



Effects of Turbidity and Visual vs. Chemical Cues on Anti-Predator Response in the Endangered Fountain Darter (*Etheostoma fonticola*)

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Abstract

Altered turbidity resulting from anthropogenic stressors is a global problem. Threatened by climate change, pollution, and increased recreational usage, the streams and rivers of central Texas are no exception. The impacts of turbidity include behavioral effects as turbidity degrades visual information, which can impair an animal's ability to accurately detect and respond to a predator. Here, we tested the impact of simulated turbidity on anti-predator response in the endangered fountain darter, *Etheostoma fonticola*. We examined the response of *E. fonticola* to four predator cue treatments (chemical, visual, chemical and visual, and no cues) using a native predator, the green sunfish (*Lepomis cyanellus*). All cue treatments were tested across two vision levels: clear and impaired, to simulate the visual effects of low turbidity (~30 NTU). Our results indicate that *E. fonticola* requires a combination of visual and chemical stimuli to respond to a native fish predator. In the absence of one or the other sensory modality, *E. fonticola* did not show an anti-predator response. Also, anti-predator response to a combination of visual and chemical stimuli was only present at the clear vision level. When vision was impaired owing to simulated turbidity, a combination of visual and chemical stimuli did not produce a significant anti-predator response. These results indicate that blocked or compromised vision hampers anti-predator response in *E. fonticola*, which may be of concern regarding the future management of this endangered species.

Introduction

Turbidity affects behavior primarily through clouding visual signals, which can make accurate signal interpretation difficult (Endler 1993). Aquatic prey animals can use a number of signal modalities for predator detection, with visual and chemical being two of the most common and well studied (Kats & Dill 1998; Collin & Whitehead 2004; Preisser et al. 2005). Visual and chemical signals can be used alone or in combination to distinguish a predator from a non-predator and to gauge an appropriate response (Ferrari et al. 2010). Although not a direct form of communication between a signaler and receiver, 'spying' by detecting and responding adaptively to signals originating from a predator may aid a prey animal in

avoiding capture (Wisenden & Stacey 2004). The specific modality used and the efficacy of single vs. multi-modal cues can vary based on the situation and environment, with multi-modal cues such as the combination of visual and chemical often providing greater interpretation accuracy (Johnstone 1996; Ward & Mehner 2010).

In addition to improving interpretation accuracy, multi-modal cues can also function as back-up signals, compensating for primary signals impaired because of environmental conditions such as turbidity (Endler 1993). If vision is impaired, then chemical back-up signals used for sensory compensation often become increasingly important (Hartman & Abrahams 2000; Leahy et al. 2011). Yet turbidity can affect anti-predator behavior in sometimes contrasting ways. Turbidity

decreases the ability of fish to generalize predator recognition (Ferrari et al. 2010), increases high-risk behavior (Miner & Stein 1996) and weakens the intensity of anti-predator response, thus increasing the risk of predation (Gregory 1993). Conversely, turbidity also increases anti-predator response through sensory compensation in the form of heightened reliance on chemical signals (Hartman & Abrahams 2000; Leahy et al. 2011). Although increased anti-predator response in turbid conditions may decrease the risk of capture and consumption, it also results in trade-offs between time allocated for other beneficial behaviors such as foraging and mating (Sih 1992), which may also be compromised in turbid conditions.

Many freshwater fish use a combination of visual and chemical signals in predator avoidance (Chivers & Smith 1998; Wisenden et al. 2003; Holmes & McCormick 2011). Aquatic prey species can use many forms of chemical information with regard to predator detection, including kairomones or chemicals emitted by a species that can be detected by another species (Kats & Dill 1998; Mathis et al. 2003), diet cues based on recent foraging activity (Chivers & Mirza 2001), or alarm cues released from an injured conspecific (Gibson & Mathis 2006; Crane et al. 2009). Kairomones alone can convey enough information for a prey animal to distinguish between a predator and a non-predator (Mathis 2003). An important organism for examining the sensory modality being used for predator recognition and the impact of turbidity is the US federally endangered fountain darter, *Etheostoma fonticola* (USFWS 1996).

