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Report

Extreme Aggression in Male Squid Induced by a β-MSP-like Pheromone

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Summary

Male-male aggression is widespread in the animal kingdom and subserves many functions related to the acquisition or retention of resources such as shelter, food, and mates. These functions have been studied widely in the context of sexual selection, yet the proximate mechanisms that trigger or strengthen aggression are not well known for many taxa. Various external sensory cues (visual, audio, chemical) acting alone or in combination stimulate the complex behavioral interactions of fighting behaviors [1]. Here we report the discovery of a 10 kDa protein, termed *Loligo* β-microseminoprotein (*Loligo* β-MSP), that immediately and dramatically changes the behavior of male squid from calm swimming and schooling to extreme fighting, even in the absence of females. Females synthesize *Loligo* β-MSP in their reproductive exocrine glands and embed the protein in the outer tunic of egg capsules, which are deposited on the open sea floor. Males are attracted to the eggs visually, but upon touching them and contacting *Loligo* β-MSP, they immediately escalate into intense physical fighting with any nearby males. *Loligo* β-MSP is a distant member of the chordate β-microseminoprotein family [2] found in mammalian reproductive secretions, suggesting that this gene family may have taxonomically widespread roles in sexual competition.

Results and Discussion

Loligo pealeii Egg Capsules Contain a Proteinaceous Contact Pheromone

Loliginid squid, like other cephalopods, are semelparous, with a life cycle of 9–12 months. Females arrive in groups at communal spawning grounds in the spring and early summer. Each female lays multiple egg capsules over several days and usually mates repeatedly during this time [3]. Male squid behavior is changed abruptly by the presence of an egg mass (which may contain tens, hundreds, or even thousands of egg capsules), and males often fight vigorously with one another to pair with fertile females [4]. Typically, the males approach the eggs (visual attraction), blow jets of water over them, and touch them with their arms and heads. Physical contact (primarily via the arm suckers, which are known to have a sensory function) with a hitherto unidentified factor in egg capsules abruptly triggers a change in their behavior from schooling to highly aggressive fighting behavior [5], including fin beating, forward lunges, and grappling (Figure 1A).

To clarify the significance of contact with eggs, we tested the behavior of individually recognizable males (n = 57 pairs). Seven behaviors were selected to assess the level of aggression because they were conspicuous, easy to score, and reliable between observers. The general sequence of increasingly high-level aggression in loliginid squid is: raised arms → splayed arms → fin beating → chase → forward lunge → forward lunge grab → grapple (see Table S1 available online for details) [4, 6]. To determine an index of aggression, once the first squid touched the stimulus, we recorded the total number of occurrences of each defined aggressive behavior [7] for a period of 10 min. Results showed that contact with eggs increased the aggression of both larger and smaller males (e.g., chase: Wilcoxon signed ranks tests, when smaller males touched the eggs more: n = 43 pairs, W = 2067.0, p = 0.00; when larger males touched the eggs more: n = 43 pairs, W = 727.0, p = 0.01), and all males that contacted the eggs more, whether larger or smaller, maintained a position in closer proximity to eggs than their opponent (Wilcoxon signed ranks tests, when smaller males touched the eggs more: n = 15 pairs, W = 115.5, p = 0.01; when larger males touched the eggs more: n = 28 pairs, W = 345.5, p = 0.01; Table S2).

Males apparently benefit from this contact signal by focusing their competitive aggression when and where it is most likely to result in fertilization; i.e., the largest and most dominant males appear to gain the greatest number of copulations, and females are more likely to use recently obtained sperm (rather than stored sperm) when fertilizing their eggs [8]. Females likely benefit by obtaining sperm from the most vigorous competitors. The factor, which we report below as a proteinaceous contact pheromone, remains active on the surface of eggs for days to weeks (unpublished data), suggesting that it is highly resistant to degradation. There is a small but growing literature supporting the use of proteins as pheromonal cues in organisms. For example, proteinaceous pheromones are necessary for egg recognition in termites [9], for mate attraction in sea slugs [10], and for mate recognition and attraction in mice [11].

