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Opercular beat rate for rainbow darters *Etheostoma caeruleum* exposed to chemical stimuli from conspecific and heterospecific fishes

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Opercular beat rates of rainbow darters *Etheostoma caeruleum* were used as a measure of a physiological response to chemical stimulation. Rainbow darters responded significantly to some chemical cues (active and ambush predators, competitors, novel stimuli and to conspecific and heterospecific alarm cues) with increases in opercular movements; neutral cues and novel alarm cues did not elicit significant changes. Changes in opercular movements may be a good bioassay for determining detection of chemical stimuli by rainbow darters.

Key words: chemical cues; *Etheostoma caeruleum*; opercular beat rate; rainbow darters.

INTRODUCTION

In natural habitats, fishes are exposed to chemical stimuli from other conspecific and heterospecific fishes. The ability to discriminate between different classes of stimuli (*e.g.* dangerous, attractive and neutral) has important consequences for fitness. Most studies have used behavioural bioassays to test hypotheses about discrimination between stimuli (Wisenden & Stacey, 2004). Preliminary observations of behavioural responses of darters (Etheostomatinae) to chemical cues suggested an apparent increase in opercular movements in response to some stimuli, even when overall movement decreased (Gibson, 2004). The current study tests whether rainbow darters *Etheostoma caeruleum* Storer change their rates of opercular movements in response to chemical stimuli from other fishes, including predators, presumed competitors (conspecific and heterospecific), novel fishes and stimuli from injured conspecific and heterospecific fishes.

Opercular beat rate has been used to provide a measure of response to stress in fishes. Jansen & Greene (1970) saw a sudden and pronounced increase in opercular movements when goldfish *Carassius auratus* (L.) were agitated by an electrical current. Opercular beat rate also increased substantially following

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noxious injections (Sneddon *et al.*, 2003) or exposure to toxic compounds such as copper (James *et al.*, 2003). Predators and competitors also are common stressors to fishes (Lima, 1998), but physiological responses to predation risk or competition have not been well studied. Based on responses to other acute stressors, the hypothesis that fishes increase opercular movements in response to exposure to predators and competitors is reasonable. One of the most common behavioural responses to biotic stressors, however, is reduced movement (Chivers & Smith, 1998), and the alternative hypothesis of decreased opercular movements would be consistent with overall reductions in activity.

MATERIALS AND METHODS

Rainbow darters were collected from the James River in Greene County, Missouri, U.S.A. (37°11′46″ N; 93°07′55″ W), in March 2004. The James River fish community includes an assortment of bass, minnows, shiners and darters. There is localized distribution of four particular species: ozark bass *Ambloplites constellatus* Cashner & Suttkus, duskystripe shiner *Luxilus chrysocephalus* Rafinesque, Arkansas saddled darter *Etheostoma euzonum* (Hubbs & Black) and yoke darter *Etheostoma juliae* Meek. The rainbow darter along with the longear sunfish *Lepomis megalotis* (Rafinesque), the fantail darter *Etheostoma flaballare* Rafinesque and the orangethroat darter *Etheostoma spectabile* (Agassiz) are not only found in the James River Basin but also elsewhere in the state. Therefore, it is a reasonable assumption that rainbow darters would have had experience with an assortment of fishes found in their natural habitat. All stimulus fishes, except bumblebee gobies *Brachygobius doriae* (Günther), were found in the James River Basin.

Rainbow darters were maintained in the laboratory in groups of 25 in 38 l aquaria. They were fed live blackworms *Lumbriculus variegatus* Jamieson daily and were kept at room temperature (range = 15–20° C) on 12L:12D cycle. Aquaria were filtered using Aqua Clear 150 power filters. Because rainbow darters were not usually in breeding condition, sex was not determined.

Stimulus fishes were maintained separately in 9·5 l aquaria containing only an aerator. Although the actual concentration of chemicals in the stimulus water was not known, variation was reduced using fewer numbers of larger stimulus fishes in each tank [see Table I for tank densities and standard lengths (L_S) of stimulus animals]. Bumblebee gobies were the exception to this rule; due to an insufficient availability of these small fishes only two were used per tank. All fishes remained in the laboratory after testing.