Study System

Headwater streams contribute significant biodiversity to river networks, often supporting a large number of endemic or endangered species and thus are particularly vulnerable to anthropogenic impacts (Meyer et al. 2007). The waters of the San Marcos and Comal rivers of central Texas, Hays and Comal CO, are no exception. These rivers are home to many endemic flora and fauna including seven threatened and endangered species such as *E. fonticola* (U.S. Fish & Wildlife Service 1996). They are also part of the Edwards Aquifer system, the source of water for over two million users and one of the most prolific artesian aquifers in the world. Turbidity levels in this system are traditionally low, ranging from 0.26 to 5.76 NTU at the headwaters up to 18 NTU downriver (Saunders et al. 2001), where 20 NTU is considered a threshold for low turbidity streams (USEPA 1999). However, natural conditions such as storm events as well as

anthropogenic impacts can cause these levels to rise substantially.

Current anthropogenic threats affecting these waters include pollution, runoff, recreational use, introduced species, and climate change, which are predicted to exacerbate water shortages in central Texas (Loaiciga et al. 2000). Water shortages owing to increased withdrawal are expected to especially impact Texas rivers and streams in the near future. The Edwards Aquifer is the primary source of drinking water for the city of San Antonio, Texas, the seventh largest city in the United States. Increasing water consumption coupled with a rapidly growing population and predicted water shortages because of drought events seriously threatens the maintenance of adequate water levels and flow in rivers sourcing from the Edwards Aquifer. Although occupying broad habitat types within their limited range, *E. fonticola* occurs primarily at the spring-fed headwaters of these rivers and is associated with vegetated stream floor habitats with relatively constant water temperature and moderate flow. As such, this species is particularly threatened by reduction of spring flow (Bonner & McDonald 2005). Such reduction of flow is associated with increased turbidity levels as are other threats to this system including recreation and runoff. Increased recreation in the form of swimming, kayaking, and tubing may be a major source of increased turbidity in the shallow waters of the San Marcos and Comal rivers during the summer months. Turbidity levels in 2010 were found to be significantly elevated in the Comal river during periods of heavy recreational use compared with periods of light recreational use (Araujo 2012). We are not aware of any previous behavioral studies of *E. fonticola*, and as many endemic fauna are exposed to similar threats, this organism may be an important model for understanding predator avoidance behavior and the effects of turbidity in this and similar freshwater systems.

Here, we investigated the effects of turbidity simulated by decreased visibility on predator avoidance in *E. fonticola*. Specifically, we tested the importance of visual cues vs. predator kairomones in the laboratory, while also examining the effects of reduced visibility on predator avoidance behavior. We tested the hypothesis that *E. fonticola* uses visual cues to detect and respond to fish predators and that simulated turbidity would decrease anti-predator response. An alternative hypothesis predicts that simulated turbidity would increase anti-predator response in *E. fonticola* when exposed to chemical cues owing to sensory compensation.

Methods

Trials were conducted at the San Marcos National Fish Hatchery and Technology Center (USFWS) in San Marcos, Texas, from Jan. to May 2011. Predator naïve first-generation, hatchery-reared adult *Etheostoma fonticola* were used for all trials. Prior to testing, individuals were housed in holding tanks with recirculating well water (23°C). Test fish were fed a diet of black worms (*Lumbriculus variegatus*), amphipods, and zooplankton once daily.

We examined the response of 120 adult *E. fonticola* in four predator cue treatments across two vision level treatments [(1) clear and (2) impaired to simulate the visual effects of turbidity]. We tested the following predator cue treatments: (1) chemical cues only ($n = 15 \times 2$ vision levels), (2) visual cues only ($n = 15 \times 2$ vision levels), (3) visual and chemical cues ($n = 15 \times 2$ vision levels), and (4) no predator cues (water control) ($n = 15 \times 2$ vision levels). We used a native predator, the green sunfish (*Lepomis cyanellus*), for all predator cue treatments. Green sunfish are opportunistic foragers (Hodgson & Kitchell 1987) that occur sympatrically with fountain darters. We acquired six adult green sunfish of similar size from hatchery stock ponds.

