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RESEARCH ARTICLE

Sperm-attractant peptide influences the spermatozoa swimming behavior in internal fertilization in *Octopus vulgaris*

Emilia De Lisa[1], Anna Maria Salzano[2], Francesco Moccia[3], Andrea Scaloni[2] and Anna Di Cosmo[1,*]

[1]Department of Structural and Functional Biology, University of Napoli 'Federico II', 80126 Napoli, Italy, [2]Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, 80147 Naples, Italy and [3]Department of Biology and Biotechnology 'Lazzaro Spallanzani', Laboratory of Physiology, University of Pavia, 27100 Pavia, Italy

*Author for correspondence (dicosmo@unina.it)

SUMMARY

Marine invertebrates exhibit both chemokinesis and chemotaxis phenomena, induced in most cases by the release of water-borne peptides or pheromones. In mollusks, several peptides released during egg-laying improve both male attraction and mating. Unlike other cephalopods, *Octopus vulgaris* adopts an indirect internal fertilization strategy. We here report on the identification and characterization of a chemoattractant peptide isolated from mature eggs of octopus females. Using two-chamber and time-lapse microscopy assays, we demonstrate that this bioactive peptide is able to increase sperm motility and induce chemotaxis by changing the octopus spermatozoa swimming behavior in a dose-dependent manner. We also provide evidence that chemotaxis in the octopus requires the presence of extracellular calcium and membrane protein phophorylation at tyrosine. This study is the first report on a sperm-activating factor in a non-free-spawning marine animal.

Key words: *Octopus vulgaris*, chemoattractant peptide, sperm swimming behavior.

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INTRODUCTION

Sperm activation and chemotaxis have mainly been reported in free-spawning marine animals (Eisenbach, 1999). They generally use external fertilization strategies, with only some species adopting internal fertilization; in the latter case, a large number of spermatozoa are directly introduced into the female reproductive tract. For other animals adopting internal fertilization, such as mammals, the phenomenon of sperm hyperactivation has also been described (Oren-Benaroya et al., 2008).

Marine invertebrates use water-borne peptides or protein pheromones to communicate among individuals and to modulate several aspects of social behavior, including reproduction. The role of chemotaxis has been explored primarily in cnidarians and echinoderms. In 1978, Miller demonstrated that the eggs of the cnidarian *Orthopyxis caliculata* not only secrete a chemotactic factor, but also regulate the timing of its release (Miller, 1978). More than 70 sperm-activating peptides (SAPs), with slight differences in their amino acid sequence, have been purified and characterized from the egg jelly of 17 sea urchin species (Suzuki and Yoshino, 1992). Similarly, 12 isoforms of astero-SAPs have been described in starfish (Nishigaki et al., 1996). These sperm-activating and sperm-attracting peptides play a crucial role in species-specific fertilization by improving sperm motility, sperm metabolism and acrosome reaction (Kupfermann and Carew, 1974). All these chemotactic events involve the activation of several ion channels; for example, resact, the SAP of the sea urchin *Arbacia punctulata*, drives the sperm swimming trajectory by causing Ca^{2+} entry into the spermatozoa by enhancing flagellar bending through a number of Ca^{2+}-permeable channels (Kaupp et al., 2008). In hydroids and one ascidian species, sperm chemotaxis also requires extracellular Ca^{2+} entry, which may be mediated by the so-called store-operated channels (Yoshida and Yoshida, 2011). In the sea cucumber *Holothuria atra*, it has been recently demonstrated that calcium plays a key role in the phosphorylation of several axonemal proteins, which might be involved in the control of flagellar beating (Morita et al., 2009). In mammals as well, Ca^{2+} plays a major role in regulating sperm motility. However, it has been demonstrated that either the release of calcium from internal stores or external Ca^{2+} influx promotes mice sperm motility (Suarez and Ho, 2003).

In the mollusk gastropods as well as cephalopods, several factors involved in spawning and chemotaxis have been identified and purified. The first water-borne pheromonal protein, namely attractin, was characterized in the sea hare *Aplysia californica*. This and other pheromones released by eggs attract animals of the *Aplysia* genus, in a non-species-specific manner, to the area of egg spawning, thus improving mating (Painter et al., 2004). The characterization of attractin and other related proteins (enticin, temptin, seduction, Alb-1, etc.) purified from the eluates of *A. californica* egg cordons suggested that they may act synergistically during egg-laying (Cummins et al., 2006; Cummins et al., 2010). An attractin-like pheromone was also identified in the mucus-secreting hypobranchial gland of the abalone *Haliotis asinina* (Kuanpradit et al., 2012).

In cephalopods, the first specific sperm attractant factor was identified and purified in *Sepia officinalis*, which is characterized by external fertilization. In this organism, several regulatory factors are involved in the successive steps of egg-laying. The release of a water-borne pheromonal peptide (ILME) by freshly spawned eggs induces the attraction of mature adults in egg-laying coastal areas (Zatylny et al., 2000). The presence of a specific hexapeptide amidated at its C terminus (PIDPGVamide), released in the external medium by the full-grown oocytes, can increase the probability of contact between gametes, thereby mobilizing the spermatozoa

(Zatylny et al., 2002). Moreover, it has been demonstrated that the presence of eggs releasing pheromones is essential to recruit conspecifics in *Sepia* and *Loligo* (Boal et al., 2010). Recently, the pheromonal protein β-MBP was isolated in *Loligo pealeii*; it triggers the most extreme male aggressive behavior observed at spawning grounds (Cummins et al., 2011). In contrast, *Nautilus pompilius* is able to detect conspecifics using excretions from the rectum (Westermann and Beuerlein, 2005).

Octopus vulgaris, unlike other cephalopod mollusks, adopts an indirect internal fertilization strategy. The males extend the hectocotylized third right arm toward the female. The tip of hectocotylized arm 'seeks' the opening of the oviduct. The spermatophores are placed at the base of the hectocotylized arm with an arched posture followed by a pumping action that sends the spermatophores down the hectocotylized arm and into the distal oviduct. The passage of spermatophores into the female reproductive tract represents just a phase of the mating behavior and does not imply fertilization. Spermatozoa delivered from spermatophore envelopes are stored in spermathecae, which are a portion of the oviducal gland. The spermatozoa penetrate deeply into the walls of the spermathecae with their spiralized acrosomes and then became immobile. When the mature and ready-to-be-fertilized eggs reach the central cavity of the oviducal glands through the proximal oviduct, unknown stimuli induce sperm detachment from the wall of spermathecae and their mobilization and attraction toward the eggs to become fertilized.

The existence of mechanisms and factors that might mobilize and attract spermatozoa toward the eggs remains unclear in this species. The only data that support the occurrence of a chemical communication process in octopus fertilization are based on the demonstration of an acrosome-like reaction inducted by progesterone (Tosti et al., 2001). In this study, we have identified a sperm chemoattractant peptide in *O. vulgaris* and we provide a functional evidence of a chemical communication between sperm and eggs in this species. In this frame, we propose the octopus as a model to investigate chemotactic mechanisms in sexual communication to unveil the detailed steps of the fertilization strategy in a non-free-spawning marine animal.

MATERIALS AND METHODS

Animals

Males and females of *Octopus vulgaris* Cuvier 1797 (with body mass in the range 0.7–1.5 kg) were captured in the bay of Naples. Animals were maintained for a maximum of 2 days in aquaria with circulating seawater at a water temperature of 16°C (8 h:16 h light:dark photoperiod). Eggs were collected from mature females in the months of March to June, which correspond to the vitellogenic period, weighed and homogenized, as previously reported (Di Cosmo et al., 2001). Male spermatophores were isolated from the Needham's sac during the whole year; a gentle mechanical stress was applied to promote spermatozoa exit. Actively moving spermatozoa were pooled, centrifuged at 200 ***g*** for 10 min in calcium-free artificial seawater (Ca^{2+}-free ASW) (0.5 mol l^{-1} NaCl, 10 mmol l^{-1} KCl, 50 mmol l^{-1} $MgSO_4$, 2.5 mmol l^{-1} $NaHCO_3$). Sperm density was quantified by using a Makler counting chamber to obtain a concentration of 1×10^6 spermatozoa ml^{-1}. Spermatozoa stored in female spermathecae of oviducal glands were gently collected and counted as above. Since there are no specific legal/ethical regulations relating to experimental work with octopus in Italy, our research activity conformed to the ethical principles of reduction, refinement and replacement (Russell and Burch, 1959). Specific attention was paid to avoiding and minimizing any suffering to animals according to Directive 2010/63/EU; thus, animals were deeply anaesthetized and killed as previously reported (Polese et al., 2012).

