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CONTACT CHEMOSENSORY CUES IN EGG BUNDLES ELICIT MALE–MALE AGONISTIC CONFLICTS IN THE SQUID *Loligo pealeii*

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Abstract—Male *Loligo pealeii* engage in frequent agonistic bouts to gain access to female mates while aggregated at communal egg beds. Male squids are attracted to eggs in the field and in the laboratory. It was recently demonstrated that visual detection followed by physical contact with egg capsules elicited male–male aggression. We tested specific physical and chemical features of the egg capsules that may cause this strong behavioral reaction. Male squids were presented with either natural or artificial egg stimuli and scored for four selected behaviors (egg touch, egg blowing, forward-lunge grab, and fin-beating), the last two of which are highly aggressive behaviors. First, squids were presented with natural eggs versus eggs sealed in agarose-coated tubes (ESACT), which eliminated both tactile and chemical stimuli. Second, males were presented with natural eggs versus eggs sealed in agarose coated tubes containing C_{18} Sep-Pak-purified extracts (TCPE) from squid egg capsules, which provided chemical cues from natural eggs without the physical stimulus of the egg capsules. Third, natural eggs versus heat-denatured eggs were tested to determine whether the active factor in natural eggs is heat-labile. Squids responded aggressively when contacting natural eggs and TCPE, whereas squids did not respond after touching ESACT or denatured eggs. These results suggest that aggressive behavior is elicited by a heat-labile factor that is embedded within squid egg capsules. This chemosensory cue appears to be a contact pheromone that stimulates the

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agonistic interactions that characterize the mating behavior of migratory squids on inshore spawning grounds.

Key Words—*Loligo pealeii*, squid, chemosensory, pheromone, agonistic behavior, mating behavior, aggression.

INTRODUCTION

Agonistic interactions are costly because of their high-energy expenditure and increased risk of predation (Huntingford and Turner, 1987). Therefore, animals will only engage in such behaviors when the costs are outweighed by some benefit, such as mate acquisition (Archer, 1988). Males of many taxa engage in agonistic encounters to gain access to females [e.g., arthropods (Berrill and Arsenault, 1984); avians, (Hirschenhauser et al., 2000; Johnsen et al., 2001) and lepidopterans, (Kemp and Wiklund, 2001)]. Male loliginid squids engage in such agonistic conflicts when competing for access to females at spawning sites (Hanlon and Messenger, 1996).

Loligo pealeii is a highly mobile species that occurs along the eastern coast of the Americas from Nova Scotia to the Gulf of Venezuela (Summers, 1983). Squids spend the winter months along the edge of the continental shelf in canyons where temperature fluctuations are minimal, and migrate inshore to warmer waters in the spring (Summers, 1983; Black et al., 1987). Inshore, mating is known to occur in aggregations where squids lay their eggs communally, often forming masses of egg capsules (Hanlon, 1998). There is a skewed operational sex ratio at the egg beds (Hanlon, 1998) (2.6 males to 1 female, unpublished data), and males must compete for valuable consortships with females. In this context, agonistic conflicts may influence the reproductive success of individual males.

Cephalopods are useful for studying agonistic behavior because they use distinct, conspicuous body patterning signals for communication during escalating agonistic encounters (Hanlon and Messenger, 1996). In addition, their polygamous mating systems create ample opportunities for agonistic bouts. However, there have been few studies of male–male agonistic behavior in cephalopods (Adamo and Hanlon, 1996; DiMarco and Hanlon, 1997; King et al., in press). DiMarco and Hanlon (1997) found that the presence of a female increased the level of aggression in agonistic conflicts in male *Loligo plei*. Recent work with *L. pealeii* has shown that males engage in frequent agonistic bouts and that egg capsules elicit such aggressive behavior even in the absence of females (King et al., in press).

Male squids are attracted to egg capsules both in the field and laboratory (Arnold, 1962; Hanlon, 1996; King et al., in press). Squids swim up to the eggs with their arms in a cone shape. Male squids touch the egg capsules with their arms, and often manipulate them by spreading their arms over a large number of egg capsules and agitate the eggs with rapid movements. Males will also often "blow on the eggs" by forcing water through their funnel over the eggs, causing them to move gently in the water (unpublished data). King et al. (in press) found that this egg-manipulating behavior elicits male–male aggression in the laboratory. In a series of experiments designed to determine the sensory stimulus necessary to elicit this behavior (i.e., visual, tactile, or chemosensory), they found that vision was important for attraction, but that squids needed to touch the egg capsules to become aggressive. Their results indicated that tactile and/or chemosensory cues may be responsible for the induction of aggressive behavior.

