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Experimental Evidence That Ovary and Oviducal Gland Extracts Influence Male Agonistic Behavior in Squids

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*Recent investigations of sensory and behavioral cues that initiate sexual selection processes in the squid *Loligo pealeii* have determined that egg capsules deposited on the substrate provide a strong visual and chemotactile stimulus to males, even in the absence of females (1, 2, 3). The visual stimulus of egg capsules attracts males to the eggs, and when the males touch the eggs, they encounter a chemical stimulus that leads to highly aggressive fighting behavior. We have recently demonstrated that egg capsule extracts implanted in artificial egg capsules elicit this aggressive behavior (4). In this communication, we present evidence that the salient chemical factor originates in the ovary and perhaps the oviducal gland of the female reproductive tract.*

Cephalopods are highly visual animals, yet recent research has shown that chemical communication plays an important role in regulating some behaviors (5). It has long been known (6) that *Loligo pealeii* is attracted visually to egg capsules (each translucent egg capsule is about 4 cm long and contains 100–300 eggs) and that females frequently lay egg capsules adjacent to existing egg capsules (7, 8). A fortuitous field observation indicated that males are visually attracted by egg capsules, but that touching the eggs was essential to evoke the intra-male competition for mates (1). Thus it appears that both visual and chemical communication play a role in triggering a change from shoaling behavior to mating behavior when *L. pealeii* migrates inshore to spawn in spring. Our ultimate goal is to identify and

characterize the compound (or compounds) that elicits this highly aggressive behavior in male squids.

Behavioral responses to natural eggs were compared with responses to artificial egg capsules coated with extracts (4) from one of four female reproductive organs or glands: ovary, oviducal gland, nidamental gland, or accessory nidamental gland. With the exception of ovary (see below), the equivalent of one-fifth of each gland was used to coat the artificial egg capsules. Six behaviors were selected to assess the level of aggression because they were conspicuous, easy to score, and reliable between observers. The general sequence of increased aggression in loliginid squids (9, 10), as shown by five of these behaviors, can be depicted as follows:

Raised arm → Fin-beating → Chase

→ Forward Lunge Grab (FLG) → Grapple

The sixth behavior, Splayed arms, is associated with defense of eggs or females and was also recorded. The frequency of any one of these six discrete behaviors was relatively low; consequently, the variable Total Aggression was computed as the sum of all occurrences of any of the six behaviors.

All experiments were conducted between May and August 2002. Squids were caught in Vineyard Sound (Falmouth, MA) using trawls or jigs. Reproductive organs were collected from 16 females. Each whole organ (except ovary, which was subsampled due to its large size) was individually extracted, centrifuged, and purified using separate C18 Sep-Pak cartridges (Waters Corp., Milford, MA) as de-

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Table 1

Mean number of behaviors recorded during the 10-min trial period for each egg stimulus

Egg stimulus	n_{pr}^*	Egg touch	Raised arm	Fin-beating	# Chases	FLG**	Grapple	Splayed arms	Total aggr.†
Natural	7	2.71	3.29	1.00	12.29	9.86	1.29	3.86	31.57
Ovary	10	3.30	2.20	0.70	5.70	0.60	0.60	3.40	13.20
Oviducal	11	5.18	0.27	0.55	7.64	2.91	0.18	1.27	12.82
Nidamental accessory	10	1.90	0.30	0.10	1.30	0.70	0.00	0.80	3.20
Nidamental	10	3.00	0.30	0.10	1.30	0.50	0.20	1.10	3.50

* n_{pr} is the number of squid pairs tested.

** FLG is the behavior Forward-Lunge-Grab.

† Total Aggression is the sum of all instances of Raised arm, Fin-beating, Chase, FLG, Grapple, and Splayed arms.

scribed previously (4); C18 Sep-Paks bind small molecules, peptides, and small proteins.

Behavioral trials were conducted in round tanks with aerated, flow-through natural seawater. Trials were conducted according to the following protocol. A pair of squids was placed in the trial tank and allowed 30 min to reach baseline behavior (*i.e.*, agonistic interactions resulted in one squid becoming dominant and occupying the center of the tank, and both squids being calm and showing normal coloration). In the pre-test, a bundle of 16–20 natural egg capsules was added to the tank. Data collection began when one of the squids touched the egg capsules. Previous experiments demonstrated that uncoated artificial eggs elicited significantly fewer egg touches than either coated artificial eggs or natural eggs (Friedman two-way ANOVA by ranks; $F_r = 7.43$, $n = 14$; $P < 0.05$). Not all squids were attracted to natural eggs; pairs in which neither squid touched the eggs were removed from the experiment. Instances of five of the six discrete behaviors (Raised arm, Fin-beating, Forward-Lunge-Grab (FLG), Grapple, Splayed arms) were recorded continuously for 10 min. Chase was frequent and continuous; consequently it was recorded at 15-s intervals. Squids that did not respond aggressively (*i.e.*, touched eggs but stayed at baseline behavior—resting or calm swimming) during the pre-test were excluded from further experiments. After a minimum of 1 h to regain baseline behavior, experimental trials commenced. The mean time to return to baseline behavior after being exposed to an egg stimulus was determined previously to be 7.9 min (range 1–40 min; $n = 11$). The egg stimulus (natural egg capsules or artificial egg capsules) was added to the tank and behaviors were scored as before.

