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Guppy Sexual Behavior as an Effect Biomarker of Estrogen Mimics

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There is widespread concern that some environmental chemicals can reduce the reproductive capability of humans and wildlife by mimicking natural estrogens and disrupting endocrine function. This potential threat to animal populations posed by xenoestrogens has, hardly surprisingly, been met by an intensive global effort to identify and develop biomarkers suitable for screening chemicals for estrogen mimicking capacity. Despite this effort, there are few biomarkers capable of linking exposure to xenoestrogens to impaired reproductive capability. The reproductive success of most animals depends strongly on the ability to perform the appropriate sexual behavior. The sexual display of the male guppy is strongly linked to reproductive success and is readily quantified under laboratory conditions. This preliminary study demonstrates that exposure of adult male guppies to water weakly contaminated with either natural estrogen (17β-estradiol) or the xenoestrogen (4-*tert*-octylphenol) causes a dramatic decrease in the rate and intensity of sexual display. It is concluded that quantitative analysis of the sexual display of male guppies holds great promise as a biomarker at the organismal level for the effects of estrogen mimicking xenobiotics. © 1999 Academic Press

Key Words: **xenoestrogen; guppy sexual behavior; biomarker; reproduction; Poecilia reticulata**.

INTRODUCTION

Recent findings that both natural and synthetic compounds present in the environment are capable of disrupting normal endocrine function have increased the concern for both human and animal populations. Of the chemicals exerting these types of effects, those mimicking the effect of natural estrogens are the focus of this concern. The most important natural estrogen in vertebrates is 17β-estradiol (Jobling and Sumpter, 1993). This steroid plays a central role in the hormonal pathways involved in the regulation and development of gametes, sexual phenotype, and sex-specific behavioral characteristics (Arcand-Hoy and Benson, 1998).

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A wide diversity of chemicals have been found capable of mimicking the action of 17β-estradiol including a number of pesticides (Chambers, 1984; Eroschenko, 1981; Johnson *et al*., 1992), phytoestrogens (Pelissero *et al*., 1989, 1991; Mellanen *et al*., 1996), and alkyl phenols (Mueller and Kim, 1978; Soto *et al*., 1991; Jobling and Sumpter, 1993; White *et al*., 1994; Sharpe *et al*., 1995; Jobling *et al*., 1996; Lech *et al*., 1996). As much as 60% of the 300,000 tons annual worldwide production of alkyl phenol ethoxylates (APEOs) has been estimated to end in surface waters and aquatic sediments (Ahel *et al*., 1987; Clark *et al*., 1992; Argese *et al*., 1994) after sewage treatment, as short chain alkyl phenol polyethoxylates, alkyl phenol carboxylic acids, or alkyl phenols (Giger *et al*., 1984, 1987). Alkyl phenolic compounds are both highly lipophilic and sorptive and have been reported to accumulate in animals (Ekelund *et al*., 1990; Ahel *et al*., 1993) and sediments (Ahel *et al*., 1994). Hence although routes of exposure to wildlife are diverse for these compounds, it has been suggested that the dangers are greatest for aquatic animals such as fish, piscivorus, birds, and mammals (Jobling *et al*., 1996), which are constantly exposed to low concentrations in the environment (White *et al*., 1994).

The potential threat to animal reproduction posed by the presence of xenoestrogens in the environment has, hardly surprisingly, been met by an intensive global effort to identify and develop biomarkers suitable for screening chemicals for their estrogen mimicking capacity. Of the few biomarkers capable of measuring estrogenic activity of xenoestrogens, most have involved *in vitro* studies at the receptor (e.g., Arnold *et al*., 1996, 1997) or cellular (e.g., Soto *et al*., 1997) level. At a slightly higher level of organization, *in vivo* vitellogenin production in male fish has been measured by a number of workers (e.g., Sumpter and Jobling, 1995). While these methodologies have proven highly sensitive to xenoestrogenic activity of chemicals and, particularly in the case of vitellogenesis, have been central in revealing the presence of these chemicals in the environment (e.g., Jobling and Sumpter, 1993; Harries *et al*., 1996), they do not provide evidence of effects on animal reproduction itself. The only known examples of potential biomarkers that directly

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indicate reduced reproductive capability, and hence effects at the population level, are those involving xenoestrogen-provoked changes in the gross morphology of male gonads (Jobling *et al*., 1996; Gimeno *et al*., 1996). Accordingly, there is clearly an urgent need for the development of biomarkers sensitive to xenoestrogens that have the capacity to directly expose deleterious effects on animal reproduction.

