Chapter 1

Egg-Laying in the Cuttlefish *Sepia officinalis*

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Additional information is available at the end of the chapter

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Abstract

This chapter reviews studies about egg-laying in the cuttlefish Sepia officinalis. Egg masses are spawned in specific mating and spawning coastal areas where mates aggregate between April and June in the English Channel and all year long in the Mediterranean Sea. Environmental cues are clearly involved in the aggregation process, but chemical communication also plays a determining role in these complex mechanisms. The successive steps of egg-laying are orchestrated by three classes of regulatory peptides: (1) neuropeptides that integrate environmental cues, (2) ovarian regulatory peptides that modulate the activity of the genital tract, and (3) sex pheromones expressed and released by the oviduct gland. After egg-laying, embryo protection is ensured for 8-10 weeks by a multilayer capsule secreted by the accessory sex glands. The oviduct gland secretes the inner layer of the egg case. The main nidamental gland secretes the main polysaccharides and glycoproteins, such as Sepia Egg Case Proteins, involved in capsule formation and in embryo protection. The accessory nidamental gland expresses specific proteins inherent in the structural organization of the gland, and hosts symbiotic bacteria. Similarly to salivary glands, this gland secretes immune factors possibly associated with gamete and/or embryo protection.

Keywords: reproduction, egg-laying, neuropeptides, ovarian peptides, egg-case, common cuttlefish

1. Introduction

Phylogenetically, anatomically, and physiologically speaking, cephalopods are indeed mollusks. Yet, they possess special characteristics that distinguish them from other molluscan classes, especially the other two major classes, gastropods and bivalves.

First of all, they are the most mobile mollusks. They come in the form of pelagic species capable of performing large-amplitude horizontal and vertical migrations, like squid. Octopoda are in turn largely territorial and therefore sedentary, while the common cuttlefish *Sepia officinalis* exhibits a nectobenthic behavior associated with low or medium migration amplitude depending on the latitude.

Unlike other classes of molluscs, cephalopods possess a cephalopodium whose eight arms play an important role in predation but also in mating and egg-laying during the formation of the egg mass (**Figure 1**). These arms are also used for handling prey during catches by capping [1].

This ability to handle prey is quite unique in the marine environment and only found in primates and in some mammals. It is probably related to the exceptional development of the central nervous system (CNS). This CNS is protected by a cartilaginous skull and is located between the eyes, which are capable of forming an image.

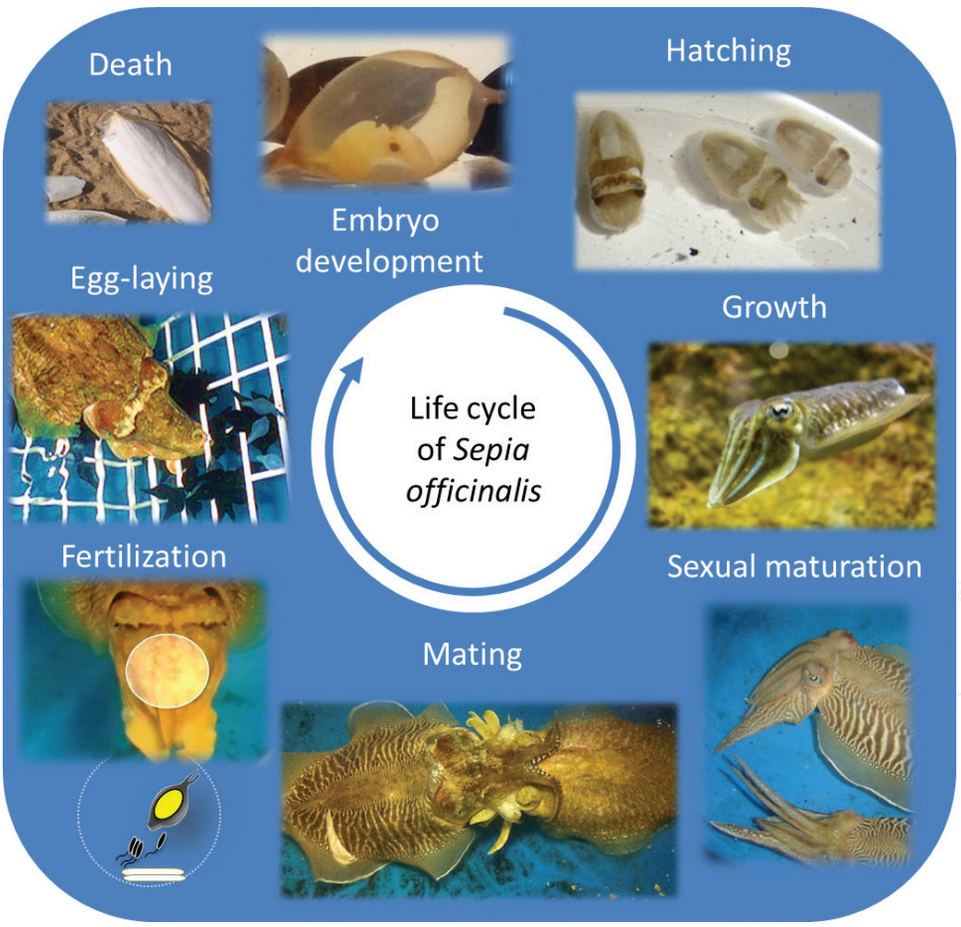

Figure 1. Main successive steps of the life cycle of the cuttlefish *Sepia officinalis*. (Photo credits: V. Cornet, J. Henry, C. Zatylny-Gaudin).

2. Egg-laying: description

The cuttlefish *Sepia officinalis* is a semelparous species with a life cycle that varies depending on the geographical location of the population: 15–16 months in the Mediterranean Sea versus 20–22 months in the English Channel. Spawning is associated with a stereotyped behavior. In aquaria, sexually mature females that have not spawned yet manipulate eggs laid earlier by conspecifics, while increasing their ventilation rate.

The different behavioral sequences leading to the laying of eggs are gradually repeated and eventually lead to the laying of the first egg (personal observation). A female can lay dozens of eggs at once, probably 150–200 eggs, which roughly corresponds to the storage capacity of the genital coelom, before a pause that allows it to replenish its stock of mature oocytes (stage V) through asynchronous gametogenesis. It also restores the capsular products secreted by the oviduct gland and the nidamental glands. Some females are then able to lay a second batch of eggs and probably several successive spawns. We do not know exactly how many eggs are laid before the programmed death of the animal. Fertility is probably quite variable among females and is very difficult to estimate.

3. Egg-laying: regulation

The first work on the contractile structures of the female reproductive system led to the identification of numerous myotropic or myosuppressor regulatory peptides. The first of them, a neuropeptide belonging to APGWamide family, was identified from a sample of optic lobes purified by rpHPLC on the basis of its myosuppressive effect on the contraction of the distal oviduct [2].

Then, new myotropic bioassays on the contractile organs of the female genital apparatus were performed, and the bases of the functional control of spawning and the related activities were laid, for example, blocking or inducing oocyte transport in the oviduct and the secretion of internal and external oocyte capsules before fertilization.

From the papers published on this topic between 1997 and 2006, it appears that the successive steps of egg-laying are mainly governed by two classes of regulatory peptides: neuropeptides involved in the integration of environmental cues and ovarian regulatory peptides that modulate the activity of the genital tract [2–8]. The recent development of "-omics" approaches based on *de novo* RNAseq and mass spectrometry led to the identification of transcripts and mature cleavage products.

Using a transcriptomic approach, Enault and collaborators [9] discovered a third category of regulatory peptides, namely the sex pheromones expressed and released by the oviduct gland, and cleaved from three protein precursors into bioactive peptides ranging between 1.3 and 8 kDa.

Thanks to the sequencing of the neurotranscriptome, several neuropeptide families involved in the regulation of egg-laying were identified on the basis of expression pattern and tissue localization out of the 38 families composing the cuttlefish neuropeptidome [10]. Finally, the RNA sequencing of ovary tissue revealed that most of the ovarian regulatory peptides involved in oocyte release were cleaved from a single yolk protein (unpublished results).