Our experimental set-up consisted of four sets of adjacent, 9.5 l drip-flow tanks (Fig. 1). The adjacent tank design allowed for separate or combined exposure to visual and chemical stimuli. We affixed glass tinting allowing 70% visible light transmission (WindowTint.com film) to two of the testing tanks on the side allowing exposure to the adjacent tank to simulate turbidity following methods of Sundin et al. (2010). Testing tanks included a fine gravel substrate and were wrapped in black plastic with the exception of the front-facing side and the side allowing visual exposure to the adjacent tank, in order to minimize disturbance to the fish. We covered the front-facing side with one-way tinting to further minimize distur-



Fig. 1: Experimental set-up. The tank housing the test fish (*Etheostoma fonticola*) is shown with syringes and airline tubing for chemical stimulus introduction (right) and the tank housing the predator species for treatments involving visual cues (left).

bance while still allowing for visibility during trials. We randomized the location of specific tanks and used hydrogen peroxide and water to clean tanks between trials. We conducted all trials between 1000 h and 1500 h.

To initiate testing, we habituated the subject fish in the test tank for ≥ 24 h. Subsequent to habituation, we measured fish movement for 10 min (pre-stimulus). We quantified movement in terms of the number of movements (darts). We then subjected the test fish to a randomly selected predator cue treatment and measured movement for another 10 min (post-stimulus). We calculated the response variable as the difference in the number post-stimulus and pre-stimulus movements so that a negative final movement score indicated reduced movement (freezing), while a positive score indicated increased movement. Reduced activity is a very common anti-predator response in aquatic prey (Skelly 1994; Vilhunen & Hirvonen 2003) including darters (Crane et al. 2009). After testing, we removed each fish and recorded the standard length (SL) and sex.

To test the response to chemical cues only (treatment 1), we placed an opaque divider between the two adjacent tanks and then removed it for the second 10-min stimulus exposure period, exposing the test fish to an empty predator tank in order to replicate the procedure followed for exposing test fish to visual cues. We next injected 50 ml of chemical stimulus material with a 60-ml syringe attached to airline tubing. The tubing was attached to the back of the tank using a suction cup, and we injected water approx. 10 cm below the surface of the water at a rate of roughly 2 ml per second. We flushed the tubing with an additional 50 ml of well water introduced at the same rate, following the methods of Epp & Gabor (2008). To test the response to visual cues only (treatment 2), we placed an individual green sunfish in the adjacent 9.5-l tank. We then removed an opaque divider prior to the post-stimulus exposure period, allowing the test fish visual access to the adjacent tank containing the predator. We randomly selected the green sunfish from the six available before each trial. We injected blank well water rather than chemical stimulus material. To test the response to visual and chemical cues (treatment 3), during the post-stimulus exposure period, we allowed visual access to an adjacent tank containing a green sunfish and injected 50 ml of chemical stimulus material flushed with 50 ml of well water following cue injection. Finally, to test the response to no predator cues (control) (treatment 4), we allowed visual access to an empty adjacent tank for the second 10 min stimulus

exposure period and injected 50 ml of blank well water into the testing tank.

We simulated turbid water in the testing tank via glass tinting following Sundin et al. (2010). Turbidity has traditionally been experimentally manipulated using some type of bentonite. However, such materials may produce odors that interfere with chemical cues or toxins that impact behavior (Engström-Öst & Candolin 2007) and the use of simulated turbidity controls for these effects. The semi-transparent tinting between tanks allowed 70% of visible light to be transmitted (70% VLT). The semi-transparent tinting was approximated to NTU by comparing light absorbency with that of known NTU standards using a spectrometer. Across the visible light spectrum (400–790 THz), the tinting corresponded with a turbidity range of 15–45 NTU. The clear vision tanks with no tinting affixed permitted for close to 100% VLT, approximately equivalent to zero NTU.

