



Ink is a conspecific alarm cue in the Caribbean reef squid, *Sepioteuthis sepioidea*

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ABSTRACT

Animals often respond to attacks from predators with behaviors directed at the predator that increase the probability of escape. Some animals also respond to attacks with behaviors that release signals or cues that are detected by conspecifics and enhance escape. Cephalopods, with their complex nervous systems, produce many behaviors, one of which is ejecting ink when attacked by predators. Ink is thought to enhance the ability of the inking cephalopod to escape from the predator, which is a reasonable idea but is lacking experimental support and description of mechanisms. Inking might also serve as an alarm cue, evoking alarm responses in conspecifics. The aim of our study was to determine if ink of the Caribbean reef squid, *Sepioteuthis sepioidea*, is a conspecific alarm cue. We studied freely behaving animals in the laboratory to determine if they produced alarm or escape behavior in response to ink. We observed that squid responded to conspecific ink introduced into their aquarium with deimatic behaviors containing chromatophore elements ('eye spots', 'all brown', and 'flashing'), cryptic behaviors with chromatophore elements ('clear', 'plaid', 'dark arms'), cryptic behaviors with postural responses ('sargassum', 'bad hair'), and protean behavior ('fin movement', 'inking', 'jetting'). Squid also responded to ink introduced into an adjacent aquarium within view, thus demonstrating that vision can contribute to the alarm response. Melanin-free ink did not evoke a significant alarm response. This is the first experimental demonstration that squid respond to ink of conspecifics as a visual alarm cue. Ink may also provide a chemical alarm cue for *S. sepioidea*, though further behavioral studies are needed to explore this.

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1. Introduction

Animals respond to predatory attacks with distinctive behaviors, signals, or displays. Some responses are directed toward the predator and function to deter the attack. Responses may make the animal cryptic or may signal warning or aggression, and may operate through any sensory channel, including visual, auditory, chemical, electrical, or others (Adamo and Hanlon, 1996; Bradbury and Vehrencamp, 1998; Brown et al., 1999; Wyatt, 2003; Murphy, 2006; Glaudas and Winne, 2007). Reactions may also be directed at or responded to by conspecifics, evoking escape responses and thus reducing their risk of predation (Smith, 1992; Seeley, 1995; Dicke and Grostal, 2001). Such stimuli can also be mediated by any sensory channel (Blum, 1985; Lima and Dill, 1990; Bradbury and Vehrencamp, 1998; Chivers and Smith, 1998; Wyatt, 2003). Depending on whether the stimuli benefit the sender and/or receivers, they are defined as signals or cues (Smith, 1992; Bradbury and Vehrencamp, 1998). If they are actively released and benefit both the sender and receiver, such as alarm pheromones

from social insects, they are considered signals. If they are passively released and benefit the receiver without clearly benefiting the releaser, such as blood oozing from a wounded animal, they are considered cues.

The coleoid cephalopods, which include squid, cuttlefish, and octopus, provide striking examples of complex responses to threats and attacks from predators. Predators of cephalopods include mammals, birds, fish, and other cephalopods (Wood et al., 2003), which are mostly active vertebrates with well developed senses and motile capability. Since coleoid cephalopods have internalized or lost their protective ancestral molluscan shell (Packard, 1972), they no longer have this means of physical protection and have evolved other defensive mechanisms. One set of defensive mechanisms involves the evolution of complex nervous systems that are adapted for exquisite sensing of predators in their environment and responding with fast and complex avoidance behaviors (Budelman, 1995). For example, their visual systems are highly developed, with large eyes and optic lobes (Young, 1963; Messenger, 1991; Hanlon and Messenger, 1996). They can detect differences in polarized light (Shashar and Hanlon, 1997), although they do not have color vision (Hanlon and Messenger, 1996). Cephalopods also have sensitive mechanosensory systems that include a lateral line analogue, enabling them to detect mechanical disturbances in the water (Corner and Moore, 1980; Hanlon and

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Table 1

Behaviors observed in this study, with a description of the associated body patterns, postural components, and movement, and the value given to each in quantification of responses