In this paper, we attempt to determine specifically which sensory cue(s) in egg capsules elicits male–male aggression. We designed a series of experiments to isolate various sensory aspects to ascertain whether chemosensory and/or tactile input elicit aggressive behavior in male squids.

In the marine mollusk *Aplysia*, several peptide pheromones that induce mating behaviors have been characterized from egg cordons (Painter et al., 1998, 1999). This prompted us to consider the possibility that similar factors in or on egg capsules may be responsible for the male–male aggression in *L. pealeii*. Therefore, we tested whether contact with C_{18} Sep-Pak-purified extracts from whole-egg capsules would stimulate male–male aggressive behavior in squids.

METHODS AND MATERIALS

Animal Collection and Care. Squids were caught from Vineyard Sound (Falmouth, Massachusetts, USA), with trawls or jigs, during the months of April through September 2001. Trawled individuals were maintained in oval tanks (360 cm long × 240 cm wide × 90 cm deep) with flow-through natural seawater (NSW) at 12°C (salinity ranged from 30 to 32 ppt). Jigged individuals were maintained in a round tank (366 cm diam. × 91 cm deep) with flow-through NSW at 16–18°C. The tanks were exposed to the ambient light cycle (ca. 14 L: 10 D). Individuals were fed 2–4 fish per day in the evening. Healthy animals, with little or no skin damage, were selected for experiments and placed in holding tanks 12 hr prior to behavioral trials.

Experimental Design for Behavioral Trials. Two behaviors—fin-beating and forward-lunge grab (Figure 1)—were used to assess the level of aggression because they were conspicuous, easy to score, and reliable between observers. Both are highly aggressive behaviors; therefore, we were measuring the strongest level of aggression. Fin-beating occurred when two males swam parallel to each other and maneuvered to beat one another's fins (Figure 1A). Fin-beating was generally preceded by some postural and behavioral patterns that we did not score here. The forward-lunge grab was a fast, forward movement in which one male lunged toward another male with arms flared (Figure 1B); in rare cases, this was followed

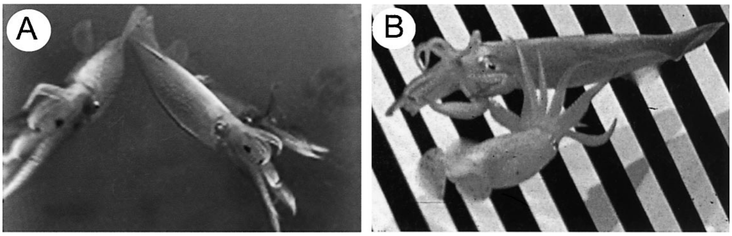

FIG. 1. (A) Two squids engaged in fin-beating. This is an aggressive behavior in which male squids swim parallel to each other and beat one another's fins. (B) A typical forward-lunge grab, the highest level of aggressive behavior. The male in front is lunging with arms flared toward the male in the back. The black and white stripes in the background are painted on the walls of the tank to discourage squids from swimming into the side. Video clips of these highly aggressive behaviors can been viewed at *http://www.mbl.edu/services/MRC/hanlon.html*.

by biting or grappling. Two other behaviors—egg blowing and egg touch—were used to determine the squid's level of interest in the eggs. Egg blowing occurred when squids swam up to an egg bundle and jetted water into it; egg touch occurred when the arms contacted the eggs.

Behavioral trials were conducted in round tanks (3 m diam. × 0.5 m deep) with aerated, flow-through NSW. Temperature was maintained at 16–18°C because previous observations indicated that temperatures above 22°C were associated with a decrease in squid responsiveness to egg capsules (King et al., in press).