4. Neuropeptides

As mentioned above, the first neuropeptide identified in cuttlefish on the basis of its ability to modulate the contractile activity of the oviduct was characterized by Henry and collaborators [2] from the optic lobes of egg-laying females (**Figure 2**).

It is the GWamide, a dipeptide that belongs to the APGWamide family and is derived from enzymatic cleavage of the APGWamide by a CNS dipeptidyl aminopeptidase (DPAP).

DPAP activity appears to be an alternative mechanism for the maturation of precursors into bioactive proteins or peptides such as peptides produced by amphibian skin, precursors of lytic peptides in honeybee venom, bactericidal peptides secreted in the insect hemolymph, or extracellular proteases in yeasts [11].

In gastropods, another molluscan class, the APGWamide is involved in the control of male behavior. In the pond snail *Lymnaea stagnalis*, this tetrapeptide detected in the penile nerve regulates penis erection [12–14].

Besides, FMRFamide-related peptides (FaRPs) also occur in the nervous fibers of the female accessory sex glands. Their occurrence was demonstrated by immunohistochemistry. Perfused FaRPs induce strong modifications of the contractile activity of the distal oviduct [3]. The involvement of two neuropeptide families—APGWa-RPs and FaRPs—in the control of egg-laying suggests a complex regulation of the successive steps leading to the formation of the egg mass.

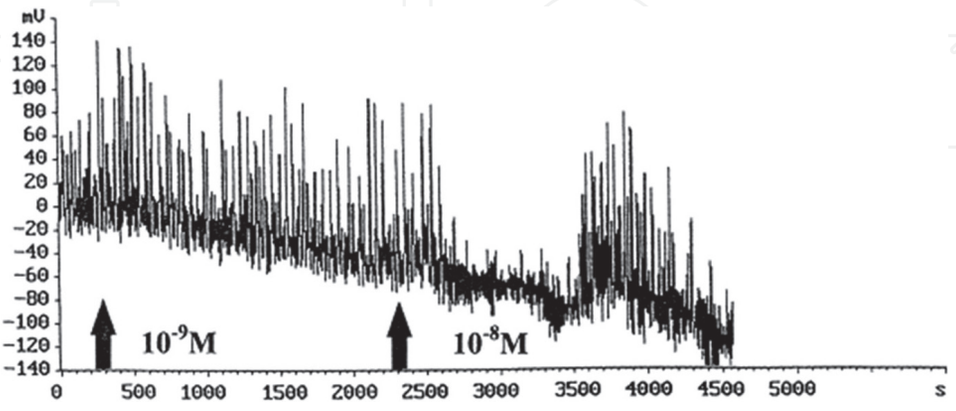

Figure 2. Decrease of the tonus, frequency, and amplitude of oviduct contractions following treatment with 10^{-8} M synthetic GWamide. A dose of 10^{-9} M GWamide did not induce contractions; therefore, the threshold for GWamide activity appears to be between 10^{-8} and 10^{-9} M [2].

The identification of the cuttlefish neuropeptidome by *de novo* RNAseq and mass spectrometry screening was the next step that provided an overview of the neuropeptidome *via* a deep structural and functional investigation [10].

Based on the filtering criteria applied to the 38 identified neuropeptide families—expression level, neuropeptide tissue mapping, and mRNA localization—seven neuropeptide families were finally selected: allatostatins, APGWamide, crustacean cardioactive peptides (CCAPs), FaRPs, FLGamide, myomodulins, and small cardioactive peptide (SCP).

Several neuropeptides cleaved from the protein precursor of **allatostatin A1** and **A2** issued from alternative splicing were detected by nanoliquid chromatography tandem mass spectrometry (nLC-MS/MS) in the oviduct gland and the main nidamental glands, suggesting a role in egg capsule secretion. In insects, **FGLamide allatostatins** (also called **allatostatins A** or **buccalins**) are involved in reproduction [15] and feeding decisions interacting with the adipokinetic hormone (AKH) and insulin-like peptides [16]. In cuttlefish, **allatostatins A1** and **A2** were also detected in the hemolymph, in accordance with the status of neurohormones that could regulate the biosynthesis of egg capsule products during vitellogenesis.

The **APGWamide** was detected by nLC-MS/MS in the CNS, and the **GWamide** was characterized from the CNS [2]. Moreover, large amounts of mRNAs were found in the OGs, MNGs, and ANGs of egg-laying females. Similar observations were reported in the pond snail *Lymnaea stagnalis* by van Minnen and Bergman [17]. High amounts of mRNAs encoding the egg-laying hormone were detected in the nerve terminals after a stimulus, as well as polyribosomes, supporting that the translation of egg-laying hormone transcripts could occur in the axonal compartment. These data are supported by recent papers revealing the occurrence of rough endoplasmic reticulum, smooth reticulum, and Golgi apparatus in the axonal compartment [18]. Otherwise, Martin and Kim [19] used Aplysia as a model to show that netrin-1, already known to promote translation in axonal growth cones [20], increased translation of subcellular mRNAs localized at the level of dendrites or axons by binding the cytoplasmic domain of the netrin-1 receptor called DCC (for deleted colorectal cancer). The rapid reaction of female cuttlefish can be related to the state of readiness of the axons that innervate the ASGs.

The three neuropeptides predicted from the protein precursor of **CCAPs** were detected by nLC-MS/MS in the CNS, the oviduct gland and the main nidamental glands [10]. **CCAPs** are also overexpressed in the sub-esophageal mass of egg-laying females (as opposed to mature males), which is the only part of the CNS that innervates the genital apparatus. This neuropeptide family is strongly suspected to regulate egg capsule secretion.

CCAPs were initially described in crustaceans. They are usually C-terminally amidated neuropeptides in arthropods [21–24], as well as in mollusks [14, 25–27], whereas the cuttlefish preprohormone predicted three nonamidated peptides confirmed by nLC-MS/MS analysis.

The four neuropeptides predicted from the protein precursor of **FaRPs** were detected by nLC-MS/MS in the CNS, and the decapeptide ALSGDAFLRFamide was the only one detected in the neurohemal area connected to the sub-esophageal mass and in the oviduct gland and main nidamental glands, confirming the immunostaining results obtained by Henry and collaborators [3]

in the ASGs of mature females. These neuropeptides are widely distributed in the animal kingdom and involved in many physiological regulation processes in mollusks such as heart activity [28], amylase secretion [29], feeding [30], and reproduction [31]. In cuttlefish, they are believed to regulate egg capsule secretion and oocyte transport in the oviduct [3] and also chromatophore control pathways [32].

FLGamide is a novel neuropeptide family never described so far in the animal kingdom although blastn revealed a similar precursor annotated "uncharacterized protein" (ELU03112) in the polychaete worm *C. teleta* [33]. Most of the eight neuropeptides predicted from the two protein precursors were detected by nLC-MS/MS in cuttlefish CNS, oviduct gland, main nidamental glands, and ovarian stroma [10]. It is the only neuropeptide family detected in the ovarian stroma, suggesting a putative involvement in the release of smooth oocytes into the genital coelom. But recent unpublished experiments show that these neuropeptides do not induce any modification on contractile activity when perfused into the ovarian stroma. As they are also detected in the hemolymph, they are thought to be involved in vitellogenesis regulation.

In the oviduct gland, recent unpublished experiments show that they regulate oocyte transport in the oviduct. They could also induce egg capsule biosynthesis and secretion.

Myomodulins were detected by nLC-MS/MS in the CNS, the oviduct gland and the main nidamental glands [10], and so were mRNAs, as described for **APGWamide** and **CCAPs**. They appear to be closely associated to accessory sex glands involved in egg capsule secretion.

In the tropical abalone *Haliotis asinine*, egg-laying is characterized by a dramatic increase in the expression of **APGWamide**, **myomodulins,** and **insulin** within 12 h of the spawning event. Expression strongly decreases 24 h after spawning, demonstrating that these neuropeptides have a regulatory role in the release of gametes [34]. In cuttlefish, the mRNAs of these three neuropeptides are recovered at the level of the oviduct gland and main nidamental glands, suggesting that they are involved in the rapid response of the genital apparatus after mating.