A behavioral bioassay was developed to experimentally test the reactions of live male squids to the presence or absence of the contact pheromone that seemingly induced aggression. Extracts of *L. pealeii* eggs were fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC; Figure 1B), and water-soluble fractions were purified and systematically administered in controlled behavioral experiments. Bioassays proceeded as described above, using small groups of six squids. The bioassay trials demonstrated that (1) the extraction

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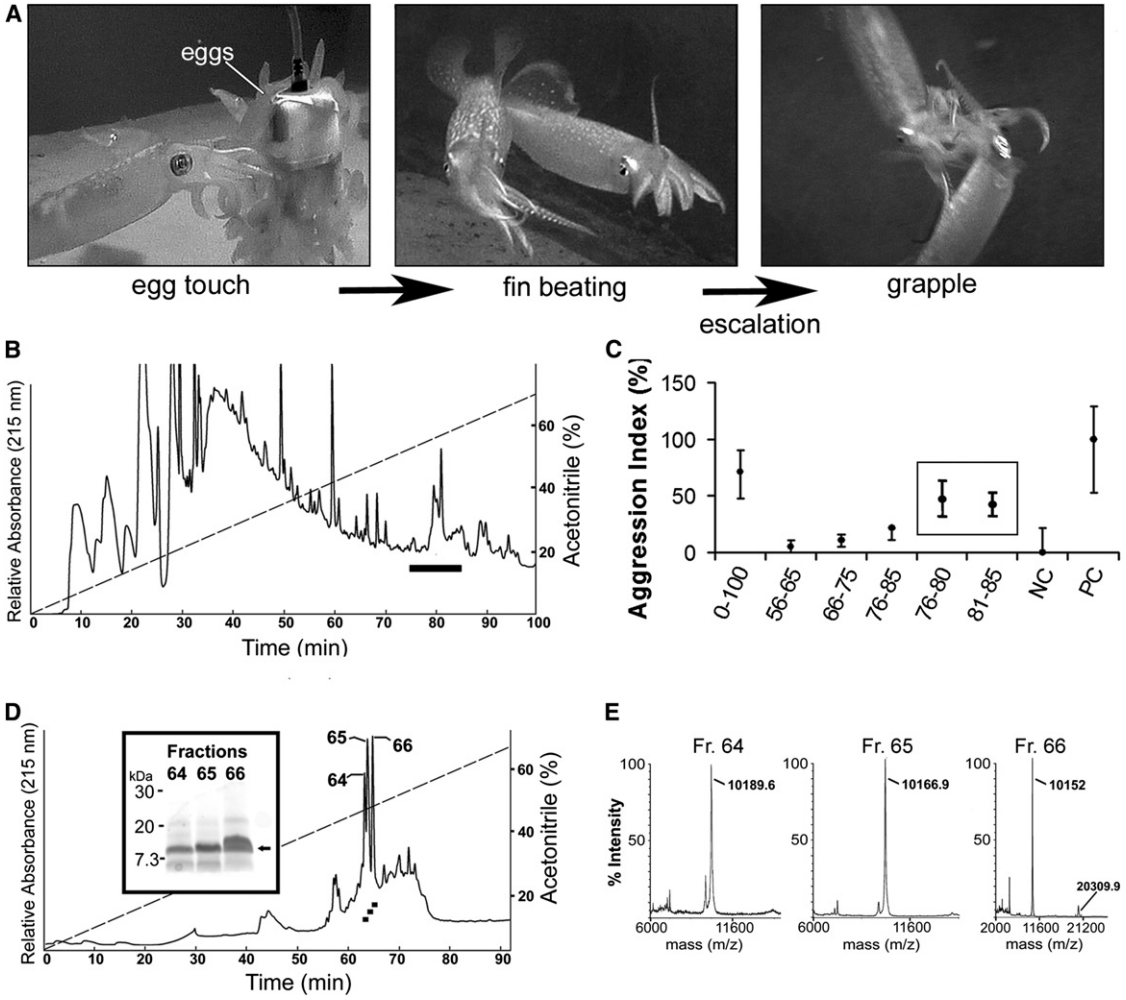

Figure 1. Purification, Bioassay, and Identification of *Loligo* β-MSP in Squid Egg Extracts

(A) Physical contact with an unidentified factor in egg capsules triggers a change in squid behavior from schooling to aggressive fighting behaviors.