The reproductive success of most animals depends strongly on the ability to perform the appropriate sexual behavior. While the exact mechanisms and hormonal pathways for the hormonal control of sexual behavior are unclear, it is known that the sex steroids play an important role in the expression of sexual behavior in fish (Arcand-Hoy and Benson, 1998). The sexual display of the male guppy toward the female, known as sigmoid display, represents one of the most thoroughly studied examples of male sexual display in the entire animal kingdom [for a thorough review of guppy sexual behavior, see Houde (1997)]. There are a number of reasons for this. First, it is extremely easy to distinguish from the rest of the behavioral repertoire. Second, sigmoid displays occur up to once every 45 s and have a duration of several seconds. Finally, the guppy itself is extremely easy to keep in captivity, and performs these sigmoid displays readily in a laboratory setting. These characteristics make the guppy and its sexual behavior an ideal object of study with respect to the effects of environmental xenoestrogens.

The hypothesis in this preliminary study was that the sexual behavior of adult male guppies would be sensitive to the action of xenoestrogens and hence appropriate as a technique to screen for the effects of xenoestrogens on animal reproduction. To test this hypothesis, adult male guppies were exposed to water containing either the alkylphenol 4-*tert*-octylphenol (OP) or the natural estrogen 17β-estradiol (EE).

MATERIALS AND METHODS

Guppy Culture

A culture of wild-type guppies (*Poecilia reticulata*) was established at this laboratory with fish imported from Columbia. Due to the suspicion that xenoestrogens may leach from plastics and even plant material, the culture was established in two 500-liter stainless-steel tanks devoid of aquarium plants. The water was constantly filtered through a 5-cm layer of aquarium gravel resting on a raised floor of stainless-steel mesh. Water flow through this filter was achieved by welding two stainless-steel "chimneys" to the steel mesh and bubbling air into the middle of each of these chimneys. This caused a flow of water through the gravel layer and up the chimneys into the main water body in the tanks. Water temperature was maintained at $25 \pm 2^{\circ}$C. Fully grown male guppies were singly removed from the culture and placed in a 5-liter aquarium containing an adult nonvirgin female. The pair was allowed to acclimate to the aquarium for 5 min and then observed for a subsequent 10-min period. If the male made no sexual displays toward the female during this 10-min period, it was rejected. In this way 60 sexually active males were chosen for experimentation.

Exposure

The 60 sexually active males were randomly divided into three groups of 20. Each group was kept in a 30-liter cast glass aquarium (Carl Hecht Gmbh, Germany) containing 16 liters of water at 25°C and with a daily flow of 39 liters provided by a peristaltic pump (Ole Dich Instrument Makers APS, Hvidovre, Denmark) with separate channels for each aquarium. The water supply for these aquaria was obtained from a 150-liter stainless-steel header tank with a retention time of approximately 24 h during which it was constantly aerated. The input water to the header tank was a mixture of deionized water, produced by a reverse osmosis system without a deionizing column (Guldager A/S, Allerød, Denmark), and chlorine-free tap water in the ratio 5:1, resulting in a pH of 7.3 and a constant conductivity of 600 μS/cm. Further, 450 mg NaCl per liter of header tank water was added. In addition, each aquarium received a constant flow of 96% alcohol from a low-revolution peristaltic pump (Ole Dich Instrument Makers), giving a total of 5.1 ml per day. This alcohol was used as the carrier for both 4-*tert*-octylphenol and 17β-estradiol. An air stone produced turbulence so that the incoming alcohol was thoroughly mixed with the 16 liters of water. The three groups of 20 fish were exposed to either 4-*tert*-octylphenol (Sigma–Aldrich, Steinheim, Germany) at a nominal aquarium concentration of 150 μg/liter, 17β-estradiol (Sigma Chemical Co., St. Louis, MO) at a nominal concentration of 10 μg/liter, or to alcohol alone as control. All plumbing in the system was either stainless steel or glass, with the exception of the 20-cm silicone tubing in each of the peristaltic pump channels. After 4 weeks of exposure, the groups were moved to three fresh cast aquaria, each containing approximately 20 liters of water with a closed-circuit bioactive stone filter receiving approximately 20 liters of water per hour. After a further 10 days in these aquaria the sexual behavior of the male guppies was quantified.

