

# Ovarian and Sperm Regulatory Peptides Regulate Ovulation in the Oyster *Crassostrea gigas*

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**ABSTRACT** For more than six decades, several studies have shown that genital products to entering the mantle cavity via the incurrent siphon, initiate in oyster, strong and rhythmic contractions of the adductor muscle (AM). In order to characterize the regulatory peptides capable of triggering AM contractions, we focused on the identification of putative myotropic peptides from genital products. Two experimental approaches were developed. The first one, based on a mass spectrometry screening of the male genital products, led to the identification of the tetrapeptide APGWamide. This neuropeptide was also detected in the seminal secretions of the cephalopod *Sepia officinalis*. In this species, APGWamide is directly involved in the oocyte transport. In *Crassostrea*, in vitro bioassay demonstrated that APGWamide modulates the AM contractions that insure the release of oocytes in the external medium. Exposure of oysters to a physiological concentration of APGWamide triggered repetitive shell closures. The second experimental approach was based on the monitoring of HPLC purification by a myotropic bioassay using the cuttlefish oviduct contractions as a target. The successive purification steps of the acidic extraction of ovaries from mature female oysters, led to the characterization of the hexapeptide PIESVD. When applied to mature female oysters, this peptide triggered the increase of shell closure frequency. The activity of these two regulatory peptides is the first experimental evidence of a peptidergic control of egg-laying in oyster. APGWamide and PIESVD could be used, in commercial and experimental hatcheries, for the identification of mature females to be selected for in vitro fertilization. *Mol. Reprod. Dev.* 73: 607–616, 2006.

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**Key Words:** molluscan; egg-laying; APGWamide; myotropin; mass spectrometry

## INTRODUCTION

In the oyster *Crassostrea gigas*, egg-laying is a complex behavior described by O'Foighil and Taylor (O'Foighil and Taylor, 2000) as "trans-ctenidial ovulation" (TCO). This behavior involves the coordinated activity of the mantle margins, gills and adductor muscle (AM) and is exclusively restricted to the spawning females. The regulation of TCO by environmental factors has been well described (Deslous-Paoli et al., 1982; Heral et al., 1986; Maurer and Borel, 1986). To date, no regulatory peptides involved in TCO have been identified in oysters, although several studies demonstrate that the addition of a sperm suspension to the water medium triggers egg-laying in mature oysters (Galtsoff, 1938b, 1964; Nelson and Allison, 1940). Furthermore, in most of the observed cases, the release of male products precedes the induction of egg-laying in females. Recently, Rice et al. (2002) identified from *C. virginica* an intrinsic sperm membrane protein involved in the regulation of egg-laying. The addition of female genital products of the same species also triggered spawning from mature females. In the cuttlefish, *Sepia officinalis*, the ovarian peptide content involved in the egg-laying regulation was investigated by a myotropic bioassay, using oviduct contractions as an endpoint (Zatylyn et al., 2000b,c; Bernay et al., 2004). This bioassay was also used to identify neuropeptides regulating the same egg-laying steps in *S. officinalis* (Henry et al., 1997, 1999). In both *C. gigas* and *S. officinalis*, these steps are clearly stereotyped. In oysters,

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the transport of oocytes through the genital tract is performed by the ciliary motion of the epithelium (Hoek, 1883; Galtsoff, 1964). Oocytes are released and stored in the exhalant chamber. Then, they are forced through the gills to the inhalant chamber by highly characteristic AM contractions (Galtsoff, 1938a; Nelson and Allison, 1940). Finally, oocytes are released into the external medium, by the AM contractions, through a small spawning window opened between the inhalant chamber mantle margins (Nelson, 1921; Galtsoff, 1930, 1938a,b, 1961, 1964; Nelson and Allison, 1940; Rice et al., 2002). This activity is thought to result in a relatively wide and uniform distribution of oocytes, improving the chances of fertilization. In the cuttlefish, the first step of egg-laying is the release by the ovary of full-grown oocytes (FGO) into the genital coelom (Dhainaut and Richard, 1976). During this period, ovarian factors released by FGO inhibit the oviducal contractions and lead to the accumulation of FGO in the genital coelom (Zatylny et al., 2000a; Bernay et al., 2004). After mating, oocytes transport is performed through the oviduct by the whole genital tract (WGT) contractions, which are modulated by neuro- and ovarian peptides (Henry et al., 1997, 1999; Zatylny et al., 2000b,c). Oocytes then received two gelatinous envelopes and are fertilized by spermatozoas stored in the female's copulatory pouch (Boletsky, 1983; Hanlon et al., 1999). Finally, the eggs are attached to a substrate until hatching. In both species studied, muscle contractions are responsible for the release of oocytes: AM contractions in oyster and WGT contractions in cuttlefish. In the last six decades, numerous studies have shown that oysters initiate a series of strong and rhythmic contractions of the AM in response to genital products entering the mantle cavity via the incurrent siphon (Galtsoff, 1938a,b, 1964; Nelson and Allison, 1940; Rice et al., 2002). In order to characterize myotropic peptides from male and female genital products in oyster, we developed two experimental approaches. The first one was based on the peptidomic analysis performed on male genital products. The peptidome was screened by means of mass spectrometry using specific sequence libraries. The second experimental approach consisted of the HPLC purification of putative ovarian peptides monitored by the cuttlefish oviduct contractions myotropic bioassay, which is a reliable and specific biological test.

## MATERIALS AND METHODS

### Animals

All the cuttlefish were trapped in the Baie of Seine between January and June. They were maintained in 1,000-L outflow tanks at  $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$  at the Marine Station of Luc sur Mer (University of Caen, France) under a natural photoperiod. Oysters come from Aber Benoît (Bretagne, France) and Saint Vaast la Hougue (Normandie, France).

### Micro LC-ESI-MS/MS Analysis

Analyses were performed with a HPLC Surveyor chain connected online to an orthogonal electrospray

source (Deca XP MS-n Thermofinnigan) operated in the positive electrospray ionization mode (ESI+). The ions were focused into an ion trap, capable of MS and MS/MS analyses. The mass spectra were acquired during 35 ms from  $m/z$  300 to 2,000. The capillary exit of the electrospray ion source was set at 70 V, the octapole at 3 V and the capillary temperature at  $200^{\circ}\text{C}$ . A counter flow of nitrogen was used as nebulizing gas. An Xcalibur data system was used to acquire the data, which were further processed with Turbo Sequest data system. The organic fraction of each extract was resuspended in 10  $\mu\text{l}$  of 0.1% formic acid in water and injected onto a C18 Thermo Hypersil column ( $50 \times 0.5\text{mm}$ , 3  $\mu\text{m}$ ) with an acetonitrile linear gradient of 3% per minute in formic acid 0.1%, from 2% to 60%. A split ratio of 30:1 was used to perfuse the column at a flow rate of 10  $\mu\text{l}/\text{min}$ . HPLC column was rinsed with 90% acetonitrile in 0.1% formic acid between each injection. The MS data was acquired in scan mode considering the positive ion signal.