The detection of the **small cardioactive peptide (SCP)** by mass spectrometry proved very difficult and was restricted to the oviduct gland. As already described for **CCAPs**, **SCP** is also overexpressed in the sub-esophageal mass of egg-laying females (as opposed to mature males). This neuropeptide could be related to the secretion of the internal layer of the egg capsule and also in the release of oocytes into the mantle cavity. The preprohormones of all these neuropeptides are presented in **Figure 3**. The OG and MNGs are closely associated in egg capsule elaboration. Just before fertilization, oocytes are embedded into two layers of egg capsule proteins: the inner layer is secreted by the oviduct gland and the outer layer by the main nidamental glands. These glands synthesize and secrete most of the egg capsule constituents. The similar function of OG and MNGs could explain why they share so many common regulatory neuropeptides. Three categories can be distinguished among them (**Table 1**): (1) **APGWamide** neuropeptides and **myomodulins** whose mRNAs are recovered in OG and MNGs, probably located in the axon ends; they can be associated to a rapid responsiveness following mating; (2) **allatostatins A**, **CCAPs**, and **SCP** neuropeptides, overexpressed in the sub-esophageal mass

of egg-laying females and detected in OG and MNGs; they stimulate the mechanical contraction of glands for egg capsule secretion when the oocyte reaches the distal oviduct embedded by the oviduct gland, just before being released into the mantle cavity; (3) **FLGamide** and **FMRFamide** neuropeptides are detected in OG and MNGs and also in the hemolymph; they modulate oviduct contractions and are probably involved in the mechanical secretion of the egg capsule. Moreover, as they are circulating peptides, they could also be involved in the

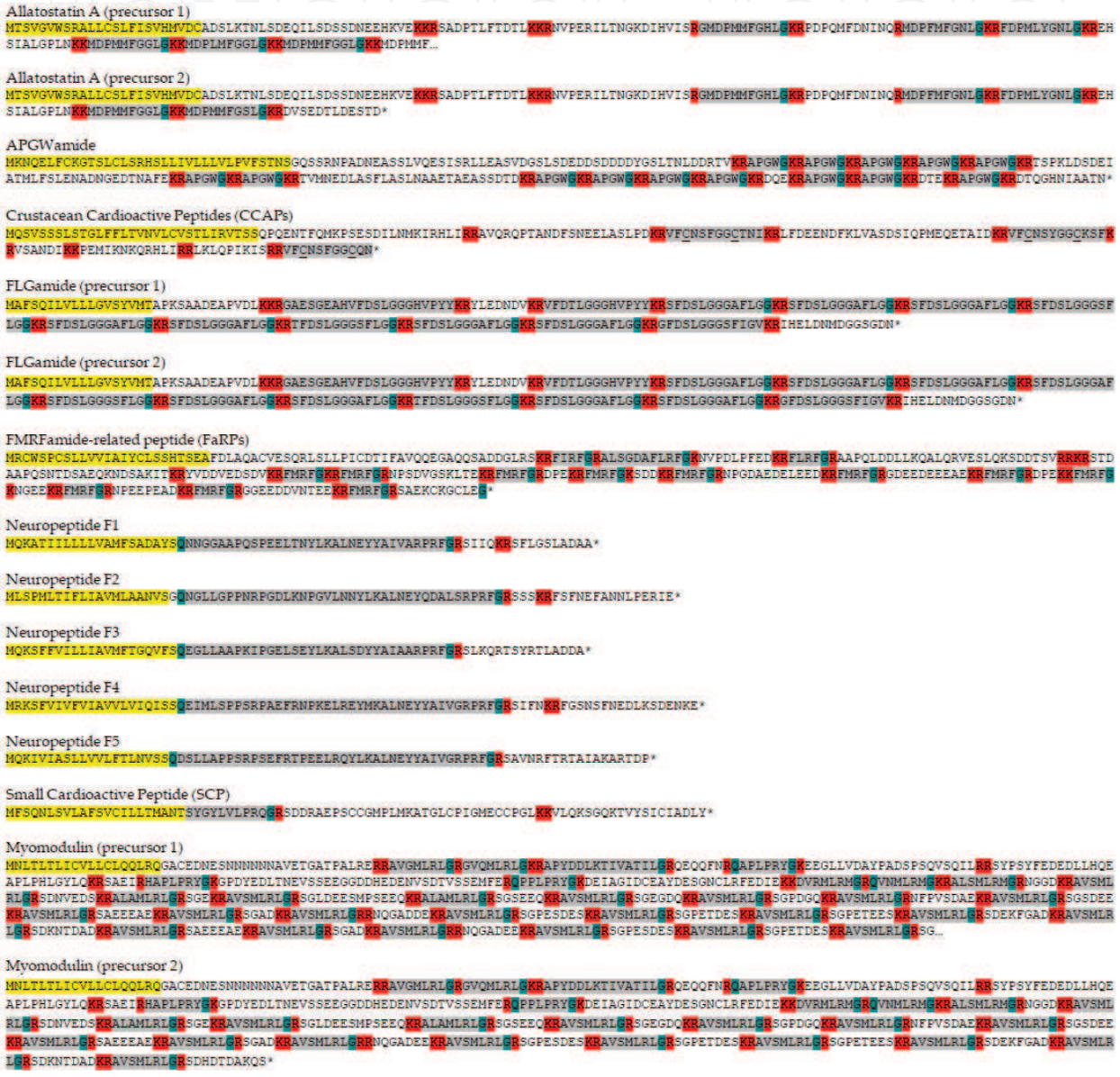

Figure 3. Preprohormones of allatostatins A1 and A2, APGWamide, crustacean cardioactive peptide, FLGamide, FMRFamide-related peptides, myomodulin, neuropeptides F1, F2, F3, F4, F5, and small cardioactive peptide, that all encode neuropeptides detected in the nerve endings and/or in the CNS of *S. officinalis* egg-laying females or by *in silico* data mining. Predicted signal peptides are highlighted in yellow, convertase cleavage sites in red, cysteines are underlined, glycine residues predicted to be converted into C-terminal amides in blue-gray, as well as glutamine residues predicted to be converted into pyroglutamate. Neuropeptides are highlighted in gray, and the stop codons at the end of the coding sequences are indicated by an asterisk [10].

regulation of the synthesis of yolk proteins and/or egg capsule proteins. In addition to the neuropeptides directly involved in the regulation of egg-laying, RNAseq revealed a substantial overexpression of neuropeptides Y (NPY), also called neuropeptides F (NPF) in protostomes because of a C-terminal tyrosine amide substituted by a phenylalanine amide (**Figure 3**). The five transcripts of cuttlefish NPF are unequally overexpressed.

The expression level of NPF 1, the most overexpressed of them, reaches 45-fold the expression level found in mature males in the same part of the CNS. NPFs are probably overexpressed in females to stimulate feeding in order to support gametogenesis and egg capsule synthesis between each spawning step. In this species, asynchronous gametogenesis allows females to resume egg-laying by replenishing their batch of mature oocytes and biosynthesizing egg capsule products until they die.

More generally, the overexpression of many neuropeptides found in the sub-esophageal mass of egg-laying females (as compared to males) could be due to the regulation associated to the production of several batches of oocytes that contain a large quantity of vitellus for embryonic development and to the mobilization of the energy needed to carry out gametogenesis. By contrast, in English Channel males, gametogenesis ends 6 months before reproduction and produces a much smaller volume of gametes than in females. The energy required for male gametogenesis is probably very low compared with the energy required for female gametogenesis.

Finally, a 36-amino-acid neuropeptide called Egg-Laying Hormone (ELH) can induce egg-laying following a single intramuscular injection in the foot of Gastropods. In cuttlefish, ELH

	Occurrence of neuropeptides			Overexpression of transcript	Localization of transcripts			Functional status
	OG	MNG	OS	subEM	OG	MNG	ANG	
APGWamide	NO	NO	NO	NO	YES	YES	YES	neuromodulator
Myomodulins	YES	YES	NO	NO	YES	YES	YES	neuromodulator
Allatostatins A	YES	YES	NO	NO	NO	NO	NO	neuromodulator
CCAPs	YES	YES	NO	YES	NO	NO	NO	neuromodulator
SCP	YES	NO	NO	YES	NO	NO	NO	neuromodulator
FaRPs	YES	YES	NO	NO	NO	NO	NO	neurohormone neuromodulator
FLGamide	YES	YES	YES	NO	NO	NO	NO	neurohormone neuromodulator

ANG: accessory nidamental gland; MNG: main nidamental gland; OG: oviduct gland; OS: ovarian stroma; SubEM: sub-esophageal mass.

