

**Evidence for the Inheritance of Alarm Substance Recognition in Johnny Darter  
(*Etheostoma nigrum*)**

**ABSTRACT.**—Naïve laboratory-spawned *Etheostoma nigrum* (johnny darter) exhibited a fright response (reduced movement) following exposure to the skin extract of wild conspecifics, indicating that the response is at least partially innate. No response was elicited by exposure to extract from lab-reared conspecifics.

INTRODUCTION

The presence of alarm pheromones in the skin of darters (Order Perciformes, Family Percidae) was discovered by Smith (1979) who found that *Etheostoma exile* and *E. nigrum* (Iowa and johnny darter, respectively) responded to an introduced solution containing exudates of the macerated skin of conspecifics. This reaction is similar (but not homologous) to the better-known “Schreckstoff” alarm pheromone response of ostariophysan fishes (Smith, 1992). Since that initial study, several darter species have been tested for their ability to recognize the alarm substance(s) of conspecifics and congeners (Smith, 1982; Commens and Mathis, 1999; Haney *et al.*, 2001). Work on darters has shown that alarm substance recognition is not always reciprocal among species, and that one species may not respond to its own alarm substance whereas a sympatric species may (Smith, 1979; Haney *et al.*, 2001).

Questions remain about the origin and function of the darter fright response system. Smith (1979) surmised that the “freeze” reaction of darters was adaptive for such cryptic benthic species and likened the response to that of benthic ostariophysan fishes. For ostariophysan fishes, elicitation of the fright response in naïve individuals demonstrates that the response is at least partially innate. However, learning may also be involved, given that some ostariophysan fishes exhibit a fright response to predator odor alone following repeated exposure to a predator odor and alarm substance mixture (Magurran, 1989; Suboski *et al.*, 1990; Mathis *et al.*, 1995), and that this association could be transferred to naïve fish tested together with experienced individuals (Suboski *et al.*, 1990).

The basis of the fright response in percids has not been formally examined (Commens and Mathis, 1999). Given that many darter species remain undescribed (Warren *et al.*, 2000) and that the family is both speciose and phylogenetically complex (Kuehne and Barbour, 1983), determining which species are innately equipped with the fright response, and then whether, and the degree to which, learning shapes these responses, will be of interest to evolutionary ecologists, systematists and conservationists alike. In the present study we test whether the alarm substance reaction is innate or learned using lab-reared naïve *Etheostoma nigrum*. Wild-caught *E. nigrum* exhibit the response (Smith, 1979) as did the wild-caught parents of the individuals we tested (Haney *et al.*, 2001).

MATERIALS AND METHODS

*Fish collection and rearing.*—Approximately 50 *Etheostoma nigrum* were collected from Hinkson Creek, Boone County, Missouri, on 23 February 2000. In the laboratory, these individuals were distributed between two 38-l aquaria which contained aged tap water and coarse gravel substrate similar to that found in Hinkson Creek, and which were maintained under a 12:12 light/dark photoperiod at 22–24 C.

Some of these fish spawned during the first 72 h of holding, and their developing eggs were clearly visible adhered to the substrate. For rearing, we transferred the egg-bearing gravel particles to separate aquaria wherein the eggs hatched and the resulting larvae were raised. The young were initially fed brine shrimp (*Artemia* spp.) nauplii and were gradually switched to adult brine shrimp and bloodworms (*Chironomus* spp.) as they grew. How many fish spawned is unknown; the eggs occurred across about half of the tank bottom, and numbered somewhere between 200 and 500, a quantity which could have derived from a single female (Grant and Colgan, 1983; Kuehne and Barbour, 1983; Carlander, 1997).

*Procedure.*—For testing for alarm substance recognition in the lab-reared fish [subadults; ca. 30 mm total length (TL)], 36 individuals were randomly allocated three each into 12, 38-liter test aquaria on 26 August 2000 and allowed to acclimate for 48 h. The test aquaria had been pre-washed and matched those in which the fish had been reared. All trials were conducted on 28 August 2000 from 12:00–6:00 PM.

