



Alarm pheromone recognition in a Missouri darter assemblage

D. C. HANEY, J. C. VOKOUN AND D. B. NOLTIE*

Department of Fisheries and Wildlife Sciences, The School of Natural Resources,
302 Anheuser-Busch Natural Resources Building, University of Missouri-Columbia,
Columbia, MO 65211-7240, U.S.A.

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The *a priori* null hypotheses that responses to donor, orangethroat darter *Etheostoma spectabile*, johnny darter *Etheostoma nigrum*, logperch *Percina caprodes*, slenderhead darter *Percina phoxocephala* and blackside darter *Percina maculata*, extracts would be absent in the three test species, orangethroat darter, johnny darter and logperch, was rejected. This was because orangethroat and johnny darter responded with decreased activity to skin extracts derived from conspecifics and from other members of the assemblage. In contrast, logperch were notable by their lack of a detectable response to any species. Similarities and differences in habitat utilization are seen as a possible basis for these responses.

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Key words: alarm pheromone; fright response; darter; Percidae; *Etheostoma* spp.; *Percina* spp.

INTRODUCTION

Various fish species use chemical signals to convey information for use in individual recognition, predator avoidance, or evaluating reproductive status (Smith, 1992; Chivers *et al.*, 1999). In some prey species, injuries inflicted by predators result in the release of an alarm pheromone, its function being to alert conspecifics of the predator's activity; other prey are capable of detecting predator odour and or the consumption-derived odours of conspecifics in predator faeces (Smith, 1992). The behavioural responses to the detection of these odours vary between species, but similarly serve to decrease predation risk.

Although darters (Percidae) are one of the most speciose families of North American freshwater fishes (Kuehne & Barbour, 1983), the chemical alarm systems of only a few species have been studied; where responses to alarm chemicals occur, they generally involve a decrease or cessation in movement (Smith, 1979, 1982, 1992; Commens & Mathis, 1999). Given that darters are generally cryptically-coloured bottom-dwellers (Pflieger, 1997), this cessation of movement is believed to further protect darters from being observed and attacked (Smith, 1979, 1982; Commens & Mathis, 1999). Darters similarly stop moving in response to detection of a live predator (Rahel & Stein, 1988; Radabaugh, 1989).

In this study, the mutual responses of five co-occurring darter species to the alarm pheromones of one another are investigated. The species tested include:

*Author to whom correspondence should be addressed. Tel.: +1 573 882 9421; fax: +1 573 884 5070; email: NoltieD@missouri.edu

orangethroat darter *Etheostoma spectabile* (Agassiz), johnny darter *Etheostoma nigrum* (Rafinesque) and logperch *Percina caprodes* (Rafinesque) as both alarm substance detectors and donors, and blackside darter *Percina maculata* (Girard) and slenderhead darter *Percina phoxocephala* (Nelson) only as donors. The *a priori* null hypothesis was that responses to the donor extracts would be absent in the three test species. Alternatively, the co-occurrence of the species, the similarities in their habitats and predators, and their phylogenetic relatedness can be considered as reasons for why inter-specific alarm pheromone recognition might occur. Although conspecific detection has been demonstrated in johnny darter (Smith, 1979; Commens, 2000) and in orangethroat darter (Smith, 1982), the present work is, as far as is known, the first testing of logperch for a fright response.

MATERIALS AND METHODS

FISHES

Pre-spawning adult-sized specimens (presumably both sexes) of all five darter species were seined live during February 2000 from Hinkson Creek in Boone County, near the city of Columbia, Missouri. The five species constitute the entire darter assemblage here; although they are sympatric (i.e. occur in the same stretches of stream), the species have differing microhabitat affinities (Pflieger, 1997). In the laboratory, the species were maintained in separate 38 l aquaria at 20–23° C, and fed to satiation daily on frozen bloodworms (*Chironomus* spp.).

TEST SOLUTIONS

Preceding each trial, fresh test solutions were prepared using methods similar to those described by Smith (1979). Each scent donor was randomly selected from the collected specimens, sacrificed in fresh tricane methane-sulphonate (MS-222) solution in a clean jar, and then thoroughly washed in de-ionized water. Following blotting and placement in a clean petri dish, both flanks of each donor were then scored with 50 shallow vertical scalpel cuts using a fresh scalpel, after which the fish was placed in 20 ml of de-ionized water and stirred for 5 min. After discarding the donor carcass, the resulting scent solution was kept refrigerated for use within 45 min of preparation. To preclude odour transference, all glassware and instruments were thoroughly washed between trials using hot water and mild detergent. It is believed that this approach for humanely euthanizing the donor fishes was unlikely to have influenced the results (i.e. via possible anaesthetic effects on the test individuals). First, any direct transfer of anaesthetic-containing water to the donor extract was minimized by rinsing the anaesthetized donors in de-ionized water and blotting them dry before they were scored. Second, although some MS-222 was likely to have permeated the donor fish tissue, the small amount of anaesthetic used to induce death minimized any resulting body burdens, and the amount of anaesthetic in the small volume of donor extract used in the tests (2 ml) was drastically diluted upon introduction into the test aquarium (38 000 ml).