(B) Reverse-phase high-performance liquid chromatography (RP-HPLC) profile of egg extracts. Fractions were initially pooled, lyophilized, and bioassayed.

(C) HPLC-purified egg extract fractions elicit aggressive behavior in male squid. HPLC samples containing fractions 76–85 (highlighted within the box) had the highest aggression index. NC denotes negative controls, PC denotes positive controls. Aggression index (median, first and third quartiles) is the sum of aggressive behaviors observed in a 5 min trial and is shown as percent of the PC. The median and first and third quartiles are illustrated.

(D) Pooled fractions 76–85 in (B) (solid bar in B) from several runs were repurified. Fractions 64, 65, and 66 showed a similar molecular mass (inset, SDS-PAGE; arrow indicates *Loligo* β-MSP) and shared the same N-terminal *Loligo* β-MSP sequence. kDa denotes kilodalton.

(E) Matrix-assisted laser desorption ionization followed by time-of-flight (MALDI-TOF) mass spectrometry analysis of purified fractions 64–66 in (D). The spectra show molecular ion species within fractions 64, 65, and 66, with masses corresponding to 10189.6 Da, 10166.9 Da, and 10152 Da, respectively. Percent intensity refers to the relative signal intensity. The most intense peak is represented at 100%. m/z denotes mass-to-charge ratio.

See also Movie S1, Figure S1, and Table S3.

procedure preserved bioactivity (i.e., it stimulated male aggressive behavior) because the aggression index for all pooled HPLC fractions 1–100 was not significantly different from that for the positive control (natural eggs; Wilcoxon-Mann-Whitney test, $W_x = 29.5$, $m = 4$, $n = 5$, $p > 0.10$; Figure 1C and Table S3) and that (2) fractions 76–80 and 81–85 stimulated the highest aggression index, were not significantly different from each other, and, when added together, were significantly different from the negative control (closed flask containing natural eggs) but not significantly different from the positive control (natural eggs; Kruskal-Wallis one-way analysis of variance with paired comparisons, $KW = 27.24$, $n = 2$, $p < 0.001$; Figure 1C and Table S3). The data suggested that the bioactive factor or factors resided in the major HPLC peak at 78–82 min (Figure 1B) and therefore was present in pooled fractions 76–80 and 81–85.

The bioactive RP-HPLC fractions (representing a major constituent of egg extracts) were then repurified, resulting in the resolution of three major peaks with similar retention times and molecular mass, as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Figure 1D). Matrix-assisted laser desorption ionization followed by time-of-flight (MALDI-TOF) mass analysis showed that these peaks share a similar mass-to-charge ratio (Figure 1E), and amino acid sequencing demonstrated that they have an identical N-terminal sequence. A remarkably long, single 90-residue N-terminal sequence for one of these fractions (66) was obtained (Figure S1; UniProt Knowledgebase: P86361). Identification of the corresponding gene sequence from RNA isolated from the ovary revealed that it encodes a 91-residue mature protein and facilitated the identification of cysteine residues and the

