Basic and Applied Herpetology 32 (2018) 71-76

Effect of pond dye on the response of Southern Leopard Frog tadpoles (*Lithobates sphenocephalus*) to Western Mosquitofish (*Gambusia affinis*) cues

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Received: 19 November 2017; returned for review: 30 April 2018; accepted: 03 July 2018.

Pollutant exposure can affect tadpole activity or affect their ability to respond to predator cues. One commonly used chemical in aquatic ecosystems, particularly those in suburban or rural areas, are dyes used to color or tint pond water. Little is known about how such dyes impact amphibians. We examined the effects of Tetra Pond Water Shade pond dye on the activity and behavioural response of Southern Leopard Frog (*Lithobates sphenocephalus*) tadpoles to cues from a potential predator, the Western Mosquitofish (*Gambusia affinis*). Tadpoles of *L. sphenocephalus* reduced activity after exposure to cues from *G. affinis*. The pond dye did not affect the activity or response of *L. sphenocephalus* tadpoles to *G. affinis* cues. Our results suggest little impact of this dye on the behavior of *L. sphenocephalus* at concentrations up to twice the recommended usage.

Key words: activity; anthropogenic chemicals; behavior; pollution; predator cues.

Given the widespread presence of chemical pollutants in ponds and lakes that serve as amphibian breeding sites, it is of great importance to understand how pollutants affect the behavior of amphibian larvae and how they respond to predators (see Ehrsam *et al.*, 2016). For example, exposure to pollutants can affect tadpole activity levels, in some cases increasing activity (Ehrsam *et al.*, 2016) and in other cases decreasing it (Bridges, 1999; Burgett *et al.*, 2007; Relyea & Edwards, 2010). Exposure to pollutants can also reduce or eliminate the ability of tadpoles to respond to predators or predator cues (e.g. Moore *et al.*, 2015; Ehrsam *et al.*, 2016; Polo-Cavia *et al.*, 2016), but not always (Burgett *et al.*, 2007; Mikó *et al.*, 2017). In addition, the simultaneous exposure to chemical pollutants and predator cues or predators can increase mortality rates (e.g. Relyea, 2005; Relyea & Edwards, 2010; Hanlon & Relyea, 2013), or in some cases, decrease mortality rates (Junges *et al.*, 2010; Relyea, 2012; Hanlon & Relyea, 2013).

These previous studies have examined the effects of pollutants, such as pesticides or fertilizers, commonly found in aquatic ecosystems where amphibians breed. Another commonly used chemical in aquatic ecosystems, particularly those in more anthropogenically altered habitats such as suburban or rural areas, are pond dyes. Such pond dyes are typically used to "beautify" ponds and decrease aquatic plant growth (Boyd & Noor, 1982; Spencer,

DOI: https://dx.doi.org/10.11160/bah..93

1984a), and are readily available to consumers. However, relatively little is known about how pond dyes impact pond life. One pond dye that is readily available to consumers is Aquashade (active ingredients: Acid Blue 9 and Acid Yellow 23; MADSEN *et al.*, 1999). Previous studies on the effects of Aquashade on pond ecosystems have been relatively limited, but the published research generally suggests little to no effect of Aquashade on fish, crayfish, and plants in these habitats (SPENCER, 1984b; BRISTOW *et al.*, 1996; LUDWIG *et al.*, 2010). However, Aquashade may affect zooplankton communities in ponds (SUSKI *et al.*, 2018) and pond dyes may have some effects on successful colonization and emergence of mosquitoes from ponds (ORTIZ-PEREA & CALLAGHAN, 2017; ORTIZ-PEREA *et al.*, 2018). We are unaware of any previous studies examining the effects of pond dyes on the physiology or behavior of aquatic vertebrates.

We examined the potential effects of another pond dye, Tetra Pond Water Shade Concentrated (active ingredient: proprietary formula; United Pet Group, Inc., Blacksburg, VA, USA) on tadpoles of the Southern Leopard Frog (*Lithobates sphenocephalus*). In particular, we determined whether exposure to this pond dye affected the activity and behavioral response of *L. sphenocephalus* tadpoles to cues from a potential predator, the invasive Western Mosquitofish (*Gambusia affinis*).

We obtained field-collected eggs of *L. sphenocephalus* from a commercial supplier (Charles D. Sullivan Co., Nashville, TN, USA). Eggs were maintained in the laboratory until they hatched (< 2 weeks). Once tadpoles reached Gosner stage 25 (GOSNER, 1960), 10 tadpoles were randomly assigned to each of four treatments: control (no dye), low dye (36 μL of Tetra Pond Water Shade Concentrated per L of water), medium dye (77 μL of Tetra Pond Water Shade Concentrated per L of water), and high dye (154 μL of Tetra Pond Water Shade Concentrated per L of water). One control tadpole died during the experiment, so N is 9 for the control treatment. The medium dye treatment was based on the manufacturer's instructions on the label. Given the short duration of the experiment, we assume the dye concentration remained relatively constant throughout the experiment. During the experiment, the room was maintained on a 12:12 light:dark regime and at a room temperature of ≈ 21°C.