### In Vitro Bioassay

The myotropic bioassay was performed with several contractile organs: the WGT, the penis and the vas deferens from *S. officinalis* and the AM from *C. gigas*. Each organ was suspended from a displacement transducer (Phymep, Bionic Instruments) connected to a computer controlling the recorder and the DATAC (Dispositif d'Acquisition et de Traitement Automatique de la Contraction). The muscle chamber was perfused at the flow rate of 0.5 ml/min with synthetic seawater (Instant Ocean) containing 1 mM glucose and maintained at  $15^{\circ}\text{C}$ . The samples were injected in the perfusing flow using a three-way valve in order to avoid mechanical and thermal stress. The flow of fractions into the muscle chamber was traced by addition of phenol red.

### In Vivo Bioassay: Shell Closure Frequency

Oysters were placed at  $4^{\circ}\text{C}$  out of water the night before the bioassay. During the experiment, each oyster was placed at  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , in a 450 ml tank containing an algal cocktail (25% *Tetraselmis* sp., 25% *T. iso*, 25% *Chaetoceros calcitrans*, and 25% *Skeletonema costatum*) adjusted to 30 cells/ $\mu\text{l}$ . Water was circulated in each tank to insure opening of the valves and filtration by the animals. Following addition of sample, the shell closure frequency was visually monitored during the subsequent assay period (10 min). Shell movements during female spawning period are so typical that they cannot be mistaken from any other type of muscular activity (Galtsoff, 1938a).

### Synthetic Peptides

Peptides were synthesized using classical Fmoc (N-[9-fluorenyl] methoxycarbonyl) solid-phase chemistry and a commercially available automated peptide synthesizer by coupling Fmoc- $\alpha$ -amino acids on preloaded Wang resin. Protected amino acids were coupled by in situ activation with diisopropylcarbodiimide (DIPCDI) and

*N*-hydroxybenzotriazole (HOBt). *N* $\alpha$ -Fmoc deprotection was performed with 20% piperidine in DMF. Side chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent B (88% trifluoroacetic acid (TFA)/5% Phenol/5% water/2% TIS) for 2h at 20°C (Sole and Barany, 1992). Peptides were purified by reversed-phase HPLC (RP-HPLC) using a waters semi-preparative HPLC system on an X Terra 10  $\mu$ m column (300  $\times$  19 mm). The elution was achieved with a linear gradient of aqueous 0.1% TFA (A) and 0.08% TFA in acetonitrile (B) at a flow rate of 10 ml/min with photodiode array detection at 210–440 nm. The purity of each peptide was controlled by analytical RP-HPLC on the same instrument with a X Terra 5  $\mu$ m column (250  $\times$  4.6 mm) using a linear gradient of 0.1% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1 ml/min. Finally, integrity of each peptide was assessed by micro LC-ESI-MS/MS analysis.

### Sperm Peptides

**Recovery of material from tissues.** Organs were harvested as follows: for *S. officinalis*: central nervous system (supra-oesophageal mass, sub-oesophageal mass, optic lobes) and genital tract (vas deferens, seminal vesicles, penis, spermatophores dissected from mature male); for *C. gigas*: oocytes, mantle edge, gills, labial palpe, smooth muscle, and striated muscle of the AM dissected from mature males and females. For micro LC-ESI-MS/MS analysis, 5 animal-equivalent of each organ studied, 80 animal-equivalent of visceral ganglions, 150 g of oocytes, and 40 g of sperm from *C. gigas* were successively homogenized in 10 volumes of 0.1N HCl at 100°C and centrifuged 30 min at 35,000g at 4°C. The supernatants were concentrated using Chromafix C18 cartridges. In *S. officinalis*, the spermatophores (complex tubular system containing spermatozoa) were removed from the spermatophoric sacs or Needham's organs and separated into two aliquots. The first aliquot was washed with artificial SW (Instant Ocean) in order to remove seminal secretions. The two aliquots were then extracted as described above.

**Identification of regulatory peptides from male genital products.** A specific fasta library containing most invertebrates regulatory peptides was created to enable the identification of peptides by MS/MS. Screenings were performed using the Sequest software (ThermoFinnigan) which can compare experimental and theoretical MS/MS spectra generated from fasta libraries. For each peptide, the amino acid sequence was definitely confirmed by the comparison between synthetic and endogenic peptide MS/MS spectra.

**Bioactivity of APGWamide.** Aliquots of synthetic APGWamide (Sigma Aldrich) ranging from 10<sup>-8</sup> to 10<sup>-6</sup> M were tested using the in vitro and in vivo bioassays in order to obtain qualitative and quantitative data associated with its myotropic activity.

**APGWamide oysters in vivo bioassay.** Two bioassays were performed with the APGWamide. The first bioassay was performed using 15 oysters and 10<sup>-7</sup> M APGWamide for the first sample and 26 oysters and

10<sup>-6</sup> M APGWamide for the second sample. The second bioassay was performed with 200 oysters and 10<sup>-6</sup> M APGWamide. For each bioassay, the shell closure events were recorded during 10 min after injection of the peptide. The experiment was followed by the sacrifice of the animals for sex determination.

**Tissue mapping by micro LC-ESI-MS/MS.** The occurrence of peptides in the samples was established by the MS/MS data of the m/z 429.5 (APGWa), 459.6 (TPGWa), 486.7 (KPGWa), 514.7 (TPGWa), and 261.2 (GWa). This method detected small amounts of peptide. Peptide identifications were obtained from the comparison of MS/MS spectra of the selected m/z with the synthetic peptides.

### Ovarian Peptides

**Recovery of material from tissues.** For HPLC purification and microLC-ESI-MS/MS analysis, 400 g of oyster ovaries were homogenized in 4 L of 0.1N HCl at 100°C and centrifuged for 30 min at 35,000g at 4°C. Supernatants were concentrated on Chromafix C18 cartridges. Moreover, the female genital products released by five oysters were extracted in 0.1N HCl at 100°C, centrifuged and concentrated on Chromafix C18 cartridges.