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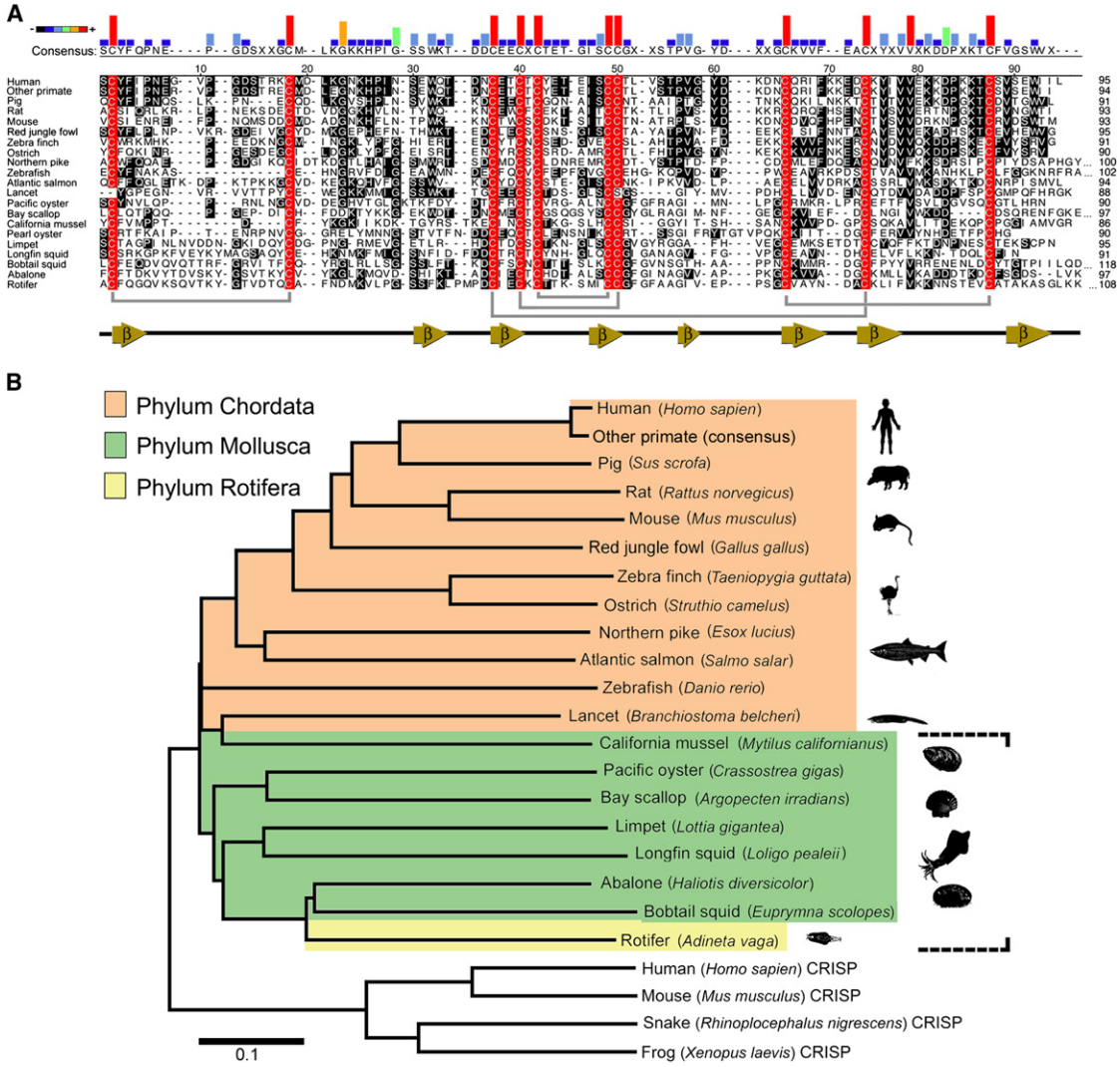

Figure 2. Squid Aggression Pheromone Has Most Similarity to the β-Microseminoprotein Family

(A) Mature protein (without signal peptide) sequence alignments are shown of known chordate β-microseminoproteins with longfin squid *Loligo* β-MSP and other nonchordate β-microseminoprotein identified in this study. Numbering is according to the human sequence. Amino acids that are identical to the consensus are shaded in black, or in red for highly conserved cysteines, and gaps have been inserted to allow proper alignment of the sequences. A histogram of consensus strength is shown, in which the relative frequency with which an amino acid appears at a given position is reflected by the color and height. The disulfide bond arrangement (Cys_2-Cys_{18}, Cys_{37}-Cys_{73}, Cys_{40}-Cys_{50}, Cys_{42}-Cys_{49}, Cys_{64}-Cys_{87}) and position of β sheets, based on the human β-microseminoprotein [26], is shown by lines joining half-cystine residues and arrows below, respectively.

