

Responses of Three Darter Species (Genus *Etheostoma*) to Chemical Alarm Cues from Conspecifics and Congeners

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Several species of darters of the genus *Etheostoma* possess a chemical alarm substance, but it is not known how widespread this substance is within the genus. We performed behavioral bioassays on Fantail Darters (*E. flabellare*), Rainbow Darters (*E. caeruleum*), and Redfin Darters (*E. whipplei*). Redfin Darters reacted with a fright response upon exposure to skin extracts from conspecifics and Rainbow Darters. Rainbow Darters, which are known to respond to conspecific extracts, responded to skin extracts from Redfin Darters. Neither Rainbow Darters nor Redfin Darters showed a significant response to Fantail Darter extract, and Fantail Darters did not respond to skin extracts from either congeners or conspecifics. Percid alarm substance is hypothesized to originate from epidermal sacciform cells. Histological examination of epidermal tissue demonstrated that sacciform cells were present in all three species. Our results demonstrate that chemical alarm signals are not ubiquitous for the genus *Etheostoma*, and that the presence of sacciform cells does not always predict the presence of the alarm response.

DETECTION of predators early in a predatory encounter increases the probability of prey survival (Lima and Dill, 1990). Although any sensory modality can be used for predator detection, chemical cues are particularly useful because they travel easily around barriers, are available in the dark, and are useful for detection of silent or visually cryptic predators (Dodson et al., 1994). One way that prey individuals are warned about danger is through chemicals (“alarm” cues) released from the skin of prey that have been wounded in a predatory attack (Chivers and Smith, 1998).

The most intensively studied alarm chemical system is that of ostariophysan fishes (about 70% of freshwater fish species, including minnows, suckers, and catfishes), a system known since the 1940s (von Frisch, 1941). Within ostariophysans, cross-species reactions to alarm chemicals are almost ubiquitous, and the alarm cue is considered to be homologous for the group (Smith, 1992). In the past decade, chemical alarm cues have been documented for numerous other species of fishes, including darters (Percidae: Smith, 1979; Commens and Mathis, 1999; Haney et al., 2001; Vokoun and Noltie, 2002), sculpins (Cottidae: Hugie et al., 1991), gobies (Gobiidae: Smith, 1989), salmonids (Salmonidae: Brown and Smith, 1997), live-bearers (Poeciliidae: Brown and Godin, 1999), and sticklebacks (Gasterosteidae: Mathis and Smith, 1993).

Although much progress has been made, much remains to be learned about alarm chemicals within these latter taxa. One question of particular interest is whether the alarm chemicals within each of these taxa represents

a homology, as is apparently the case in the ostariophysans, or whether the alarm chemicals have evolved independently multiple times within a lineage. The answer to this question requires data on numerous species, including reactions to alarm chemicals from both conspecifics and closely related heterospecifics. Our study focuses on species in the most diverse genera of percids, the genus *Etheostoma*.

The family Percidae includes approximately 183 species of darters, with over 136 species in the genus *Etheostoma* (Page, 1983; Nelson et al., 2004). When the skin of a darter is damaged, chemicals are released from the skin and conspecific (and sometimes heterospecific) receivers typically respond with a decrease or cessation of movement (Radabaugh, 1989; Commens and Mathis, 1999). For darters, the alarm chemical is hypothesized to be produced in large vacuolated cells in the epidermis (Smith, 1982). The sacciform cells are ductless so that chemical release occurs only following mechanical damage to the skin, such as occurs when the fish is attacked by a predator. To date, responses to conspecific alarm chemicals are documented in five species of *Etheostoma* (Table 1), all of which yielded positive results (Smith, 1979, 1982; Commens and Mathis, 1999; Haney et al., 2001).

If the alarm chemical is homologous within the genus *Etheostoma*, cross-reactions should occur whereby fishes exhibit fright responses when exposed to alarm chemicals from congeners. Cross-species responses are documented between three different *Etheostoma* species (Commens and Mathis, 1999; Haney et al., 2001; Gibson and Mathis, 2006). To further examine responses to

TABLE 1. RESULTS OF STUDIES TESTING WHETHER DARTERS OF THE GENUS *Etheostoma* RESPONDED OR FAILED TO RESPOND TO CONSPECIFIC (BOLD) AND HETEROSPECIFIC SKIN EXTRACTS.