Pairs of male squids were selected whose mantle lengths differed by fewer than 2 cm (population range: 12.0–30.0 cm). Individual squids were isolated in tanks for at least 12 hr prior to the trial, one in the trial tank (the resident squid) and another in a smaller holding tank (132 cm long × 76 cm wide × 43 cm deep; intruder) due to space constraints. Trials were conducted with the following protocol (Figure 2A). To begin the trial, the intruder squid and an egg stimulus were added to the resident squid's tank. Data collection began when one of the squids touched the egg stimulus, and the four behaviors were scored for a period of 20 min. Observers were visible to the animals but care was taken to minimize movement. After 20 min, the intruder squid was removed from the trial tank and placed back into the small holding tank; the stimulus was also removed. Squids remained in their individual holding tanks for 4 hr before the second half of the trial began. The second half of the trial was conducted as described above but with a different stimulus (e.g., if natural eggs were used as a stimulus in the first part of the trial, eggs sealed in agarose-coated tubes were used in the second part of the trial). Treatment order was randomized to minimize effects of the time of day since there may be a circadian rhythm to these agonistic behaviors.

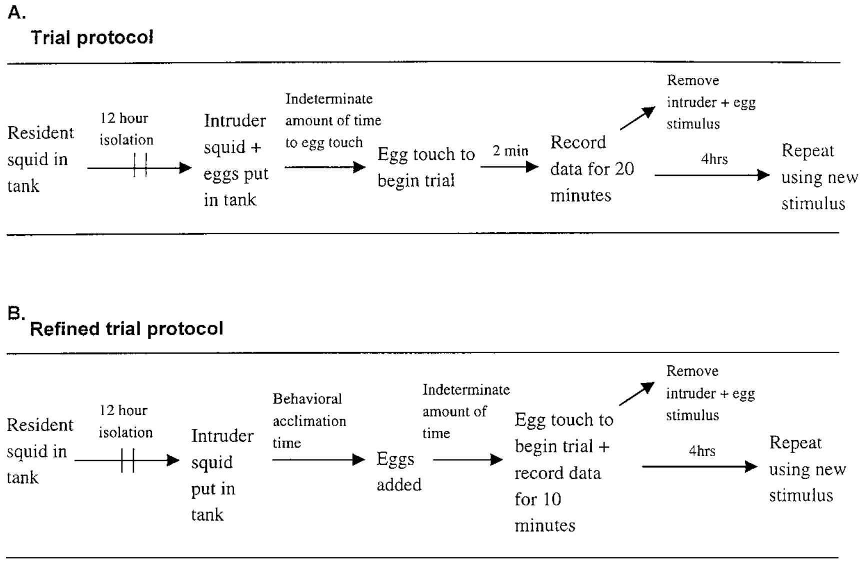

FIG. 2. Protocol A was used in May. The protocol was changed to the one shown in B because of what was learned with the previous protocol; it was used June–September.

The trial protocol was refined (Figure 2B) based on results at the beginning of the study; most trials were conducted using the refined protocol ($N = 50$ of 74). Two differences were noteworthy. First, the egg stimulus and the intruder squid were not added simultaneously. The intruder was added prior to the egg stimulus, and squids were monitored until their behavior reached baseline (i.e., no overt aggression). An egg stimulus was then added to the tank. Second, data were recorded for only 10 min after the first egg touch. To correct for differences in observation time in the two protocols, the data for egg touch, egg blowing, forward-lunge grab, and fin-beating collected from the original trial protocol were divided by two. We found that most of the agonistic behaviors occurred in the first 10 min of data collection; therefore, this should be a conservative estimate.

C_{18} Sep-Pak Purification of Egg Capsule Extracts. Squids were induced to mate and lay eggs by placing day-old eggs into a tank in which they had been isolated for 12 hr. Freshly-laid eggs were collected from several females, frozen on dry ice, and stored at –80°C until extraction. Samples from each egg were individually extracted at 4°C in 20 ml of 0.1% heptafluorobutyric acid (HFBA) using a Brinkmann Polytron homogenizer, and sonicated. Each extract was centrifuged at 48,000*g* for 20 min at 4°C, and the supernatants were purified on separate C_{18} Sep-Pak cartridges (Waters Associates, Milford, Massachusetts, USA); Sep-Paks were pretreated with 3 ml of 100% acetonitrile (CH_3CN) containing 0.1% HFBA

and rinsed with 20 ml of 0.1% HFBA. The bound sample was eluted with 5 ml of 50% CH_3CN containing 0.1% HFBA, divided into 1 ml aliquots, and lyophilized in siliconized 1.5 ml microfuge tubes.

The eluted sample was resuspended in 1.0 ml of Tris-buffered (20 mM, pH 8.0) artificial seawater. Resuspended samples were added to the agarose slurry after it cooled to 39°C so that heat-sensitive factors would not be denatured. Each test tube was coated with agarose containing the extracts from 1/6 of a squid egg capsule, meaning that each artificial egg bundle contained extracts from one egg capsule.