We acquired the chemical stimulus material of two *L. cyanellus* individuals that were maintained in aquaria and fed a neutral diet of earthworms for 6 d to eliminate the potential effects of diet cues. We then determined the volume of each stimulus animal by displacement and placed each individual in a separate 75.7 l tank with an amount of aerated well water equal to approx. 230 ml per 1 ml of stimulus animal. After 24 h, we removed animals from the aquaria and combined and stirred equal proportions of water from each tank following standard protocols (Ferrari et al. 2007; Epp & Gabor 2008; Brown et al. 2011). We froze the stimulus material in quantities of 75 ml to be thawed immediately prior to testing.

We analyzed anti-predator response measured by the number of movements using a two-factor ANOVA as the data met the assumptions of parametric analyses. We conducted *post hoc* comparisons between treatment groups using Tukey's honest significant difference (HSD) test. All analyses were carried out using JMP 9 software (SAS Institute Inc., Cary, NC, USA).

Results

To test the effects of visual vs. chemical cues and simulated turbidity on anti-predator response, we used a two-factor ANOVA and found a significant difference across predator cue treatments ($F_{7,109} = 3.39$, $p = 0.02$) but not vision level ($F_{7,109} = 1.82$, $p = 0.18$) or an interaction between cue treatment and vision level ($F_{7,109} = 1.55$, $p = 0.20$).

Post hoc comparisons between all predator cue treatments revealed only one significant difference between the control and the visual plus chemical

combination cue treatment (Tukey's HSD, $p = 0.02$). When *post hoc* analyses were separated by vision level, this difference was only present in the clear vision (Fig. 2a, b). When included as a factor in the model, there was no affect of sex on movement score.

Discussion

Our data indicate that a combination of visual and chemical stimuli is necessary to fountain darters for predator detection. Only the visual plus chemical treatment (treatment 3) differed from the control. The lack of anti-predator response (freezing) when only one signal (visual or chemical) was presented indicates that *E. fonticola* is unable to increase reliance on another modality when one signal is lost. This result is consistent with previous work documenting the importance of multi-modal cues in interpretation accuracy (Johnstone 1996; Ward & Mehner 2010). For instance, freshwater fish have been shown to perceive visual signals faster than chemical signals but rely on chemical signals for more detailed information

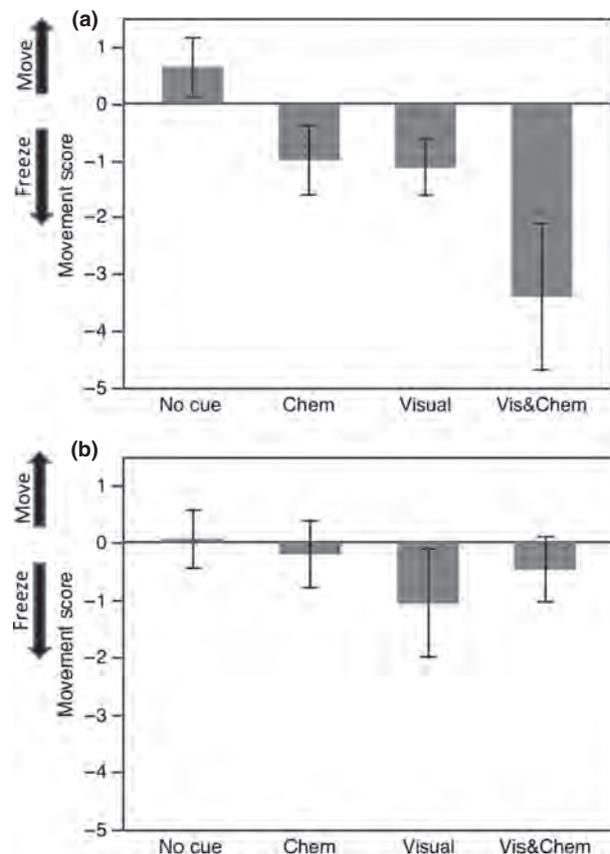


Fig. 2: Mean \pm 1 SE movement score for (a) clear vision and (b) impaired vision (~30 NTU).