Peptide purification and characterization

Eggs in the vitellogenic phase were homogenized using an Ultra-Turrax T25 homogenizer in a 90:9:1 (v/v/v) ratio of methanol:filtered seawater (FSW):acetic acid supplemented with a protease inhibitor cocktail (Complete mini plus EDTA, Roche, Basel, Switzerland). The homogenate was centrifuged at 25,000 ***g*** for 20 min at 4°C, and the supernatant was then loaded on a C_{18} Sep-Pak Vac cartridge (Waters, Milford, MA, USA), which was previously equilibrated according to the manufacturer's recommendations. The cartridge was washed with 0.1% trifluoroacetic acid (TFA) and the bound material was then eluted with 50% MeOH containing 0.1% TFA and lyophilized. This material was then solved in 0.1% TFA, filtered and resolved by reversed-phase HPLC on a Vydac C_8 analytical column (0.46 cm×25 cm×5 μm), and then eluted with a linear gradient (from 5 to 90%) of acetonitrile with 0.1% TFA over 60 min at a flow rate of 1 ml min^{-1}, and monitored at 214 nm. Fractions (1 ml) were collected, dried and evaluated for a portion (20%) of the collected material using the chemotaxis assay reported below. The most active fraction was then solved in 0.1% TFA and resolved by reversed-phase HPLC on a Vydac C_{18} analytical column; this fraction was eluted at 5% for 10 min, with a gradient from 5 to 35% for 10 min, and then from 35 to 60% (for 40 min) of acetonitrile with 0.1% TFA at a flow rate of 1 ml min^{-1}, and monitored at 214 nm. Fractions (1 ml) were collected, dried and evaluated for a portion (20%) of the collected material using the chemotaxis assay reported below.

MALDI-TOF mass spectrometry analysis was performed using a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA, USA). Bioactive fractions (1 μl) from chromatography were loaded on the instrument target using the dried droplet technique and α-cyano-4-hydroxycinnamic (5 mg ml^{-1} in 50% acetonitrile, 5% formic acid as matrix), as previously reported (Calvello et al., 2003). Spectra were acquired in linear mode with delayed extraction, and calibrated by either external or internal calibration using the molecular ions from insulin (bovine), thioredoxin (*E. coli*) and apomyoglobin (horse). Data are reported as average masses.

Bioactive fractions from chromatography were also subjected to sequential Edman degradation using a Procise 491 protein sequencer (Applied Biosystems) equipped with a 140C microgradient apparatus and a 785A UV detector (Applied Biosystems) for the automated identification of phenylthiohydantoin-amino acids (Rodriguez de la Vega et al., 2004).

Bioactive fractions were also added with sample buffer containing dithiothreitol, boiled and separated by SDS-PAGE on a 15% acrylamide gel (14 cm×16 cm×0.75 mm) run in a SE600 system (Hoefer, Holliston, MA, USA) at a constant current of 25 mA. After running, polypeptides were visualized by colloidal Coomassie staining.

Chemotaxis assay

Sperm chemotaxis was assayed using a 96-well chemotaxis chamber system (ChemoTx, Neuroprobe, Gaithersburg, MD, USA). Bottom wells were filled with the chemotactic agent solved in chemotaxis buffer. A framed 8-μm-pore diameter polycarbonate membrane filter was then placed over the wells, and 1×10^5 cells were added to the top of the filter, over each well. Plates were incubated at 22°C for 15 min. The top of the filter was then scraped to remove residual

cells and rinsed. The plate was centrifuged at 100 ***g***, for 5 min, the filter was then removed and Hoechst-labeled sperm migration was quantified using a Perkin Elmer Wallac Victor^3 1420 microplate reader (PerkinElmer, Santa Clara, CA, USA), with excitation at 485 nm and emission detection at 530 nm. Wells with no cells added were used as negative control. Each experiment was performed in triplicate.

Peptide cloning

On the basis of the amino acid sequence determined, two degenerate primers were designed to amplify the cDNA encoding the sperm chemoattractant peptide. Total RNA was isolated from octopus eggs in full vitellogenic phase using the E.Z.N.A. mollusk RNA kit (Omega Bio-Tek, Doraville, GA, USA), and then treated with RNase-free Dnase I (Promega, Madison, WI, USA) according to the manufacturers' guidelines. The cDNA was generated by reverse transcription of total RNAs using the oligodT primer and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, followed by RNase H treatment. The resulting first-strand cDNAs were amplified and then cloned by insertion into a pGEM-T Easy Vector (Promega) following the manufacturer's guidelines, and finally sequenced at PRIMM s.r.l. (Naples, Italy). Resulting nucleotide sequences were translated and analyzed for post-translational modification motifs using the corresponding Expasy tool at http://expasy.org/tools/.

Recording and analysis of sperm cell swimming behavior

Sperm direction and motility were recorded with a 10× objective on a Nikon ECLIPSE TI inverted microscope in a Neuroprobe Zigmond chamber, following the manufacturer's protocol. Spermatozoa were placed in the left well, whereas the right well was filled with the chemoattractant peptide. The movement of cells in the capillary space (~10 µm) between the coverslip and the partition wall separating the wells was recorded at 10× in the middle of the field between both compartments. Spermatozoa tracks were recorded 5 min after the sealing of the chamber, when the distribution of the cells was stable. Recordings were carried out by acquiring one image every 0.016 s (continuous running), at room temperature, for a total time of 15 min. Images were collected with an Andor iXonEM + CCD camera (Andor Technology, South Windsor, CT, USA); each frame was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) using the 'manual tracking' and 'chemotaxis and migration' tools. One hundred cells were tracked and subsequently analyzed relative to the direction of the chemical gradient (defined as the *x*-axis).

Tyrosine phosphorylation of sperm proteins during chemotaxis

Octopus sperm membrane proteins were isolated using the Mem-PER Membrane protein extraction reagent kit (Pierce Biotechnology, Rockford, IL, USA); protein concentration was determined by the BCA method (Smith et al., 1985). Twenty micrograms of extracted proteins were subjected to 12% SDS-PAGE and then electrotransferred on Immobilon PVDF membrane (Millipore, Billerica, MA, USA), at 200 mA, overnight. Non-specific membrane binding was blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 1 h at 25°C. Membranes were incubated with monoclonal anti-phosphotyrosine (mouse IgG2b isotype) (Sigma-Aldrich, St Louis, MO, USA) at dilution of 1:5000 in PBS containing 0.1% Tween 20, for 1 h at 25°C. After several washings with PBS containing 0.1% Tween 20, a peroxidase-conjugated goat anti-mouse secondary antibody in PBS containing 0.1% Tween 20 was applied to the membrane at a dilution of 1:10,000, for 1 h at 25°C. Immunopositive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate, according to the manufacture's protocols (Pierce Biotechnology), and a Chemidoc EQ System (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analyses

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) with one-way ANOVA followed by Tukey's *post hoc* test. *P*-values <0.05 were considered significant.