Statistical Analyses. All treatments were compared with natural egg treatments using a Wilcoxon's signed rank test (Sokal and Rohlf, 1995). We anticipated substantial variation in squid responsiveness; therefore, we designed the experiments to use paired analyses. In the beginning of the study, we did not have enough space to hold many animals. Thus, we were restricted to two treatments per animal. This experimental setup did not facilitate direct comparisons between experiments; differences can only be inferred.

RESULTS

Squid Responsiveness. There was considerable individual variation in squid responsiveness to the presence of natural egg capsules. Some individuals did not attempt to touch the eggs (4.0%), while others touched but did not exhibit aggressive behavior (16.2%). Data (Figure 3) were only analyzed from trials in which squids touched the natural egg stimulus. Fin-beating and egg blowing did not occur with enough frequency to compare between treatments.

Natural Eggs versus ESACT. Do male squids exhibit as many agonistic behaviors when exposed to eggs sealed in agarose-coated tubes (ESACT), which have no scent, as when exposed to natural eggs that have a scent ($N = 23$)? This experiment was designed to remove all chemosensory aspects of the egg capsules while still allowing the squids to have the visual stimulus of an egg bundle. A natural egg bundle consisting of 16–20 egg capsules was used as a behavioral control. Egg capsules were attached to an airstone to weight them to the bottom of the tank. To create an artificial egg bundle, 16 egg capsules were placed in 5 ml test tubes that were sealed with rubber stoppers or corks. The "egg bundle" was tied together with fishing line, and a weight was added to allow the tubes to easily rest on the bottom of the tank. The outsides of six of the test tubes were coated with 5% low-melting-point agarose (Sigma) to replicate the number of coated capsules that were embedded with C_{18} Sep-Pak-purified extracts (below).

Squids responded to the visual stimulus of ESACT by approaching the tubes and touching them. Some individuals repeatedly touched the tubes, while others touched only once. This behavior paralleled the response to natural egg

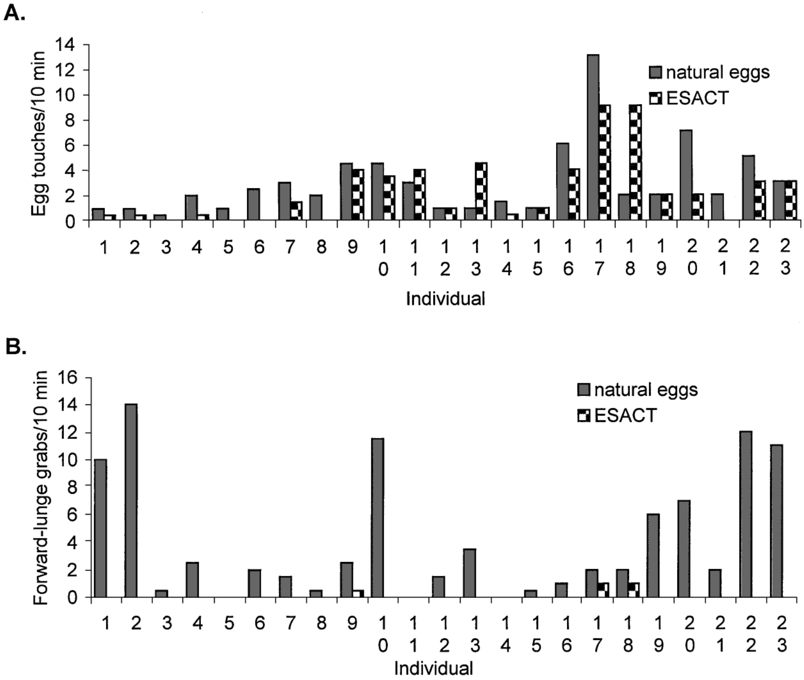

FIG. 3. Chemosensory and/or tactile cues from eggs increase the number of agonistic behaviors exhibited by individual male *Loligo*. The number of egg touches (A) was not significantly different in response to natural eggs and ESACT ($N = 23$, $P = 0.03$). The number of forward-lunge grabs (B) per 10-min period was significantly higher in individual males when exposed to natural eggs than when exposed to eggs in sealed agarose-coated tubes (ESACT; $N = 23$, $P < 0.001$).

bundles—instances of egg touch were similar between treatments (Figure 3A; Wilcoxon's sign rank test $T = 41.5$, $P = 0.03$). There was a dramatic difference in the agonistic response to natural eggs versus ESACT; squids responded much more aggressively (greater number of forward-lunge grabs) after touching natural eggs (Figure 3B; Wilcoxon's signed rank test $T = 0.00$, $P < 0.001$).