Receiver	Sender	Response	Reference
<i>E. caeruleum</i>	<i>E. caeruleum</i>	Yes	Commens and Mathis, 1999
	<i>E. blennioides</i>	No	Gibson and Mathis, 2006
	<i>E. flabellare</i>	No	This study
	<i>E. juliae</i>	Yes	Commens and Mathis, 1999; Gibson and Mathis, 2006
	<i>E. whipplei</i>	Yes	This study
	<i>E. zonale</i>	Yes	Gibson and Mathis, 2006
<i>E. exile</i>	<i>E. exile</i>	Yes	Smith, 1979, 1981; McPherson et al., 2004
<i>E. flabellare</i>	<i>E. flabellare</i>	No	This study
	<i>E. caeruleum</i>	No	This study
	<i>E. whipplei</i>	No	This study
<i>E. nigrum</i>	<i>E. nigrum</i>	Yes	Smith, 1979; Haney et al., 2001; Vokoun and Noltie, 2002
	<i>E. spectabile</i>	No	Haney et al., 2001
	<i>P. caprodes</i>	No	Haney et al., 2001
	<i>P. maculata</i>	No	Haney et al., 2001
	<i>P. phoxocephala</i>	Yes	Haney et al., 2001
<i>E. spectabile</i>	<i>E. spectabile</i>	Yes	Haney et al., 2001
	<i>E. nigrum</i>	Yes	Haney et al., 2001
	<i>P. caprodes</i>	Yes	Haney et al., 2001
	<i>P. maculata</i>	No	Haney et al., 2001
	<i>P. phoxocephala</i>	Yes	Haney et al., 2001
<i>E. swaini</i>	<i>E. swaini</i>	Yes	Smith, 1982
	<i>Ammocrypta beani</i>	Yes	Smith, 1982
	<i>Percina nigrofasciata</i>	Yes	Smith, 1982
<i>E. whipplei</i>	<i>E. whipplei</i>	Yes	This study
	<i>E. caeruleum</i>	Yes	This study
	<i>E. flabellare</i>	No	This study

alarm chemicals within the genus *Etheostoma*, we tested responses of *Etheostoma whipplei* (Redfin Darters), *E. caeruleum* (Rainbow Darters), and *E. flabellare* (Fantail Darters) to conspecific and congeneric skin extracts.

We tested the following predictions about behavioral responses to alarm chemicals found in skin extracts: For all three species a fright response (=decreased activity) will occur following exposure to skin extracts from damaged conspecifics, indicating the presence of alarm chemicals in the skin; fright responses will not occur in response to the water or goby treatments, indicating that the alarm chemicals are not general responses to disturbance or chemicals found in the skin of distantly related fishes (i.e., some degree of specificity); and fright responses will occur in response to skin extracts from congeners, indicating a possible homology across the genus. In addition, we examined histological sections of the skin of each species for the presence of sacciform cells, the hypothesized site of alarm chemical production.

MATERIALS AND METHODS

We collected Redfin Darters from the Ouachita River drainage (34°45'43"N, 93°04'14"W) in Saline County, Arkansas. We collected Fantail Darters from the White River drainage (36°51'41"N, 93°13'33"W) in Christian County, Missouri, and we collected Rainbow Darters from the White River drainage (37°11'40"N, 93°07'29"W) in Greene County, Missouri. Although the Rainbow and Fantail Darters used in this experiment were collected from different streams, they are syntopic over large parts of their respective ranges (Pflieger, 1997). We collected Redfin Darters from an area where they do not co-occur with either Rainbow Darters or Fantail Darters (Robison and Buchanan, 1984). As a control stimulus, we used skin extracts from *Brachygobius sabanus* (Bumblebee Gobies: Family Gobiidae). We selected this control species because it is not closely related to darters, it does not co-occur with darters, and in previous studies it served as an effective control in tests

with Rainbow Darters (Commens and Mathis, 1999; Gibson and Mathis, 2006).

We maintained darters in groups of 10–25 in 38 L aquaria at ambient temperatures (17–22 C). We obtained tropical Bumblebee Gobies (*Brachy-gobius sabanus*) commercially, for use as our control stimulus, and kept them in groups of 5–20 in 38 L aquaria at 25–26 C. We fed all fishes frozen brine shrimp (*Artemia* sp.) occasionally supplemented with blackworms (*Lumbriculus variegatus*).