The control solution (scent blank) used was prepared by stirring a freshly opened scalpel blade in 20 ml of de-ionized water for 5 min. This, too, was kept refrigerated for use within 45 min of preparation. Because the responses of test individuals might constitute detection simply of fish tissue ichor, some researchers use as a control the extract from a donor species that lacks alarm chemical (usually a phylogenetically distant species). In the present study it was elected not to follow suit out of concern that a test species' lack of response (interpreted as non-detection of the control) could instead be attributable simply to it 'ignoring' the unfamiliar. Because it could not be guaranteed

that the preparation of an extract was completely free of alarm substance, it was decided not to use, as a control, a preparation of skin-free tissue derived from a more relevant donor.

PROCEDURE

Trials were conducted in 381 aquaria (51 long \times 25 wide \times 30 cm high) containing washed coarse to medium gravel similar to that found in Hinkson Creek, and filled with aged tap water (20–23°C) aerated continuously with an airstone. Each tank and its gravel were washed between trials.

For each trial, five test individuals of the same species were placed into an aquarium 30–60 min before testing. To preclude handling-related alarm pheromone releases, small aquarium nets were used to transfer the fish between tanks (Smith, 1979). Each fish was tested in one trial only to avoid the effects of prior experience.

Following Commens & Mathis (1999), reductions in activity were used as an indicator of a fright response. Darters naturally move in 'hops' (single, quick, short-distance displacements from one substratum location to another, separated by longer periods of on-bottom station-holding); consequently, any movement was counted as a hop where the fish either left the bottom or executed a quick whole-body change in the direction it was facing (or both). Darters responding to macerated skin extract exhibit a distinct 'alert' posture (back arched, head up, dorsal fins erect; Smith, 1979); the trials in which this response was observed were also noted.

Three replicate trials (five different individuals each trial) were completed for each test of species' detection (e.g. johnny darter exposure to slenderhead darter extract). For each trial, a baseline activity level was measured by recording the total number of hops performed by all five fish for 3 min. Thereafter, a previously unused syringe was used to inject 2 ml of control solution into the aquarium (near the airstone to accelerate dispersion), after which another 3 min recording was completed. Finally, 2 ml of donor extract solution was injected into the aquarium water, followed by another 3 min observation period. The observer (DCH in all trials) sat between 0.67 and 1 m from the test aquaria. To minimize visibility of the observer to the test subjects, all trials were conducted in the dark under aquarium lighting (a single 15 W fluorescent bulb 46 cm long \times 2.5 cm diameter located above the aquarium in an aquarium hood). All trials were conducted between 1200 and 1700 hours in random order, and were completed within 5 weeks of the fishes' collection.

ANALYSIS

For each test species, tests were made as to whether the control and extract solution responses differed respectively from baseline responses during a 1-way repeated-measures analysis of variance (PROC GLM in SAS; SAS Institute, 1989) using as data the total number of movements by the five darters collectively recorded over each 3 min interval, and trials as the replicates. Data normality was confirmed using normal probability plots, stem-leaf plots and the Shapiro-Wilk statistic; sphericity was confirmed using Mauchly's criterion applied to orthogonal components (Hatcher & Stephanski, 1994).

It was intended to test the response of each of the five species to each of the four remaining species. Unfortunately, the low natural abundance of blackside and slenderhead darters in Hinkson Creek only allowed these species to be used as scent donors, and not as potential alarm pheromone detectors. Thus, the experimental design was not completely reciprocal.

RESULTS

Overall, the results dictated rejection of the null hypothesis. Substantial reductions in movement rate (indicative of alarm substance discernment) were observed when orangethroat darters were exposed to the skin extracts of conspecifics, a congener (johnny darter), and two confamilials (logperch and

TABLE I. Alarm responses in darter species from Hinkson Creek, Boone County, central Missouri. A significant ($P < 0.05$) reduction in test subject movement frequency relative to a pre-treatment observation period is interpreted as detection (+), whereas a non-significant change is interpreted as non-detection (-)

Test species	Extract species				
	Orangethroat (<i>E. spectabile</i>)	Johnny (<i>E. nigrum</i>)	Logperch (<i>P. caprodes</i>)	Blackside (<i>P. maculata</i>)	Slenderhead (<i>P. phoxocephala</i>)
Orangethroat	+	+	+	-	+
Johnny	-	+	-	-	+
Logperch	-	-	-	-	-

slenderhead, but not to blackside darter; Table I). In comparison, johnny darters exhibited significant activity reductions only to the extracts of conspecifics and of one confamilial (slenderhead darter). The non-significant logperch responses to all extracts were indicative of non-discernment (Table I).