(B) Evolutionary origins and conservation of β-microseminoproteins. A tree shows phylogenetic relationships among the sequences, with analysis conducted using the neighbor-joining method (Poisson correction) with the MEGA version 4.0 software (http://www.megasoftware.net/). The scale bar represents the number of substitutions per site. β-MSPs identified in this study are represented within the dotted lines. The phylogeny was rooted using the cysteine-rich secretory proteins (CRISPs).

See also Supplemental Experimental Procedures for details of β-microseminoproteins, as well as Figure S2 and Table S4.

C-terminal residue (Figure S1; GenBank GQ906708). The presence of ten cysteine residues, which can potentially form five intramolecular disulfide bonds, is consistent with the protein being compact and resistant to proteolytic degradation, which presumably prolongs its activity on the egg surface for days to weeks. Similarly, the water-borne protein pheromones released by the mollusc *Aplysia* are resistant to rapid degradation and contain multiple intramolecular disulfide bonds [10, 12–14].

Purified Egg Capsule Protein Is a Distant Member of the β-Microseminoprotein Family

The purified egg capsule protein has highest overall sequence identity with β-microseminoproteins (β-MSPs), which were originally discovered in human seminal plasma and prostatic fluids [15] and only described in other vertebrates [16–20] and the basal chordate amphioxus [21]. Although highly variable (only 45% of residues are conserved between human and rat β-MSP [22]), chordate β-MSPs are typically 88–95 amino acids in length, possess ten spatially conserved cysteine residues, are resistant to proteolytic cleavage, and are secreted, yet they do not appear to be glycosylated [21, 23, 24]. *Loligo* β-MSP shares these key features, and phylogenetic analyses support *Loligo* β-MSP being a member of the β-MSP family and being distinct from the cysteine-rich secretory protein family (Figures 2A and 2B). This conservation in disparate bilaterians and our identification of β-*MSPs* in the

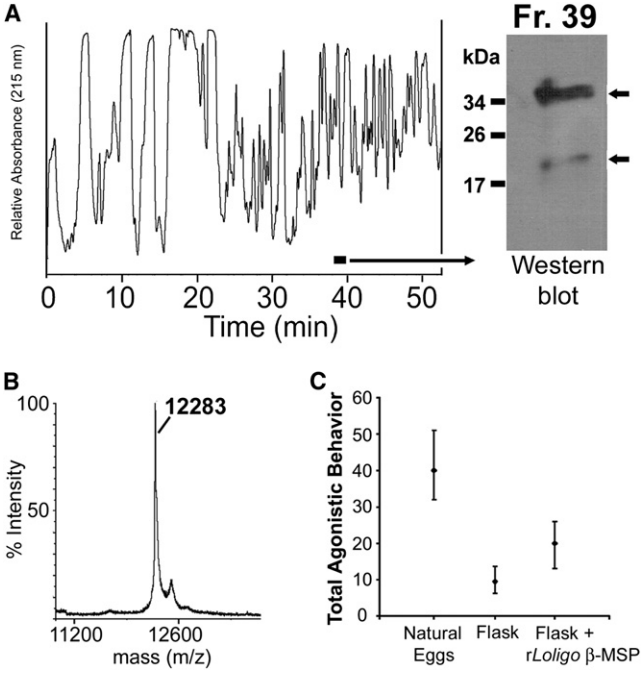

Figure 3. Recombinant Protein Expression, Purification, and Bioassay

(A) *Loligo* β-*MSP* cDNA was inserted into the insect expression vector pIEx/Bac-3 and was expressed in Sf9 cells, then prepurified on C_{18} Sep-Pak Vac cartridges. Chromatogram shows C_{18} RP-HPLC separation of insect cell extract. SDS-PAGE and western blot analysis of RP-HPLC fraction 39 (bold line) using anti-*Loligo* β-MSP shows two immunoreactive bands (arrows). The difference in mobility on western blot is probably a reflection of difference in SDS binding rather than size. kDa denotes kilodaltons; r*Loligo* β-MSP denotes recombinant *Loligo* β-MSP.