We prepared skin extracts using the methods of Smith (1982). We sacrificed donor fish with a blow to the head and made 25 shallow vertical cuts with a razor blade on each side of the body. We then immediately placed the donor fish in 50 mL of dechlorinated water that was stirred on an automatic stir plate for five minutes. We removed and measured the body of the donor fish, and placed the solution on ice until observations were made. We used each for two consecutive observations within 30 min of preparation. According to a “personal observation” from R. J. F. Smith cited in Chivers et al. (1995a), the darter alarm cues begin to lose efficiency after 1 hour. We prepared a blank control (dechlorinated tap water) by rinsing a clean razor blade with dechlorinated water into an additional 20 mL of dechlorinated water, which was stirred for five minutes on an automatic stir plate and then placed on ice.

We exposed test individuals of each darter species to stimuli in the following five treatments: blank control (dechlorinated water), skin extracts from conspecifics, both congeners, and gobies. However, we did not expose Rainbow Darters to conspecific extract because previous work documented a decreased activity response to conspecific skin extract (Commens and Mathis, 1999). We tested 14 individuals in each treatment, using each individual only once.

We placed test fish of each species individually in randomly selected 7.6 L testing tanks 2–5 days prior to testing. Testing tanks were aerated via an airstone at the back of the tank, and the bottom was lined with small rocks. We inserted separate plastic tubing for injection of the stimulus into the tank near the airstone so that the stimulus would disperse rapidly. We conducted trials between 0800 and 2000 hours at a water temperature of 17–22 C. Mean total lengths (\pm SD) of test individuals were: Rainbow Darters 55.2 ± 9.57 mm; Redfin Darters 58.6 ± 9.65 mm; Fantail Darters 48.8 ± 8.18 mm; and Bumblebee Gobies 30.7 ± 6.29 mm.

We used a testing protocol identical to that of Commens and Mathis (1999). We conducted

observations through a one-way mirror to minimize disturbance. We estimated activity as the number of moves made by the test fish, with a move scored when the fish either left the tank bottom or changed direction. To establish baseline activity, we recorded the number of moves for 8 min prior to stimulus injection. We then injected 20 mL of a randomly selected stimulus solution (conspecific, heterospecific, or blank) into the test tank through the stimulus introduction tube. After 1 min (to allow for dispersion and detection of the stimulus), we recorded activity for an additional 8 min. We calculated a response index for each fish as the difference between post-stimulus and pre-stimulus activity. Negative indices indicate decreased activity and positive indices indicate increased activity with respect to baseline activity. We compared indices for the treatments using a nonparametric Kruskal–Wallis test by ranks, followed by Nemenyi’s test contrasting each stimulus against the water treatment control (Zar, 1984).

We prepared histological sections of the skin for each darter species. We removed skin tissue from the anterior dorsal area of the body and fixed it in formalin, embedded it in paraffin, and cut it into 4 μ m sections. We stained the sections with eosin and Harris hematoxylin stains and examined them for sacciform cells under a light microscope.

RESULTS

Responses of Redfin Darters differed significantly among treatments ($H = 13.22$, $df = 4$, $P \leq 0.010$, Fig. 1). Relative to the control, activity decreased significantly in the presence of both conspecific skin extract ($Q' = 2.995$, $P < 0.01$) and Rainbow Darter skin extract ($Q' = 2.457$, $P < 0.05$). No differences were detected between the control treatment and either the goby ($Q' = 0.689$, $P \leq 0.25$) or Fantail Darter ($Q' = 0.598$, $P \leq 0.25$) treatments.

Rainbow Darter activity responses were similar to those of Redfin Darters. Significant differences occurred among treatments ($H = 18.16$, $df = 3$, $P < 0.001$, Fig. 1). Activity decreased significantly in response to Redfin Darter skin extract relative to the control treatment ($Q' = 3.407$, $P < 0.001$). No differences were detected between the control treatment and either the goby ($Q' = 0.990$, $P \leq 0.25$) or Fantail Darter ($Q' = 0.503$, $P \leq 0.25$) treatments. In contrast to congeners, Fantail Darters showed no significant effects of treatment ($H = 1.60$, $df = 4$, $P < 0.808$, Fig. 1). Sacciform cells were visible in the epidermis of all three darter species (Fig. 2).

