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Impact of benzyl butyl phthalate on shoaling behavior in *Fundulus heteroclitus* (mummichog) populations

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ABSTRACT

Fundulus heteroclitus preference for association with familiar conspecifics of similar body length was impacted by benzyl butyl phthalate (BBP); this was found to be a statically significant result with a $p < 0.0001$. When presented with equally sized shoals consisting of either large or small fish, the majority of unexposed (84%) and acetone exposed control (82%) fish selected the shoal of large fish. A small number of control fish chose either the shoal of small fish (6% and 10%) or the neutral zone (10% and 8%) where they were clear morphological outliers. Fish exposed to 0.1 mg/L BBP exposure daily for four weeks selected the shoal of small fish more often than unexposed or acetone controls (7.5- and 4.5-fold respectively). They also remained in the neutral zone and displayed agitation at levels more than twice that of control. Agitation and shoal choice disruption are quantifiable behavioral responses that support the use of *F. heteroclitus* as a model for detecting sub-lethal BBP exposure.

1. Introduction

Environmental pollutants pose substantial risk to aquatic organisms, and accordingly, a great deal of research has been focused on characterizing the physiological mechanisms of toxicity in animals exposed to a variety of inorganic and organic contaminants (Scott and Sloman, 2004). Since behavior serves as a link between physiological and ecological processes, there has been increasing interest, both at the scientific and regulatory levels, in the use of animal behavior models for evaluation of the environmental impact of pollutants (Kulig et al., 1996; Scott and Sloman, 2004).

Fish provide an excellent model system for ecologically relevant monitoring of environmental contamination in aquatic systems. Interactions associated with predator avoidance and social behaviors, for example, form an important part of a successful, adaptive life history strategy for many fish species. Aggregation, which is one specific grouping behavior that is exhibited by many fish species (Dugatkin and Wilson, 1993), may manifest as schooling or shoaling. These behaviors lack the uniformity of orientation and polarized movement present in schooling (Hoare et al., 2000). Field studies demonstrate that the composition of free-ranging fish shoals is largely consistent with laboratory predictions (Krause and Ruxton, 2002; Peuhkuri et al., 1997). Fish shoals are strongly assorted by body length where individuals prefer to associate with familiar conspecifics (Ranta et al., 1992; Svensson et al., 2000). Shoaling behavior, which are fish staying in a group for social reasons where the structure of the group is of secondary importance (Krause and Ruxton, 2002), appears to provide an anti-predator function (Pitcher and Parrish, 1993). The increased homogeneity in terms of individual fish size, often found in shoals, is considered an efficient anti-predator adaptation (Peuhkuri, 1997; Peuhkuri et al., 1997; Ranta et al., 1992). Shoaling may also increase foraging success (Pitcher and Parrish, 1993).

Individual fish of certain species show a preference for forming shoals of familiar individuals that suggests development of familiarity among the group members may be beneficial to the

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individual fish. In many fish species (as well as other animal species), aggregation can be a fairly rapid response to simultaneously experienced fright stimuli caused by an approaching predator (Tegeder and Krause, 1995). Size-assortative shoaling among sticklebacks (*Gasterosteus aculeatus*) populations theoretically serves as a mechanism to reduce predation risk resulting from the oddity effect (being an outlier) or minimize competition for food (Peuhkuri et al., 1997). Studies with European minnows (*Phoxinus phoximus*) suggest that the benefits associated with selection of familiar shoal mates are equivalent to those associated with doubling shoal size (Barber and Wright, 2001).

Toxic effects of aquatic contaminants have been shown to impair survival by inducing inappropriate behavioral responses to environmental and/or physiological stimuli (Kane et al., 2005; Weber and Spieler, 1994). Since many contaminants disrupt normal fish behavior at much lower doses than those causing mortality, behavioral indicators may be ideal for assessing sub-lethal exposure to pollutants. In fact, shoaling behavior of fish has been used to a moderate extent as a bioindicator of environmental pollution (O'Connor et al., 2000; Vogl et al., 1999; Weis and Weis, 1974; Wibe et al., 2002).

Benzyl butyl phthalate (BBP) is a phthalic acid ester used to make plastic products such as toys, packaging materials, and vinyl flooring more pliable and durable (Wibe et al., 2002). Since phthalate esters are not chemically bonded within the polymer matrix, they may migrate from the plastic composite into the environment (Adams et al., 1995). Phthalates have been detected in all aspects of the environmental: water, air, sediment, biota, marine, and freshwater ecosystems (Fatoki and Vernon, 1990; Giam et al., 1978). Many BBP metabolites (mono butyl phthalate (MBuP), mono benzyl phthalate (MBeP), hippuric acid, phthalic acid, benzoic acid, and a ω-oxidized metabolite) are proposed toxins (Ema et al., 1995; Nativelle et al., 1999; Parkerton and Konkel, 2000). Produced commercially in large quantities, phthalate esters are also thought to be members of the estrogenic group of endocrine disruptors (Jobling et al., 1995; Sonneschein et al., 1995; Soto et al., 1995). Thus, the environmental fate and toxicology of phthalates are of great interest (Wibe et al., 2004).