Table 1. Tissue mapping of neuropeptides and mRNAs in the female cuttlefish.

remains unknown despite *in silico* data mining from transcriptomic data associated to nLC-MS/MS screenings of the CNS of egg-laying females. Considering that cephalopods are the only class among the three main molluscan classes in which no ELH was ever identified, we can hypothesize that the reason is the loss of the ELH gene or more probably a low level of structural conservation leading to a failure of the data mining strategy or an insufficient depth of RNAseq. Further deeper RNA sequencing will probably allow for the identification of this neuropeptide in cephalopods.

5. Sex pheromones

During a short life cycle of about 22 months, English Channel cuttlefish can perform four horizontal migrations from the Normandy coasts to the western part of the English Channel [35]. After a last migration to reach specific mating and spawning coastal areas, cuttlefish aggregate for mating and egg-laying between April and June on the Normandy coasts. This behavior suggests the occurrence of some kind of chemical communication *via* waterborne molecules that induce the aggregation of mates. Chemical communication in cuttlefish was first demonstrated by Boal and collaborators [36, 37] using y-mazes. They showed that recently laid eggs, ovary extracts and nidamental glands, induced an increase in ventilation rate and the attraction of sexually mature cuttlefish in the arm of the y-maze containing purified extract *versus* artificial sea water. In the same way, Cummins and collaborators [38] identified a 10 kDa protein in Loligo termed Loligo β-microsemino-protein (Loligo β-MSP) that immediately changes the behavior of male squid from calm swimming and schooling to extreme fighting. Loligo β-MSP is synthesized in the accessory sex gland of females—the oviduct gland, the main and accessory nidamental glands—and is secreted with the proteins of the outer tunic of egg capsules. When a male is attracted to the eggs visually, upon touching them and contacting Loligo β-MSP, it immediately escalates into intense physical fighting with any nearby males.

Loligo β-MSP was originally discovered in human seminal plasma and prostatic fluids [39]. It is only described in other vertebrates [40–44] and in the basal chordate amphioxus [45]. It is a highly variable 91-amino-acid protein, with 10 spatially conserved cysteine residues than can potentially form five intramolecular disulfide bonds, giving resistance to proteolytic cleavage to prolong its activity on the egg surface.

In *Sepia officinalis*, Enault and collaborators [9] identified three major related transcripts encoding secreted peptides and expressed in the oviduct gland. RT-PCR and mass spectrometry analyses revealed that transcripts and expression products were co-localized in the oviduct gland. The two very similar protein precursors termed SPα and SPα′ (**Figure 4**) diverge by only four amino acids in the α3 and α3′ peptides. They yield seven putative expression products ranging from 1.3 kDa (α5) to 7 kDa (α3 and α3′).

All peptides except α1 contained at least one cysteine, and two of them, α3 and α3′, are C-terminally amidated like many bioactive peptides (**Figure 5A**).

The third protein precursor, termed SPβ, shares 56.7% similarity with SPα and SPα′ (**Figure 4**) and yields five putative expression products ranging from 1.1 kDa (peptide β1) to 8.3 kDa

Figure 4. Amino acid alignments of the three protein precursors SPα-α′ and SP β. Red asterisks indicate conserved cysteines. Predicted signal sequences are highlighted in yellow, the conserved sequence between SPs in green, differences between Spα and SPα′ in blue, and potential basic residue cleavage sites in red [9].

(peptide β3), with C-terminal amidation (peptide β2), disulfide bonds (peptides β3, β4, β5), or N-glycosylation (peptide β3) (**Figure 5B**).

For most of the expression products derived from SPα-α′ and SPβ, predicted post-translational modifications such as C-terminal amidation and disulfide bonds have been confirmed by nLC-MS/MS analysis. These modifications can provide a strong protection against protease and peptidase activity and can be expected to confer the peptides a long life in marine environments. As most of the predicted peptides were recovered by nLC-MS/MS analysis, the processing of SPα-α′ and SPβ should lead to the release of a cocktail of waterborne pheromones. Peptides α3 and α2 strongly stimulate the contraction of the penis and the gills when they are applied on these parts (**Figure 6**).

Therefore, peptides expressed and secreted by a female's accessory sex gland can modulate the activity of a male's genital apparatus. Recent unpublished data show that the protein precursors SPα and SPβ are also able to release a second batch of high-molecular-weight (22–26 kDa) pheromones secreted with the egg capsule proteins and integrated to the inner layer of the egg capsule. Finally, they are detected in the sea water around egg masses once they have crossed the outer layer of the egg capsule. The presence of these high-molecular-weight pheromones identified by proteomic analysis of the oviduct gland and egg capsule also implies the presence of high-molecular-weight polypeptides/proteins derived from SP precursors. These analytical results demonstrate that two modes

of cleavage of SP precursors coexist and generate low-molecular-weight peptides (prohormone convertase cleavages) and also 22–26 kDa polypeptides/proteins released by the eggs into the surrounding medium.

The mechanism that leads to the release of both low- and high-molecular-weight pheromones processed from a same protein precursor has to be elucidated. The occurrence of C-terminal amidation for peptides β2 and α3 demonstrates that two distinct processings are performed in the Golgi apparatus, which means that low-molecular-weight pheromones (LMWPs) are not degradation products of high-molecular-weight pheromones (HMWPs).

The present functional hypothesis could be that LMWPs induce mating and the release of oocytes into the mantle cavity, and that HMWPs, as described in *Aplysia*, facilitate the