In no case was a non-significant overall model test accompanied by a significant paired comparison against the baseline data; similarly, significant overall model test outcomes were generally accompanied by a significant baseline *v.* extract comparison (Table II). Two exceptions to the latter were in evidence: the overall significance of the johnny darter to blackside extract and of the orangethroat darter to logperch extract which was largely attributable to a baseline *v.* control effect (Table II). In both cases, however, the decrease in movement following introduction of the control solution was anomalous, and test individuals did not exhibit the alert posture. In contrast, logperch extract prompted a significant reduction in orangethroat darter movement and the test subjects displayed alert posturing (back arched, head up, dorsal fins erect), suggesting a true fright response. Because the alert posture (Smith, 1979) was readily observed in all trials yielding significant outcomes, the results appear realistic despite the multiple tests employed. However, a spurious result is always more probable under multiple testing situations.

DISCUSSION

The number of darter species tested for alarm pheromone recognition has been small (Smith, 1992), and the present findings build upon this base. Most notable was evidence that orangethroat darters could detect the skin extracts of a congener (johnny darter) and two confamilials (logperch and slenderhead, but not blackside darter) and that johnny darters responded only to the extracts of a single confamilial (slenderhead darter). The outcome of the test of johnny darter responses to conspecific extracts is consistent with Smith's (1979) finding of decreased or discontinued movement, and the orangethroat darter responses to conspecific extracts parallel unpublished results cited by Smith (1982); re-obtaining these results gave confidence to the validity of these findings.

Consistent with the approaches adopted by other researchers (Smith, 1982; Commens & Mathis, 1999), the present study focused on reduced movement as the behavioural response variable because it is considered indicative of

TABLE II. Aggregate number of hops by five darters, means (\pm s.e.) over three trials (different darters each trial), under three test conditions, together with the results of 1-way repeated-measures ANOVAs on the raw per-trial hop numbers

Test species	Extract species	Treatment		Extract	Overall model		Baseline v. control		Baseline v. extract	
		Control	Extract		$F_{2,4}$	P	$F_{1,2}$	P	$F_{1,2}$	P
Orangethroat	Orangethroat	101.3 \pm 4.1	91.0 \pm 17.9	35.0 \pm 7.6	14.99	0.01	0.34	0.62	139.93	0.007
	Johnny	123.3 \pm 9.6	101.0 \pm 11.4	35.7 \pm 11.2	15.95	0.01	1.22	0.38	21.22	0.04
	Logperch	130.3 \pm 9.5	101.7 \pm 11.3	55.3 \pm 9.5	46.99	0.002	238.58	0.004	65.92	0.02
	Blackside	121.7 \pm 16.1	103.0 \pm 19.7	100.3 \pm 12.3	2.49	0.19	6.60	0.12	7.74	0.11
	Slenderhead	157.0 \pm 9.0	132.7 \pm 14.7	97.7 \pm 5.2	31.60	0.004	16.86	0.06	161.65	0.006
Johnny	Orangethroat	87.0 \pm 53.5	96.0 \pm 23.1	93.7 \pm 29.7	0.08	0.92	0.09	0.80	0.08	0.81
	Johnny	69.0 \pm 11.8	64.7 \pm 17.9	31.0 \pm 4.7	9.66	0.03	0.49	0.56	26.58	0.04
	Logperch	128.3 \pm 17.9	115.7 \pm 14.2	97.7 \pm 6.8	1.55	0.32	0.94	0.44	1.8	0.31
	Blackside	27.7 \pm 6.9	46.7 \pm 12.0	24.3 \pm 7.6	14.30	0.02	11.90	0.08	1.32	0.37
	Slenderhead	103.3 \pm 30.2	94.3 \pm 27.4	67.0 \pm 22.3	6.53	0.05	0.63	0.51	20.81	0.04
Logperch	Orangethroat	107.7 \pm 23.9	106.3 \pm 17.0	118.0 \pm 4.7	0.18	0.84	0.01	0.93	0.14	0.75
	Johnny	112.7 \pm 40.6	104.7 \pm 38.5	153.3 \pm 30.2	3.37	0.14	0.79	0.47	3.75	0.19
	Logperch	172.7 \pm 26.1	169.3 \pm 18.5	135.0 \pm 37.6	3.39	0.14	0.12	0.76	4.76	0.16
	Blackside	163.0 \pm 6.2	158.3 \pm 10.5	97.7 \pm 28.9	4.51	0.09	0.08	0.80	4.06	0.18
	Slenderhead	137.0 \pm 26.5	109.0 \pm 52.9	103.7 \pm 53.9	0.89	0.48	0.77	0.47	0.98	0.43