Fundulus heteroclitus, aeuryhaline, estuarine fish species found along the Atlantic coast from Nova Scotia to Florida (Abraham, 1985; Smith, 1985), is a good choice as in indicator of estuarine contamination because it is easily collected and transported, tolerant of captivity, ecologically relevant, ubiquitous in the nearshore environment, and responsive to environmental perturbation. *F. heteroclitus* feed on a variety of invertebrates and small fishes and grow at a rate of 35–50 mm per year which makes approximate age determination relatively simple. Unlike some fish models, *F. heteroclitus* exhibit a restricted home range of 30–40 m. Large populations are regularly found from late spring to early fall near tidal marshes at the mouths of all watercourses in Connecticut that empty into the Long Island Sound Estuary (Whitworth, 1996). *Fundulus* species do exhibit social groupings (Kavaliers, 1980) with a size dependent sorting mechanism (Blakeslee et al., 2009). *F. heteroclitus* not only demonstrates schooling and shoaling (Hoare et al., 2000), but also disruption of the behavior (to the point where fish avoid conspecifics) in response to low-level exposure to certain chemicals such as 4-Nonylphenol (Ward et al., 2008).

Since low-level environmental contamination may disrupt shoaling and negatively impact both fitness and transmission of social information (Reader et al., 2003), the focus of this study was to examine the influence of BBP on shoaling behavior in *F. heteroclitus* to determine if the model (fish and behavior) can be utilized as a bioindicator of sub-lethal BBP exposure.

2. Materials and methods

2.1. Fish collection and maintenance

F. heteroclitus were collected in early September at Milford Point Estuary, Long Island Sound using minnow traps. The fish were transported back to the laboratory in ambient water (16 ppt salinity). The fish were separated by size (large and small fish). Large *F. heteroclitus* were divided into four groups: fish for the shoal of large fish (SLF), untreated control focal fish (UC), acetone treated control focal fish (AC), and BBP exposed focal fish (BBP). Small *F. heteroclitus* were housed separately from large fish, and used to generate the shoal of small fish (SSF).

Fish were distributed by group and housed in 110 L glass aquaria with gravel and air supplementation (30–45 fish per aquarium); all fish were depurated for eight days. Throughout depuration and experimentation, fish were fed daily with Tetramin® Fish Flake Food. Food that remained in the tank after a 10-min period was removed. Salinity (16 ppt), temperature (20 °C), and photoperiod were consistent with the environmental conditions for both season and the field collection site.

2.2. Exposure protocol

Exposure of all focal fish groups (UC, AC, and BBP exposed) began after an eight-day depuration period. No water changes were made during the exposure period. All aquaria were dosed daily on six consecutive days per week for a total of four weeks. AC fish ($N = 47$) were dosed daily with 0.1 mg/L acetone, and UC fish ($N = 30$) were dosed daily with an equivalent volume of 16 ppt Instant Ocean® water. A BBP stock solution (10 g/L) was prepared using benzyl butyl phthalate (CAS-85-68-7, Sigma–Aldrich) and acetone as the solvent. Stock solution was added daily to the aquaria water housing the BBP exposed focal fish ($N = 53$) to produce an approximate dosage of 0.1 mg/L BBP.

2.3. Chemical sampling protocol

Water from the aquaria of all focal fish groups (UC, AC, and BBP exposed fish) was tested for the presence of BBP at various time intervals over the four-week dosing period. Immediately after dosing, the aquarium water was gently stirred with a fish net for approximately 15 s. Water samples were collected at various time intervals, ranging from 5 min to 3 h by dipping 60 mL Qorpak amber glass bottles (Fischer Scientific, # 033208B) to 10 cm below the water surface. After collection, the bottles were kept at 4 °C until analysis.

2.4. Chemical analysis

Analyses of all samples were performed using an Agilent Technologies Model 6850 Gas Chromatograph attached to an Agilent 5973N Mass Spectrometric Detector (GC–MSD). An Agilent Technologies HP-5MS, 20 m × 0.25 μm capillary column equipped with a Merlin High Pressure Septum (Supelco Co., 24816-U) was used for analyte separation. Experimental conditions were as follows: initial temperature of 110 °C with a ramp of 5 °C/min, to 250 °C, and a total run time of 40 min. Extraction of all water samples was performed using a polydimethylsiloxane (PDMS-DVB) divinylbenzene fiber (65 μm) with a fused silica/ss core (Supelco Inc., 57346-U) solid phase microextraction (SPME) assembly (Supelco Inc., 57330U) that was capable of specific adsorption of BBP in the water. Prior to sample extraction and analysis, the SPME was conditioned by placing the SPME in the GC injection port of the GC–MSD and the 40-min sample program was run.