Behavior	Description	Value
Chromatophore Elements		
Eye spots (Deimatic)	Distinct eye spots on fins; either one pair or two; distinct from overall body patterns, such as Plaid	1
Clear (Cryptic)	Body becomes translucent; lasting for >1 sec	1
All Brown (Deimatic)	Single dark brown color over entire body, lasting >1 sec	1
Plaid (Cryptic)	Vertical stripes on a lighter background, lasting >1 sec	1
Dark Arms (Cryptic)	Only arms and tentacles are a black or dark brown color, while rest of body is lightly-colored or clear	1
Flashing (Deimatic)	Brief rapid color change, lasting <1 sec	1
Postural Elements		
Sargassum (Cryptic)	Arms separated into two distinct groups; ca. 80° apart, extended diagonally outward from mantle	1
Bad Hair (Cryptic)	Arms individually separated, oriented toward surface while in a crinkled or curlieue arrangement	1
Movement Away		
Fin movement (Protean)	Slow to moderate movement using fins	1
Jetting (Protean)	Use of jet propulsion to move	2

Budelmann, 1987). Their chemosensory abilities are more poorly understood, but there are indications that they can be used for defense and other functions (Gilly and Lucero, 1992; Lucero et al., 1994; Hanlon and Messenger, 1996; Boal and Golden, 1999).

Cephalopods' sensory abilities provide information that enables them to quickly process environmental information and produce quick and coordinated responses (Budelmann, 1995). They can change shape, color, and texture, through their complex skin structure that includes chromatophores, leucophores, iridophores, and papillae (Hanlon and Messenger, 1996; Mather, 2004). They produce a variety of antipredatory behaviors (Packard, 1972; Hanlon and Messenger, 1996; Messenger, 2001; Hanlon, 2007), some of which are summarized in Table 1. One type is crypsis, which can involve changes in coloration, texture, and body shape. Crypsis is thought to be cephalopods' primary defense against predators (Hanlon and Messenger, 1996). A second type of response is deimatic behaviors, which include actions or body patterns that threaten, startle, frighten, or bluff predators (Hanlon and Messenger, 1996; King and Adamo, 2006). Demonstration of a role of deimatic behaviors in escape from predators comes from a laboratory study of cuttlefish, in which deimatic behaviors were only observed in trials with a simulated predator, not in trials without this stimulus (Adamo et al., 2006). A third type is protean behaviors, which include unpredictable erratic escape behavior such as jetting and inking, or less intense fin movement. Inking involves the ejection of a mass of black chemicals that can take different forms. It may be a diffuse plume, a gelatinous mass called a pseudomorph, or intermediate forms (Shimek, 1983; Anderson and Mather, 1996; Hanlon and Messenger, 1996; Bush and Robison, 2007).

Cephalopod ink is typically thought to have a defensive function through its effect on visual attacks by predators (Bush and Robison, 2007). Ink may act as a smokescreen behind which the cephalopod can hide or escape from a predator (Adamo et al., 2006), or as a decoy that attracts the attention of the predator (Caldwell, 2005), or as some unknown stimulus that startles or distracts the predator. Molluscan ink has been hypothesized to act against predators through the chemical pathways using deterrent chemicals, sensory disruption, or phagomimicry (Derby, 2007). Some of these suggestions stem from the presence of millimolar concentrations of amino acids in cephalopod ink (Derby et al., 2007). (See Table 2 for amino acid content of ink of *S. sepioidea*). However, the evidence for a defensive function of ink, either visually or chemically, against predators is almost entirely observational or anecdotal (Eibl-Eibesfeldt and Scheer, 1962; MacGintie and MacGintie, 1968; Fox, 1974; Moynihan and Rodaniche, 1982;

Shimek, 1983; Hanlon and Messenger, 1988, 1996; Anderson and Mather, 1996; Grüniger, 1997; Forsythe et al., 1999; Hanlon et al., 1999; Caldwell, 2005). For example, no experiments have been performed using cephalopods devoid of ink to demonstrate that ink aids in their escape as has been done in other inking molluscs (Nolen et al., 1995; Kicklighter et al., 2005).