CHEMICAL CUES FROM UNINJURED FISHES

Stimuli from uninjured fishes were categorized as predatory or non-predatory (see Table I for tank densities and L_S of stimulus animals). The two predatory species banded sculpin *Cottus carolinae* (Gill) and ozark bass were fed fathead minnows *Pimephales promelas* Cope. Non-predatory species greenside darters *Etheostoma blenniodes* Rafinesque, yoke darters, banded darters *Etheostoma zonale* (Cope), fathead minnows, bumblebee gobies and conspecifics were fed blackworms. Fishes were fed daily until the feeding response slowed. Any remaining food was removed from the tank. All stimulus fishes were fasted for 48 h before the stimulus was extracted. Water in stimulus tanks was changed within 12 h after fasting began to reduce any food residue. A blank control of de-chlorinated water was also used.

CHEMICAL CUES FROM INJURED FISHES

Chemical stimuli from injured fishes were categorized as conspecific skin extracts or heterospecific skin extracts (yoke, banded and greenside darters, fathead minnows and

TABLE I. Species used as chemical stimuli for exposure with rainbow darters. All stimulus fishes were housed separately in 9·5 l aquaria with an aerator. Numbers in tanks were based on relative size or availability

Species	Number in tank	L_S ± S.E. (mm)
Fathead minnow *Pimephales promelas*	4	62·8 ± 4·4
Bumblebee goby *Brachygobius doriae*	2	27·5 ± 1·8
Rainbow darter *Etheostoma caeruleum*	6	51·8 ± 1·1
Banded darter *Etheostoma zonale*	6	52·3 ± 1·7
Yoke darter *Etheostoma juliae*	6	59 ± 1·2
Greenside darter *Etheostoma blenniodes*	3	89·3 ± 2·8
Ozark bass *Ambloplites constellatus*	2	107·5 ± 8·5
Banded sculpin *Cottus carolinae*	2	135 ± 9·9

L_S, standard length.

bumblebee gobies). For rainbow darters, skin extracts of conspecifics and some heterospecifics can act as alarm cues (Commens & Mathis, 1999). Skin extract preparation (IACUC #98H) followed the methods of Commens & Mathis (1999). Donor fishes were killed with a blow to the head. Using a razor blade, 25 shallow vertical cuts were made into the carcass of the stimulus fish. The carcass was then placed in 50 ml of de-chlorinated water and stirred with a stir bar for 5 min, after which the carcass was removed from the water and measured. The solution was placed on ice until use (10 min).

TESTING PROTOCOL

Testing protocol was the same for both sets of experiments. Rainbow darters (48·0 ± 0·86 mm L_S) were placed individually into 700 ml clear glass jars (height: 16 cm, width: 9 cm) containing 500 ml of de-chlorinated water. The relatively small diameter of the jars restricted swimming activity, allowing for accurate observation of opercular movements. Aeration was not provided during the short time frame of the experiment, which may have led to high overall rates of opercular movements; however, this variable should have affected all treatments equally. After a 1 h acclimation period, the number of opercular movements in 2 min was recorded for each individual to serve as a baseline measurement. The randomly assigned chemical stimulus (12 ml) was introduced onto the surface of the water using a syringe. The stimulus diffused through the water column for 30 s, and then post-exposure opercular beat rate was recorded for another 2 min. Order of presentation was not completely random because stimulus fishes were not all available at the same time. Tests occurred in runs of three randomized stimulus treatments (12 darters per treatment × 15 treatments = 180 rainbow darters tested). Each rainbow darter was tested only once.

Because order of stimulus presentation was not fully randomized and temperature differences may have influenced the magnitude of responses by rainbow darters, separate statistical analyses were preformed for each stimulus, using paired *t*-tests ($\alpha = 0{\cdot}05$) on baseline *v.* post-exposure data.