We expected to examine the differences in responses of squids (experimental trial response minus pre-test response) to control for variation in responses between squids; however, the variances in the calculated differences were higher than the variances of responses for either pre-test trials or experimental trials (*e.g.*, the variances for total aggression with real eggs were pre-test, 398; experimental trials, 369;

differences, 692; $n = 7$). Consequently, only data from experimental trials were analyzed further. A Kruskal-Wallis analysis of variance by ranks (data were unitless and not normally distributed) was performed to determine which female reproductive organ elicited an agonistic response comparable to natural eggs. Multiple comparisons of treatments with the control were then performed. Note that statistical significance indicates that a treatment was not as effective as real eggs.

A total of 54 Raised arms, 22 Fin-beatings, 253 Chases, 119 FLGs, 19 Grapples, and 94 Splayed arms were recorded throughout the experiment ($n = 48$ pairs; means are listed in Table 1). Aggressive behavior (“Total Aggression” in Table 1) differed significantly between egg stimuli ($\chi^2 = 15.5$, $df = 4$, $P < 0.01$). Squids responded with the most aggression in response to natural eggs and with the least aggression in response to extracts from nidamental and accessory nidamental glands. Comparisons (11) of treatments *versus* the control (*i.e.*, natural eggs) revealed that aggressive responses to extracts from nidamental and accessory nidamental glands were significantly lower than responses to natural eggs. If we assume that more egg touches provide a greater stimulus for aggression, then it is reasonable to consider the aggression observed per egg touch. In this case, aggressive behavior again differed significantly between egg stimuli ($\chi^2 = 12.72$, $df = 4$, $P < 0.05$); however, comparisons (11) of treatments *versus* the control (*i.e.*, natural eggs) show that only extracts from ovaries elicited aggression statistically indistinguishable from that of real eggs (Fig. 1).

These results indicate that a chemical factor that induces agonistic interactions is produced in the ovary, and perhaps also in the oviducal gland, but not in the nidamental or accessory nidamental glands. The reversible binding of the chemical to C18 Sep-Pak cartridges suggests strongly that it is a soluble factor. Eggs are produced in the ovary and are transported into the oviducal gland, which is a specialized segment of the oviduct that is involved with secretion (12). The oviducal gland produces the inner jelly of the egg mass

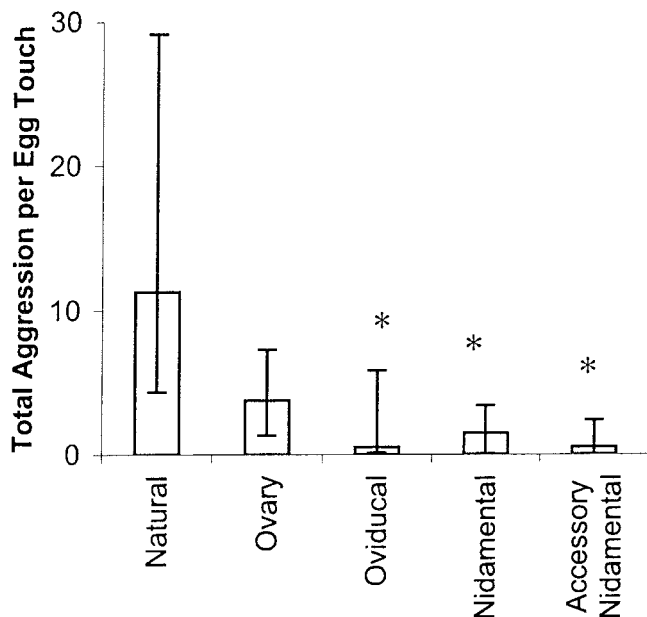


Figure 1. The median numbers of aggressive behaviors per egg touch (error bars indicate first and third quartiles) of squids after contact with natural eggs or contact with extracts from female reproductive glands ($n = 48$ pairs). An asterisk indicates that responses were significantly different from responses to natural eggs ($\chi^2 = 12.72$, $df = 4$, $P < 0.05$).

of *L. pealeii* (6). In contrast, the nidamental gland produces the outer coating of the egg capsule (12), and the accessory nidamental gland is responsible for coating the eggs with bacteria that may deter pathogens or reduce predation (13). There may be several chemical factors responsible for inducing agonistic behavior—for example, chemicals in the eggs themselves and perhaps in the inner jelly of the egg capsule secreted by the oviducal gland. If the chemical is indeed produced in the ovary, as suggested by these results, there must be some mechanism (*e.g.*, diffusion) by which the compound reaches the outer jelly coats of the egg capsule so that male squids can detect the compound when they touch the eggs.

Pheromones are key mediators of reproductive behaviors, and an understanding of their roles is essential to understanding the ecology and evolution of populations and species (14). Aquatic pheromones are particularly difficult to characterize because they are rapidly degraded (15); consequently, few invertebrate pheromones have been characterized in aquatic animals. However, a family of structurally related peptide pheromonal attractants (“attractins”) has recently been characterized in five species of the opisthobranch *Aplysia* (16, 17), and the three-dimensional NMR solution structure of *A. californica* attractin has been determined (18). These peptide pheromones are secreted by the albumen gland, a large exocrine gland that packages the eggs into a cordon. Our results with squids suggest that the ovary and oviducal gland should be tested further, and that

chemical factors in those organs should be chemically and behaviorally characterized. Clearly, more research is required to understand the mechanisms and functions of multiple sensory cues that play a critical role in initiating the sexual selection processes in *Loligo pealeii*.

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