(B) When fraction 39 was tested in MALDI-TOF mass analysis, a single 12283 Da peptide was observed (correlates to recombinant *Loligo* β-MSP with its fusion tag MAHHHHHHHHHHGALEVLFQGPG). Percent intensity refers to the relative signal intensity. The most intense peak is represented at 100%. m/z denotes mass-to-charge ratio.

(C) Total agonistic behavior (median, first and third quartiles) of groups of six squids was significantly greater after contact with a flask that contained natural eggs, and was coated on the outside with recombinant *Loligo* β-MSP embedded in agarose ("Flask + r*Loligo* β-MSP," n = 15), than after contact with a similar flask coated with agarose alone ("Flask," n = 10). The median and first and third quartiles are illustrated.

See also Movie S1.

genomes of other molluscs (i.e., the limpet *Lottia gigantea*) and the bdelloid rotifer, *Adineta vaga* (Figure 2B), indicates that the origin of this gene family antedates the last common ancestor to deuterostomes and protostomes.

Despite their similarities, chordate β-MSPs are rapidly evolving, with limited sequence identity between species [21] (Table S4). This divergence is not surprising, given that high nonsynonymous to synonymous substitution ratios (greater than 1) have been found in this family of genes, suggesting that they have experienced adaptive evolution in at least some lineages, as previously identified in primates [25]. Significantly, an estimated 42% of the vertebrate β-MSP codons appear to be under positive selection and are located uniformly on the protein surface. Therefore, no distinct functional regions can be inferred, even among vertebrate groups. Given this observation, it can be expected that the β-MSP-like *Loligo* β-MSP only shares structural features with its vertebrate counterparts. The nuclear magnetic resonance solution structure of human β-MSP shows two distinct beta sheet domains [26], and, if the disulfide-bonding arrangement is conserved, a *Loligo* β-MSP homology model predicts that it also contains two beta-sheet domains (Figure S2).

Recombinant *Loligo* β-MSP Also Induces Aggressive Male-Male Behavior

A recombinant *Loligo* β-MSP was obtained using the baculovirus cell expression system, and the RP-HPLC fraction containing recombinant *Loligo* β-MSP was verified by western blot analysis (Figure 3A). This was further validated by MALDI-MS (Figure 3B). In behavioral bioassays of recombinant *Loligo* β-MSP, males showed more total agonistic behavior after contacting glass flasks streaked with agarose embedded with *Loligo* β-MSP than they did after contact with flasks coated with agarose alone (Wilcoxon signed ranks test, $z = -1.886$, $m = 10$, $n = 15$, $p = 0.0294$; Figure 3C and Movie S1). Thus, the presence of *Loligo* β-MSP (either in natural egg capsules or embedded in transparent agarose on glass flasks containing squid egg capsules) accounted for the highest levels of aggression in male squid. We conclude that *Loligo* β-MSP is a protein contact pheromone that triggers the most extreme male aggressive behavior observed at spawning grounds. This pheromone works in tandem with the visual stimulus that first attracts male squids to the eggs from a short distance and indicates a multimodal sensory system in which vision plays the initial role; in addition, a chemical/touch stimulus enhances the immediate escalation of intraspecific male aggression to its highest levels. Critical triggers of agonistic behavior allow males to target such behavior to times and places when fertile, receptive females are available for mating and egg laying. Moreover, a contact pheromone may have evolved into a species-specific cue, given that sympatric squid species share parts of the same range. To confirm this, future research should target the identification of orthologs from other squid species.

Loligo β-MSP Is Synthesized in Accessory Exocrine Glands

Prior to spawning, eggs must first pass from the ovary through the oviducal gland, which is specialized to produce the inner jelly of the egg capsule [27, 28]. Subsequently, the accessory nidamental gland coats eggs and jelly with bacteria that are thought to deter pathogens or reduce predation, and then the nidamental gland produces the outer coating of egg capsules [29]. An RT-PCR screen of these tissues detected *Loligo* β-*MSP* transcripts in the ovary, nidamental, and accessory nidamental glands (Figure 4A), and its corresponding protein could be localized to the ciliated epithelium of the nidamental gland and secretory-like cells throughout the accessory nidamental gland (Figures 4B and 4C and Figure S3); no *Loligo* β-MSP protein was detected in the ovary (Figure S3). This observation is consistent with our previous observations suggesting that the active factor for aggression was incorporated into the eggs as they passed through the accessory glands [7].