Natural Eggs versus TCPE. Do male squids exhibit as many agonistic behaviors when exposed to eggs sealed in agarose-coated tubes containing C_{18} Sep-Pak-purified extracts from squid egg capsules (TCPE), which possess the scent but not the mechanosensory qualities of natural eggs, as when exposed to natural eggs ($N = 35$)? This experiment was designed to determine if these purified extracts would elicit an agonistic response comparable to natural eggs. An artificial egg bundle was embedded with the purified extract from natural egg capsules. A natural egg bundle was used as a behavioral control as above.

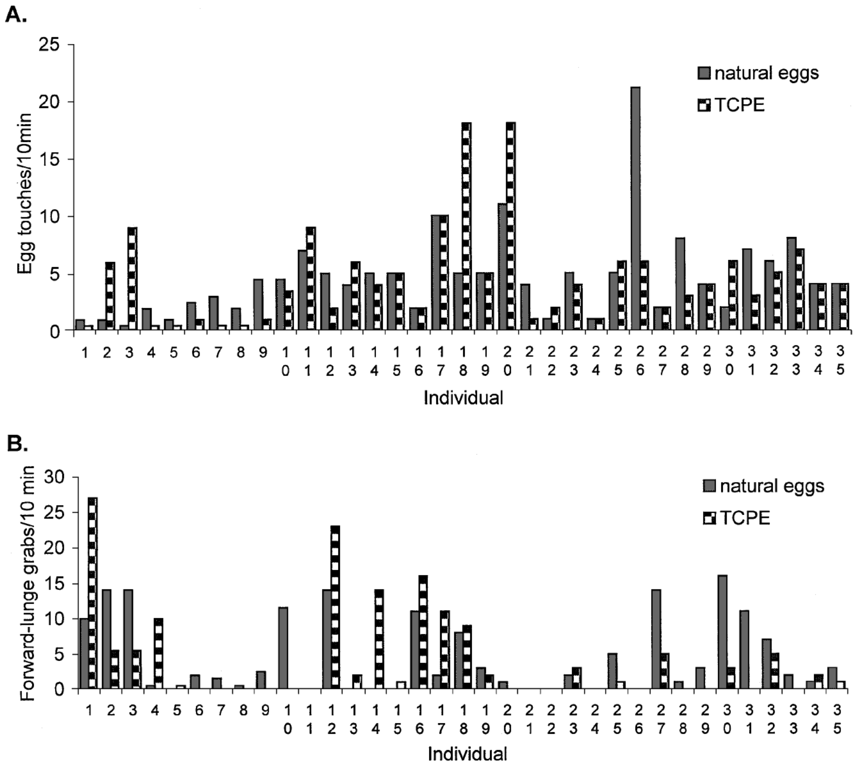

FIG. 4. Individual male *Loligo* exhibit a similar number of agonistic behaviors following physical contact with either eggs or with a C_{18} Sep-Pak-purified extract of eggs. The number of egg touches (A) was not significantly different in response to natural eggs and TCPE ($N = 35$, $P = 0.55$). The number of forward-lunge grabs (B) per 10-min period did not differ significantly in individual males that have come in physical contact with either natural eggs or TCPE ($N = 35$, $P = 0.35$).

Squids responded to the TCPE by approaching the artificial egg bundle and touching the outside of the tubes. In this experiment, they touched the TCPE with the same frequency as they touched natural eggs (Figure 4A; $T = 152.0$, $P = 0.55$). Touching TCPE also elicited an aggressive response (forward-lunge grabs) similar to responses to natural eggs (Figure 4B; $T = 187.5$, $P = 0.35$).

Natural Eggs versus Denatured Eggs. Could the chemical component be a peptide or a protein, as indicated by a loss of activity following heat-denaturation ($N = 8$) or treatment with trypsin ($N = 8$)? This experiment was designed to determine whether activity could be destroyed by heat or by treatment with trypsin. A natural egg bundle was used as a control. An egg bundle containing 16–20 egg capsules was treated in one of two ways: (1) Egg capsules were heated to 70–100°C for 30–45 min (egg capsules remained intact after this treatment); or (2) egg capsules were incubated in a seawater–trypsin solution (100 μg/ml) at 37°C for 2 hr.