Quantification of Male Sexual Display

For the quantification of male sexual display, two sand-blown 3-liter aquaria each containing 1.8 liters of 25°C water from the culture aquarium were placed on a sheet of glass 50 cm over diffusely lit white paper (100×100 cm). The two arenas were recorded from above using a B/W video camera (Bischke CCD 502, Neumünster, Germany) connected to a Panasonic AG5700 SVHS video recorder. The

entire setup was enclosed in a metal frame covered with a blackout curtain. This arrangement resulted in clear silhouettes of the two fish, which were easily distinguishable because of the size difference. A male fish was paired with a 4-month-old nonreceptive female in each of the two aquaria. Nonreceptive females were chosen to ensure constant female responses toward all males. The pairs was left undisturbed for 5 min after which the scenario was taped for 10 min. The resulting videotapes were subsequently analyzed for number and duration of male sexual display toward the female.

When performing sigmoid display, the male moves into the female's field of vision. His body assumes an arched "S" or "C" form, from which the name of the behavior is derived, and vibrates while he swims sideways. He either moves along the length of the female while facing her to come into position for a copulation, or he moves away from the female, remaining in her field of view to entice her to follow. This behavioral pattern, in particular the vibration, is so characteristic that determining the number and duration of sigmoid displays presents few problems for even inexperienced observers.

Quantification of Locomotor Behavior

Subsequently, the spontaneous locomotor behavior of the male guppies was quantified using the Gipstra (Image House Inc., Denmark) computer-automated video tracking system, described previously (Baatrup and Bayley, 1993; Bayley, 1995). The same experimental environment was used for these measurements as described above for quantification of sexual behavior, except that six arenas were monitored simultaneously and no female was present in any of the arenas. Again, male fish were transferred individually to test arenas, allowed to acclimate for 5 min, and tracked for a further 10 min. The Gipstra tracking system produces a list of x,y, time coordinates which are transformed by Motio software (Institute of Biological Sciences, University of Aarhus, Denmark) into vectors from which locomotor parameters are compiled. These include the path length of the animals, their mean velocity while moving, turning rate, and time spent in locomotor activity.

RESULTS

During the first week of exposure, the male guppies in all three treatments appeared territorial and were fairly evenly spaced in the aquaria. Further, they were observed to make occasional sigmoid displays toward each other. During the final week of exposure, these behaviors remained unchanged in the control aquarium receiving alcohol alone. In contrast, fish in both the EE and OP aquaria demonstrated a clearly reduced frequency of sigmoid displays, swam noticeably closer to each other, and often clustered together in a corner of the aquarium. In addition, while both the controls and the guppies receiving OP maintained their bright orange coloration, the color patterns in the group receiving EE were noticeably faded.

During the last week of exposure, five fish died in each of the two aquaria containing OP and EE and eight controls died. There was no further mortality during the 10 days following exposure in the fresh aquaria.

Quantification of the male sexual display indicated that both males exposed to EE and those exposed to OP were less sexually active than the controls (Table 1). Thus, while controls displayed on average once a minute during the 10-min analysis period, those exposed to OP performed less than half the number of sigmoids ($P < 0.05$) and those exposed to EE exhibited no displays at all ($P < 0.001$). In addition, the displays of males exposed to OP were on average less intense, lasting only 2.7 s in comparison to the 4.5-s average of controls. This difference was, however, not significant at the 5% level.