RESULTS

CHEMICAL CUES FROM UNINJURED FISHES

Opercular movements increased significantly (*t*-tests) following exposure to stimuli from both species of predatory fishes (banded sculpin: d.f. = 11, $P < 0{\cdot}05$ and ozark bass: d.f. = 11, $P < 0{\cdot}01$; Fig. 1), and to stimuli from yoke darter (d.f. = 11, $P < 0{\cdot}05$; Fig. 2), banded darter (d.f. = 11, $P < 0{\cdot}05$; Fig. 2) and bumblebee goby (d.f. = 11, $P < 0{\cdot}05$; Fig. 2). Exposure to stimuli from conspecifics, fathead minnow, greenside darter and to the blank control did not significantly (*t*-tests) influence opercular movements [conspecifics: d.f. = 11, $P > 0{\cdot}05$, fathead minnow: d.f. = 11, $P > 0{\cdot}05$, greenside darter: d.f. = 11, $P > 0{\cdot}05$ (Fig. 2) and blank: d.f. = 11, $P > 0{\cdot}05$ (Fig. 1)].

CHEMICAL CUES FROM INJURED FISHES

Opercular movements significantly (*t*-tests) increased following exposure to skin extracts from injured conspecifics, yoke darters, banded darters and fathead minnows (conspecifics: d.f. = 11, $P < 0{\cdot}01$, yoke darter: d.f. = 11, $P < 0{\cdot}001$, banded darter: d.f. = 11, $P < 0{\cdot}05$ and fathead minnow: d.f. = 11, $P < 0{\cdot}01$; Fig. 3). Although opercular rates also showed an average increase following exposure to chemical stimuli from injured greenside darters and bumblebee gobies, this difference was not significant (greenside darter: d.f. = 11, $P > 0{\cdot}05$ and bumblebee goby: d.f. = 11, $P > 0{\cdot}05$; Fig. 3).

Because the non-significant results are important to the overall interpretation of the data, *post hoc t*-tests (with Bonferroni corrections) were preformed to confirm that the responses to greenside darters and bumblebee gobies differed from the responses to the other darters. The significant increases in opercular movement following exposure to stimuli from conspecifics and banded and yoke darters averaged 35% whereas the per cent change was 15% to stimuli from greenside darters and bumblebee gobies. These differences were significantly different (greenside darter: d.f. = 17, $P = 0{\cdot}01$ and bumblebee goby: d.f. = 20, $P < 0{\cdot}05$, $\alpha = 0{\cdot}025$).

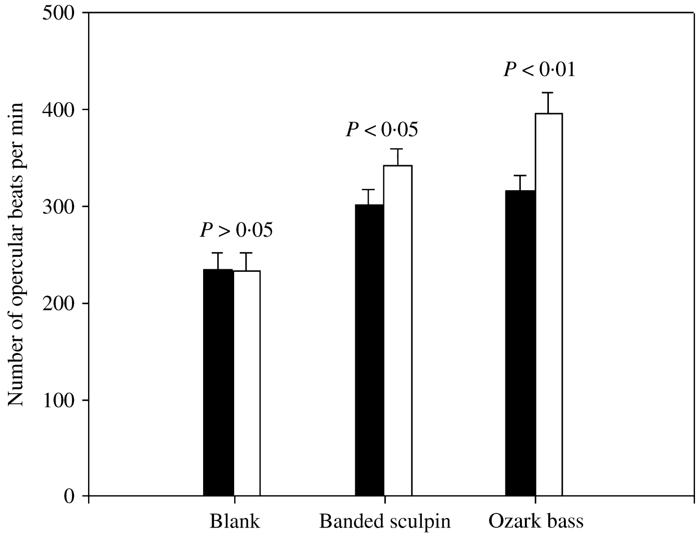

FIG. 1. Mean ± S.E. number of opercular beats in 1 min by rainbow darters before (■) and after (□) exposure to chemical stimuli (water) from tanks housing predatory fishes and to a blank control. *P* values are for two-tailed paired *t*-tests, $n = 12$.