RESULTS

Purification and characterization of a sperm chemoattractant peptide from octopus

With the aim to identify sperm chemoattractant molecules released by the octopus female, eggs homogenates were roughly purified by a single-step extraction over a Sep-Pak cartridge, which was eluted as described in the Materials and methods. This material was then fractionated by reverse-phase HPLC on a C_8 column; the corresponding chromatographic profile showed a well-resolved peak that coincided with most of the measured sperm chemoattractant activity (Fig. 1A), as assayed in a chemotaxis chamber (see Materials and methods for details). Corresponding bioactive material was further purified by reversed-phase HPLC over a C_{18} column. Two fractions were associated with chemoattractant activity (Fig. 1B); in particular, peaks 1 and 2 corresponded to 100% and 19% of the measured relative chemotaxis, respectively. The most active fraction (peak 1) was then subjected to SDS-PAGE, which demonstrated the protein nature of this material, which occurred as a wide polypeptide band migrating at approximately 11 kDa (Fig. 1C). N-terminal sequencing analysis provided a unique succession, namely ENKTDITRYIP…, which suggested the presence of a single polypeptide component in this purified material, which was here named octopus sperm-attractant peptide (Octo-SAP). In contrast, MALDI-TOF-MS analysis demonstrated its heterogenic nature, with various MH^+ signal pairs eventually differing for mass values associable with glycan units (i.e. Δm=203 Da) (Fig. 1D). Additional LC-ESI-LIT-MS analysis in the *m*/*z* range 200–2000 demonstrated the absence of eventual non-peptide components in this fraction, which should have been coming through the purification process (data not shown). A related heterogenic mass spectrometry pattern was also observed for the protein material corresponding to peak 2 in Fig. 1B, which showed a SDS-PAGE profile with a peptide band migrating at approximately 7 kDa (data not shown) and yielded a unique sequence when subjected to Edman degradation, namely GPIPTKKKSL…. In both cases, no significant sequence similarity to other protein entries present in the non-redundant NCBI database was observed.

Octopus sperm chemoattractant peptide cloning

In order to fully characterize Octo-SAP, which was associated with most of the measured sperm chemoattractant activity, the corresponding amino acid sequence ascertained by Edman degradation was then used to design degenerated oligonucleotides; these oligonucleotides were then utilized to amplify the cDNA encoding for this molecule, starting from total RNA from female eggs. We amplified a 116 bp PCR product encoding for a 37-amino-acid-long sequence to be associated with this sperm chemoattractant peptide (Fig. 2A), where amino acids revealed by direct N-terminal sequencing occur at the protein C terminus. BLAST analysis

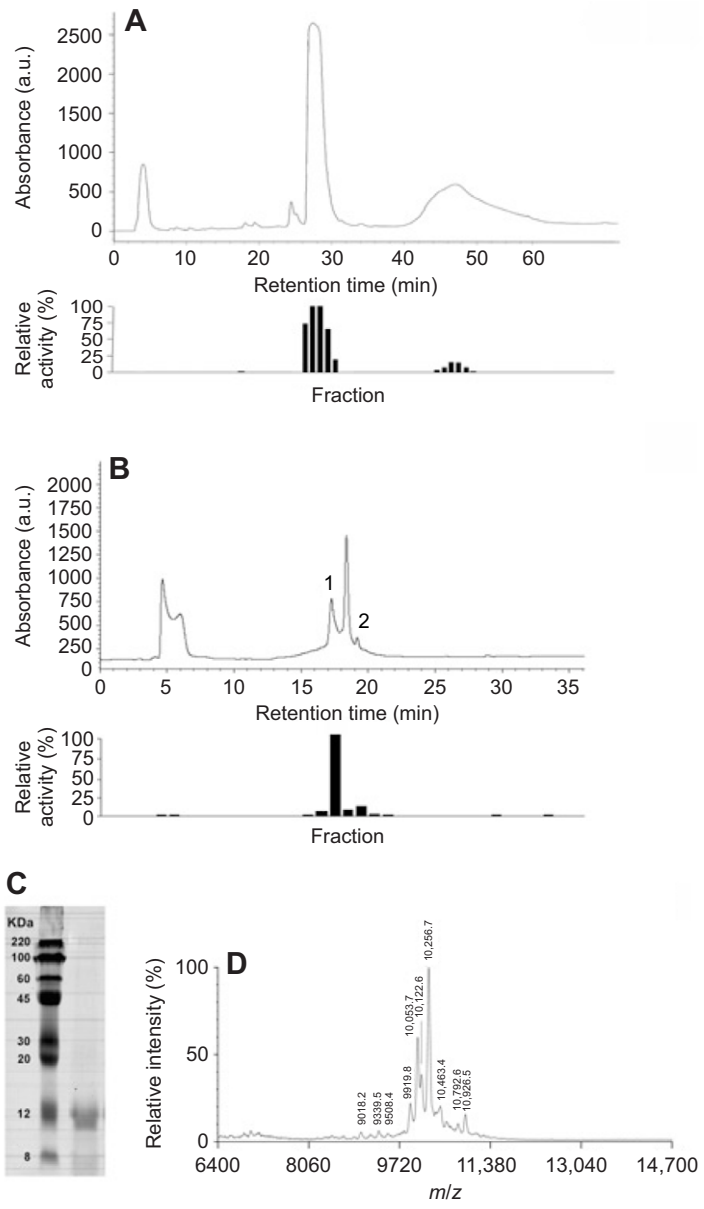

Fig. 1. Isolation and characterization of the sperm chemoattractant peptide Octo-SAP from *Octopus vulgaris* eggs. (A) Egg homogenate material from a Sep-Pak cartridge was fractionated by reverse-phase HPLC on a C_8 column; eluted fractions were then assayed for their sperm chemoattractant activity, which is reported in the corresponding histogram. (B) The most active fractions from C_8 chromatography were pooled and further purified by reversed-phase HPLC over a C_{18} column; eluted material was then assayed for its sperm chemoattractant activity, which is reported in the corresponding histogram. A main portion (labeled as peak 1) was associated with most of the chemoattractant activity. (C) SDS-PAGE analysis of the material corresponding to peak 1 in B. (D) MALDI-TOF-MS analysis of the material corresponding to peak 1 in B.

indicated that this nucleotide/amino acid sequence does not show any similarity to other identified entries in the non-redundant NCBI database, including EST ones, which was strongly indicative of the species-specificity of this peptide, due to the very poor number of octopus entries present therein. Significant homology with allatotropin from *Spodoptera frugiperda* was only detected in the signal sequence (Fig. 2B). Furthermore, scanning whole Octo-SAP sequence with PROSITE, a predictor of putative post-translational modifications (http://expasy.org/tools), identified Asn25 and Thr35 as putative sites of N-glycosylation and phosphorylation, respectively (Fig. 2A). These residues occur within the sequence of the mature bioactive peptide purified in this study. This finding was suggestive of the eventual occurrence of post-translational modifications in the processed Octo-SAP, which are claimed to justify the peak pattern and the mass values observed during MALDI-TOF-MS analysis of this peptide, and have to be detailed in future studies. Their presence hampered a further characterization of Octo-SAP biological properties by performing assays on a synthetic peptide.

Chemotaxis assay

In order to further assess the chemotactic activity of Octo-SAP, spermatozoa (1×10^5) collected from spermatophores of mature animal males were incubated in a chemotaxis chamber with different amounts of this purified peptide solved in ASW or with ASW alone (control). After 15 min of incubation for the stabilization of the chemotactic gradient, sperm migration was assessed as described in the Materials and methods. As shown in Fig. 3, the sperm chemotactic response showed a peptide concentration-dependent profile; a significant response ($P<0.05$) was detected at dilution 1:10. The time of exposure to peptide did not affect the sperm migration (data not shown).