Random assignment was used to allocate three of the 12 aquaria to each of the four treatments:

- (1) the procedural control treatment, into which no solution was introduced;
- (2) the scent-blank control treatment, into which 5 ml of aged tap water was injected;
- (3) the lab-reared fish extract treatment, into which we injected 5 ml of aged tap water in which the macerated skin of lab-reared *Etheostoma nigrum* had been soaked; and
- (4) the wild fish extract treatment, into which we injected 5 ml of aged tap water in which the macerated skin of wild-caught *E. nigrum* had been soaked.

The injection of each aquarium was made out of sight of the fish using a syringe joined to a pre-positioned length of rinsed aquarium tubing that emptied into the rear of the tank beside a bubbling airstone that facilitated rapid solution dispersal, as per Commens and Mathis (1999).

*Solution preparation.*—The scent-blank control solution was prepared by stirring an unused scalpel blade in 20 ml of aged tap water for 5 min. The two extract solutions were prepared following the methods used by Smith (1979). The lab-reared fish extract was derived from two mid-sized donor individuals (ca. 30 mm TL), whereas the wild-caught fish extract was derived from a single larger donor (ca. 65 mm TL).

To prepare these extracts, the donors were anesthetized to death in separate washed containers of tricane methane-sulfonate (MS-222) solution and then rinsed thoroughly in deionized water and blotted dry. Fifty shallow vertical cuts were then scored into each flank of the donor individuals using a new scalpel blade, after which the donors were stirred for 5 min in 20 ml of aged tap water. The resulting test solutions were then refrigerated and used within 45 min of preparation.

We followed the rationale of Haney *et al.* (2001) in deciding to anesthetize the donor fish. By using a low euthanizing concentration of MS-222, followed by a rinse in de-ionized water and blotting to dryness, the amount of MS-222 left in the donor tissues was minimized. Of that small quantity, whatever was then transferred to the extract solutions was diluted many times over when the aquaria received the solution injections (5 ml treatment solution vs. 3800 ml aquarium volume). Consequently, anesthesia effects on the test subjects were unlikely.

As in Commens and Mathis (1999) and Haney *et al.* (2001), we used reduction in the activity of our study fish to indicate alarm substance detection. Darters typically move in “hops”; consequently, we tallied for 5 min postinjection the total number of instances that any of the three fish either left the bottom or changed orientation on the substrate. These activity observations were made from a distance of 1 m in a dark room with the aquarium lights on to reduce visibility of the observer (JCV in all cases) to the test subjects.

*Analysis.*—Between-treatment differences in activity level were detected using a one-way analysis of variance (ANOVA) on the replicate movement totals from the 5 min of observation of each tank. Each replicate involved three *Etheostoma nigrum* (different individuals and aquaria each trial); three replicate trials were completed for each treatment; the treatments were the control, lab-reared extract and wild-caught extract solution injections. Tukey’s honestly significant difference (HSD) mean separation procedure was used to detect differences in specific comparisons. The assumption of data normality was assessed using normal probability plots, stem-leaf plots and the Shapiro-Wilk statistic (Zar, 1984; Hatcher and Stepanski, 1994). Bartlett’s test was used to assess homogeneity of variance.

#### RESULTS AND DISCUSSION

Naive laboratory-spawned and -reared *Etheostoma nigrum* differed across the four treatments in their postinjection activity levels ( $F = 10.2$ ,  $df = 3,8$ ,  $P = 0.004$ ). The lab-reared test subjects moved significantly less frequently compared to scent-blank control treatments when exposed to the extract of wild-caught conspecifics to which they had never been exposed, but no less often when exposed to the extract of lab-spawned conspecifics with whom they had been raised (Fig. 1; Tukey’s HSD,  $P < 0.05$ ). There was no significant difference in activity between fish in the procedural control treatments and the scent-blank control treatments (the former wherein test subjects were observed without adding liquid to the aquaria), indicating that our method of treatment solution delivery alone did not prompt the observed response. In overview, these results provide evidence that responding to alarm substance with a reduction in activity is at least partially innate in *E. nigrum*: opportunities for learning were precluded because our test subjects had never been in the presence of a predator, nor had they been injured or handled in ways that would have caused an alarm substance release (Smith, 1979).