**UV-HPLC purification.** HPLC analyses were performed with a Varian 4050 integrator connected to a Varian 9012 solvent delivery system and a Varian 9050 wavelength UV-VIS detector set at 214 nm. The ovary extract was resuspended in 0.1% formic acid in water and injected onto a Nucleodur C18 column (250  $\times$  10 mm, 20  $\mu$ m) with an acetonitrile linear gradient of 1.33% per minute in 0.1% formic acid at a flow rate of 5 ml/min. A bioactive fraction eluted at 29 min was injected onto a Nucleosil C18 column (250  $\times$  3 mm, 7  $\mu$ m) with an acetonitrile linear gradient of 1.05% per minute in 10 mM ammonium acetate at a flow rate of 1 ml/min. The third purification step was performed on a Supelcosil C18 column (150  $\times$  4.6 mm, 3  $\mu$ m) with an acetonitrile linear gradient of 1.33% per minute in 0.1% formic acid at a flow rate of 1 ml/min. Finally, the last step of purification was performed in LC-ESI-MS/MS on a Nucleosil C18 column (120  $\times$  4 mm, 3  $\mu$ m) with an acetonitrile linear gradient of 0.66% per minute in 0.1% formic acid at a flow rate of 600  $\mu$ l/min. A split ratio of 100:1 was used to perfuse the electrospray source at a flow rate of 10  $\mu$ l/min. One minute fractions of each step were collected, dried, and stored at 4°C until use.

**Amino acid sequencing.** Fasta libraries were generated with the software Aaseq 5.0 (Zanuttini and Henry, University of Caen). Screenings were performed using Sequest software (ThermoFinnigan), which is able to compare experimental MS/MS spectra and theoretical MS/MS spectra generated from fasta libraries. The MS/MS spectrum of the amino acid sequence was then checked using MS-Tag and MS-Product (Protein Prospector 3.4.1, University of California). Finally, primary structures were verified by Edman degradation. N-terminal sequence analyses were performed using an Applied Biosystems Model 477 A protein sequencer and

amino acid phenylthiohydantoin derivatives were identified and quantified online with a Model 120A HPLC system, as recommended by the manufacturer.

**PIESVD oysters in vivo bioassay.** The shell closure bioassay was performed in the middle of June with oysters from Saint Vaast la Hougue, matured in SMEL (Syndicat Mixte pour l'Équipement du Littoral, Blainville). The first bioassay was performed with PIESVD  $10^{-7}$  M on a sample of eight oysters and the second on a sample of 39 oysters with PIESVD  $10^{-6}$  M. Shell closure events were visually recorded during 10 min. The experiment was followed by the sacrifice of animals for sex determination.

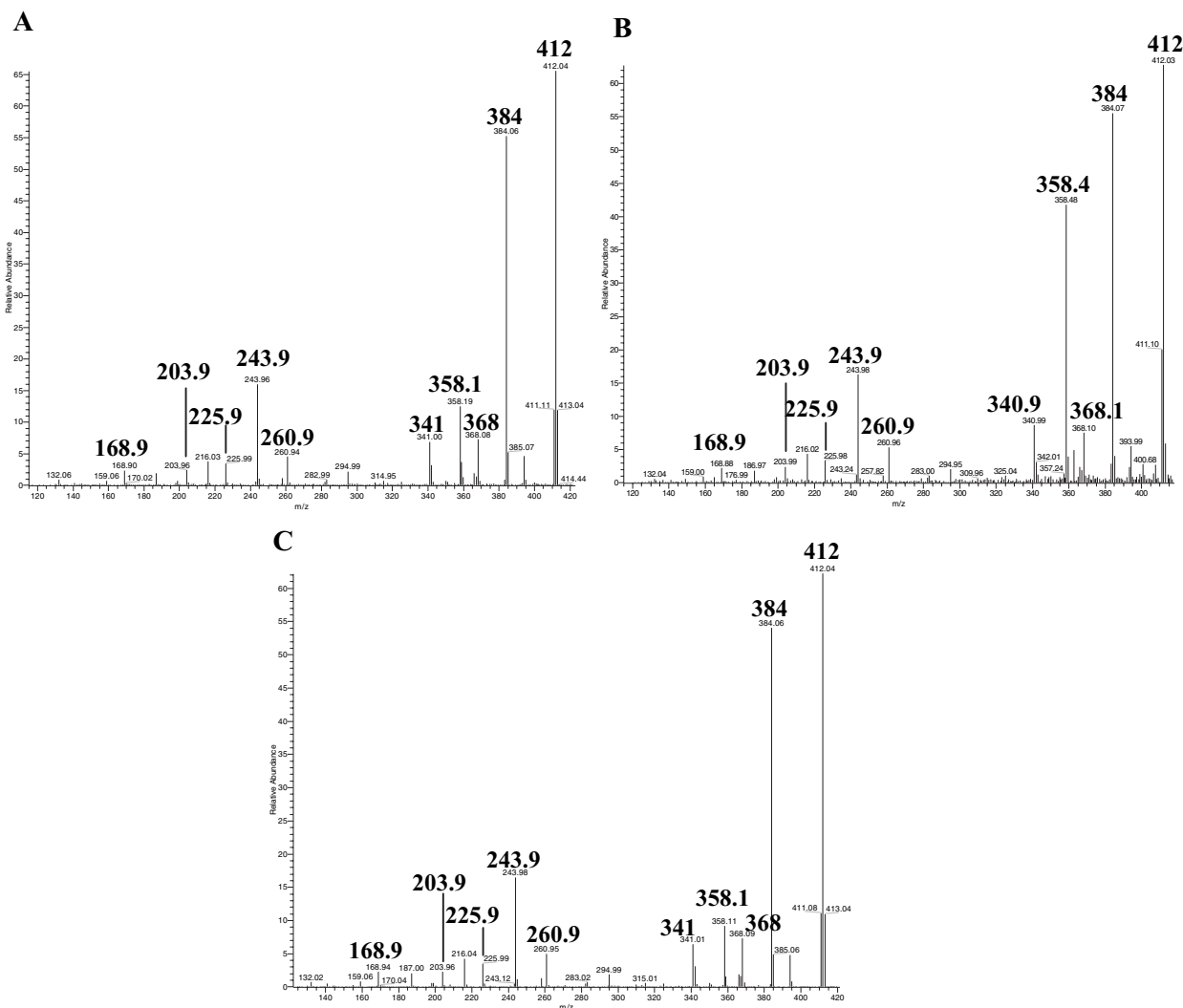
**Statistics.** Significant differences between treatments were tested by one-way analysis of variance (ANOVA) using the Statistica software. The method used to discriminate among the means was the Student's Newman Keuls procedure. Results were deemed significant for  $P < 0.05$ .