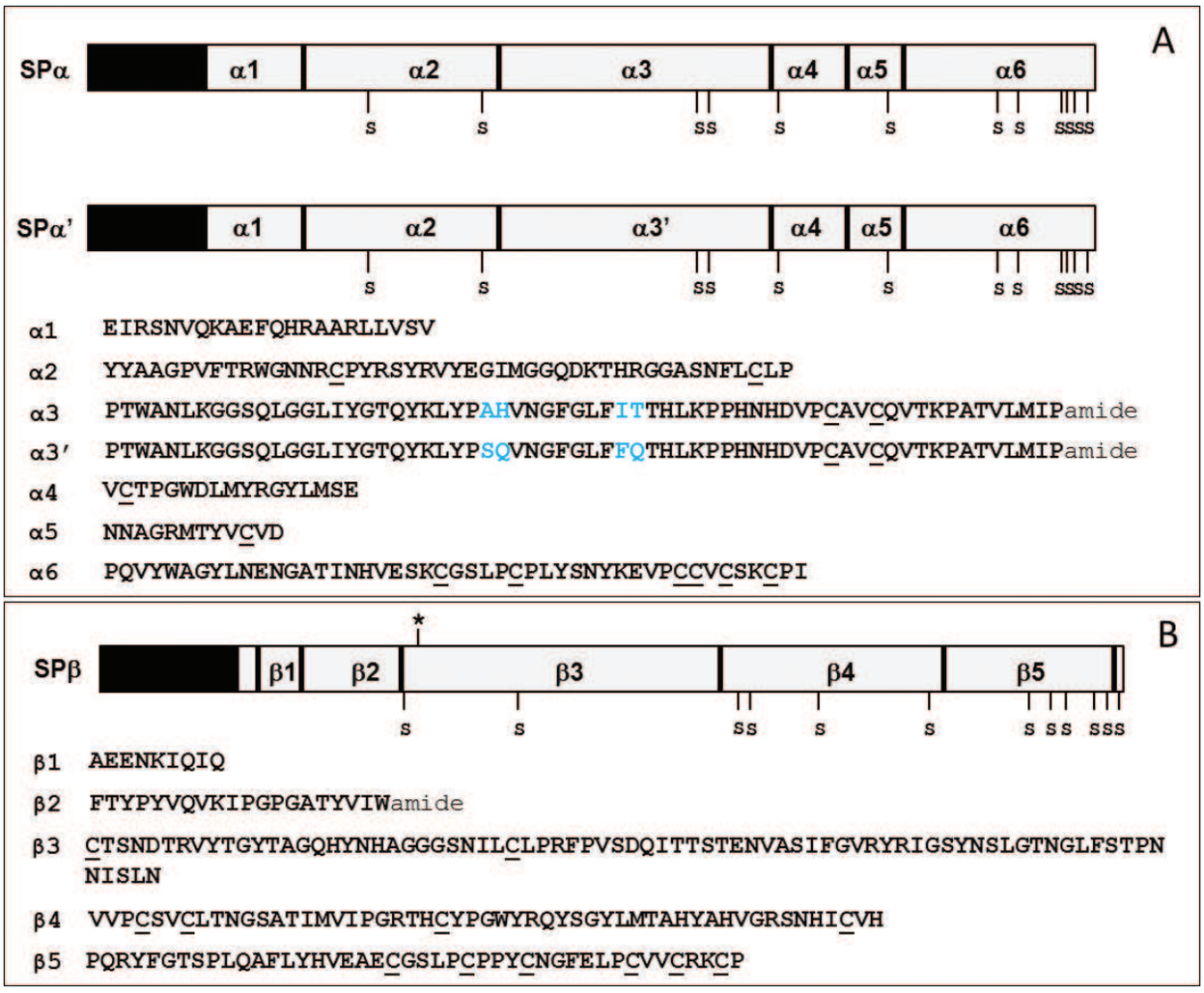

Figure 5. Schematic diagrams showing the organization of *Sepia officinalis* pheromone precursors (A) SP α and α'; (B) SPβ. Precursors encode a complex cocktail of peptides and polypeptides resulting from dibasic cleavages. Black box, signal peptide; vertical black line, potential dibasic residue cleavage site; asterisk, predicted N-linked glycosylation site; S, Cys residue [9].

aggregation of mature cuttlefish in the coastal egg-laying areas. All these data confirm that cuttlefish eggs are a source of pheromones, as described in other mollusks such as marine gastropods of the genus *Aplysia* [46–48]. Behavioral tests now have to be performed to clarify the mechanism of action of LMWPs and HMWPs in sexually mature cuttlefish.

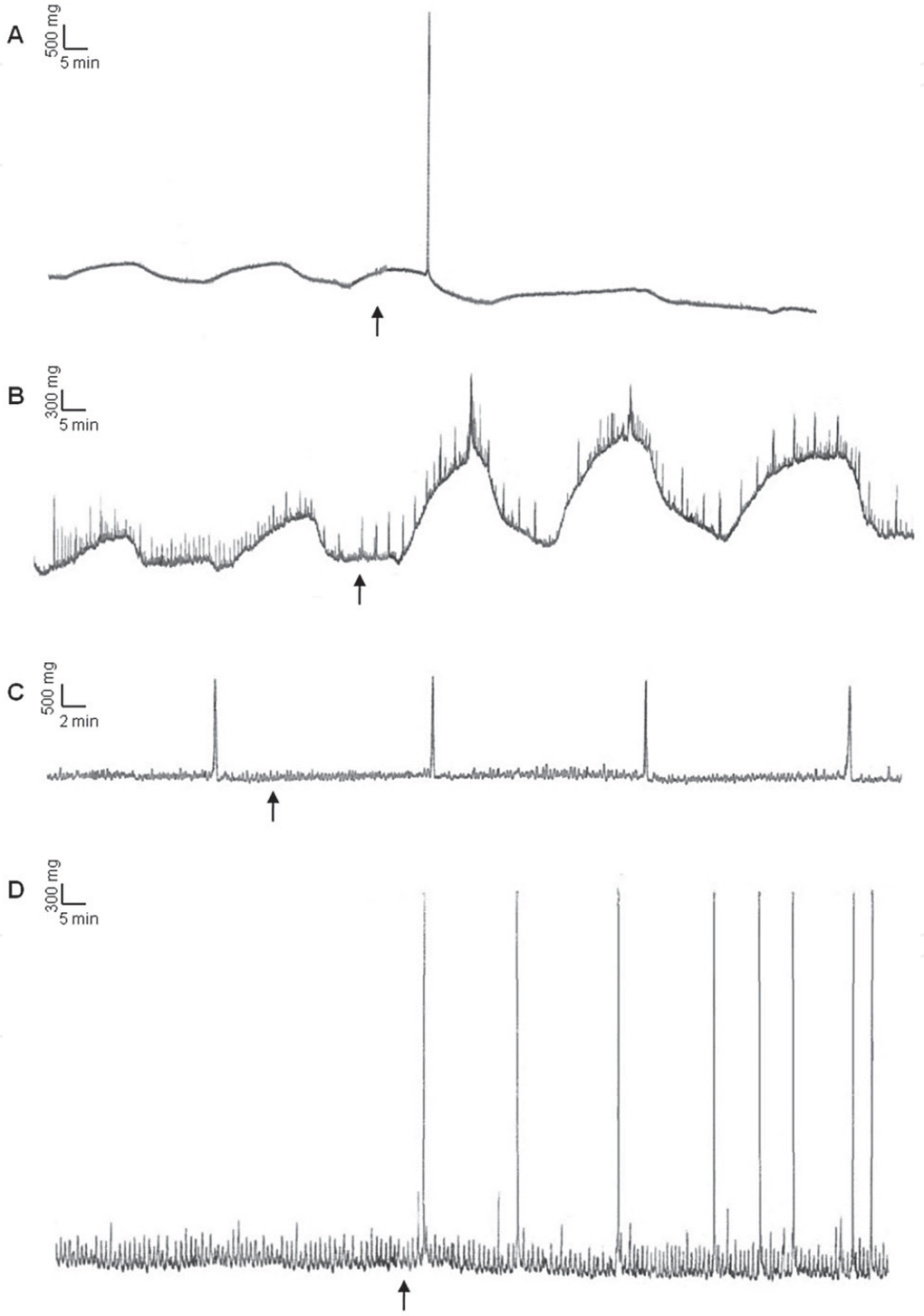

Figure 6. Bio-activity of synthetic β2 and α3 peptides. β2-induced contractions on (A) female gill and (B) penis from a threshold of 10^{-8} M. No activity on (C) rectum. α3-induced contractions on (D) penis from a threshold of 10^{-9} M [25].

6. Ovarian regulatory peptides

The role of the ovary in the regulation of the synthesis of capsular products secreted by MNGs was highlighted for the first time by Henry and Boucaud-Camou [49]. Ovary extract stimulated the incorporation of ^{3}HLeucine and ^{14}CGlucose into the proteins and polysaccharides of primocultures of glandular cells from main nidamental glands.

Seawater used for incubating oocytes also modified the contractile activity when applied on perfused oviduct (**Figure 7A**). The first ovarian regulatory peptide ever characterized was the tetrapeptide ILME [4], followed by SepOvotropin [5], SepCRPs (Sepia Capsule Releasing Peptides) [6, 8], and OJPs (Ovarian Jelly Peptides) [7]. All these peptides modulate the contraction of the distal oviduct, and some of them also regulate the contraction of the main nidamental glands (**Figures 7B**, **C**, and **8A–E**). They are expressed in vitellogenic follicles and smooth oocytes and secreted into the lumen of the oviduct during egg-laying to regulate the contractions that permit oocyte transport to the mantle cavity. They are suspected to be key-players in the synchronization of the accessory sex glands and oviduct. This regulation takes into account the number of oocytes stored in the genital coelom, which substantially fluctuates according to the successive spawning events.

A recent transcriptomic approach showed that SepOvotropin, SepCRPs, and OJPs are cleaved from a single large protein precursor of 1634 amino acids expressed in the ovarian follicle and smooth oocytes and as yet never described in the animal kingdom (**Figure 9A**).

