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RAPID COMMUNICATION

Sexually Mature Cuttlefish are Attracted to the Eggs of Conspecifics

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Abstract Cuttlefish typically are solitary, but form aggregations to spawn. We tested the hypothesis that bioactive factors in the eggs of conspecifics may facilitate the formation of spawning groups of *Sepia officinalis*. Cuttlefish detected odors from cuttlefish eggs, resulting in an increased ventilation rate. Extracts from female ovaries induced the largest increase in ventilation rate, suggesting that this organ could be a potential source of the bioactive chemicals. In y-maze assays, sexually mature, but not subadult, cuttlefish, were attracted to odors of cuttlefish eggs. These data suggest that *Sepia* eggs could be a source of reproductive pheromones.

Key Words Chemoattraction · Reproductive behavior · Spatial orientation · Invertebrate · Mollusk

Introduction

Many species of decapod cephalopods (cuttlefishes, squids) form large, near-shore aggregations for spawning, and lay their eggs in large, communal masses (Hanlon and Messenger 1996). The mechanisms that facilitate this behavior are largely unknown. Cuttlefishes typically are solitary or semi-solitary and semelparous; spawning occurs inshore when they are 1 year old to 2 years old.

Chemical attractants facilitate reproduction in many mollusks (Susswein and Nagle 2004), and cuttlefish are capable of chemoreception over a distance (Budelmann et al. 1997). Here, we tested the hypothesis that *Sepia officinalis* eggs could provide a source of chemoattractants (Zatylny et al. 2000). First, we evaluated whether cuttlefish detect odors from freshly laid eggs, measuring ventilation rate in a bioassay (Boal and Golden 1999). This method has been used previously to show that cuttlefish detect a wide range of odors, including those of food (Boal and Golden 1999). Second, we recorded ventilation responses to eggs laid ~36–48 h previously, and to extracts of female reproductive organs, to determine the potential source(s) of bioactive factor(s). Third, we investigated whether cuttlefish were attracted to egg odors when presented in a simple y-maze.

Methods and Materials

Subjects were first generation *S. officinalis* (National Resource Center for Cephalopods, Galveston, Texas). Sexual maturity was determined behaviorally; sex was confirmed by necropsy. Housing and experimental tanks were interconnected within a 57,000-l marine system of recirculating artificial seawater (ASW; salinity 34±2 ppt, temperature 17.5±1.5°C; Hvorecny et al. 2007).

Ventilation rate Trials were conducted in Plexiglas tanks (38.1×19.6×26.7 cm, 18.5 l) with flow rates of 91±3.8 ml/sec (Boal and Golden 1999). In dye tests, red food coloring almost instantly spread evenly throughout the tank, with no color remaining after ~10 min. Subjects were sub-adults (5–7 mo post-hatching, approx. 9.0–13.0 cm mantle length) for

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tests of newly laid eggs. In the remaining trials, these same subjects were sexually mature adults (eight female, six male; 8–10 mo post-hatching).

A single cuttlefish was placed into an experimental tank and allowed to settle (typically 20–30 min). After this, ventilations were counted for 20 sec. in each minute. If the number of ventilations was steady for 5 consecutive min., the sampling period was increased to 30 sec. If the number of ventilations remained steady for a further 5 min., an odor sample was added to the water supply line and ventilations were counted (30 sec/min) for 10 min. Changes in ventilation rate (VR: ventilations/min) were calculated by subtracting the average VR, in the 5-min. period before addition of odor, from peak VR in the 5 min. after addition of odor. Changes in VR among treatments were compared by using a single Friedman two-way analysis of variance by ranks, followed by multiple comparisons tests.

Y-Maze The y-maze base compartment (49×63×20 cm) was connected to two arms (each 24.5×70×20 cm), separated by a 60-degree angle, with a perforated gate between the base and arms (Boal and Marsh 1998). During trials, the sides and top of the maze were covered with dark towels. Cuttlefish were viewed in mirrors beneath the transparent maze floor. Cuttlefish rarely looked down toward the mirrors.

The y-maze was filled to a depth of 15 cm; seawater entered the distal ends of the arms and drained at the opposite end of the maze. During trials, odor solutions were dripped into the seawater inflow (1 drop/sec) using intravenous solution bags. It took 1.5 min (early trials) or 1 min (later trials) for control dye to travel from the inflow to the drain. Trials typically were separated by many hours, and the location (left/right) of odors added to the maze was randomized.

At the trial start, one cuttlefish was placed in the base compartment. After a 30-sec delay, the odor drips were switched on. After a further delay (1.5 min, early trials; 1 min, later trials), behavioral monitoring began. Once the cuttlefish reached the behavioral criteria (see below), an overhead light was switched on and the gate raised, allowing it access to the y-maze arms. A cuttlefish was considered to have made a choice when its entire body entered one maze arm. Two criteria were used to ensure that a cuttlefish was exposed to odors from each maze arm before it gained access to the arms: it had to travel to both sides (left and right) of the base compartment, and then be positioned at least half-way back in the base compartment so that it would not move simply into the nearest maze arm once the gate was raised.