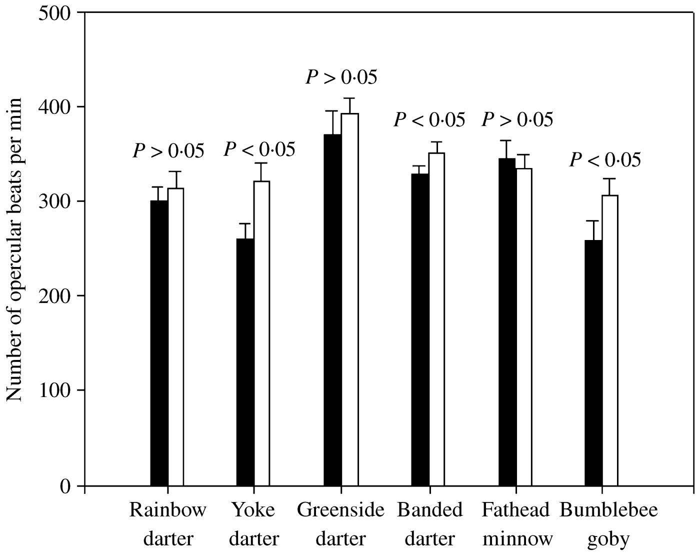

FIG. 2. Mean ± S.E. number of opercular beats in 1 min by rainbow darters before (■) and after (□) exposure to chemical stimuli (water) from tanks housing conspecific and heterospecific non-predatory species of fishes. *P* values are for two-tailed paired *t*-tests, $n = 12$.

DISCUSSION

Are there adaptive consequences to increased opercular beat rates following exposure to chemical stimuli from conspecifics and heterospecifics? Two possible functional explanations are proposed. First, increased opercular activity can

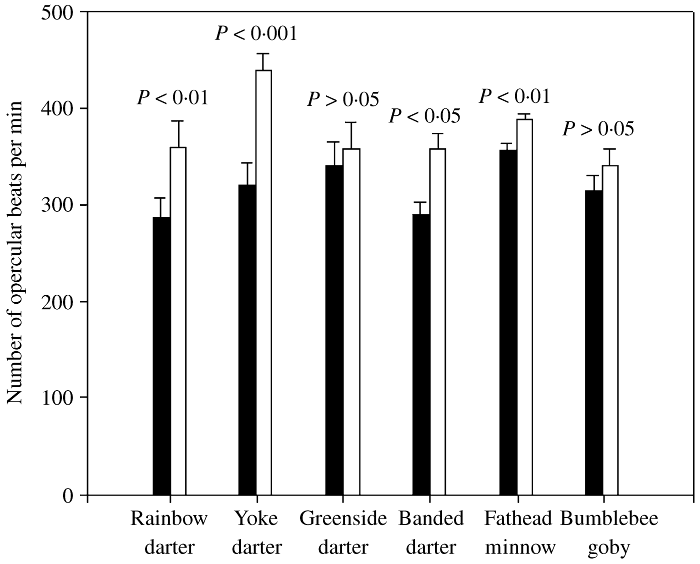

FIG. 3. Mean ± S.E. number of opercular beats in 1 min by rainbow darters before (■) and after (□) exposure to chemical stimuli from injured conspecific and heterospecific fishes (potential alarm cues). *P* values are for two-tailed paired *t*-tests, $n = 12$.

lead to increased oxygen consumption (Dalla Valle *et al.*, 2003), which could be preparatory to flight, offensive or defensive movements, or exploratory behaviour (Rottmann *et al.*, 1992). Second, increased opercular movement could increase water flow towards the head and olfactory epithelia, potentially increasing the efficiency of chemoreception while allowing the fish to remain inactive and retain the benefits of crypsis (Wisenden & Chivers, 2006).

Because increased opercular beat rates were detected in a variety of contexts, use of opercular beat rate as an assay for chemoreception is limited. For example, it cannot be determined whether rainbow darters distinguished between predatory stimuli and novel stimuli because both yielded similar increases in opercular beat rates (*e.g.* ozark bass = 20% increase and bumblebee goby = 25% increase). In this respect, use of opercular beat rate as a bioassay may be analogous to the tongue-flick assay used by researchers who study squamate reptiles (snakes and lizards). Because squamates can show similar increased tongue-flick rates in response to a variety of chemical stimuli, increased tongue flicking is interpreted only as evidence of 'interest' in the stimuli (Mason *et al.*, 2003). Although the functional interpretation of increased opercular beat rate in fishes should be made with caution, differences in opercular rates following exposure to different stimuli can be clear evidence of discrimination.