pheromone detection. Present interest was not in assessing the presence or degree of a fright response, so exhibition of the alert posture (number of individual fish displaying the posture; posture frequency, intensity, or duration) was not quantified more fully. Noting those trials in which the alert posture was exhibited proved to be important in interpreting those spurious cases where a significant baseline *v.* control effect suggested that the scent blank was itself eliciting a response. Consequently, it is suggested that future studies similar to this one incorporate a more formal consideration of the alert posture.

The logperch results are believed to be the first to have been reported for this species. The absence of responses in logperch was unanticipated: logperch possess on their flanks the sacciform cells hypothesized to be the alarm pheromone source (Smith, 1979, 1992). However, Commens (2000) similarly found that fantail darter *Etheostoma flabellare* Rafinesque did not respond to conspecific skin extracts, despite their retention of the sacciform cells. Nonetheless, it cannot be ruled out that logperch respond to darter species that were not considered. Because observations of the logperch in their holding and testing aquaria showed that they moved much more frequently and for longer distances than did the other darters that were tested, it is recommended that tests on this species be repeated, looking for differences in 'hop distance' rather than frequency. Should logperch truly lack a conspecific fright response, its loss may be a consequence of their feeding habits: they forage for benthic invertebrates by turning over stones after wedging their pointed snouts under the stone edges (Pflieger, 1997). This behaviour may result in frequent abrasion of the skin, with the consequence being akin to 'crying wolf' too frequently. Given the apparent absence of data, an interesting correlate to test for would be a reduction (numbers, sizes, sensitivity, output) in the sacciform cells on logperch snouts and bodies relative to congenics.

Interspecific differences and similarities in habitat use suggest a possible interpretation of the patterns of alarm pheromone recognition that the Hinkson Creek darters exhibited. Qualitatively and statistically, orangethroat darters respond most strongly to conspecifics and slenderhead darter, logperch, and then johnny darter, but did not respond to blackside darter. Similarly, orangethroat and slenderhead darter habitat preferences are the most similar (Pflieger, 1997). Logperch and blackside darter habitat preferences most resemble those of the johnny darter, with the blackside being 'less a bottom fish than many darters, swimming for extended periods in midwater and sometimes rising to the surface' (Pflieger, 1997). The limited degree to which orangethroat and blackside darters share habitat may provide a reason for the apparent lack of alarm pheromone recognition between the two species.

Whether or to what degree interspecific alarm pheromone recognition in darters is learned or an inherited trait is unknown; in some cyprinid species, responses to intraspecific skin extract are innate but are also influenced by experience (Chivers & Smith, 1994). For co-occurring darters, similarities in habitat use would provide opportunities to be exposed to the alarm pheromones of other species. Phylogenetic relatedness and similarities in pheromone chemistry may also influence the likelihood of interspecific recognition. Darters provide an interesting group against which to test ideas generated from the results of research on the ostariophysid fishes. The present results also suggest

that mutual or differential habitat use may also be important to consider when interpreting interspecific differences in alarm pheromone response.

It is recognized that the sample sizes used resulted in a lessening of the ability to detect subtler differences (lowered power), particularly where the fish responses were more variable. These sample sizes reflect attempts to use a realistic test condition (multiple individuals together in sight of one another above the substratum, as occurs in the wild) and to ensure independence (non re-use of individual test animals). The use of groups of animals as the experimental unit was also intended to ameliorate possible effects of atypically high or low individual animal response rates; these would have been more influential had individual fish been the sample units. The logistical constraints of collecting and maintaining hundreds of animals was also a consideration.

Assuming the verity of the present findings and the absence of other complicating factors, the lack of a johnny darter response to orangethroat darter extract suggests that the alarm substances of the two congeners differ. Similarly, Commens (2000) found that fantail darter retain the sacciform cells but do not exhibit an alarm response to conspecific extracts. Together, the evidence of apparent response losses implies that the evolutionary history of darter alarm substance may be phylogenetically more complex than is the case in the ostariophysan fishes.

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