Another possible antipredatory mechanism of ink is as an alarm signal for conspecifics. Ink might act as a visual alarm cue, though there is no experimental evidence supporting this. The case for chemical alarm signaling in cephalopods receives strongest support from laboratory studies of the squid *Doryteuthis opalescens*, in which animals immobilized in a dish increase the frequency of jet escape responses when conspecific ink or one of its components, L-dopa, was pipetted directly into the squid's mantle cavity next to the olfactory organ (Gilly and Lucero, 1992; Lucero et al., 1994). The cuttlefish *Sepia officinalis* increased its ventilation rate following chemoreception of conspecific ink and could thus detect it, but whether or not ink is an alarm cue was not tested (Boal and Golden, 1999).

The aim of our study was to examine if ink is a conspecific alarm cue in the Caribbean reef squid *S. sepioidea*. During the day, these squid aggregate in shallow coastal waters. They are gregarious and appear to have a specialized visual communication system and flexible but ordered social grouping (Moynihan and Rodaniche, 1982). Thus, *S. sepioidea* is a likely candidate among the cephalopods to use ink as an alarm cue. We performed laboratory experiments on freely behaving animals, to determine if animals respond with alarm or escape behavior – color changes, protean or deimatic behaviors – to ink, and if so, whether the responses are mediated through its vision, chemoreception, or both. Our results show that squid respond to conspecific ink as a visual alarm cue.

2. Materials and Methods

2.1. Animals

Caribbean reef squid, *Sepiotheuthis sepioidea* (Blainville, 1823), were collected in local waters using seine and hand nets per Bermuda Government Permit No. SP051002. Squid weighed 43.7 ± 17.0 gm (mean \pm S.D.) and had a ventral mantle length of 68 ± 14 mm (mean \pm S.D.) upon capture. Each squid was tagged with visual implant elastomer (Replinger and Wood, 2007) so they could be individually identified. Squid were fed an *ad libitum* supply of live

Table 2

Free Amino Acid Composition of Ink of Caribbean reef squid, *Sepiotheuthis sepioidea*

Chemical	Conc (μM)	Relative Conc (% total)
Taurine	17207	34.7
L-Aspartic acid	858	1.7
L-Threonine	804	1.6
L-Serine	888	1.8
L-Asparagine	57	0.1
L-Glutamic acid	1170	2.4
L-Glutamine	104	0.2
L-Proline	8013	16.2
L-Glycine	10650	21.5
L-Alanine	5750	11.6
L-Valine	611	1.2
L-Cystine	80	0.2
L-Methionine	503	1.0
L-Isoleucine	540	1.1
L-Leucine	978	2.0
L-Tyrosine	40	0.1
L-Phenylalanine	448	0.9
L-Tryptophan	8	0.0
L-Lysine	444	0.9
L-Histidine	93	0.2
L-Arginine	344	0.7
TOTAL	49590	100

locally collected silversides, including hogmouth fry *Anchoa choerostoma*, blue fry *Jenkinsia lamprotaenia*, rush fry *Allanetta harringtonensis*, pilchard *Harengula humeralis*, and anchovy *Sardinella anchovia*. An individual squid was used for approximately 5–10 days in trials. In our holding conditions of 23.7 ± 2.1 °C, squid had an instantaneous relative growth rate (Forsythe and Van Heukelem, 1987) of $2.18 \pm 0.89\%$ body mass per day (mean \pm S.D.).

2.2. Collection of ink and preparation of stimuli

Stimuli were selected for use in our study with the aim of determining if ink is a chemical alarm cue, a visual alarm cue, or both. Stimuli included 'frozen ink', 'fresh ink', 'melanin-free ink', and 'seawater', as described below. When introduced into the aquarium with the squid, 'frozen ink' and 'fresh ink', which are colored, provide a chemical and visual stimulus to the squid, and 'melanin-free ink', which is colorless and provides a chemical stimulus only. We also introduced frozen ink into an aquarium located adjacent to the aquarium with the squid ('ink in adjacent tank'), which provides a purely visual stimulus.