Strong increases in opercular movements followed exposure to cues from both ambush (banded sculpin) and active predators (ozark bass). Therefore, increased opercular rates can now be added to the list of documented behavioural responses of fishes to predators (Kats & Dill, 1998). It is not likely that the increased opercular movements resulted directly from increased overall activity. Although swimming behaviour in this study was not quantified, focal rainbow darters were fairly sedentary in the observation chambers, and decreased rather than increased activity is one of the most common responses of darters to predatory stimuli (Smith, 1979, 1982; Wisenden *et al.*, 1995; Commens & Mathis, 1999; Haney *et al.*, 2001).

Exposure to chemical cues from both banded and yoke darters also led to increased opercular movements. Rainbow darters may view these two species as competitors for food or habitat. Both species are sympatric with rainbow darters and consume similar prey (Pflieger, 1997). Banded darters are known to be particularly aggressive towards other species of darters (Gray & Stauffer, 2001), and typically are found in similar microhabitats (*e.g.* between rocks) as rainbow darters (Welsh & Perry, 1998). Surprisingly, stimuli from greenside darters did not elicit a significant response even though they also co-occur with rainbow darters and consume similar invertebrate prey (Pflieger, 1997). The potential for competition between greenside and rainbow darters, however, may be reduced by differences in microhabitat: rainbow darters occupy deeper water with smaller rocks and faster velocities, and greenside darters tend to forage on top of rocks, while rainbow darters tend to forage between the rocks (Welsh & Perry, 1998).

Rainbow darters increased opercular beat rates following exposure to stimuli from bumblebee gobies. The Gobiidae has no native species in North America, and the increased opercular movements may represent a functional response to novel stimuli. Novel stimuli often elicit increased activity and exploratory behaviour from fishes (Hugie *et al.*, 1991; Gelowitz *et al.*, 1993), and increased opercular movements are consistent with this response.

There was no change in opercular movements in response to chemical cues from non-damaged conspecifics, fathead minnows or to the blank control. Therefore, the increases in rates of opercular movements to the other species were not general disturbance responses or a general response to stimuli from other fishes.

In the experiments using cues from injured conspecifics, rainbow darters increased their opercular beat rates following exposure to stimuli from yoke and banded darters. The response to chemicals from injured conspecifics and yoke darters are consistent with the results of previous behavioural activity assays (Commens & Mathis, 1999; Commens, 2000). This is the first report that rainbow darters respond to alarm cues from banded darters. Cross-species reactions to alarm cues have been reported for darters in other studies (Smith, 1982; Commens & Mathis, 1999; Commens, 2000; Haney *et al.*, 2001), indicating that: (1) there is some degree of homology across the family and (2) darters respond to alarm cues produced by other members of the same prey guild.

Rainbow darters failed to respond to chemical cues from injured greenside darters. As discussed above, greenside darters differ somewhat in microhabitat from rainbow darters, so damage-released cues of greenside darters may not be a reliable indicator of danger. Conversely, the lack of a cross-reaction between the two species may indicate an evolutionary change in the molecular make-up of the cue.

Rainbow darters also increased opercular movements following exposure to alarm cues from fathead minnows (Smith, 1992). Because darters and minnows are not closely related taxonomically, and because there are some differences in the putative cells that are the source of the alarm cue (Smith, 1979), it seems unlikely that this response represents an evolutionary homology. Therefore, the most likely explanation of this cross-reaction is that danger to minnows is a good indicator of danger to darters. Other studies have documented behavioural responses to alarm cues from fathead minnows by taxonomically distant members of the same prey guild (Mathis & Smith, 1993; Wisenden *et al.*, 1997). Such within-guild cross-reactions can be learned (Pollock *et al.*, 2003).

In agreement with other studies using activity assays (Commens & Mathis, 1999; Commens, 2000), rainbow darters did not respond to damage-released chemicals from bumblebee gobies, indicating that the positive results were not merely a result of disturbance or response to blood or other general by-products of tissue damage. It is interesting that rainbow darters increased their opercular movements in response to stimuli from non-injured bumblebee gobies but not from injured bumblebee gobies; the reason for this difference is unknown.

Opercular beat rate as an assay for chemoreception alone is limited due to the responsiveness in a variety of contexts. This behaviour, however, appears to be another reliable response in predator recognition, exploratory behaviour and as a physiological tool for studying discrimination abilities in darters.

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