Analysis of sperm swimming trajectories

In order to analyze octopus spermatozoa migration in response to the purified Octo-SAP, we evaluated the cell's ability to migrate in a planar space using a Zigmond chemotaxis chamber (Zigmond, 1977) and time-lapse video microscopy. All experiments were repeated at least three times; data obtained from an exemplifying experiment are shown in Fig. 4. In the absence of Octo-SAP, spermatozoa migration was homogeneously distributed in all possible directions (Fig. 4A), in agreement with the Brownian motions exhibited by a small particles in two dimensions; cells exhibited a low motility and were distributed uniformly in both wells (Rayleigh test, $P=0.169$), as demonstrated by the number of left and right counts shown in Fig. 4B. Spermatozoa showed the well-known 'turn and run' phenomenon already observed in diverse marine organisms (Böhmer et al., 2005; Kaupp et al., 2003), associated with a movement pattern that is not very circular, probably because of the long cellular flagella. In the presence of purified Octo-SAP, spermatozoa redirected their swimming paths towards the source of the chemoattractant molecule and a polarization of cell migration vectors was observed (Rayleigh test, $P=1.02E{-}16$; Fig. 4C). A higher percentage of spermatozoa was found in the right well (right counts > left counts) (Fig. 4D). These results are in agreement with a deviation from uniformity or random migration.

We then analyzed spermatozoa three-directionality-based parameters according to Fabro and co-workers (Fabro et al., 2002), i.e. by evaluating the mean of the net distance travelled along the chemoattractant gradient (ΔX), the percentage of cells with a net distance greater than zero ($\Delta X>0$) and the percentage of cells with the quotient between the net distance in X over the absolute value of the net distance in Y higher than the unit ($\Delta X/|\Delta Y|>1$). ΔX demonstrated a statistically significant difference when experiments were performed in the presence of purified Octo-SAP, with respect to the expected values in the case of random movement ($P<0.05$; Fig. 5A). These cells migrated predominantly up to the chemoattractant gradient. The percentage of spermatozoa that

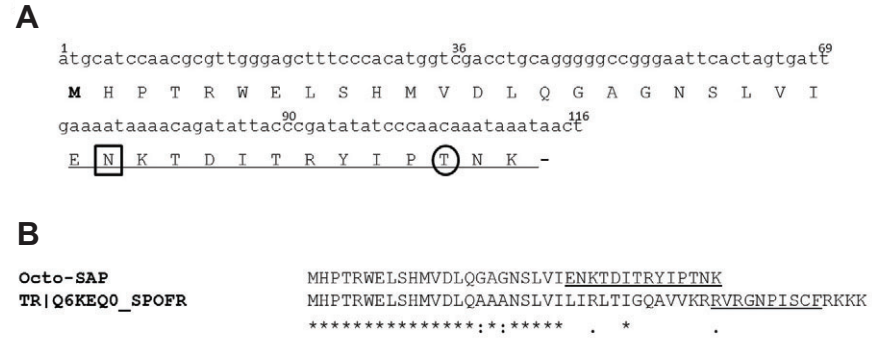

Fig. 2. Nucleotide and deduced amino acid sequence of the sperm chemoattractant peptide Octo-SAP from *Octopus vulgaris* eggs. (A) Nucleotide and deduced amino acid sequence of Octo-SAP. N-terminal Met (M) at protein N-terminus in the non-processed polypeptide chain, mature peptide and putative N-glycosylation (N) and phosphorylation (T) sites are reported as bold, boxed and circled amino acids, respectively. (B) Results from amino acid sequence alignment. The sequence of allatotropin from *Spodoptera frugiperda* is shown as aligned with Octo-SAP. Processed bioactive peptides are underlined.

migrated along the *x*-axis toward the chemoattractant gradient showing a $\Delta X>0$ was statistically significant (>50%) with respect to the expected values in the case of random movement ($P<0.05$). The analysis of the spermatozoa trajectories, whose projection on the *x*-axis was >1 and longer than the projection on the *y*-axis, demonstrated that the percentage of cells with $\Delta X/|\Delta Y|>1$ was statistically significant (50%), with respect to the expected values in the case of random movement (>25%; Fig. 5B). In contrast, 50% of the spermatozoa showed a $\Delta X>0$ in the absence of the peptide; these cells migrated in a random manner (Fig. 6). In conclusion, these parameters demonstrated that Octo-SAP clearly affects the direction of the sperm migration as induced by a chemoattractant gradient.

Tyrosine phosphorylation of sperm membrane proteins is induced by extracellular Ca^{2+} entry during chemotaxis

It has long been known that sperm attractant peptides induce spermatozoa migration by eliciting a Ca^{2+} inflow across the plasma membrane (Kaupp et al., 2008). Consistent with this notion, the chemotactic response to purified Octo-SAP diminished by approximately 50% at all peptide dilutions tested ($P<0.05$) in the absence of extracellular Ca^{2+} (Fig. 7).

As shown in Fig. 7, the presence of extracellular calcium at concentration in normal seawater (11 mmol l^{-1}) did not affect sperm migration.

In the presence of the high-voltage-activated (HVA) Ca^{2+} channel blocker nifedipine (50 μmol l^{-1}) (Stork and Cocks, 1994), spermatozoa migration was similar to that observed in the presence of the peptide, at all peptide dilutions applied (data not shown).

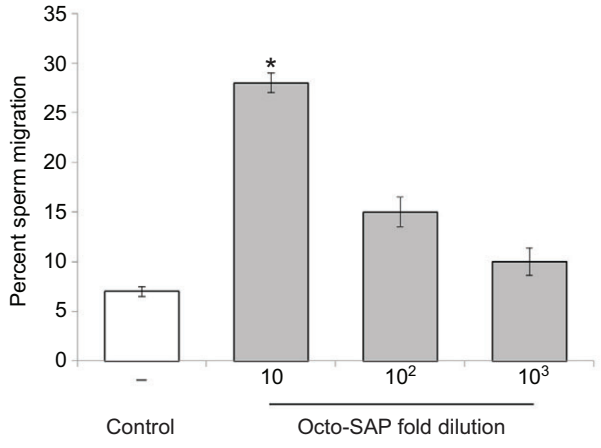

Fig. 3. Concentration-dependent profile of sperm chemotaxis elicited by Octo-SAP. The graph illustrates the percentage (mean ± s.d.) of octopus spermatozoa migration towards a peptide gradient. Experiments were performed in triplicate in the presence or absence of the purified peptide. Asterisk indicates a significant difference from control ($P<0.05$).

In mammalian sperm, progesterone is able to determine a rapid increase in intracellular calcium concentration ($[Ca^{2+}]_i$), protein phosphorylation at Tyr residues and chloride efflux *via* non-genomic receptors (Torres-Flores et al., 2008). On the basis of previous results from our laboratory that demonstrate the presence of progesterone receptors on the acrosomal region of octopus spermatozoa (Tosti et al., 2001) and in the nuclei of the cells within the glandular compartment of oviducal gland (Di Cosmo and Di Cristo, 1998), we performed chemotaxis assays in the presence of progesterone. In the presence of 15 μmol l^{-1} progesterone in ASW (Tosti et al., 2001), the sperm migration was similar to that observed in the absence of the peptide, at all peptide dilutions applied (data not shown). Each experiment was performed five times on spermatozoa collected from the spermatophores of five different octopuses. The same experiments, performed also on spermatozoa collected from distal oviducts of inseminated females, gave similar results. On this basis, we can conclude that progesterone does not serve as chemoattractant molecule for octopus spermatozoa.

The signal transduction pathway activated downstream from Ca^{2+} influx was investigated by examining the protein phosphorylation at the sperm plasma membrane *via* western blot analysis. After incubation with purified Octo-SAP at a 1:10 dilution, we observed an increase of Tyr phosphorylation for a protein migrating at approximately 75 kDa, as compared with control experiments performed in the absence of the peptide (Fig. 8A). Western blotting analysis was then performed in the absence of calcium and in the presence of 15 μmol l^{-1} progesterone. As shown in Fig. 8B, we observed a faint immunopositive staining of the same band reported above in the absence of calcium (lane 1), while an increase in protein Tyr phosphorylation was observed in the presence of progesterone (lane 2), as compared with control experiments conducted in the presence of Octo-SAP (1:10 dilution) (lane 3).