Tadpoles were maintained individually in the experimental containers (i.e. 40 containers; originally 10 replicates of each treatment). Experimental containers were plastic containers (19 cm x 14 cm x 10 cm) containing 1.42 L of aged tap water, with a grid on the bottom of each tank to use as a means of quantifying tadpole activity (see below). We fed the tadpoles ground alfalfa pellets twice a week *ad libitum* and changed the water after each behavioral trial (refreshing the dye treatments). To gather chemical cues from fish, 20 *G. affinis* individuals were kept in a 37.8 L aquarium and fed dried bloodworms *ad libitum*. We conducted behavioral trials on days 5, 7, and 9 of exposure. During each trial, we counted the number of grid lines the tadpole crossed in two minutes as an index of how active the tadpole was (i.e. the more lines crossed, the more active the tadpole). After two minutes, we gently added 15 mL of cue water taken from the aquarium housing *G. affinis* into the tadpole's container. Immediately following cue addition, we again counted the number of lines crossed by the tadpole in two minutes. We averaged the difference in the number of lines crossed between before and after cue addition (after - before; negative numbers indicate reduction in activity, positive numbers indicate increased activity).

We used a Kruskal-Wallis test to compare the mean activity level before addition of the *G. affinis* cue and the mean change in activity among the four treatments. We compared the overall mean change in activity to a hypothesized mean of zero (i.e. no change in activity) using a one-sample t-test. We used JMP Pro 12 (SAS Institute Inc., Cary, NC, USA) for all statistical analyses. Means are given ± 1 S.E.

Mean activity prior to the introduction of the *G. affinis* cue did not differ among the treatments (Table 1; $H_3 = 5.46$, $P = 0.14$). Mean change in activity did not differ among dye treatments (Table 1; $H_3 = 2.44$, $P = 0.48$). The mean change in activity before and after *G. affinis* cue was added (mean = -1.11 ± 0.54) was significantly lower than zero ($t_{36} = -2.06$, $P < 0.05$).

Tadpoles of *L. sphenocephalus* in our study reduced their activity after exposure to chemical cues from *G. affinis* fed blood worms. The mean change in activity by tadpoles of *L. sphenocephalus* did not differ among dye treatments, suggesting that the pond dye did not affect the response of *L. sphenocephalus* tadpoles to *G. affinis* cues. Tetra Pond Water Shade also had no effect on tadpole activity levels prior to exposure to *G. affinis* cues. Our results are consistent with the conclusion that Tetra Pond Water Shade, even at twice the recommended level, had very little, if any, effect on the antipredator response of *L. sphenocephalus* tadpoles to cues from the invasive *G. affinis*. Our results therefore match most previous observations that another pond dye, Aquashade, has little effect on aquatic organisms, including fish (BOYD & NOOR, 1982; BRISTOW *et al*., 1996; LUDWIG *et al*., 2010) and crayfish (SPENCER, 1984b). To our knowledge, this is the first examination of potential effects of a pond dye on amphibians. We do caution that our experiment lasted only 10 days and took place in simple laboratory conditions. Experiments in mesocosms or simulating natural conditions are needed, as well as experiments on other species, to confirm our results.

Our results indicate that *L. sphenocephalus* tadpoles reduced their activity after the addition of chemical cues from *G. affinis*. *Gambusia affinis* is an invasive species,

Table 1: Mean (± 1 SE) activity of *Lithobates sphenocephalus* tadpoles before exposure to *Gambusia affinis* cues and mean change in activity of tadpoles after exposure to *G. affinis* cues in the different treatments (control and three concentrations of pond dye).

Treatment	Activity before cue exposure (lines crossed)	Change in activity after cue exposure
Control (N = 9)	1.54 ± 0.85	0.00 ± 0.88
Low dye (N = 10)	3.85 ± 0.90	-0.85 ± 1.44
Medium dye (N = 10)	2.77 ± 0.70	-1.07 ± 0.77
High dye (N = 10)	3.07 ± 1.39	-2.27 ± 1.13

but the native range of *G. affinis* includes Tennessee (RAUCHENBERGER, 1989) where the *L. sphenocephalus* eggs used in this experiment were obtained. Thus, it is possible that *L. sphenocephalus* has an evolutionary history with *G. affinis*, and may have evolved a response to *G. affinis*, as these fish are also potential predators on *L. sphenocephalus* (GREGOIRE & GUNZBURGER, 2008). Other studies have also shown that *L. sphenocephalus* tadpoles respond to cues from *Gambusia*. *Lithobates sphenocephalus* tadpoles increased hiding when exposed to *G. holbrooki* (GREGOIRE & GUNZBURGER, 2008). *Lithobates spehnocephalus* tadpoles exposed to visual cues and chemical cues of mosquitofish grew slower than control tadpoles or those exposed only to chemical cues (COLLIER *et al*., 2008). In addition, other anuran tadpoles have been shown to decrease their activity in response to cues from *G. affinis* (LAWLER *et al*., 1999; SMITH *et al*., 2010, 2011), although not all species show such a response (SMITH *et al*., 2008, 2009; BUTTERMORE *et al*., 2011).

In conclusion, our experiment demonstrates minimal impact of Tetra Pond Water Shade pond dye on the behavior and growth of *L. sphenocephalus* at concentrations up to twice the label recommended level. *Lithobates sphenocephalus* tadpoles reduced their activity after the addition of cues from *G. affinis*, but the pond dye did not affect the response of *L. sphenocephalus* tadpoles to cues from *G. affinis*.

Acknowledgement

Experimental procedures were approved by the Denison University IACUC (Permit 10-003). We thank two anonymous reviewers for helpful comments on the manuscript.

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