Squids responded to eggs that had been either heat-denatured or trypsin-treated by approaching the eggs and manipulating them with their arms as they would with natural eggs. The frequency of egg touching was the same among heat-denatured, trypsin-treated, and natural eggs (Wilcoxon's signed rank test, Figure 5A; heat $T = 17.5$, $P = 0.94$; Figure 6A; trypsin $T = 6.50$, $P = 0.20$). Heat-denaturing the eggs effectively eliminated or reduced the agonistic response that was present with natural eggs (Figure 5B; Wilcoxon's signed rank test

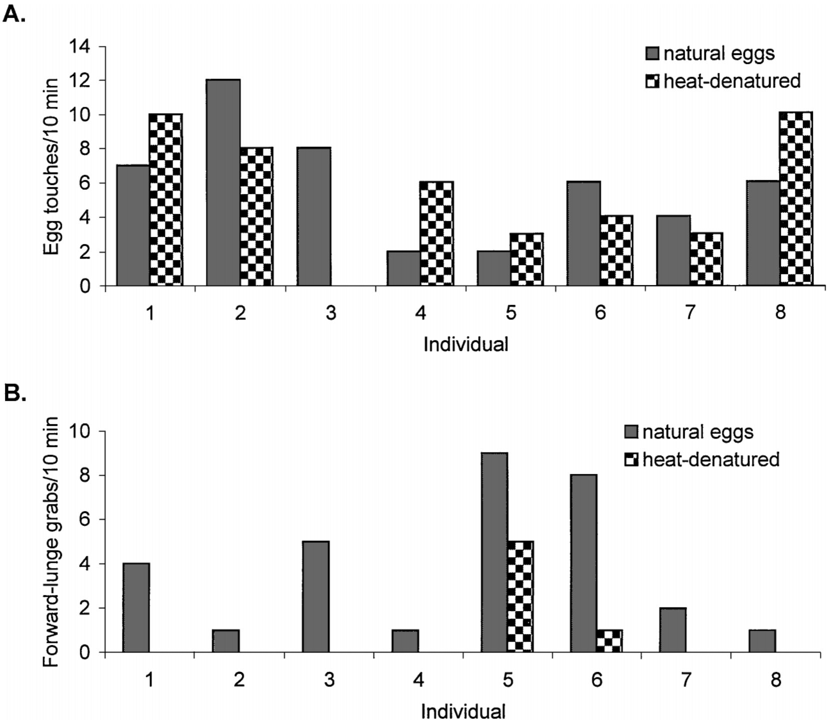

FIG. 5. The chemical that makes animals exhibit more agonistic behaviors is heat-labile. Approximately the same number of egg touches occur (A) per 10-min period when male *Loligo* make contact with either heat-denatured eggs or natural eggs ($N = 8$, $P = 0.94$). When male *Loligo* come into contact with heat-denatured eggs, they exhibit significantly fewer forward-lunge grabs (B) per 10-min period than when they contact natural eggs ($N = 8$, $P = 0.01$).