The occurrence of a signal peptide reveals that the expression products released by this protein precursor are secreted. The spatial and temporal expression patterns of the transcripts show that it is probably a yolk protein (unpublished results: **Figure 9B**) involved in embryo development. This implies that yolk proteins could be submitted to successive processes, leading to the release of regulatory peptides. A comparison of the protein precursors with the primary sequences obtained from MS/MS analysis, and Edman degradation revealed some mistakes probably due to the tool used to determine molecular weights (ionic trap) and to analyze MS/MS spectra by a *de novo* strategy. In SepCRPs, there was a mistake about the

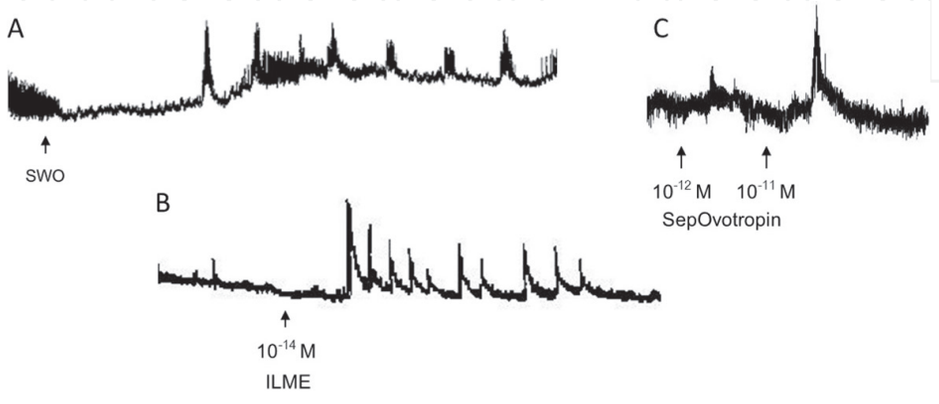

Figure 7. Perfusion of distal oviduct with (A) seawater used for incubating mature oocytes (SWO), (B) the synthetic peptide ILME and (C) synthetic SepOvotropin [4, 5].

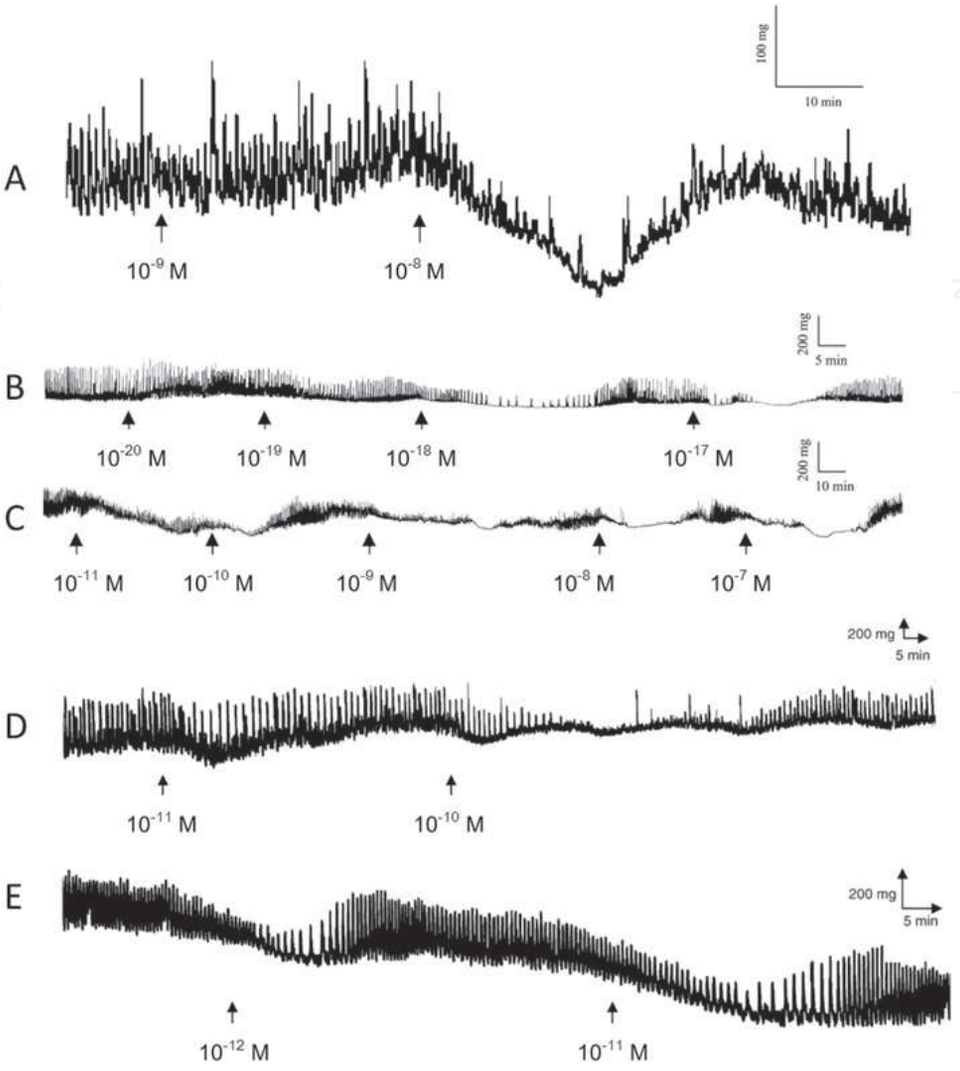

Figure 8. Effects of increasing concentrations of SepCRP on (A) the main nidamental gland and the whole female genital tract, (B) before the laying of a first batch of eggs, and (C) after the laying of a first batch of eggs. Effects of increasing concentrations of DQVKIVL on the whole female genital tract (D) and on the main nidamental gland (E) [6–8].

amino acid in position 5: aspartate (D) should be replaced by asparagine (N) (EISLNKDEVK instead of EISLNKD). In OJPs, the same amino acids should be swapped in the C-terminal moiety (EISLNKDEVK instead of EISLNKD), and in position 2, glutamate (E) should be replaced by glutamine (Q). In each case, the amino acids involved have very similar molecular masses that only diverge by 1 Da.

Finally, SepCRPs and OJPs are smaller families initially described in [6–8].

A similar mistake was made at the level of SepOvotropin because the sequence PKDSML LLQVPVYamide has the same molecular weight as PKDSMoxLLLQVPVMox. The primary sequence of SepOvotropin released by the protein precursor is PKDSMLLLQVPVM. The corrected sequences of SepOvotropin, SepCRPs and OJPs are summarized in **Table 2**. They reveal the occurrence of a conserved domain suggesting that they could bind the same receptor.