(Endler 1993). Our finding is significant in that it suggests that *E. fonticola* may not be able to adjust signal usage to rely exclusively on chemical cues when vision is significantly impaired because of turbidity. This has significant conservation implications for an endangered species living in a habitat highly subject to varying turbidity levels because of recreation, flood or drought events.

Although we did not detect a significant interaction between predator cue treatment and vision level in our analysis, only fish at the clear vision level demonstrated a stronger anti-predator response when exposed to visual and chemical cues. At the impaired vision level, anti-predator response in fish exposed to visual and chemical cues did not differ from the control. These results indicate that *E. fonticola* requires both visual and chemical cues to respond to a fish predator. When there were no visual cues or vision was impaired because of simulated turbidity, *E. fonticola* was not able to respond to a predator using chemical stimuli alone. One hypothesis for the absence of a significant interaction between cue treatment and vision level is that the simulated turbidity level was too low. The tint used to simulate turbidity allowed for 70% VLT and was found to be comparable with a turbidity range of 15–45 NTU (Saunders et al. 2001). This level was chosen to maintain consistency with turbidity levels naturally occurring in *E. fonticola* habitat where turbidity downstream from the headwaters can exceed 18 NTU in normal conditions. However, a darker film (i.e., greater NTU) may have produced a significant effect. Such an increase may still have been biologically relevant as turbidity levels can far exceed normal ranges during heavy storm events and high recreational use. A follow-up study examining the specific level of impairment required to produce a significant interaction would be of interest.

The use of chemical and visual cues in anti-predator response has not been tested previously in *E. fonticola*. However, response to kairomones (Gibson & Mathis 2006), disturbance pheromones (Wisenden et al. 1995) and alarm cues (Commens-Carson & Mathis 2007) has been documented in other species of *Etheostoma*. It is possible that using another form of chemical signal such as alarm cues would have produced a stronger anti-predator response, potentially allowing for sensory compensation when vision was blocked or impaired. Alternatively, manipulating turbidity with bentonite as opposed to simulating turbidity with tinting may have produced a stronger or weaker anti-predator response. Suspended solids can alter chemical properties in the water in ways that either enhance (Reddy 1981) or degrade (Engström-

Öst & Candolin 2007) chemical signals. Specifically, we aimed to target the visual impacts of turbidity and chose to eliminate these potential effects in addition to the possible confounding effects on behavior resulting from introduced suspended solids.

Although our results indicated that *E. fonticola* has an innate ability to respond to a native fish predator, innate vs. learned response exists on a continuum in many species (Ferrari et al. 2010). Previous studies have shown that a baseline innate response in fishes can be heightened in the laboratory through conditioning with conspecific chemical alarm cues presented simultaneously with visual or chemical predator stimuli (Berejikian et al. 2003). Thus, using wild-caught *E. fonticola* may have resulted in a more dramatic anti-predator response or reliance on one modality in the absence of another. However, using predator naïve fish eliminated the potential effects of differential predator experience levels present in wild populations and maternal effects.

In conclusion, we experimentally demonstrate that visual and chemical signals combined were required to produce an anti-predator response in *E. fonticola*. Blocked vision when only chemical signals were presented weakened anti-predator response so that it did not differ from the water control. The effect of visual and chemical cues on anti-predator response was only present at the clear vision level. When vision was impaired because of simulated turbidity, anti-predator response to visual and chemical cues did not differ from the water control. Thus, we found no evidence of sensory compensation when one signal modality is removed or altered. These findings highlight the need for further study of the effects of turbidity on this endangered, endemic species as an indication of the impact on these darters and other similar endemics living in habitats currently threatened by fluctuating turbidity levels because of anthropogenic impacts.

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