## RESULTS

### Regulatory Peptides From Sperm

**Detection of APGWamide by Micro LC-ESI-MS/MS in male genital products of both studied species.** Putative regulatory peptides were investigated by Sequest software using the sequence library of invertebrate regulatory peptides. This approach led to the identification of APGWamide in male genital products of both *S. officinalis* and *C. gigas*. The comparison of MS/MS data obtained from synthetic and endogenic peptides confirmed the amino acid sequence (Fig. 1). Furthermore, in the cuttlefish, APGWamide was detected in the seminal fluid coating of spermatophores but not in washed spermatophores demonstrating that APGWamide was associated with the seminal fluid.

**Bioactivity of APGWamide on *S. officinalis* male genital tract.** For concentrations ranging from



**Fig. 1.** MS/MS spectra of synthetic APGWa (A) and endogenic APGWa detected in male products of *Crassostrea gigas* (B) and *Sepia officinalis* (C).

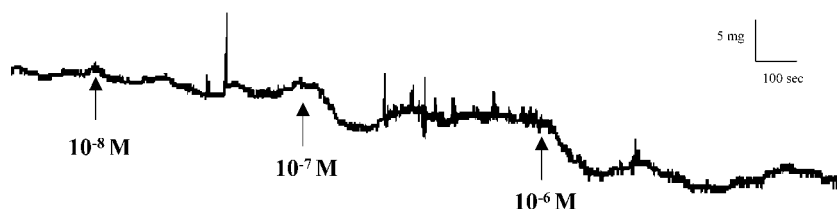


Fig. 2. Dose dependent APGWamide-induced contractions of the adductor muscle of *Crassostrea gigas*.

$10^{-8}$  to  $10^{-6}$  M, APGWamide had no effect on penis and proximal vas deferens contractions.

**Bioactivity of APGWamide on *S. officinalis* female WGT.** The synthetic peptide was tested on the WGT of mature and immature females. From a threshold at  $10^{-7}$  M, APGWamide inhibited the contraction of this tract, decreasing the amplitude and the intensity of the contractions in mature females (data not shown). This biological activity was previously described in (Henry et al., 1997). In immature females, APGWamide had no effect on WGT contractions.

**Bioactivity of APGWamide on oyster AM.** The synthetic peptide was tested on the AM of mature female oysters. From  $10^{-7}$  M, APGWamide decreased the intensity of the contractions. In a dose-response experiment, the increase of concentration appeared to be correlated with the increase of biological activity (Fig. 2).

**Shell closure in vivo biological assay.** Egg-laying was never triggered during experiments. For the first bioassay, oysters were exposed to  $10^{-7}$  M or  $10^{-6}$  M APGWamide and shell closure events were recorded during 10 min. After dissection, we noticed that APGWamide increased shell closure frequency from a threshold of  $10^{-7}$  M on mature females. This peptide had no significant effect on mature males and immature oysters (Fig. 3). A second experiment was performed by exposing 200 oysters to  $10^{-6}$  M APGWamide. Shell closures were recorded during 10 min. Shell closures ( $\geq 4$ ) were displayed by 48% of oysters. Sex was confirmed after dissection. APGWamide induced shell closure events in 6% of immature oysters, 30% of males and 67% of females. Results are summarized in Figure 4.

**APGWa-RPs tissue-mapping by microLC-ESI-MS/MS.** Standard MS/MS spectra were acquired from

the injection of 1 ng of each of the APGWamide-related-peptides (APGWa-RPs). These spectra were used for the identification of APGWa-RPs in the tissues of *S. officinalis* and *C. gigas*. Table 1 summarizes the results obtained for the tissue-mapping performed in the male genital tract and central nervous system (CNS) of *S. officinalis*. In the male genital tract, APGWa was detected in the spermatophoric glands and TPGWa in the vas deferens. In the CNS, APGWa, TPGWa, and GWa were detected in the optic lobes, supra- and sub-oesophageal mass. No APGWamide-related peptides were detected in the hemolymph. Table 2 summarizes the results obtained for the tissue-mapping performed in males and females of *C. gigas*. Only APGWamide was detected in the sperm and visceral ganglions. APGWa-RPs were not detected in somatic tissues.

### Ovarian Regulatory Factors

**HPLC purification.** The purification of oyster oocytes extract was monitored by the cuttlefish oviduct bioassay. After the first purification step, a myotropic activity was detected in the 28–29 min fraction. After two further steps of purification, the activity was concentrated in an apparently pure peak with a retention time of 17–18 min (Fig. 5). The last purification step was performed in LC-ESI-MS/MS.

**LC-ESI-MS/MS purification.** The 17–18 min HPLC fraction, containing the myotropic activity was submitted to LC-ESI-MS/MS purification. The spectrum revealed a pure peak at  $m/z$   $659 \pm 0.5$  (Fig. 6). This peak was concentrated to be submitted to Edman degradation.

**Amino-acid sequence determination.** Aaseq 5.0 software was used to build a sequence library ranging from 657 to 660 Da. The primary sequence of the peptide was partially determined by Sequest software, MS-Tag and MS-Product: PI/LESVD. The primary sequence of this peptide was definitively

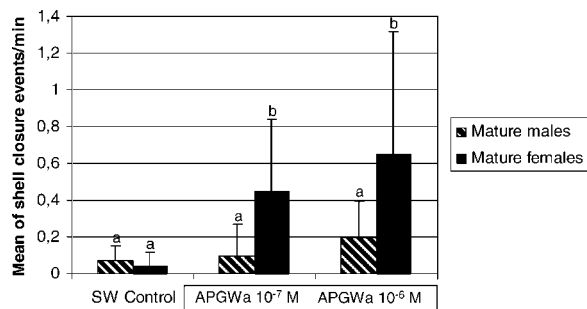


Fig. 3. Shell closure in vivo bioassay. APGWamide-induced contraction of the female adductor muscle of *Crassostrea gigas* at a threshold of  $10^{-7}$  M. Letters indicate a significant difference between treatments (mean  $\pm$  SD; ANOVA,  $P < 0.05$ ).



Fig. 4. Shell closure in vivo bioassay performed with APGWamide  $10^{-6}$  M. APGWamide triggers more shell closures in mature females than in males or immature oysters.