DISCUSSION

Reproductive success in free-spawning marine invertebrates (Riffell et al., 2002) is guaranteed by the chemical communication between sperm and eggs, a well-known phenomenon called chemotaxis. A key role in this communication is played by oocyte-released molecular signals and components of the oocyte external layers. These signals, in turn, could induce processes such as sperm chemokinesis/chemotaxis and the acrosome reaction. While sperm chemotaxis in free-spawning marine species has been well described, sperm chemotaxis in animals with internal fertilization has been established only in a few cases. The main reason for resistance to the concept of sperm chemotaxis in animals with internal fertilization resides in the fact that a very large number of spermatozoa are

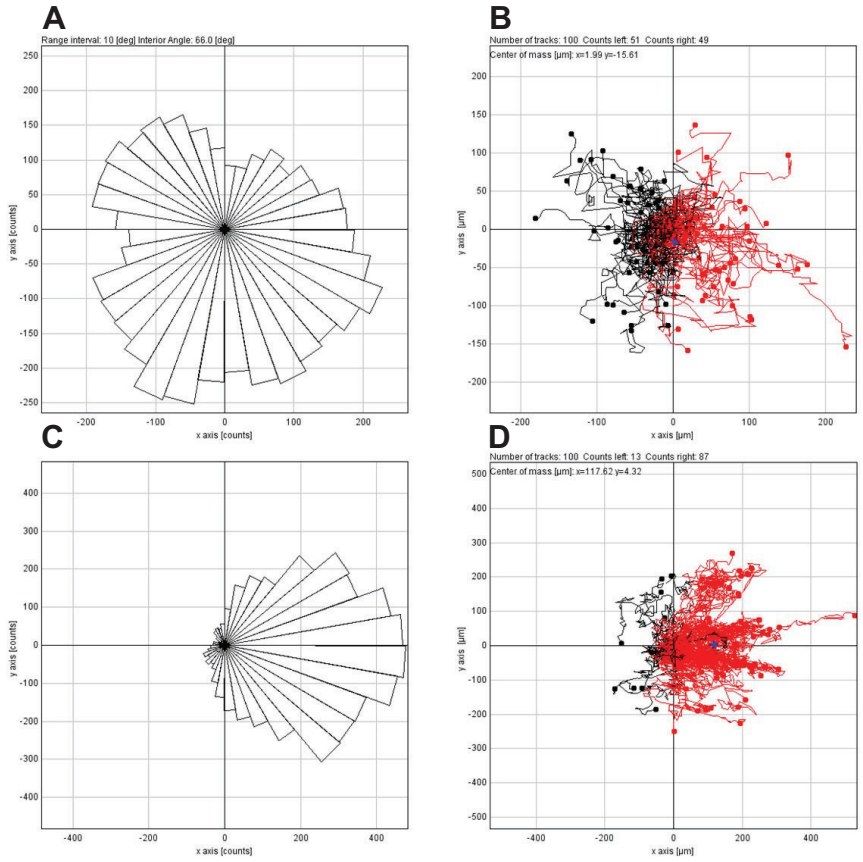

Fig. 4. Directional migration and representative spermatocyte trajectory plots in the absence (A,B) and presence (C,D) of the purified Octo-SAP peptide. (A) Rose diagrams showing the distribution of migration angles in the absence of the Octo-SAP peptide in an experiment during 15 min; the migration angles were calculated from *x*,*y* coordinates at the beginning and the end of the cell tracks (*N*=100), and were grouped into 10 deg intervals, with the radius of each wedge indicating the cell number. A Rayleigh uniformity test confirmed that the distribution of migration angles was uniform (*P*=0.169). (B) Representative cell trajectory plots in the time period from 0 to 15 min. A chemotaxis experiment was performed in the absence of the Octo-SAP peptide. No directed migration was observed and a smaller displacement of the center of mass occurred. (C) As in A, but in the presence of the Octo-SAP peptide. A Rayleigh uniformity test confirmed that the distribution of migration angles was not uniform (*P*=1.02E–16). (D) Representative cell trajectory plots in the time period from 0 to 15 min. A chemotaxis experiment was performed using a dilution of 1:10 of the Octo-SAP peptide. Spermatocytes moved in the direction of the *x*-axis, which is by definition the direction of the gradient with higher displacement of center of mass.

ejaculated or introduced directly into the female reproductive tract, as the case of the *O. vulgaris*, where many cells may reach the eggs by chance, avoiding the need for sperm chemotaxis. However, even in animals characterized by internal fertilization, such as mammals, the phenomenon of sperm hyperactivation, physiologically very similar to chemotaxis, seems to be induced by factors released from follicles. Very uncommonly for cephalopods, *O. vulgaris* has an internal fertilization that takes place in the oviducal gland. The secretory activity of the oviducal gland is under the control of neuropeptides and sex steroid hormones (Di Cosmo and Di Cristo, 1998; Di Cosmo et al., 2001; Di Cristo et al., 2002; Di Cristo et al., 2005; Di Cristo et al., 2008). An octopus male introduces a large amount of spermatozoa in the female oviducts through a hectocotylized arm; these cells are enveloped in spermatophores. Once free from the spermatophore envelope, spermatozoa are immobilized in the mucosa of the oviducal gland by their spiralized acrosome until the mature rich-yolk eggs, ready to be fertilized, fall through the proximal oviduct in the fertilization chamber of the gland (Hanlon and Messenger, 1996; Di Cosmo et al., 2001; Bertolucci et al., 2002).

The present study was undertaken to fill the gap in the knowledge of the fertilization strategy in octopus and to identify eventual soluble factor(s) able to mobilize and rapidly activate the immobilized spermatozoa toward the eggs, enabling fertilization. Here we report on a peptide of approximately 11 kDa that we termed Octo-SAP, which was originally purified and cloned from octopus eggs. Alignment investigations over the mature peptide sequence demonstrated no significant similarities

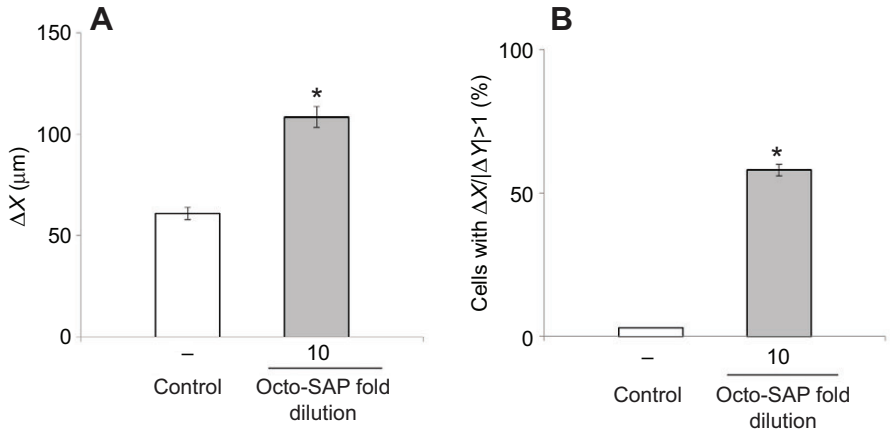

Fig. 5. Spermatocyte three-directionality-based parameters in the absence or in the presence of the purified Octo-SAP peptide. (A) Mean net distance travelled along the chemoattractant gradient (Δ*X*) in the absence (control) or in the presence of the chemoattractant peptide at a dilution of 1:10 (Octo-SAP). (B) Percentage of spermatocyte cells with the quotient between the net distance in *X* over the absolute value of the net distance in *Y* higher than the unit Δ*X*/|Δ*Y*|>1 in the absence (control) and in presence of the chemoattractant peptide at dilution 1:10 (Octo-SAP). Asterisks indicate a significant difference from control (*P*<0.05).