Ink was obtained from individual live squid, *S. sepioidea*, with the goal of keeping it as concentrated as possible. An individual squid was placed in a $32 \times 18 \times 20$ cm acrylic aquarium with seawater and induced to release ink by striking the aquarium. Ejected ink in the pseudomorph form is viscous and held together by mucus. This ink was collected using a turkey baster and placed in a finger bowl. Sea water surrounding the ink was then removed using a 10-ml syringe. This ink was then either tested immediately (= 'fresh ink') or kept on ice until approximately 500 ml of ink was collected from 12–20 animals, vigorously stirred, divided into aliquots, and stored in a -70 °C freezer (= 'frozen ink'). Prior to freezing at -70 °C, a portion was set aside for the 'melanin-free ink'. This treatment was used as a colorless chemical stimulus, with the aim of dissociating the chemical and visual components of ink. This stimulus was prepared by centrifuging the ink at $50,000 \times g$ for 2 hr at 4 °C (Russo et al., 2003). This procedure separated a pellet containing the pigmented melanin granules from the colorless, melanin-free supernatant.

There were qualitative differences in the physical nature of 'fresh ink' and 'frozen ink'. The homogenized solution of 'frozen ink' lost its viscous consistency when thawed, and it dispersed more quickly than 'fresh ink' when it was introduced into the aquarium. 'Fresh ink' provided a more cohesive and sustained visual stimulus, similar to an ink pseudomorph.

2.3. Behaviors and their quantification

Responses of squid to stimuli were characterized and quantified using behaviors established in the literature (Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1996; Messenger, 2001; Mather, 2004; Adamo et al., 2006). Behaviors included those associated with changes in chromatophore activity ('eye spots', 'clear', 'all brown', 'plaid', 'dark arms', 'flashing'), posture ('sargassum', 'bad hair'), movement ('fin movement', 'jetting'), or inking (Table 1). These behaviors can also be categorized according to function, as protean, deimatic, or cryptic (Table 1). Protean behaviors are those facilitating escape, and include 'fin movement', 'inking' and 'jetting'. Deimatic behaviors are those that threaten, startle or frighten the predator (Hanlon and Messenger, 1996; Adamo et al., 2006) and include 'eye spots', 'all brown', and 'flashing'. 'Eye spots' is a component of deimatic display and can be superimposed on a variety of lighter body colorations. 'Eye spots' are usually provoked by other species (Moynihan and Rodaniche, 1982). All brown, termed 'dark' by Moynihan and Rodaniche (1982), was reported to be used in moderately alarming situations such as approach by humans or large, non-threatening fish. Flashing is a rapid change or series of changes in body pattern. Cryptic behaviors hide the squid from detection and include 'clear', 'plaid', 'dark arms', 'sargassum', and 'bad hair'.

Behavioral responses were identified from analysis of the video recordings by a person who was experienced in scoring squid behaviors but unaware of the treatments and the details of this experiment. Behaviors were quantified for 1 min after presentation of a stimulus. Each behavior was assigned a value of 1 except for 'jetting', which was received a value of 2 because of its greater energetic costs compared to other behaviors (O'Dor and Wells, 1987; Hanlon and Messenger, 1996). A 'composite behavioral score' was calculated for the response of each animal to each stimulus by adding up the points for all behaviors that occurred during that trial. The scores were analyzed using the non-parametric Kruskal-Wallis tests with the tied ranks correction factor (Zar, 1999). Post-hoc analysis was performed to compare the sea water control vs. each of the ink stimuli, using four Mann-Whitney U tests with a Bonferroni adjusted alpha value of 0.0125. We assessed observer reliability of scoring by having the person who originally scored the videos rescore them in random order several months after the initial scoring. Reliability was very high, as total composite scores deviated by a standard deviation of ± 1.07 between the first and second assessments. Observer reliability was additionally tested with a Spearman's correlation between both sets of scores, which yielded an r^2 of 0.755.