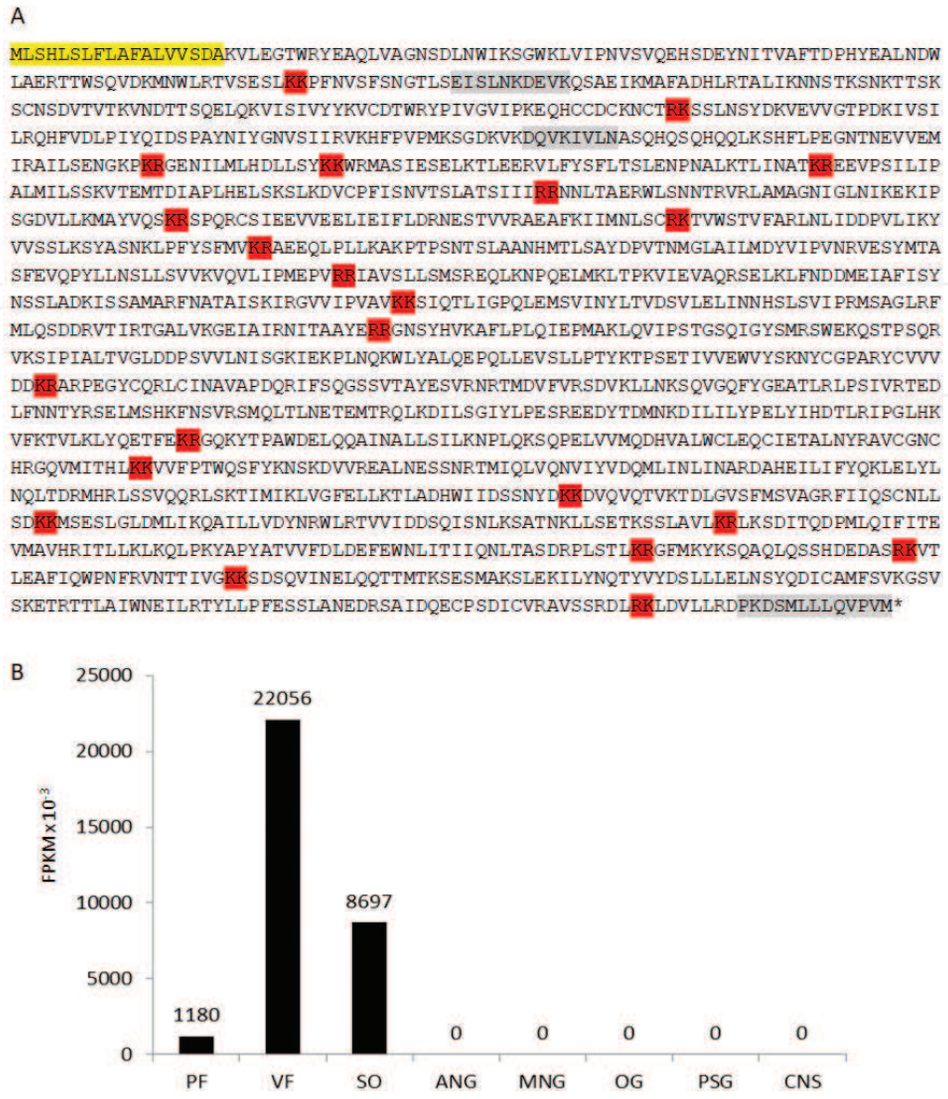

Figure 9. (A) Protein precursor of yolk-protein-releasing SepOvotropin, SepCRPs, and OJPs. The predicted signal peptide is highlighted in yellow and the convertase cleavage sites in red. Ovarian regulatory peptides are highlighted in gray, and the stop codon at the end of the coding sequence is indicated by an asterisk. (B) Expression pattern of the yolk protein. PF: previtellogenic follicles; VF: vitellogenic follicles; SO: smooth oocytes; ANG: accessory nidamental gland; MNG: main nidamental gland; OG: oviduct gland; PSG: posterior salivary gland; CNS: central nervous system, FPKM: fragments per kilobase of exon per million fragments mapped x 10^{-3}.

As for tetrapeptide ILME, it could be cleaved from the protein precursor of a retinol-binding protein (**Figure 10**) expressed in the ovarian follicles and the oocytes. *In silico,* data mining showed that it was the only protein precursor expressed in the ovary and containing the sequence ILME. The specificity of ovarian regulatory peptides lies in the fact that they come from the secondary cleavage of functional proteins. As they are cleaved at atypical cleavage sites, this makes it difficult to predict their primary sequence on the basis of protein precursor structure.

Similar regulatory peptides have been described in insects, such as TMOFs for "Trypsin-Modulating-Oostatic Factors." Bioactive peptides cleaved from vitellin membrane proteins [50] control egg development [51] and inhibit ecdysone biosynthesis [52].

Peptides	Primary sequences
SepOvotropin	PKDSMLLLQVPVM
SepCRPs	SLNKD
	ISLNKD
	EISLNKD
	EISLNKDEVK
	ESLNKDEV
	ISLNKDEV
	EISLNKDEV
OJPs	DQVKIVL
	DQVKIVLN

Table 2. Primary sequences of ovarian regulatory peptides.

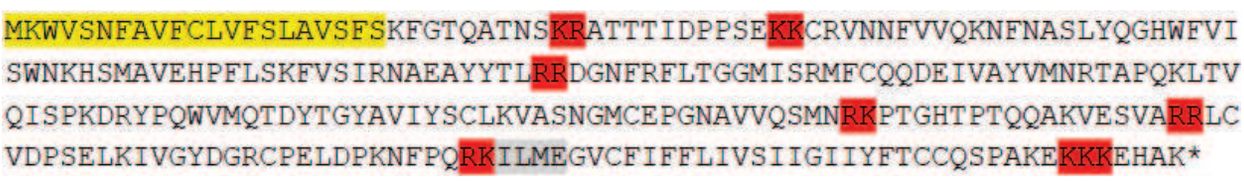

Figure 10. Protein precursor of a retinol-binding protein able to release the tetra-peptide ILME. The predicted signal peptide is highlighted in yellow and the convertase cleavage sites in red. ILME is highlighted in gray, and the stop codon at the end of the coding sequence is indicated by an asterisk.

Egg-laying regulation in cuttlefish is a complex mechanism that involves peptide and protein regulatory factors of different nature produced by the central nervous system, the ovary, and the ASGs.

The neuropeptides trigger egg-laying by integrating environmental stimuli across a neurosensory network. The ovarian regulatory peptides synchronize oocyte transport and egg capsule secretion, and their concentration is correlated to the number of smooth oocytes stored in the genital coelom. As they are short and unprotected peptides, they have a short life time after secretion, hence a very dynamic regulation.

The waterborne sex pheromones cleaved from three protein precursors overexpressed in the oviduct gland stimulate and facilitate mating and reproduction behaviors by aggregating mates in egg-laying areas. Short pheromones participate to the release of oocytes in the mantle cavity, and large pheromones are suspected to modulate reproduction behaviors by aggregating mates in egg-laying areas.

These multiple regulatory layers can be correlated with the complexity of the successive steps of the egg-laying mechanism that involves the ovary and ASGs and is performed thanks to a

stereotyped behavior: (1) ovulation, with the release of mature oocytes in the genital coelom, (2) oocyte transport by the oviduct, (3) secretion of the inner egg capsule by the OG, (4) secretion of the outer egg capsule by the MNGs, (5) black pigmentation of the egg capsule by the ink bag, (6) fertilization of oocytes by the sperm stored in the female's copulatory pouch, and (7) attachment of eggs to the sea bottom to form an egg mass.

7. The egg case: structure and function during embryo development

After the spawning period, the genitors die and leave their eggs in the marine environment without any parental protection. Thus, the sustainability of the species depends on the reproduction success and more precisely on the ability of the eggs to complete their development.