The first group of cuttlefish (N=15; eight females, seven males) was tested at 7- and 9-mo post-hatching; the second group (N=16; six females, ten males) was tested at 11-mo post-hatching. Not all cuttlefish responded by choosing an arm.

Odor preparation Sepia officinalis eggs (fresh <1 h, N=10; old=36–48 h, N=16) and reproductive tract organs from two anesthetized individuals were frozen on dry ice. Eggs (individually), ovaries (30 g, 90 g), nidamental glands (50 g, 46 g), and accessory nidamental glands (3.6 g, 7.0 g) were extracted and purified on C18 Sep-Pak Plus cartridges, as previously described (Buresch et al. 2003). Peptides and proteins were eluted with 5 ml (eggs) or 15 ml (reproductive organs) of 50% acetonitrile/0.1% heptafluorobutyric acid. The eluates (new eggs: 50 ml; old eggs: 80 ml; ovaries: 45 ml or 135 ml; nidamental glands: 60 ml or 75 ml; accessory nidamental glands: 15 ml or 30 ml) were pooled, and divided into 1-ml aliquots (eggs) or ten equal aliquots (organs), and lyophilized.

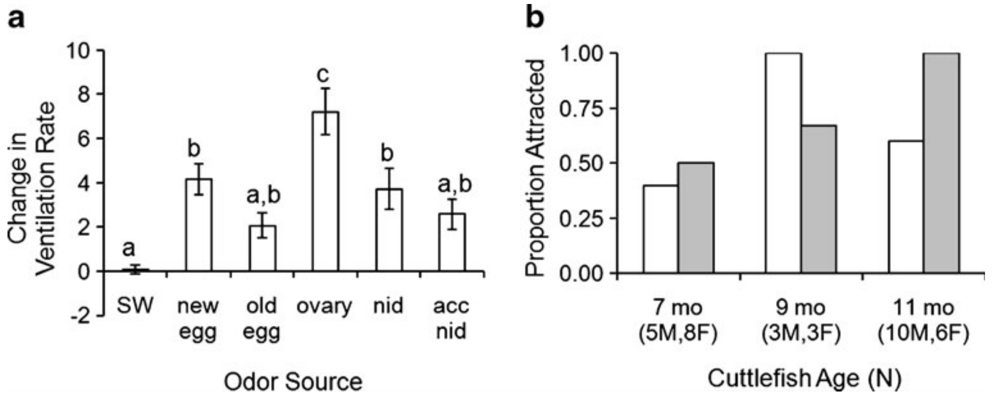

Fig. 1 *Sepia officinalis* responses to various extracts of eggs and reproductive tissue. **a** Ventilation responses (±SEM) to purified extracts of freshly laid eggs (new egg), 36–48 h-old eggs (old egg), ovary, nidamental glands (nid), and accessory nidamental glands (acc nid), or seawater controls (SW). **b** Attraction of males (*open bars*) and females (*shaded bars*), of different ages, to purified extracts of 36–48 h-old eggs. Different letters atop bars indicate means that were significantly different ($P<0.05$; Friedman analysis of variance by ranks)

In all experiments, fresh ASW was a control. ASW (100 ml) containing a mashed shrimp, then filtered to remove particulates, was the food control. Odor samples were prepared by re-suspending 1 aliquot of gland or egg extract in 15 ml ASW (VR experiments) or 20 ml ASW (attraction experiment). In each trial, 5 ml (VR experiments) or 20 ml (attraction experiment) of the resulting suspension were used.

Results and Discussion

Cuttlefish increased their ventilation rate in response to odors from recently laid eggs, extracts of ovary, and nidamental glands, but not to odors from older eggs or extracts from accessory nidamental glands (Friedman analysis of variance by ranks, $Fr=31.43$, $N=14$, $k=6$, $P<0.001$; Fig. 1a). Responses to extracts of ovaries were significantly greater than responses to all other odors tested, suggesting that the stimulatory chemicals in eggs might originate there.

In the y-maze, more sexually mature cuttlefish were attracted to the arm containing purified extracts of freshly laid eggs than to the arm containing only ASW (16 of 22 attracted, $X^2=4.54$, $df=1$, $P<0.05$; Fig. 1b; c.f., food control, four of 13 attracted; ASW control, 7 to left arm, 6 to right arm), indicating their attraction to odors from eggs. Previously, Zatylny et al. (2000) isolated a peptide from eggs of S. *officinalis* that stimulated contractions of the female genital tract. Although we do not know whether this compound is the same or related to the compound(s) that mediate VR rate and attraction in our study, it is likely that eggs contain a number of bioactive factors (Susswein and Nagle 2004) that stimulate different responses from cuttlefish.

Our results indicate that in *Sepia*, as in *Loligo* (Buresch et al. 2003) and *Aplysia* (Painter et al. 1998), eggs are a source of pheromones that play a role in coordinating essential reproductive behavior.

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