2.4. Experimental testing

The test aquarium was 74 cm long \times 30 cm wide \times 30 cm deep and was surrounded by a blind to prevent squid from being disturbed by unwanted visual stimulation. The aquarium was illuminated from above by two halogen lamps and fluorescent ceiling lights. Squid behavior was recorded from above using a Sony® HDR-HC7 HDV 1080i high definition video camera with an Impact® DVP-SWA38-37 super wide angle converter lens and a Sony® RM-VD1 remote. During the experiment, squid were viewed from behind a screen using either a monitor attached to a small camera or the LCD viewfinder of the Sony® HD camera. Sea water from the Bermuda Institute of Ocean Science's open system flowed into one end the experimental tank at a rate of 5 liter/min through a large siphon and drained from the other end via an excurrent siphon. The experimental conditions – size of the experimental tank and size of animals – were a compromise between providing enough space for animals to move freely and express alarm and escape responses, and restricting animals sufficiently so that all behaviors could be recorded and quantified.

Each trial consisted of placing a squid in the aquarium and allowing it to acclimate for 20 min or until it achieved a behavioral score lower than 3 (see Table 1), whichever was longer. Then, 5 ml of a stimulus – 'fresh ink', 'frozen ink', 'melanin-free ink', or 'seawater' – was released into a second, much smaller siphon (inner diameter of 4 mm) at the

Table 3

Sum of responses from the 12 animals for each of the 11 behaviors to the five stimuli

Behavior	Fresh ink	Frozen ink	Melanin free ink	Ink in adjacent tank	Sea water	TOTAL
Chromatophore Elements						
Eye spots (Deimatic)	10	6	1		5	23
Clear (Cryptic)	3	1	0		4	9
All Brown (Deimatic)	5	3	0		1	10
Plaid (Cryptic)	7	3	2		2	14
Dark Arms (Cryptic)	5	1	0		2	8
Flashing (Deimatic)	6	6	3		6	23
Postural Elements						
Sargassum (Cryptic)	2	0	0		0	2
Bad Hair (Cryptic)	3	1	0		0	4
Movement Away						
Fin movement (Protean)	5	8	3		6	24
Jetting (Protean)	5	1	0		1	7
Inking						
Inking (Protean)	1	0	0		0	1
TOTAL	52	30	9		27	125

upstream end of the aquarium. Behavioral data were collected over a 60-sec period, beginning with introduction of the stimulus. Although most of squid initially reacted in the first seconds, squid also responded beyond this time, and we could not discriminate between continued responses to the initial stimulus or new responses to the prolonged stimulus. Thus, we used 60-sec to insure that all reactions were recorded. The order of the stimulus presentations was random. After each trial, the aquarium was fully drained and wiped clean. If the squid inked before the trial began, the trial was terminated and the aquarium was drained and cleaned before proceeding to the next trial. Each squid was used in only one trial per day, and only once per stimulus.

The aquarium for trials in which ink was presented as a purely visual stimulus was slightly different, for logistical reasons. The ink was introduced within clear view of the squid in a separate smaller acrylic aquarium 28 cm long×17 cm wide×17 cm deep placed lengthwise adjacent to the main aquarium housing the squid. 5 ml of 'frozen ink' was injected into the intake siphon of the adjacent tank. Otherwise, all other conditions were identical to the other trials.

3. Results

The responses of 12 animals to each of the five stimuli were included in our analysis (Table 3). The stimuli evoked all 11 behaviors at least once. Deimatic behaviors with chromatophore elements were most commonly evoked. These are 'eye spots', 'all brown', and 'flashing' (56 total events). Cryptic behaviors with chromatophore elements were also common: 'clear', 'plaid', 'dark arms' (31 total events). Cryptic behaviors with postural responses – 'sargassum', 'bad hair' – occurred, but infrequently (6 total events). Low intensity protean behavior – 'fin movement' – was common (24 total events), but high intensity protean behaviors ('inking', 'jetting') were infrequent: only 1 'inking' event and 7 'jetting' events. The 'inking' event and 5 of the 7 'jetting' events were to 'fresh ink'. Thus, deimatic and cryptic behaviors with chromatophore elements were most commonly evoked in squid. Examples of these responses are shown in Supplemental Videos 1–10 available online at doi: 10.1016/j.jembe.2008.08.004 and The Cephalopod Page <<http://www.thecephalopodpage.org/squideo/index.php>>.