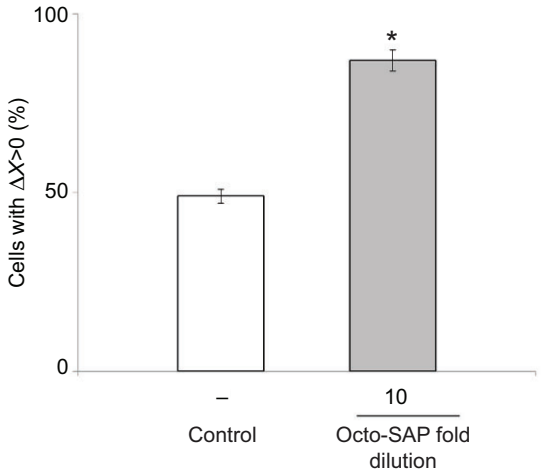

Fig. 6. Spermatocyte three-directionality-based parameters in the absence or in the presence of the purified Octo-SAP peptide. The graph shows the percentage of cells with a net distance greater than zero ($\Delta X>0$) in the absence (control) or in the presence of chemoattractant peptide at dilution 1:10 (Octo-SAP). Asterisk indicates a significant difference from control ($P<0.05$).

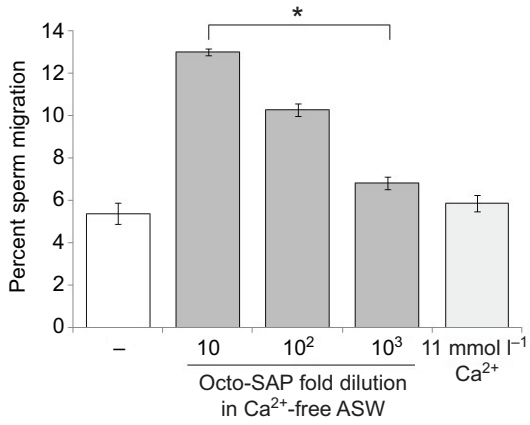

Fig. 7. Concentration-dependent profile of sperm chemotaxis elicited by Octo-SAP in Ca^{2+}-free artificial seawater (ASW). The graph illustrates the percentage of octopus spermatozoa migration towards a peptide gradient in a Ca^{2+}-free medium. The presence of extracellular Ca^{2+} at the concentration in normal seawater (11 mmol l^{-1}) did not affect sperm migration. Experiments were performed in triplicate in the presence or absence of the purified peptide. Asterisk indicates a significant difference from control ($P<0.05$).

with other polypeptides from other organisms, thus suggesting a species-specificity of this molecule. Currently, species-specific sperm responses to egg extracts have been reported for eight non-mammalian species, such as ascidians (Yoshida et al., 2002), coral (Coll et al., 1994), sea urchins (Guerrero et al., 2010), a starfish (Böhmer et al., 2005), a cuttlefish (Zatylny et al., 2002), an abalone (Riffell et al., 2002) and an amphibian (Olson et al., 2001). In the mollusk cephalopod *S. officinalis*, various sperm attractant factors have been purified; in this case, external fertilization occurs within the arm bundle that creates a 'protected' site for gamete fusion (Zatylny et al., 2002). These chemotactic molecules were characterized as being small peptides synthesized in the embedded oocytes during vitellogenesis, released in the external media during egg-laying and able to facilitate fertilization by increasing the chance of gamete collision. Our finding that Octo-SAP is selectively expressed in the egg mass of adult octopus females in the vitellogenic phase fits well with the proposal for its key role during the early stage of fertilization.

Furthermore, we have shown here that octopus spermatozoa exposed to an Octo-SAP gradient passed through the porous membrane of a Zigmond chamber in a dose-dependent manner and that this phenomenon was time-independent. This feature is consistent with the chemoattractant properties of this peptide. Analysis of the chemotactic responses of octopus spermatozoa further demonstrated that, in the absence of Octo-SAP, spermatozoa exhibit a circular movement with low motility, according with Brownian motion. By contrast, spermatozoa changed their swimming behavior in the presence of Octo-SAP, showing a significant polarization of migration vectors towards the chemoattractant source. These analyses demonstrated that this peptide is able to stimulate sperm motility, acting as a chemoattractant factor.

The hallmarks of the signalling pathways activated by sperm-activating and sperm-attracting peptides in marine species include the induction of Ca^{2+} entry from extracellular space and phosphorylation/dephosphorylation of spermatozoa proteins that initiate flagellar beating (Kaupp et al., 2008; Morita et al., 2009; Yoshida and Yoshida, 2011). Ca^{2+} influx and Tyr phosphorylation of axonemal sperm proteins have also been associated with chemotaxis in mammal species where internal fertilization occurs (Inaba, 2011). Consistently, we found that octopus sperm migration diminished by approximately 50% at all peptide dilutions applied in the absence of extracellular Ca^{2+}. Furthermore, western blotting carried out with an anti-Tyr antibody demonstrated the phosphorylation of a 75 kDa membrane protein in spermatozoa exposed to Octo-SAP.

Ca^{2+}-sensitive decoders translating ion influx into flagellar movement are far from being fully elucidated (Inaba, 2011; Kaupp et al., 2008; Yoshida and Yoshida, 2011); however, it has been shown that Ca^{2+}-dependent phosphorylation (Morita et al., 2009) and Ca^{2+}-dependent dephosphorylation events may regulate flagellar motility in various organisms. In particular, the Ca^{2+}-dependent calmodulin (CaM)-dependent kinase IV (CaMKIV) phosphorylation cascade enhances the ^{32}P-uptake of a 45 kDa protein that might regulate flagellar waveform in sperm from *H. atra* (Morita et al., 2009). Similarly, microtubule sliding movement in tilapia sperm flagella was associated with the CaM/CaMKIV-mediated phosphorylation of 48, 75, 120, 200, 250, 380 and 400 kDa axonemal proteins. Intriguingly, only a faint immunopositive 45 kDa band was observed in octopus spermatozoa pretreated with Octo-SAP in the absence of extracellular Ca^{2+}. Future experiments will be devoted to unveiling the molecular mechanisms recruited by this peptide in this organism. More specifically, the phosphorylation pathway(s) mediated by Ca^{2+} entry, yielding the phosphorylated 75 kDa protein, are currently under investigation. Data reported in this study show that dihydropyridine nifedipine, a selective HVA blocker, did not affect either spermatozoa migration or protein phosphorylation in response to Octo-SAP. The same feature was found for the ascidian spermatozoa exposed to the sperm-activating and -attracting factor (SAAF), which stimulates a chemotactic behavior by activating the store-operated Ca^{2+} channels (Yoshida et al., 2003). Conversely, HVA channels have been shown to contribute to the Ca^{2+} entry evoked by either resact or speract (Wood et al., 2007) in sea urchin sperm cells.