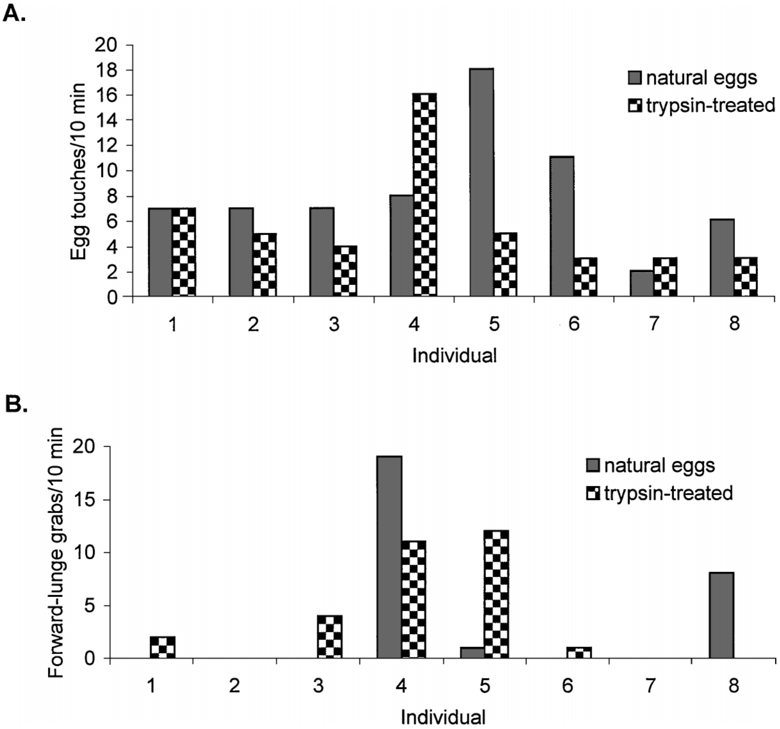

FIG. 6. Enzymatic digestion of eggs with trypsin does not reduce the frequency of agonistic behavior of male squid relative to natural eggs. Approximately the same number of egg touches occur (A) per 10-min period when male *Loligo* make contact with either trypsin-treated eggs or natural eggs ($N = 8$, $P = 0.24$). When male *Loligo* come into contact with trypsin-treated eggs, they exhibit approximately the same number of forward-lunge grabs (B) per 10-min period as they do when they contact natural eggs ($N = 8$, $P = 0.75$).

$T = 0.0$, $P = 0.01$); however, trypsin-treated eggs still elicited an aggressive response (forward-lunge grabs) similar to natural eggs (Figure 6B; Wilcoxon's signed rank test $T = 9.0$, $P = 0.75$).

DISCUSSION

Our data support the hypothesis that contact chemoreception is responsible for increased agonistic behavior. First, we showed that squids are visually stimulated to touch an artificial egg bundle but that contacting the artificial bundle does not elicit an aggressive response. Next, we showed that contact with an artificial egg bundle containing C_{18} Sep-Pak-purified extracts from squid egg capsules elicits male–male agonistic interactions comparable to natural eggs. Finally, we demonstrated that heat denaturation of natural egg capsules greatly reduces the aggressive response. Thus, we conclude that squid egg capsules contain a heat-labile factor that functions as a contact pheromone, which in turn induces male–male aggressive behavior.

The presence of either natural eggs, ESACT, TCPE, heat-denatured, or trypsin-treated eggs stimulated squids to swim up to the egg capsules and touch them, indicating that squids were attracted visually to all treatment types. However, only treatments with intact chemosensory stimuli caused squids to become aggressive after touching the eggs or artificial egg bundles, thus revealing that chemosensory stimuli are responsible for the increased levels of agonistic behavior.

Squid Responsiveness. In contrast to the results of King et al. (in press), the squids in our experiments were not always responsive to egg capsules; i.e., they were not always attracted to them visually and did not always respond aggressively when they did make contact with the egg capsules. We are uncertain of the reasons for this. It is possible that differences resulted from unmeasured environmental effects that differed between two seasons (1999 vs. 2001) when the experiments were conducted. Nonetheless, most squids in our experiments (80%) that responded to natural eggs (by swimming up to them and manipulating them in the characteristic manner) displayed aggressive behavior.

Visual Stimuli. The experiment testing natural eggs versus ESACT illustrated the importance of visual and chemosensory stimuli in squid responsiveness to egg capsules. Squids were attracted to ESACT; however, this stimulus alone was not sufficient to cause an escalation of aggressive behavior similar to natural eggs. As expected, vision played an important role. Since placing eggs in sealed tubes eliminated any chemosensory stimuli from the artificial egg bundle, other sensory stimuli must be responsible for inducing the aggressive behavior that resulted from touching the eggs.

Chemical Stimuli. The experiment testing natural eggs versus TCPE demonstrated the importance of chemosensory stimuli to elicit aggressive interactions among males. This experiment removed chemical stimuli from the eggs (placing them in sealed tubes) but artificially restored their chemical properties by applying C_{18} Sep-Pak-purified extracts from eggs to the outside of the tubes. Squids responded similarly to natural eggs and TCPE, indicating that the agonistic response was elicited by chemical cues. However, this design did not remove the tactile stimulus entirely because squids still “touched” eggs, although they were unable to manipulate the TCPE in the same manner as natural eggs, and the TCPE did not have the same mechanosensory qualities as natural eggs.