The stimuli evoked different combinations of responses, resulting in different response intensities. This is most obvious from Fig. 1, which shows the median composite behavioral scores to the stimuli.

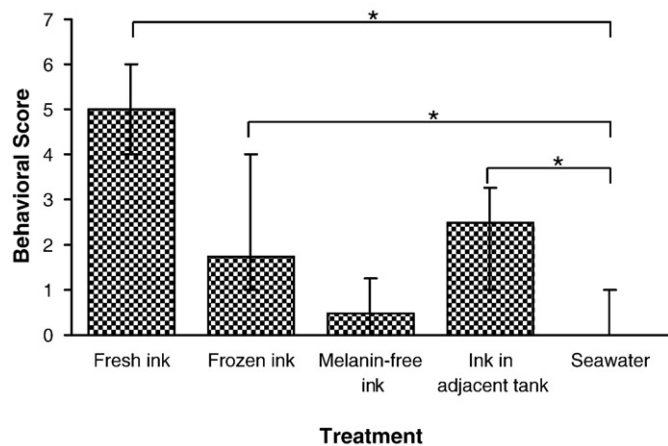


Fig. 1. Responses of squid to ink stimuli. Responses are medians ± interquartile ranges of the composite behavioral scores. N=12 squid, each tested with all stimuli. Horizontal bars topped by an asterisk indicate significant statistical comparisons of ink vs. seawater, using a Kruskal-Wallis test with a tied ranks correction factor followed by post-hoc testing with Mann-Whitney U tests and Bonferroni correction, $\alpha=0.0125$.

There was a significant treatment effect, demonstrating that squid respond differently to these stimuli (Kruskal-Wallis test, $p<0.001$). Post-hoc analysis comparing each of the four ink stimuli to the sea water control (Mann-Whitney U-test, $\alpha=0.0125$) showed that 'fresh ink', 'frozen ink' and 'ink in adjacent tank' evoked significantly greater responses than 'seawater', but 'melanin-free ink did not.'

4. Discussion

Our results show that Caribbean reef squid, *Sepioteuthis sepioidea*, respond to conspecific ink with a variety of behaviors. Responses most commonly produced include deimatic behaviors which threaten, startle, frighten or bluff ('eye spots', 'flashing') and cryptic behaviors with chromatophore elements ('clear', 'plaid', 'dark arms'). Cryptic behaviors with postural elements ('sargassum', 'bad hair') and protean behaviors ('inking', 'jetting'), which are more metabolically costly, were also produced, though less frequently. These behaviors can enhance escape from predators, and thus ink can be considered conspecific alarm cues (Smith, 1992; Dicke and Grostal, 2001). This conclusion is supported by field observations that ink pseudomorphs produced by a member of a school of *S. sepioidea* in response to a predator typically result in squid jetting away from the predator (Wood, pers. obs.). We conclude that squid ink acts as an alarm cue.

The quality of ink influenced its effectiveness as an alarm cue. 'Fresh' or 'frozen ink' introduced to the same aquarium as the squid produced significant alarm responses (Fig. 1, Table 3). These stimuli, which are pigmented due to the presence of melanin (Russo et al., 2003; Fiore et al., 2004), provided both chemical and visual features to the squid, so the modality through which the alarm cues operated was not determined. 'Fresh ink' evoked a qualitatively greater response than 'frozen ink'. 'Fresh ink' also released almost all (5 of the 7) 'jetting' events, which is the most energetically expensive behavior (O'Dor and Wells, 1987), and the single 'inking' event, which requires the use of a limited resource. The greater efficacy of 'fresh' vs. 'frozen ink' might be due to chemical differences, since freezing removes the mucus fraction and any chemicals in it. The difference between 'fresh ink' and 'frozen ink' may also be due to differences in their consistency and visual appearance. 'Fresh ink' is held together by mucus and takes the form of a pseudomorph, which in nature can act as a visual decoy (Caldwell, 2005; Bush and Robison, 2007). This physical difference might account for 'fresh ink's' greater efficacy as an alarm stimulus.