Most remarkable is the localization of β-MSP and *Loligo* β-MSP in accessory sex glands, fluids, and structures documented in male-to-male competition: mammalian seminal fluid [15] and cephalopod egg masses (this study). This correlation, along with the strong signal of positive selection, is similar to that observed in other sex-related genes across the animal kingdom [25, 30, 31]. To date, a biological role for β-MSP in mammalian aggression has not been explored; however,

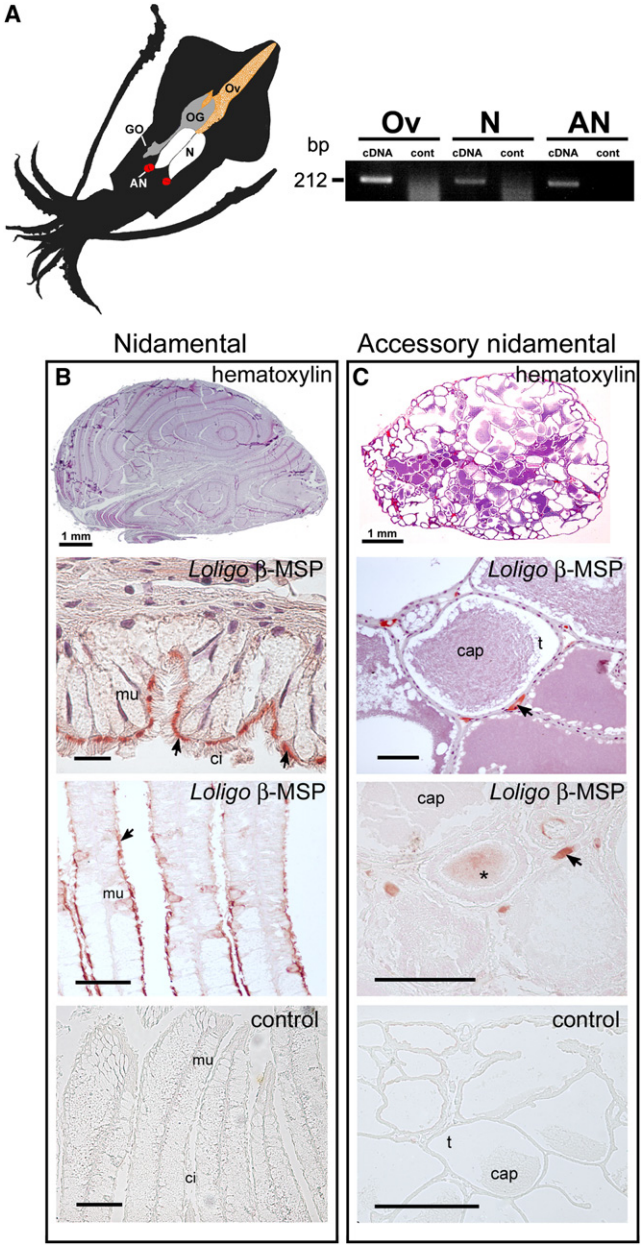

Figure 4. Expression and Localization of *Loligo pealeii* β-MSP in the Female Reproductive Tract

(A) Diagram showing female reproductive tract and the relative position of the ovary (Ov), oviducal gland (OG), gonadal opening (GO), nidamental gland (N), and accessory nidamental gland (AN). Ovary, nidamental gland, and accessory nidamental gland total RNA were used to amplify *Loligo* β-*MSP* cDNA by RT-PCR. Agarose gel electrophoresis shows *Loligo* β-*MSP* expression (212 bp) within the ovary, nidamental gland, and accessory nidamental gland. Negative controls (without cDNA; cont) show no amplification. bp denotes base pairs.

(B) Paraffin cross-section through the nidamental gland was treated with hematoxylin (purple), revealing distinct tubular structures relating to their secretory function. Micrographs show immunolocalization of *Loligo* β-MSP (arrows), restricted to ciliated epithelial cells of nidamental gland. Control, using preimmune serum, showed no staining.