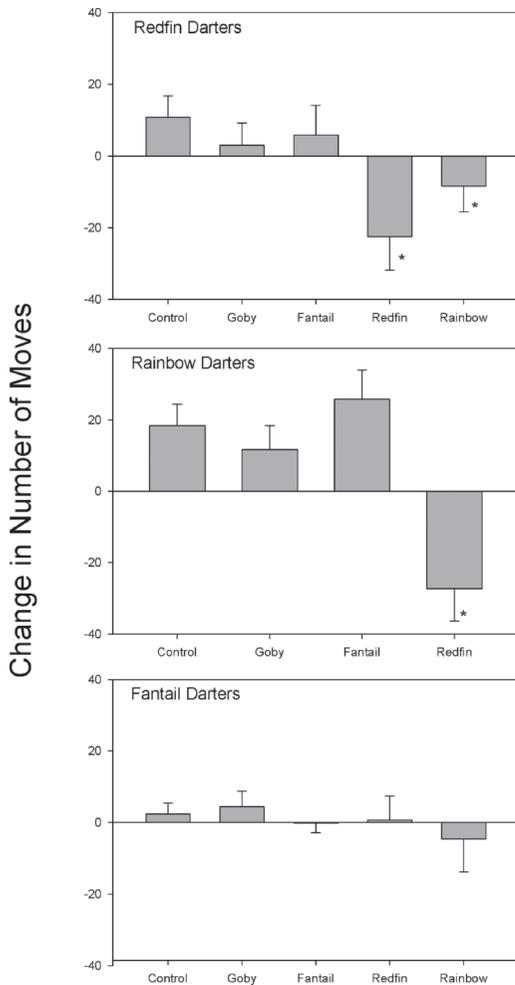


Fig. 1. Mean change in activity (± 1 SE) by darters following exposure to water and to skin extracts from Bumblebee Gobies, congeners, and conspecifics. Redfin Darters, Kruskal–Wallis $P = 0.01$. Rainbow Darters, Kruskal–Wallis $P < 0.001$. Fantail Darters, Kruskal–Wallis $P = 0.808$. * indicates a significant difference from the control; $n = 14$ for each treatment.

DISCUSSION

Redfin Darters are the sixth species of the genus *Etheostoma* to respond to conspecific alarm chemicals (Table 1). Redfin Darters did not exhibit a response to skin extracts from Bumblebee Gobies or to the water treatment, indicating the alarm responses are more specific than just a general response to injured fish or to disturbance.

Redfin and Rainbow Darters responded to skin extracts of each other similarly to their responses to conspecific skin extracts. Reciprocal responses are consistent with the hypothesis that the alarm chemicals of these two species are homologous.

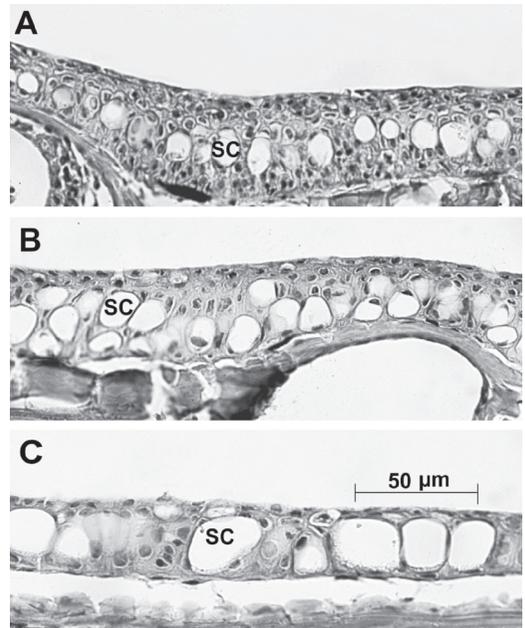


Fig. 2. Cross section of the epidermis of a (A) Redfin Darter, (B) Rainbow Darter, and (C) Fantail Darter. Epidermis is stained with eosin and Harris hematoxylin stain. $40\times$ magnification. SC = Sacciform cell.