No significant differences were seen in the level of spontaneous locomotion in the three groups of males (Table 2). All three groups of fish swam between 14 and 15 m during the 10-min tracking period. The speed of movement of EE-exposed fish was nonsignificantly reduced in comparison to the other two groups, but these fish were active for a greater proportion of the available time. Also, no significant differences were found in the turning rates of the three groups of fish.

DISCUSSION

It is clear from the results that both the natural estrogen 17β-estradiol and the estrogen analogue 4-*tert*-octylphenol caused major disruption in the sexual display of the male guppy. A principal question is whether this response was purely a result of the action of these chemicals on estrogen

TABLE 1
Effects of 17β-Estradiol and 4-*tert*-Octylphenol on the Sexual Behavior of the Male Guppy

	Total duration of sigmoid display (s)	Number of sigmoid displays	Average duration of sigmoid displays (s)
Controls	44 (27)[a]	10 (7)[a]	4.5 (1.8)[a]
OP	19 (20)[a]	5 (6)[a]	2.7 (2.9)[b]
EE	0 (0)[a]	0 (0)[a]	—

Note. The sexual display of adult guppies toward nonreceptive adult females during a 10-min monitoring period was quantified. Values are means (SD). When comparing figures within a column, numbers followed by the same letter are significantly different using the Tukey test for honestly significant difference. $N = 12$ for controls; $N = 15$ for OP and EE groups.

TABLE 2
Spontaneous Locomotor Behavior in Exposed and Control Guppies

	Velocity (mm/s)	Turn rate (°/s)	Path length (m)	Activity time (s)
Controls	40.2 (11.2)	75.8 (14.2)	14.9 (5.2)	478 (66.5)
OP	42.3 (12.6)	78.2 (12.5)	15.0 (3.7)	475 (71.7)
EE	30.8 (10.0)	73.9 (14.5)	14.1 (5.6)	516 (57.6)

Note. The levels of spontaneous locomotion in male guppies in a 10-min interval were measured after the observation of sexual display. Values are means (SD). There was no significant difference between any of the groups for any parameter (Tukey test for honestly significant difference). $N = 12$ for controls; $N = 15$ for OP and EE groups.

receptors, or whether the observed effect was, at least in part, the result of a general toxic response. It is important to address this question in terms of this behavioral biomarker's potential for revealing the estrogen mimicking properties of xenobiotics.

There was some mortality in the fish exposed to both chemicals which might be attributable to the general toxic action of these chemicals. However, mortality was actually higher in the control group and can probably be attributed to an unfortunate growth of bacteria in all three aquaria during the last few days of exposure. Although there was no awareness of problems associated with the use of alcohol as a vehicle when designing the experiment, it has previously been reported to give rise to bacterial growth in experimental aquatic toxicological studies (Granmo *et al*., 1989). Accordingly, in subsequent experiments acetone was used instead.

Analysis of the guppies' spontaneous locomotor behavior did not reveal any differences between the groups, suggesting that the OP- and EE-exposed fish did not suffer from general toxic stress. Although animals often exhibit a considerable natural variance in locomotor activity and moving patterns, the three groups of guppies were remarkably similar in this respect. There is an extensive body of evidence that spontaneous swimming behavior is an extremely sensitive indicator of toxic stress. In a review of studies including a very wide range of xenobiotics, Little and Finger (1990) found that swimming behavior was a more sensitive measure of toxic stress than either lethality or growth. The development of swimming behavior as a general biomarker of toxic stress has now reached the stage where it has been included in the American test battery for aquatic toxicology (ASTM, 1994, 1995). In conclusion, there was no evidence of general toxic effects on the exposed fish in the present study.