The lack of response to fellow lab-reared conspecifics was notable, and we offer three hypotheses

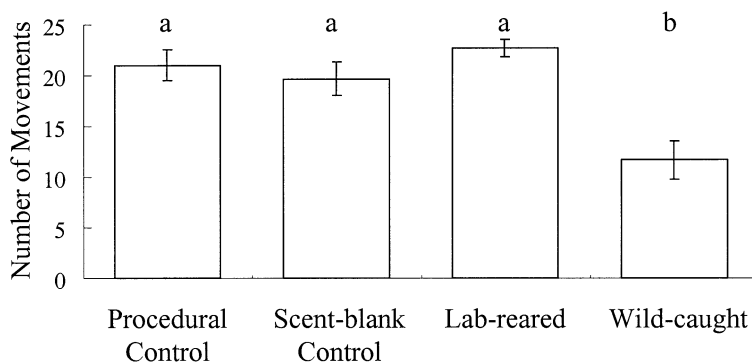


FIG. 1.—The mean ( $\pm 1$  SE) aggregate number of movements made by groups of three *Etheostoma nigrum* in three replicate trials following exposure to one of four treatments: (1) no solution (procedural control), (2) a solvent blank of tap water (scent-blank control), (3) skin extract solution from lab-reared conspecifics, (4) skin extract solution from wild-caught conspecifics. Bars labeled with different lowercase letters indicate significant differences (Tukey's HSD:  $P < 0.05$ )

that merit further investigation. First, our test subjects were young-of-the-year and were approximately half their adult size. Perhaps alarm substance production is ontogenetic and occurs later in life. Alternatively, the substance may be produced in younger fish, but not in quantities sufficient to elicit a conspecific response. Our concern about total skin surface area and the confounding effects of potency (Smith, 1982) explains why we used two lab-reared sub-adults but just one larger wild-caught adult darter when making the donor extract solutions. That species extracts have a threshold potency below which a conspecific fright response is not elicited has been demonstrated in the ostariophysid species *Pimephales promelas* (Lawrence and Smith, 1989). A second possibility is that darter alarm substance contains chemicals that derive from or are found in the fish's diets. Since the diversity of the diets of our lab-reared fish was limited (although the fish grew well), perhaps their alarm substance differed from that of wild fish in quality or quantity. Third, our lab-reared individuals were full sibs (all from the same male and female) or/and half-sibs (single father/multiple mothers; single mother/multiple fathers). In addition, their levels of mutual familiarity were potentially high after having been reared together from hatch. One would be inclined to argue that heightened relatedness or/and familiarity should result in a more pronounced alarm response. However, it might be the case that prolonged mutual exposure in a confined space resulted in habituation, a phenomenon we have not seen addressed in the literature.

We recognize that the sample sizes used in this study may have resulted in a lessening of statistical power. Most importantly, our decision to use fish triads rather than individuals as the experimental unit had a biological basis: three darters atop the substrate within sight of one another seemed a more realistic representation of circumstances in nature than did lone individuals, especially given that social factors (learning or transmission) may be involved. Secondly, our subjects (approximately 40) were limited to those whose parents we knew reacted to conspecific alarm substance.

Much remains to be determined about the origin and transmission of the fright response observed in the percid darters. Our results suggest a heritable component, but the lack of response to lab-reared conspecifics raises new questions about the ontogeny, production and development of the alarm pheromone substance.

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