TABLE 1. Tissue Mapping of APGWamide-RPs in Male *Sepia officinalis*

Occurrence	APGWa	RPGWa	KPGWa	TPGWa	GWa
Sub-oesophageal mass	d <sup>b</sup>	nd	nd	d	d
Supra-oesophageal mass	d	nd	nd	d	d
Optic lobes	d	nd	nd	d	d
Male genital tract	d	nd	nd	d	nd
Spermatophores	nd <sup>a</sup>	nd	nd	nd	nd
Seminal fluid	d	nd	nd	nd	nd
Penis	nd	nd	nd	nd	nd
Seminal vesicles	d	nd	nd	nd	nd
Vas deferens	nd	nd	nd	d	nd

<sup>a</sup>nd, not detected.

<sup>b</sup>d, detected.

established by Edman degradation as PIESVD and checked by MS/MS analysis of the synthetic peptide.

**Bioactivity of synthetic peptide.** Increasing concentrations of synthetic PIESVD were tested on the WGT of cuttlefish. From a threshold of  $10^{-6}$  M, the peptide decreased the intensity of the contractions (Fig. 7).

**In vivo bioassay: shell closure frequency.** Oysters were exposed to increasing concentrations of synthetic PIESVD. Shell closure frequency was visually recorded. From a threshold at  $10^{-6}$  M, PIESVD induced a significant increase of shell closure frequency compared to the control in the population of mature females. The peptide had no significant effect on mature males (Fig. 8).

**Tissue-mapping by microLC-ESI-MS/MS.** Mapping was performed in MS/MS mode based on the m/z 659. Analysis performed in the male genital tract, hemolymph and nervous system (visceral ganglions) did not reveal any trace of this peptide. PIESVD was restricted to the ovary. Furthermore, the analysis of the genital products of spawning females clearly established the occurrence of a release of PIESVD during egg-laying.

## DISCUSSION

Two regulatory peptides isolated from *C. gigas* were identified by mean of mass spectrometry screening and RP-HPLC purification and provide the first experimental evidence of a peptidergic control of egg-laying in oyster. The first approach, based on a peptidomic analysis, led to the identification of the tetrapeptide APGWamide in male genital products of *C. gigas*. Subsequently, this neuropeptide was detected in semi-

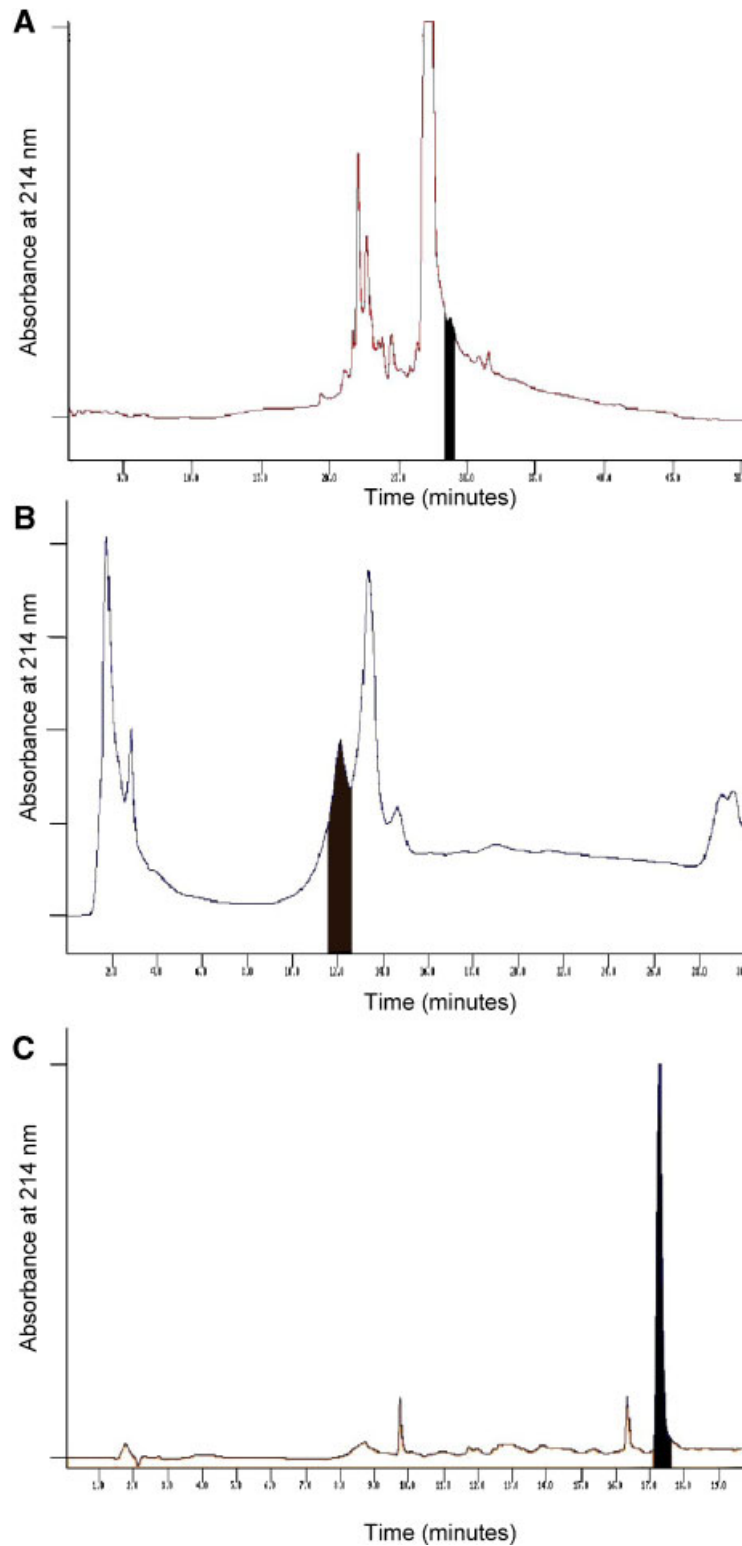
nal secretions of the cuttlefish *S. officinalis*. MicroLC-ESI-MS/MS tissue mapping demonstrated that this peptide is located in the CNS and male genital tract in both oyster and cuttlefish, as well as, according to previous studies, in gastropods, bivalves, and cephalopods (Li et al., 1992; Smit et al., 1992; Smith et al., 1997; Favrel and Mathieu, 1996; Fan et al., 1997; Henry and Zatylny, 2002). In cuttlefish, APGWamide is directly involved in oocyte transport (Henry et al., 1997), whereas in gastropods, APGWamide detected in the male genital tract modulates the male mating behavior (Li et al., 1992; van Golen et al., 1995b). In *S. officinalis*, in vitro bioassays clearly demonstrate that APGWamide has no effect on penis and vas deferens contractions. The APGWamide detected in seminal fluid is deposited with spermatophores in the female copulatory pouch during mating. This pouch contains seminal receptacles surrounded by muscle fibers, which could be involved in the storage of spermatophores (Hanlon et al., 1999) and may be modulated by APGWamide. We speculate that the APGWamide detected in the oyster CNS (visceral ganglions) could target the AM of mature females, as is described in the sea scallop (Smith et al., 1997). Bioassays performed on the AM, which is responsible for the release of oocytes into the external medium, demonstrated that APGWamide induces AM contractions from  $10^{-7}$  M. When oysters were exposed in vivo, a physiological concentration of APGWamide triggered repetitive shell closures. The in vivo bioassay also demonstrated that a majority of mature females appear to be sensitive to APGWamide (67% of females compared to only 30% of males). Moreover, when shell closures were observed, the frequency was higher in females.