In an attempt to dissociate chemical and visual components of ink, we presented two additional stimuli. Ink added to an adjacent aquarium, which provided a visual but not a chemical stimulus, evoked significant alarm responses when compared with the 'seawater control'. 'Melanin-free ink', which is not pigmented and thus provides a chemical but not a visual stimulus, did not evoke significant responses when compared to the control. Thus, our results demonstrate that ink is a visual alarm signal. Given the keen vision of cephalopods, especially *S. sepioidea* with their large optic lobes (Young, 1963; Hanlon and Messenger, 1996; Messenger, 2001), this is not surprising. Vision also appears to play a role in the intraspecific behavior of other cephalopods (Boal, 2006). Examples including the squid *Loligo pealeii*, where visual, but not chemical, access to egg mops evokes intraspecific aggression by males (King et al., 2003), and the cuttlefish *Sepia officinalis*, where the visual presence of conspecifics affects body patterns (Boal et al., 2004; Boal, 2006; Palmer et al., 2006).

Our experiments do not provide conclusive evidence that ink is a chemical alarm cue, but neither do they rule it out. Squid did not significantly respond to 'melanin-free ink', which would have been the clearest evidence to conclude that ink contains chemical alarm cues. However, the process of collecting 'melanin-free ink' included centrifuging for 2 hr, which removed or altered chemical components such as the mucus fraction of the ink and melanin. In this process, we may have removed alarm cues bound to them. For example, dopamine adsorbs onto melanin granules of ejected cuttlefish ink (Fiore et al.,

2004). Other possibilities are that melanin itself serves as a chemical cue, or that mucus possesses chemical factors eliciting a behavioral response. The processing of ink required freezing, which also may have affected the bioactivity of some alarm cues. Therefore, the lack of a significant response to melanin-free ink does not rule out the possibility of alarm cues in whole ink. Previous studies on *Doryteuthis opalescens* showed that its ink and one compound in ink – L-dopa – elicit jetting in animals secured in test chambers in the laboratory, suggesting the presence of chemical alarm cues in some squid species (Gilly and Lucero, 1992; Lucero et al., 1994). We also know that *S. sepioidea* ink contains substantial amounts of at least one type of chemical that squid are likely to detect – 50 mM total free amino acids (see Table 2). A role for chemical cues in eliciting behavior in other cephalopods is also known (Boal and Golden, 1999; Boal et al., 2000). Determining if ink of *S. sepioidea* contains chemicals that act as alarm cues requires further study.

The alarm response of squid to ink may not be specific to conspecific ink. They may respond with alarm to other dark and looming objects or to the ink of heterospecifics. Squid may have evolved to react strongly to a variety of sources of sudden change in their environment, and conspecific ink is one of these. Testing of a greater diversity of stimuli will be necessary to evaluate fully the specificity of conspecific ink in evoking alarm responses.

Ink provides cephalopods with a defense against predators. Over time, squid that react to ink of conspecifics with alarm responses probably accrue a selective advantage in survival. We believe that inking originally evolved to deter predatory attacks, as a visual decoy and perhaps also as a chemical stimulus, and then secondarily acquired the function of alarm cue. Ink might be considered a signal, since it is a limited resource that is actively released under neural control and may benefit both the sender and receiver. Many organisms including fish assess predation risk using alarm cues from injured conspecifics (Bradbury and Vehrencamp, 1998; Wyatt, 2003; Brown et al., 2004). Responding to alarm cues should be advantageous to the receiver because cues that are released from prey are reliable indicators of increased predation risk. Thus, squid that can identify and appropriately respond to these cues have a survival advantage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jembe.2008.08.004.

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