The experiment testing real eggs versus heat-denatured or trypsin-treated eggs revealed that removing the chemosensory stimulus eliminates the aggressive response. Squids responded to the visual stimulus of heat-denatured eggs by approaching and touching them as they would natural eggs. Touching and manipulating heat-denatured eggs, however, failed to elicit an aggressive response. This clearly indicated that it was a chemical stimulus—not physical—that was responsible for the increase in male–male aggression and that it was a heat-labile factor. We further tested this hypothesis in a subset of trials. We tested the ability of trypsin to destroy the activity responsible for increased aggression. Interestingly, the trypsin-treated eggs failed to eliminate an agonistic response, and squids responded as they would to natural eggs. This result may be due to the specificity of trypsin, which cleaves peptide bonds at arginine and lysine residues (Walsh, 1970) and may have left the active site(s) intact. It is possible that the pheromone(s) does not have these residues or does not have them in a position accessible to the enzyme.

Chemical Stimuli and Mating Behavior. Chemosensory stimuli operate in several taxa to elicit different types of mating behaviors. Several species of copepods use both water-borne and contact chemical cues for mate recognition (Lonsdale et al., 1998). In a study with a design similar to ours, the mating response of the rotifer *Asplanchna sieboldi* was induced by stimulation with agarose spheres impregnated with extracts from females (Joanidopoulos and Marwan, 1999). Recent work has illustrated the presence of a water-borne pheromone, attractin, in the marine mollusk *Aplysia*. It is secreted into the egg cordons when eggs are being prepared for deposition and then elutes into the surrounding seawater. It attracts *Aplysia* to the egg cordons and induces them to mate as males when they arrive (Painter et al., 1998, 1999). There are also contact pheromones on the eggs, which cause other animals to lay eggs (Begnoche et al., 1996). Boal (1997) suggested that female cuttlefish (*Sepia officinalis*) use chemosensory cues to select the most recently mated males as their own mates. Chemosensory cues may be important in *L. pealeii*. The presence of eggs may indicate proximity of receptive females (King et al., in press). The reproductive benefits of mating with and guarding a female would outweigh the cost of male–male agonistic interactions at this time. Chemical cues embedded in egg capsules may be responsible for allowing male–male agonistic interactions to be restricted to a time when receptive females are present. DiMarco and Hanlon (1997) found that the presence of mature females induced fighting behavior in *L. plei* males. They did not test the effect of egg bundles on fighting behavior; however, it is possible that this cue may be present in other loliginids.

We have noticed that the presence of eggs not only elicits male–male agonistic interactions but also stimulates mating behavior between males and females (unpublished observations). These observations suggest that the factor in egg capsules that is responsible for increased male aggression either has multiple effects or that several pheromonal cues are present in squid egg capsules.

This chemical stimulus (or another one embedded in egg capsules) may also act to attract squids to the egg beds, as in *Aplysia*. If the “agonistic factor” acts as an attractant to the egg beds, then it must be a water-borne pheromone rather than a contact pheromone. More work is necessary to determine whether the agonistic factor is solely a contact pheromone or whether it could also be waterborne.

Potential Sites of Chemoreception. Squids have eight arms and two tentacles, and average adult male *L. pealeii* have an average of 63 suckers on each arm. The site responsible for contact chemoreception may be the chemoreceptors in the suckers or in the buccal mass, since squids, octopuses, and cuttlefish are known to have chemoreceptors in the lips and on the suckers (Emery, 1975; Hanlon and Messenger, 1996). When male squids manipulate egg capsules, they place their heads directly into the egg mass and manipulate the capsules with their arms. Manipulating the eggs with their arms would allow chemoreceptors on the suckers to come into contact with pheromone(s) present on the surface of the egg capsules. Placing their heads into the egg mass may cause receptors in the buccal mass to come into contact with the egg capsules, and hence the pheromone(s) embedded in them. Squids also often blow on the eggs by forcing water over them through their funnel. This may cause pheromone(s) embedded in the egg capsules to be released into the water and come into contact with chemoreceptors in the suckers and/or in the buccal area.

An alternative site for chemoreception may be the olfactory organ located beneath the eye. One report indicates that *Loligo opalescens* can detect water-borne chemicals via this organ (Gilly and Lucero, 1992). Woodhams and Messenger (1974) have suggested that octopus olfactory organs may be sensitive to sexual pheromones from conspecifics.

It is obvious that males would benefit from limiting costly agonistic interactions to a time when receptive females are present. To the best of our knowledge, using chemosensory cues from egg capsules as an indirect signal to elicit agonistic interactions is unique to this cephalopod mating system. Further investigation is necessary to determine the precise mechanism by which this chemosensory cue operates.

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