TABLE 2. Tissue Mapping of APGWamide-RPs in *Crassostrea gigas*

Occurrence	APGWa	RPGWa	KPGWa	TPGWa	GWa
Male and female gills	nd <sup>a</sup>	nd	nd	nd	nd
Male and female mantle edge	nd	nd	nd	nd	nd
Male and female smooth muscle	nd	nd	nd	nd	nd
Male and female striated muscle	nd	nd	nd	nd	nd
Male and female labial palpes	nd	nd	nd	nd	nd
Male and female visceral ganglions	d <sup>b</sup>	nd	nd	nd	nd
Female genital products	nd	nd	nd	nd	nd
Male genital products	d	nd	nd	nd	nd

<sup>a</sup>nd, not detected.

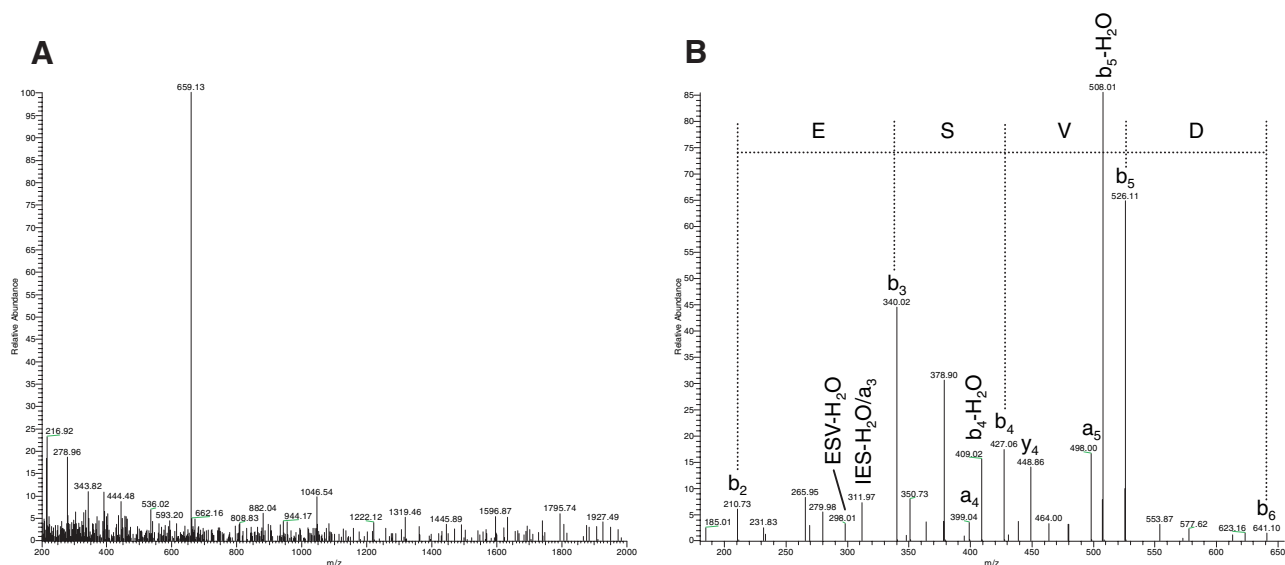
<sup>b</sup>d, detected.



**Fig. 5.** First (A), second (B) and third (C) purification steps of oyster oocyte extract. Myotropic fractions are indicated in black.

Contractions observed in the male population could be a consequence of the successive hermaphroditism of *C. gigas* (Amemiya, 1929; Galtsoff, 1961). Galtsoff demonstrated that although sex change was characterized by

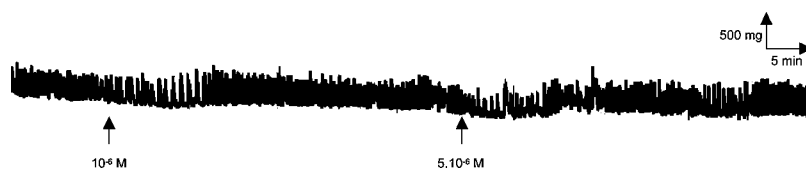
modifications in the spawning behavior, a developed female reaction could appear in sex-reversed males (Galtsoff, 1961). Furthermore, we suspect that the APGWamide detected in the visceral ganglions targets



**Fig. 6.** ESI-MS spectrum of the myotropic HPLC fraction obtained after three steps of purification (A). MS/MS spectra of the  $m/z$  659 from oyster oocytes (B).

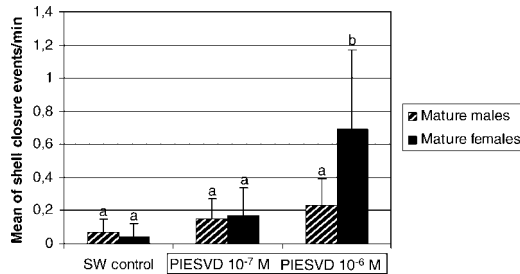
longitudinal and circular muscle fibers of the genital channel (Galtsoff, 1964). A similar observation was made in the sea scallop, where APGWamide immunoreactivity was found around the gonoduct (Smith et al., 1997). In this species, similar investigations led to the detection of 5-HT immunoreactivity around this channel (Matsutani and Nomura, 1986). In vivo experiments clearly demonstrated the involvement of 5-HT in the enhancement of oocytes release in bivalves (Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Braley, 1985; Khotimchenko and Deridovich, 1991; Ram et al., 1993; Fong et al., 1994, 1998). Thus, we speculate that APGWamide and 5-HT could be associated in the regulation of oocyte transport through this genital channel. APGWamide could also be involved in the regulation of egg-laying through a second pathway. APGWamide released with male genital products, and detected by mean of mass spectrometry screening, is suspected to target the AM of mature oysters through a pheromonal pathway. However, the effective concentration of  $10^{-7}$  M established with synthetic peptide is much higher than those observed for other water-borne peptides identified in some invertebrates (Suzuki, 1995; Zatylny et al., 2000b,c, 2002; Bernay et al., 2004; Cummins et al., 2004, 2005a,b). Yet, it is similar to that observed in the polychaete worm *Nereis* for the water-borne peptide nereithione involved in male nuptial