Recent studies conducted on mammal species revealed that progesterone may induce sperm chemotaxis and hyperactivation;

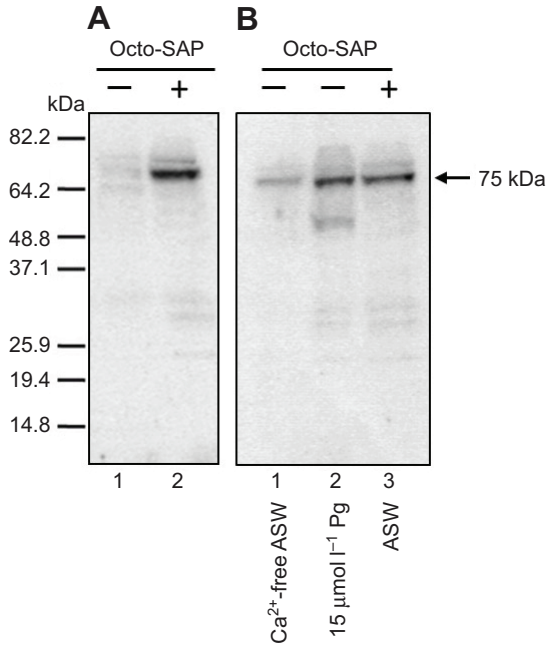

Fig. 8. Western blotting of sperm membrane phosphotyrosine-containing proteins in the absence or in the presence of the purified Octo-SAP peptide. (A) Analysis was carried out using a monoclonal anti-phosphotyrosine antibody in the absence (lane 1) or in the presence of the chemoattractant peptide in ASW (lane 2). (B) Analysis was carried out using a monoclonal anti-phosphotyrosine antibody in absence of calcium (lane 1), in presence of 15 µmol l^{-1} progesterone (lane 2) or in presence of the chemoattractant peptide (lane 3).

this hormone triggers the acrosome reaction by activating Ca^{2+} entry into the sperm flagellum (Lishko et al., 2011). Our previous results on *O. vulgaris* showed that the stimulation of spermatozoa collected from the spermatophores in the male reproductive tracts with progesterone or the Ca^{2+}-ionophore A23187 induces the breakdown of the membranes overlapping with the acrosomal region, thus exposing a spiralized acrosome (Tosti et al., 2001). These events resemble an acrosome-like reaction. Supporting the hypothesis of a pre-activation mediated by factor(s) released by female reproductive tracts, spermatozoa stored in the spermathecae of the female oviducal gland do not show the membranes covering the acrosomal region present in the male spermatozoa (Tosti et al., 2001). As the oviducal gland and non-activated spermatozoa are both targets of progesterone, as demonstrated by the presence of a nuclear and membrane receptors of this sex steroid, respectively (Tosti et al., 2001; Di Cosmo and Di Cristo, 1998), we believe that progesterone may play a key role in the activation (or better pre-activation) of the octopus spermatozoa within the female spermathecae. Surprisingly, progesterone did not affect spermatozoa migration when probed at a concentration (i.e. 15 µmol l^{-1}) that is known to induce the acrosome-like reaction. Experiments conducted on spermatozoa collected from the spermatophores in the male reproductive tracts or from the distal oviduct of inseminated females gave similar results. These findings suggest that progesterone, unlike mammalian sperm, does not act as a proper chemoattractant factor; by contrast, it may induce processes on spermatozoa, such as capacitation, acrosome reaction or protein phosphorylation in the oviducal gland, where these male cells are immobilized and stored.

In light of all previous data on octopus and those presented in the present study, we can conclude that male sperm seems to pass throughout the female distal oviduct and is immobilized in the mucosa of the oviducal gland. Endogenous progesterone induces an increase of the intracellular calcium necessary for spermatozoa pre-activation (Tosti et al., 2001). When the eggs are ready to be fertilized by falling in the fertilization chamber, a release of the chemoattractant factor Octo-SAP induces spermatozoa mobilization towards a concentration gradient, which is followed by Ca^{2+} mobilization and membrane protein Tyr phosphorylation. These events facilitate egg fertilization, although the molecular mechanisms underlying this event are far from being fully characterized.

Conclusions

Our results shed lights on mechanisms and factors that mobilize and attract sperm toward eggs in *O. vulgaris*, which, unlike other cephalopods, adopts an indirect internal fertilization strategy. These comprise just one piece of the complex puzzle that is the reproductive behavior exhibited by this species, which comprises multiple mates for both sexes, resulting in multiple paternity.

This scenario calls for many questions, starting with: (1) how many sperm are stored in the spermathecae of the female; and (2) because the females are promiscuous, how does consequent sperm competition affect reproductive success?

Studies that address these specific questions are lacking but are needed to extend our knowledge not only on reproductive strategies in cephalopods, but also on population and conservation genetics, which might be useful for repopulation strategies for depleted octopus fisheries, considering that a male-biased contribution will increase effective population size.

AUTHOR CONTRIBUTIONS

E.D.L. designed and performed experiments; A.M.S. and A.S. characterized the chemoattractant peptide; F.M. contributed to the signalling pathway experiments; and A.D.C. designed experiments, analysed data and wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Bertolucci, E., Conti, M., Di Cosmo, A., Maiorino, M., Mettivier, G., Montesi, M. C., Paolella, G., Pecorella, T., Russo, P. and Scognamiglio, R. (2002). Real time β-imaging with silicon hybrid pixel detectors: kinetic measurement with C-14 amino acids and P-32 nucleotides. *IEEE Trans. Med. Imaging* **49**, 2213-2217.

Boal, J. G., Prosser, K. N., Holm, J. B., Simmons, T. L., Haas, R. E. and Nagle, G. T. (2010). Sexually mature cuttlefish are attracted to the eggs of conspecifics. *J. Chem. Ecol.* **36**, 834-836.

Böhmer, M., Van, Q., Weyand, I., Hagen, V., Beyermann, M., Matsumoto, M., Hoshi, M., Hildebrand, E. and Kaupp, U. B. (2005). Ca^{2+} spikes in the flagellum control chemotactic behavior of sperm. *EMBO J.* **24**, 2741-2752.

Calvello, M., Guerra, N., Brandazza, A., D'Ambrosio, C., Scaloni, A., Dani, F. R., Turillazzi, S. and Pelosi, P. (2003). Soluble proteins of chemical communication in the social wasp *Polistes dominulus*. *Cell. Mol. Life Sci.* **60**, 1933-1943.

Coll, J. C., Bowden, B. F., Meehan, G. V., Konig, G. M., Carroll, A. R., Tapiolas, D. M., Alin, P. M., Heaton, A., De Nys, R., Leone, P. A. et al. (1994). Chemical aspects of mass spawning in corals. I. Sperm-attractant molecules in the eggs of the scleractinian coral *Montipora digitata*. *Mar. Biol.* **118**, 177-182.

Cummins, S. F., Nichols, A. E., Schein, C. H. and Nagle, G. T. (2006). Newly identified water-borne protein pheromones interact with attractin to stimulate mate attraction in *Aplysia*. *Peptides* **27**, 597-606.

Cummins, S. F., Nuurai, P., Nagle, G. T. and Degnan, B. M. (2010). Conservation of the egg-laying hormone neuropeptide and attractin pheromone in the spotted sea hare, *Aplysia dactylomela*. *Peptides* **31**, 394-401.

Cummins, S. F., Boal, J. G., Buresch, K. C., Kuanpradit, C., Sobhon, P., Holm, J. B., Degnan, B. M., Nagle, G. T. and Hanlon, R. T. (2011). Extreme aggression in male squid induced by a β-MSP-like pheromone. *Curr. Biol.* **21**, 322-327.