(C) Paraffin cross-section through the accessory nidamental gland was treated with hematoxylin (purple), revealing distinct tubular structures relating to their secretory function. Micrographs show immunolocalization of *Loligo* β-MSP (arrows), restricted to secretory-like cells of the accessory nidamental gland. The asterisk indicates *Loligo* β-MSP within capsular material. Control, using preimmune serum, showed no staining.

Unless stated, scale bars are 100 μm. ci denotes cilia, mu denotes mucous cell, t denotes tuberculi, cap denotes capsular material. See also Figure S3.

induced aggressive behavior has been linked to pheromones isolated from biological fluids such as urine and saliva. In mice, for example, male-male aggression is triggered by urine-derived pheromones comprising both specialized lipocalin proteins of high molecular weight and by unknown low-molecular-weight biomolecules [32]. Also, chemical cues of an unknown nature are released from the preputial and lacrimal glands that are capable of stimulating aggression between male mice [33], likely supporting the maintenance of social status and increasing mating success.

Conclusions

Pheromones are widespread throughout the animal kingdom [34]. Here we report multisensory cues (visual followed by chemotactile) in which a protein serves as a contact pheromone that triggers immediate and extreme aggression in the context of sexual selection. Our demonstration of the role of *Loligo* β-MSP in extreme male aggression in the squid *Loligo* raises the prospect of the β-MSP family encoding conserved pheromones that have been co-opted into a diversity of secondary sexual contexts in different animal phyla. It would be of interest to learn whether β-MSPs can induce hormonal and associated behavioral responses, including aggression and sperm competition, in vertebrates.

Experimental Procedures

Animals

Live squids were trawled from Vineyard Sound (Falmouth, MA) and housed in one half of a large round tank (3.67 m × 1 m) with a continuous supply of aerated flow-through natural seawater (May–June) or recirculating chilled seawater (34 parts per thousand, 14.5°C–16.5°C; July). The following morning, behavioral bioassays were performed on groups of six male squids. At the end of the day, the squids were removed, and fresh squids were placed in the tank for bioassay (details of bioassay can be found in the Supplemental Experimental Procedures).

Protein Purification

L. pealeii eggs were collected at Woods Hole, MA, frozen on dry ice, and stored at −80°C until they were used. Eggs (2.6–4.2 g) were extracted at 4°C in 25 ml 0.1% heptafluorobutyric acid (HFBA) using a Polytron homogenizer (Brinkmann Instruments) and sonicated. The extract was centrifuged (20,000 × g; 20 min; 4°C), and the supernatant was purified on C_{18} Sep-Pak Vac cartridges (5 g; Waters Corporation). Peptides and proteins were eluted with 70% acetonitrile (CH_3CN) containing 0.1% HFBA and lyophilized in preparation for RP-HPLC (details of RP-HPLC are in the Supplemental Experimental Procedures).

Immunohistochemistry

Immunohistochemical staining was performed on paraffin sections of *L. pealeii* ovary, nidamental, and accessory nidamental glands. Anti-*Loligo* β-MSP (rabbit polyclonal against SRKGPKFVEYKYMAC) was used at a dilution of 1:400 (1 mg/ml), and detection was performed with goat anti-rabbit Ig HRP followed by aminoethylcarbazole visualization.

Comparative Sequence Identification and Phylogenetic Analysis

Amino acid sequences and/or nucleotide sequences were processed using EditSeq (Lasergene version 7.1; DNASTAR) and further analyzed using the MegAlign (Lasergene version 7.1) with the ClustalW algorithm. A phylogenetic tree was constructed using MEGA version 4.0 software built using the neighbor-joining method [35], and the confidence levels for the groups defined in the topology were assessed by bootstrap and interior branch tests (1000 replicates).

Accession Numbers

The nucleotide sequence for the *Loligo* β-micoseminoprotein gene has been deposited in the GenBank database under the accession number GQ906708.

Supplemental Information

Supplemental Information includes three figures, four tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at doi:10.1016/j.cub.2011.01.038.

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