behavior and sperm release (Zeeck et al., 1998; Ram et al., 1999). Similarly, the proximity and high density of mature adults could lead to high concentrations of genital products in the environment surrounding mature females and participate in the regulation of egg-laying in oysters. Hence, the release of oocytes is probably induced by the action of multiple regulatory factors and APGWamide is only one of them. The results from this study using tissue mapping as well as biological activities as endpoints, clearly establish the involvement of APGWamide in the regulation of physiological mechanisms related to egg-laying in *C. gigas* and *S. officinalis*. In addition to sperm-linked regulatory peptides, we also focused on the identification of ovarian regulatory peptides, based on regulatory pathways established in the cuttlefish (Zatylny et al., 2000a,b,c). Following successive steps of UV-HPLC and microLC-ESI-MS/MS purification, a new hexapeptide was characterized from the ovary of mature females based on its ability to modulate the cuttlefish oviduct contractions. The primary structure of this ovarian peptide was established by MS/MS analysis and Edman degradation as PIESVD. MicroLC-ESI-MS/MS tissue mapping revealed that this peptide was restricted to the ovary. The occurrence of a release with female genital products in seawater was clearly established by MS/MS analysis. Furthermore, when synthetic PIESVD was



**Fig. 7.** PIESVD-induced contractions of the cuttlefish whole genital tract at a threshold of  $10^{-6}$  M.





**Fig. 8.** PIESVD-induced contractions of oyster adductor muscle at a threshold of  $10^{-6}$  M. Letters indicate significant difference between treatments (mean  $\pm$  SD.; ANOVA,  $P < 0.05$ ).

added to a rearing tank containing a population of oysters, shell closure frequency increases from a concentration of  $10^{-6}$  M. This biological activity appears to be restricted to mature females. Thus, we speculate that PIESVD triggers AM contractions through two pathways: (i) when released with oocytes in the exhalant chamber, this peptide targets the AM and insures the release of oocytes into the external medium, and (ii) through a pheromonal pathway, the PIESVD released by spawning females with genital products could induce the egg-laying behavior of other mature females in the immediate vicinity. Although the activity threshold of PIESVD is relatively high, the proximity and density of mature oyster populations is consistent with pheromonal activity. The biological activity of PIESVD on the cuttlefish WGT demonstrates that some ovarian regulatory peptides could be highly conserved between cephalopods and bivalves and probably among the seven molluscan classes. Indeed, concerning oysters, *in vitro* bioassays are not reliable enough to monitor the purification of a peptide and *in vivo* bioassays require large quantities of biological material. Thus, the purification and characterization of regulatory peptides appears to be possible using interspecies bioassays. Finally, APGWamide and PIESVD could be used in commercial as well as in experimental hatcheries, for non-destructive sex and maturity stage determination in oysters. Statistical analysis clearly established that the main targets of APGWamide and PIESVD are the mature females in which they induce an important increase of shell closures. APGWamide and PIESVD, could be used to select mature females in order to provide pure gametes for use in genetic manipulation and *in vitro* fertilization.

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#### REFERENCES

- Amemiya I. 1929. On the sex-change of the Japanese common oyster, *Ostrea gigas* Thunberg. Proc Imperial Acad (of Japan) 5:284–286.
- Bernay B, Gagnon J, Henry J. 2004. Egg capsule secretion in invertebrates: A new ovarian regulatory peptide identified by mass spectrometry comparative screening in *Sepia officinalis*. Biochem Biophys Res Commun 314:215–222.
- Boletsky S. 1983. *Sepia officinalis*. In: Boyle P, editor. Cephalopods life cycles. London: Academic Press. pp 31–52.
- Braley R. 1985. Serotonin-induced spawning in giant clams (bivalvia: tridacnidae). Aquaculture 47:321–325.
- Cummins S, Nichols A, Amare A, Hummon A, Sweedler J, Nagle G. 2004. Characterization of *Aplysia* enticin and temptin, two novel water-borne protein pheromones that act in concert with attractin to stimulate mate attraction. J Biol Chem 279:25614–25622.
- Cummins SF, Nichols AE, Warso CJ, Nagle GT. 2005a. *Aplysia* seductin is a water-borne protein pheromone that acts in concert with attractin to stimulate mate attraction. Peptides 26:351–359.
- Cummins SF, Schein CH, Xu Y, Braun W, Nagle GT. 2005b. Molluscan attractins, a family of water-borne protein pheromones with interspecific attractiveness. Peptides 26:121–129.
- Deslous-Paoli J, Heral M, Berthome J, Razet D, Garnier J. 1982. Reproduction naturelle de *Crassostrea gigas* Thunberg dans le bassin de Marennes-Oleron en 1979 et 1981: Aspects biochimiques et energetiques. Rev Trav Inst Pêches marit 45:319–327.
- Dhainaut A, Richard A. 1976. Vitellogenèse chez les céphalopodes décapodes. Evolution de l'ovocyte et des cellules folliculaires au cours de la maturation génitale. Arch Anat Micr Morph Exp 65:183–208.
- Fan X, Croll R, Wu B, Fang L, Shen Q, Painter SD, Nagle GT. 1997. Molecular cloning of a cDNA encoding the neuropeptide APGWamide and cerebral peptide 1: Localization of APGWamide-like immunoreactivity in the central nervous system and male reproductive organs of *Aplysia*. J Comp Neurol 387:53–62.
- Favrel P, Mathieu M. 1996. Molecular cloning of a cDNA encoding the precursor of Ala-Pro-Gly-Trp amide-related neuropeptides from the bivalve mollusc *Mytilus edulis*. Neurosci Lett 205:210–214.
- Fong P, Duncan J, Ram J. 1994. Inhibition and sex specific induction of spawning by serotonergic ligands in the zebra mussel *Dreissena polymorpha* (Pallas). Experientia 50:506–509.
- Fong P, Huminski P, D'Urso L. 1998. Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re-uptake inhibitors (SSRIs). J Exp Zool 280:260–264.
- Galtsoff P. 1930. The role of chemical stimulation in the spawning reactions of *Ostrea virginica* and *Ostrea gigas*. Proc Natl Acad Sci USA 16:555–559.
- Galtsoff P. 1938a. Physiology of reproduction of *Ostrea virginica* I. Spawning reactions of the female and male. Biol Bull 74:461–486.
- Galtsoff P. 1938b. Physiology of reproduction of *Ostrea virginica* II. Stimulation of spawning in the female oyster. Biol Bull 75:286–307.
- Galtsoff P. 1961. Physiology of reproduction in molluscs. Am Zoologist 1:273–289.
- Galtsoff P. 1964. The American oyster *Crassostrea virginica* Gmelin: Fish Bull US. pp 1–480.
- Gibbons MC, Castagna M. 1984. Serotonin as an inducer of spawning in six bivalve species. Aquaculture 40:189–191.
- Hanlon R, Ament S, Gabr H. 1999. Behavioral aspects of sperm competition in cuttlefish, *Sepia officinalis* (Sepioidea: Cephalopoda). Mar Biol 134:719–728.
- Henry J, Zatylny C. 2002. Identification and tissue mapping of APGWamide-related peptides in *Sepia officinalis* using LC-ESI-MS/MS. Peptides 23:1031–1037.
- Henry J, Favrel P, Boucaud-Camou E. 1997. Isolation and identification of a novel Ala-Pro-Gly-Trp-amide-related peptide inhibiting the motility of the mature oviduct in the cuttlefish, *Sepia officinalis*. Peptides 18:1469–1474.
- Henry J, Zatylny C, Boucaud-Camou E. 1999. Peptidergic control of egg-laying in the cephalopod *Sepia officinalis*: Involvement of FMRFamide and FMRFamide-related peptides. Peptides 20:1061–1070.