The sexual display of male guppies has previously been suggested as an indicator of the action of general toxic stress (Schröder and Peters, 1988a, b). These studies were performed before there was any widespread awareness of the presence of hormone analogues in the environment, and involved the exposure of guppies to either low concentrations of lindane (1,2,3,4,5,6-hexachlorocyclohexane) or water contaminated with effluent from a sewage treatment works in Munich, Germany. Lindane has been found to interfere with estrogen pathways in both domestic ducks (Chakravarty and Lahiri, 1986) and rats (Lahiri *et al*., 1985; Cooper *et al*., 1989) and to induce the production of vitellogenin in the rainbow trout (Flouriot *et al*., 1995). The presence of estrogen analogues in water leaving municipal sewage plants has been repeatedly demonstrated through the induction of vitellogenin in fish collected or kept near municipal sewage works (Jobling and Sumpter, 1993; Sumpter, 1995; Sumpter and Jobling, 1995; Harries *et al*., 1996; Folmar *et al*., 1996; Lye *et al*., 1997), and this problem lies at the very core of the present concern. In hindsight, it therefore seems more likely that the reductions in male guppy sexual display observed by Schröder and Peters (1988a,b) were in fact a result of the estrogen mimicking properties of lindane and of chemicals present in the sewage effluent.

It is also important to consider whether the observed inhibition of male sexual display can be interpreted as *de facto* reductions in the animals' Darwinian fitness and, therefore, as an indicator of population health. The male guppy has two strategies for transferring sperm to females. The first involves stimulating the female to willingly perform sexual intercourse through the use of the sexual display quantified in this study. The second strategy, termed sneaky copulations, involves a rapid approach to the female from behind, followed by an attempt at insemination. However, it has been demonstrated that the proportion of sneaky copulation attempts resulting in successful insemination is small (Clark and Aronson, 1951) and according to Houde (1997), most successful inseminations are preceded by sigmoid display. Further, there is considerable evidence that performance of sigmoid display increases the risk of predation (see Houde, 1997, for a review). The trade-off involved in performing this display at the risk of predation must therefore involve an increase in fitness. Matthews *et al*. (1997) recently reported that the intensity of sexual display performed by a male guppy is correlated to sperm quality, hence providing further evidence of the direct links between the intensity of a male guppy"s sexual display and its Darwinian fitness.

The nominal concentrations of chemicals used in this preliminary study were high when compared with the concentrations producing a vitellogenin response (Jobling *et al*. 1996). Vitellogenin induction in trout has been demonstrated at concentrations above 4.8 μg OP/liter water and inhibition of testicular growth at about 38 μg OP/liter water (Jobling *et al*., 1996). No attempt to measure the actual concentrations of either OP or EE in the water column were made in the present study. However, in an almost identical experiment with the same nominal concentration of OP

(150 μg/liter) set up by collaborative partners at the University of Odense, Denmark, analysis of water samples indicated that the actual concentration of OP in the water column was only 42 μg OP/liter water (K. Pedersen, University of Odense, personal communication). With this in mind the changes in sexual display measured in the present study are of the same order of sensitivity as the inhibition of testicular growth and may be able to approach the sensitivity of vitellogenin in the future using a greater number of replicates. Further, the sensitivity of testicular growth, measured by Jobling *et al.* (1996), was found only when exposing maturing male trout. When mature or regressing trout were exposed later in the season, this inhibition of testicular growth was absent. The behavioral biomarker presented in the present study is independent of season and the study was performed on fully matured animals. It is quite likely that sensitivity could be greatly increased if male guppies were exposed as juveniles. These studies are presently in progress.

CONCLUSION

The use of quantitative analysis of the sexual display of male guppies has great promise as a screening biomarker for the estrogen mimicking properties of xenobiotics. There is little doubt that a response in this biomarker is intimately linked to changes in the fitness of the animal and therefore can be used to predict effects at the population level. This preliminary study poses a number of questions requiring further experimentation. These include dose–response studies with this biomarker; studies to determine whether alterations in this biomarker induced by estrogen mimics are associated with changes in the sperm quality of the male, and experiments to determine whether responses in this biomarker are specific to exposure to estrogen mimicking xenobiotics. Finally, a computerized video system is currently being developed that is capable of automatically quantifying the sexual behavior of guppies.

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