Cross-species reactions have been documented for several, but not all, species of *Etheostoma* tested (Table 1). Attempts to produce phylogenies of the genus typically have led to variable results with poor resolution among species; however, based on the current phylogenetic analyses (Sloss et al., 2004), cross-species reactions apparently are not limited to specific lineages within the genus. As a working hypothesis, we propose that darter alarm chemicals are homologous at least within the genus *Etheostoma*. An alternative hypothesis is that cross-species reactions are the result of selection for responses to alarm cues by individuals in the same prey guild (Wisenden et al., 1994; Chivers et al., 1995a). However, in this study we observed cross-species reactions to alarm chemicals by two species that did not co-occur (Redfin and Rainbow Darters), so the prey guild hypothesis seems unlikely for these species. Some cross-generic tests have yielded negative results (Table 1), suggesting that either alarm chemicals are not homologous across the sub-family Etheostominae or that some species have lost the ability to produce or respond to the alarm chemicals. Cross-genus reactions have been documented between some species, including *Etheostoma* and *Percina*, *Etheostoma* and *Ammocrypta* (Table 1), and *Percina* and *Ammocrypta* (Smith, 1982); because the species that were tested all co-occurred, these

reactions are consistent with the prey-guild hypothesis.

Of the species of *Etheostoma* that have been tested to date, Fantail Darters are the first to fail to respond to conspecific extracts with decreased activity (Table 1). This result could be explained as either a failure of Fantail Darters to respond to alarm chemicals by reducing activity or by the absence of alarm chemicals in the epidermis of Fantail Darters. The latter explanation seems to be the most likely because Rainbow Darters and Redfin Darters also failed to respond to Fantail Darter extracts.

In addition to failure to respond to conspecific skin extract, Fantail Darters did not respond to the alarm chemicals produced by Redfin and Rainbow Darters. In some fishes, response to heterospecific alarm pheromone can be learned (Chivers et al., 1995b). However, Vokoun and Noltie (2002) found that the response to conspecific alarm substance is innate in at least one darter species. The Fantail Darters used in our study were collected from an area where they do not co-occur with Redfin Darters, and Rainbow Darters are seldom collected from this site, so the opportunity for learning may have been inadequate. The lack of response also could indicate that nearby predation on other species of darters is not a good indicator of risk for Fantail Darters because of differences among the species in microhabitat use; Fantail Darters may be more likely to hide among rock crevices than other darters (Pflieger, 1997). In addition, Fantail Darters appeared to be less active than the other species in this study (mean number of moves \pm 1 SE prior to stimulus introduction: Fantail Darters = 26.3 ± 8.53 , Rainbow Darters = 46.2 ± 9.72 , Redfin Darters = 49.3 ± 8.96), so changes in activity would be more difficult to detect in this species. Additional experiments using other response variables may reveal changes in activity that we could not detect.

We found epidermal sacciform cells, the hypothesized site of alarm chemical production (Smith, 1979), in the epidermis of all three species tested (Fig. 2). For Fantail Darters, the presence of sacciform cells and the apparent absence of alarm chemicals appear incongruent with their hypothesized function. We offer three potential explanations for our results. First, sacciform cells may not be the site of production of alarm chemicals for darters. For example, Zaccone et al. (1988) hypothesized that these cells may facilitate wound healing through antipathogenic secretions. Even so, we cannot at this time offer a hypothesis for an alternate site for production of alarm chemicals. Second, the function of the sacciform cells may vary across

species of darters with alarm chemicals being produced by the cells of some species but not others. The cells would remain present (perhaps with a different function) even though the alarm chemical is no longer produced. Third, the presence of sacciform cells may vary seasonally in Fantail Darters. Males of some species of ostariophysan fishes lose their alarm cells during the breeding season when skin damage due to spawning activity is common (Smith, 1982). Fantail Darters differ from other species tested in this study in that the males provide parental care by building nests and guarding the eggs throughout development (Lake, 1936). Interestingly, in another guarding species, *Etheostoma nigrum*, alarm responses were not detected by congeners, but conspecific responses did occur (Grant and Colgan, 1984; Table 1). Alarm cells in Fantail Darters may be lost or altered during the breeding season to avoid incidental release of the alarm substance caused by nest maintenance. In this study, we tested responses of Fantail Darters to alarm chemicals during their breeding season (April and May) but present the histological section (Fig. 2) from darters collected in the nonbreeding season when sacciform cells were obviously present. We also examined sections of male and female Fantail Darters during the breeding season (unpubl. data) and the sacciform cells were still present, suggesting that breeding season differences do not manifest as a complete absence of the cells. We recommend that future studies include more fine-scale analysis of seasonal changes, including an assessment of possible changes in secretory chemicals.

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