- Heral M, Deslous-Paoli J, Prou J. 1986. Influence du climat sur le recrutement et sur la production d'huîtres cultivées (*C. angulata* et *C. gigas*) dans le bassin de Marennes-Oléron. *Haliotis* 15:193–207.
- Hoek P. 1883. Researches on the generative organs of the oyster (*O. edulis*). *Bulletin of the US Fish Commission* 2:343–345.
- Khotimchenko Y, Deridovich I. 1991. Monoaminergic and cholinergic mechanisms of reproduction control in marine bivalve molluscs and echinoderms: A review. *Comp Biochem Physiol C* 100:311–317.
- Li K, Smit A, Geraerts W. 1992. Structural and functional characterization of neuropeptides involved in the control of male mating behavior of *Lymnaea stagnalis*. *Peptides* 13:633–638.
- Matsutani T, Nomura T. 1982. Induction of spawning by serotonin in the scallop *Pactinopecten yessoensis* (jay). *Mar Biol Letters* 3:353–358.
- Matsutani T, Nomura T. 1986. Serotonin-like immunoreactivity in the central nervous system and gonad of the scallop, *Pactinopecten yessoensis*. *Cell Tissue Res* 244:515–517.
- Maurer D, Borel M. 1986. Croissance, engraissement et cycle sexuel de *C. gigas* dans le bassin d'Arcachon: Comparaison de huîtres âgées de 1 et 2 ans. *Haliotis* 15:217–228.
- Nelson T. 1921. Aids to successful oyster culture. I. Procuring the seed. *N J Agr Exp Sta ed. Vol. Bull* 351.
- Nelson T, Allison J. 1940. On the nature and action of diantlin; a new hormone-like substance carried by the spermatozoa of the oyster. *J Exp Zool* 85:299–338.
- O'Foighil D, Taylor D. 2000. Evolution of parental care and ovulation behavior in oysters. *Mol Phylogenet Evol* 15:301–313.
- Ram J, Crawford G, Walker J, Mojares J, Patel N, Fong P, Kyozuka K. 1993. Spawning in the zebra mussel (*Dreissena polymorpha*): Activation by internal or external application of serotonin. *J Exp Zool* 265:587–598.
- Ram J, Muller C, Beckmann M, Hardege J. 1999. The spawning pheromone cysteine-glutathione disulfide ('nereithione') arouses a multicomponent nuptial behavior and electrophysiological activity in *Nereis succinea* males. *FASEB J* 13:945–952.
- Rice P, Ray SM, Painter SD, Nagle GT. 2002. An intrinsic membrane protein in oyster sperm stimulates spawning behaviors in *Crassostrea virginica*: Implications for aquaculture. *J Shellfish Res* 21:715–718.
- Smit A, Jimenez C, Dirks R, Croll R, Geraerts W. 1992. Characterization of a cDNA clone encoding multiple copies of the neuropeptide APGWamide in the mollusk *Lymnaea stagnalis*. *J Neurosci* 12:1709–1715.
- Smith S, Nason J, Croll R. 1997. Detection of APGWamide-like immunoreactivity in the sea scallop, *Placopecten magellanicus*. *Neuropeptides* 31:155–165.
- Sole N, Barany G. 1992. Optimization of solid-phase synthesis of [Ala<sup>8</sup>]-dynorphin A. *J Org Chem* 57:5399–5403.
- Suzuki N. 1995. Structure, function and biosynthesis of sperm-activating peptides and fucose sulfate glycoconjugate in the extracellular coat of sea urchin eggs. *Zool Sci* 12:13–27.
- van Golen F, Li K, De Lange R, Van Kesteren R, Van Der Schors R, Geraerts W. 1995b. Co-localized neuropeptides conopressin and ALA-PRO-GLY-TRP-NH<sub>2</sub> have antagonistic effects on the vas deferens of *Lymnaea*. *Neuroscience* 69:1275–1287.
- Zatylny C, Durantou F, Boucaud-Camou E, Henry J. 2000a. Evidence of 5-hydroxytryptamine synthesis in the follicles of *Sepia officinalis* and direct involvement in the control of egg-laying. *Mol Reprod Dev* 55:182–188.
- Zatylny C, Gagnon J, Boucaud-Camou E, Henry J. 2000b. ILME: A waterborne pheromonal peptide released by the eggs of *Sepia officinalis*. *Biochem Biophys Res Commun* 275:217–222.
- Zatylny C, Gagnon J, Boucaud-Camou E, Henry J. 2000c. The SepOvotropin: A new ovarian peptide regulating oocyte transport in *Sepia officinalis*. *Biochem Biophys Res Commun* 276:1013–1018.
- Zatylny C, Marvin L, Gagnon J, Henry J. 2002. Fertilization in *Sepia officinalis*: The first mollusk sperm-attracting peptide. *Biochem Biophys Res Commun* 296:1186–1193.
- Zeeck E, Müller C, Beckmann M, Hardege J, Papke U, Sinnwell V, Schroeder F, Francke W. 1998. Cysteine-glutathione disulfide, the sperm-release pheromone of the marine polychaete *Nereis succinea* (Annelida: Polychaeta). *Chemoecology* 8:33–38.