Di Cosmo, A. and Di Cristo, C. (1998). Neuropeptidergic control of the optic gland of *Octopus vulgaris*: FMRF-amide and GnRH immunoreactivity. *J. Comp. Neurol.* **398**, 1-12.
Di Cosmo, A., Di Cristo, C. and Paolucci, M. (2001). Sex steroid hormone fluctuations and morphological changes of the reproductive system of the female of *Octopus vulgaris* throughout the annual cycle. *J. Exp. Zool.* **289**, 33-47.
Di Cristo, C., Paolucci, M., Iglesias, J., Sanchez, J. and Di Cosmo, A. (2002). Presence of two neuropeptides in the fusiform ganglion and reproductive ducts of *Octopus vulgaris*: FMRFamide and gonadotropin-releasing hormone (GnRH). *J. Exp. Zool.* **292**, 267-276.
Di Cristo, C., Van Minnen, J. and Di Cosmo, A. (2005). The presence of APGWamide in *Octopus vulgaris*: a possible role in the reproductive behavior. *Peptides* **26**, 53-62.
Di Cristo, C., Paolucci, M. and Di Cosmo, A. (2008). Progesterone affects vitellogenesis in *Octopus vulgaris*. *Open Zool. J.* **1**, 29-36.
Eisenbach, M. (1999). Sperm chemotaxis. *Rev. Reprod.* **4**, 56-66.
Fabro, G., Rovasio, R. A., Civalero, S., Frenkel, A., Caplan, S. R., Eisenbach, M. and Giojalas, L. C. (2002). Chemotaxis of capacitated rabbit spermatozoa to follicular fluid revealed by a novel directionality-based assay. *Biol. Reprod.* **67**, 1565-1571.
Guerrero, A., Wood, C. D., Nishigaki, T., Carneiro, J. and Darszon, A. (2010). Tuning sperm chemotaxis. *Biochem. Soc. Trans.* **38**, 1270-1274.
Hanlon, R. T. and Messenger, J. B. (1996). *Cephalopod Behavior.* Cambridge: Cambridge University Press.
Inaba, K. (2011). Sperm flagella: comparative and phylogenetic perspectives of protein components. *Mol. Hum. Reprod.* **17**, 524-538.
Kaupp, U. B., Solzin, J., Hildebrand, E., Brown, J. E., Helbig, A., Hagen, V., Beyermann, M., Pampaloni, F. and Weyand, I. (2003). The signal flow and motor response controling chemotaxis of sea urchin sperm. *Nat. Cell Biol.* **5**, 109-117.
Kaupp, U. B., Kashikar, N. D. and Weyand, I. (2008). Mechanisms of sperm chemotaxis. *Annu. Rev. Physiol.* **70**, 93-117.
Kuanpradit, C., Stewart, M. J., York, P. S., Degnan, B. M., Sobhon, P., Hanna, P. J., Chavadej, J. and Cummins, S. F. (2012). Characterization of mucus associated proteins from abalone (*Haliotis*): candidates for chemical signaling. *FEBS J.* **279**, 437-450.
Kupfermann, I. and Carew, T. J. (1974). Behavior patterns of *Aplysia californica* in its natural environment. *Behav. Biol.* **12**, 317-337.
Lishko, P. V., Botchkina, I. L. and Kirichok, Y. (2011). Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* **471**, 387-391.
Miller, R. L. (1978). Site-specific sperm agglutination and the timed release of a sperm chemo-attractant by the egg of the leptomedusan, *Orthopyxis caliculata*. *J. Exp. Zool.* **205**, 385-391.
Morita, M., Kitamura, M., Nakajima, A., Sri Susilo, E., Takemura, A. and Okuno, M. (2009). Regulation of sperm flagellar motility activation and chemotaxis caused by egg-derived substance(s) in sea cucumber. *Cell Motil. Cytoskeleton* **66**, 202-214.
Nishigaki, T., Chiba, K., Miki, W. and Hoshi, M. (1996). Structure and function of asterosaps, sperm-activating peptides from the jelly coat of starfish eggs. *Zygote* **4**, 237-245.
Olson, J. H., Xiang, X. Y., Ziegert, T., Kittelson, A., Rawls, A., Bieber, A. L. and Chandler, D. E. (2001). Allurin, a 21-kDa sperm chemoattractant from *Xenopus* egg jelly, is related to mammalian sperm-binding proteins. *Proc. Natl. Acad. Sci. USA* **98**, 11205-11210.
Oren-Benaroya, R., Orvieto, R., Gakamsky, A., Pinchasov, M. and Eisenbach, M. (2008). The sperm chemoattractant secreted from human cumulus cells is progesterone. *Hum. Reprod.* **23**, 2339-2345.
Painter, S. D., Cummins, S. F., Nichols, A. E., Akalal, D. B. G., Schein, C. H., Braun, W., Smith, J. S., Susswein, A. J., Levy, M., de Boer, P. A. C. M. et al. (2004). Structural and functional analysis of Aplysia attractins, a family of water-borne protein pheromones with interspecific attractiveness. *Proc. Natl. Acad. Sci. USA* **101**, 6929-6933.
Polese, G., Ulloa Severino, F. P., Willow, W. and Di Cosmo, A. (2012). Anesthesia of *Octopus vulgaris* with the volatile general anesthetic isoflurane. In *Cephalopod International Advisory Council Symposium*. Florianopolis, Santa Catarina, Brazil.
Riffell, J. A., Krug, P. J. and Zimmer, R. K. (2002). Fertilization in the sea: the chemical identity of an abalone sperm attractant. *J. Exp. Biol.* **205**, 1439-1450.
Rodriguez de la Vega, R. C., Garcia, B. I., D'Ambrosio, C., Diego-Garcia, E., Scaloni, A. and Possani, L. D. (2004). Antimicrobial peptide inductionin the hoemolymph of the Mexican scorpion *Centruroides limpidus limpidus* in response to septic injury. *Cell. Mol. Life Sci.* **61**, 1507-1519.
Russell, W. M. S. and Burch, R. L. (1959). *The Principles of Humane Experimental Technique.* London: Methuen.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.
Stork, A. P. and Cocks, T. M. (1994). Pharmacological reactivity of human epicardial coronary arteries: phasic and tonic responses to vasoconstrictor agents differentiated by nifedipine. *Br. J. Pharmacol.* **113**, 1093-1098.
Suarez, S. S. and Ho, H. C. (2003). Hyperactivated motility in sperm. *Reprod. Domest. Anim.* **38**, 119-124.
Suzuki, N. and Yoshino, K. (1992). The relationship between amino acid sequences of sperm-activating peptides and the taxonomy of echinoids. *Comp. Biochem. Physiol. B* **102**, 679-690.
Torres-Flores, V., Hernández-Rueda, Y. L., Neri-Vidaurri, P. C., Jiménez-Trejo, F., Calderón-Salinas, V., Molina-Guarneros, J. A. and González-Martínez, M. T. (2008). Activation of protein kinase A stimulates the progesterone-induced calcium influx in human sperm exposed to the phosphodiesterase inhibitor papaverine. *J. Androl.* **29**, 549-557.
Tosti, E., Di Cosmo, A., Cuomo, A., Di Cristo, C. and Gragnaniello, G. (2001). Progesterone induces activation in *Octopus vulgaris* spermatozoa. *Mol. Reprod. Dev.* **59**, 97-105.
Westermann, B. and Beuerlein, K. (2005). Y-maze experiments on the chemotactic behaviour of the tetrabranchiate cephalopod *Nautilus pompilius* (Mollusca). *Mar. Biol.* **147**, 145-151.
Wood, C. D., Nishigaki, T., Tatsu, Y., Yumoto, N., Baba, S. A., Whitaker, M. and Darszon, A. (2007). Altering the speract-induced ion permeability changes that generate flagellar Ca^{2+} spikes regulates their kinetics and sea urchin sperm motility. *Dev. Biol.* **306**, 525-537.
Yoshida, M. and Yoshida, K. (2011). Sperm chemotaxis and regulation of flagellar movement by Ca^{2+}. *Mol. Hum. Reprod.* **17**, 457-465.
Yoshida, M., Murata, M., Inaba, K. and Morisawa, M. (2002). A chemoattractant for ascidian spermatozoa is a sulfated steroid. *Proc. Natl. Acad. Sci. USA* **99**, 14831-14836.
Yoshida, M., Ishikawa, M., Izumi, H., De Santis, R. and Morisawa, M. (2003). Store-operated calcium channel regulates the chemotactic behavior of ascidian sperm. *Proc. Natl. Acad. Sci. USA* **100**, 149-154.
Zatylny, C., Gagnon, J., Boucaud-Camou, E. and Henry, J. (2000). ILME: a waterborne pheromonal peptide released by the eggs of *Sepia officinalis*. *Biochem. Biophys. Res. Commun.* **275**, 217-222.
Zatylny, C., Marvin, L., Gagnon, J. and Henry, J. (2002). Fertilization in *Sepia officinalis*: the first mollusk sperm-attracting peptide. *Biochem. Biophys. Res. Commun.* **296**, 1186-1193.
Zigmond, S. H. (1977). Ability of polymorphonuclear leukocytes to orient in gradients of chemotactic factors. *J. Cell Biol.* **75**, 606-616.