male mosquitofish.

microbially-degraded papermill wastes (Conner et al. 1976). A normal, control female mosquitofish anal fin is seen in Figure 1-A. Figure 1-B shows the masculinized anal-fin of a female mosquitofish treated with acetone-solubilized androstenedione (10 mg/L). Since our acute lethality studies (see below) indicate that 10.5 mg/L is the 96-h LC_{50} for androstenedione, some fish died. However, in the surviving fish, consistent and uniform masculinization was seen within 2 wk. Based upon our present studies, we have seen masculinization effects with concentrations of androstenedione as low as 8 mg/L. We have also masculinized female mosquitofish using this acetone delivery procedure with androstanol, methyltestosterone, and spironolactone.

The acetone solubilization delivery method has allowed us to obtain an LD_{50} value for ADD of 10.5 mg/L (95% CI = 10.00 to 11.03 mg/L; single test). In control tanks containing 5 mL of acetone, but no ADD, there was no lethality.

A major consideration in any steroid delivery system is how well the fish can tolerate the solvent. We have found that volumes up to 100 mL acetone per 10 L aquaria have no acute lethal effect. The actual 96-h LC_{50} for acetone was determined to be 140 mL/10 L aquaria water (95% CI = 128.70 to 152.25 mL/10 L; single test). In practice, we have seen no reason to use over 5 mL acetone/10 L aquaria water. With these low volumes (i.e., nearly 30 times below the acetone LC_{50}), acetone causes no obvious stress to the fish , acutely, and we have observed no long-term adverse effects in fish that had received 4 wk of treatment in acetone-solubilized steroids. Except for the masculinization effects of the steroids on the anal fin, they appeared normal in all other respects and survived for observation periods as long as 8 mon. Based upon these observations, we believe that our delivery method would be ideal for use by other researchers studying the effects of similar compounds on aquatic organisms.

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