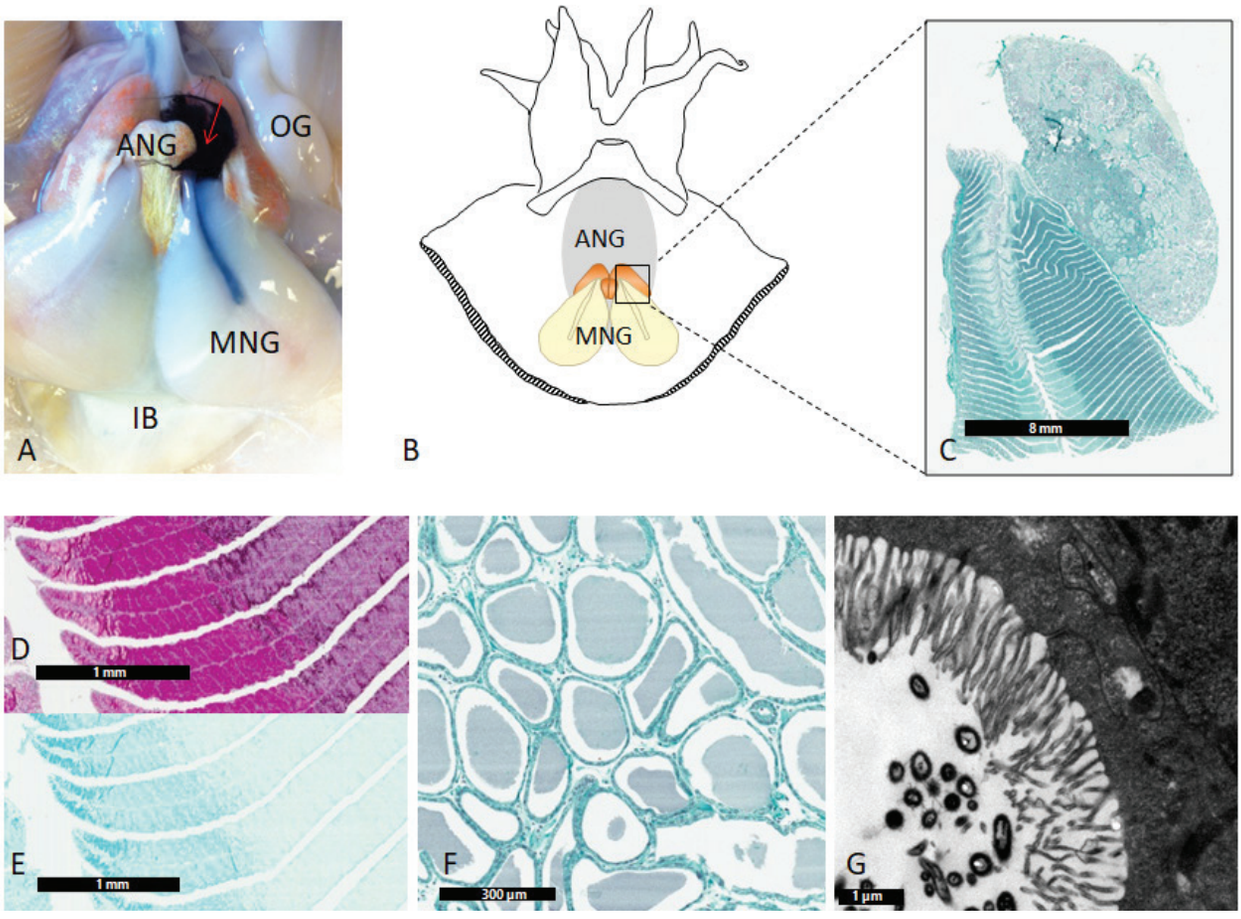

Figure 11. (A) Photograph of female reproductive glands during secretion of the egg case (red arrow). ANG, accessory nidamental gland; IB, ink bag; MNG, main nidamental gland; OG, oviduct gland. (B) Schematic representation of a mature female cuttlefish in ventral view showing the localization of the MNG and ANG. (C) Longitudinal section of the MNG and ANG stained in Prenant-Gabe triple staining. Longitudinal section of the MNG lamellae stained in alcian blue and periodic acid of Schiff highlighting the secretion of acid mucopolysaccharides (D), and neutral mucopolysaccharides and glycoproteins (E). (F) Longitudinal section of the ANG stained in Prenant-Gabe triple staining showing that tubules are composed of a single layer of ciliated epithelium and filled with bacteria in the lumen. (G) Thin section of the lumenal surface of accessory nidamental gland tubules showing a single layer of ciliated epithelium with microvilli and a few lumenal bacteria in TEM (x 12,000). (Photo credits: V. Cornet. D. Goux).

Cuttlefish eggs are large oocytes containing all the nutrient reserves required for embryo development. To withstand physical and microbial threats, mature oocytes are enclosed within a protective egg case produced by secretions of the female genital apparatus [53, 54]. This egg case is composed of two distinct envelopes. The inner layer is in direct contact with the chorion surrounding the oocyte; it is formed by secretions added, while the egg passes through the oviduct gland [53]. The oviduct gland secretes proteins and polypeptides. The main proteins secreted by this gland correspond to sex pheromones. Afterward, the oocyte is released inside the mantle cavity and embedded with an outer layer secreted by the two nidamental glands and stained with ink (**Figure 11A**).

8. Nidamental glands: a specificity in decabrachia cephalopods

The genital apparatus of *Sepia officinalis* contains two pairs of accessory reproductive glands partly involved in egg case formation (**Figure 11**). The main nidamental glands (MNGs) are related to the accessory nidamental glands (ANGs). The two paired glands are located on the ventral side of the visceral mass. The histological structure of these glands in cuttlefish is similar to the structure of squid (*Loligo forbesi*) nidamental glands [55].

The main nidamental gland and the oviduct gland both present a lamellar structure (**Figure 11C**). Each lamella consists of a central lamina of connective tissue covered with a glandular epithelium at the origin of the polysaccharides labeled by periodic acid-Schiff (PAS)-positive deposits (**Figure 11D**). The cells located at the free end of the lamellae produce particularly acid mucopolysaccharides and glycoprotein secretions revealed by alcian blue (**Figure 11E**), while the other cells secrete neutral mucopolysaccharides. During egg case formation, the secretions are released into the lumen and are led out through a duct opening onto the mantle cavity at the anterior end of the gland (**Figure 11A**). MNG and ANG structures substantially differ.

The ANG is divided into four lobes attached to the anterior end of the MNG by conjunctive tissue. Histological observations of ANG reveal a tubular gland harboring symbiotic bacteria. These symbionts are enclosed in the lumen of tubular structures that nearly completely fill the gland (**Figure 11F**). The wall of each tubule appears to be composed of a single layer of ciliated epithelium with microvilli (**Figure 11G**). The role of this gland in reproduction is unclear. Some clues suggest its involvement in egg case formation at the spawning period. During sexual maturation, the ANG indeed increases in size and changes in color from white to bright orange at the time of spawning (**Figure 11A**). It also harbors a dense consortium of bacteria that secrete carotenoids at the origin of the intense orange color of ANGs in mature females [56].

Using 16S RNA gene sequencing, many bacterial taxa were identified in ANGs, including *Agrobacterium*, *Roseobacter*, *Sporichthya*, *Rhodobium*, *Xanthobacter*, and *Clostridium* [57]. The origin of the bacterial symbionts in cuttlefish remains undetermined. Although the presence of bacteria in the egg capsule suggests vertical transmission, we cannot exclude horizontal transmission as in *Loligo opalescens* [58]. In squid, ANGs develop only a few months after hatching from a single layer of cells containing many cilia and microvilli [58].

The conserved innate immune Toll/NF-κB pathway was described for the first time in *Sepia officinalis* ANG [59]. The transcriptomic analysis of ANG led to the identification of different constitutive elements of the Toll/NF-κB pathway. Five related Toll receptors (TLRs) have been characterized. Among them, TLRα shares 89% sequence identity with the unique TLR found in the light organ of *E. scolopes*. In addition, eight phosphorylation cascade elements have been demonstrated such as IRAK, TRAF6, and Rel/NF-κB. These immune pathway proteins (α2-macroglobulin-like protein, CD-63 antigen, transferrin) are probably involved in the establishment and maintenance of the bacterial symbionts like those in the light organ of *E. scolopes* [60]. Although several studies have been carried out about the subject, the real function of this ANG and its symbionts remains unknown. Several studies in squid suggest protection of the egg via the secretion of antimicrobial or antifouling compounds by ANG or its symbionts [61, 62], but no molecule has been characterized yet.

The function of the main nidamental gland (MNG) in egg case formation is clearer (**Figure 11A**). This white gland secretes the mucopolysaccharides and glycoproteins that form the egg case.

A recent unpublished analysis of the MNG proteome reveals the occurrence of proteins involved in glycolysis/gluconeogenesis (6-phosphofructo-2-kinase, type-2 Hexokinase, Pyruvate kinase, Glyceraldehyde 3-phosphate dehydrogenase, Fructose-1,6-bisphosphatase, fructose-bisphosphate aldolase) and in glycogenolysis/glycogenesis (Glycogen phosphorylase, Glycogen synthase). These results indicate a large amount of energy production and consumption by the MNG due to an intense production and secretion of egg case components. Some of the identified proteins are also involved in the metabolism of polysaccharides or glycoproteins, like glycosyltransferases, which catalyze the transfer of oligosaccharide moieties from activated nucleotide sugars to nucleophilic glycosyl acceptor molecules or GDP-mannose pyrophosphorylase, involved in the production of N-linked oligosaccharides. Finally, the MNG secretes the main capsular components, the Egg Case Proteins, involved in the formation of a narrow mesh that provides elasticity and resistance properties to the egg case [63].

9. The oral cavity: completion of the eggs

At the time of fertilization in the oral cavity of the female cuttlefish, the oocytes are already wrapped in the thick and complex egg case. The female's arms form a chamber to keep the freshly embedded oocytes near the oral copulatory pouch where spermatophores have been deposited by the male during mating (**Figure 1**). Fertilization of the oocytes by spermatozoa is facilitated by a diffusible chemoattractant factor: SepSAP (Sepia Attracting Sperm Peptide). This hexapeptide is expressed in the vitellogenic follicles and released by embedded oocytes through the various capsular envelopes to facilitate fertilization by increasing chances of gamete collision. SepSAP has an attractant effect on sperm from low concentrations around 10^{-17} M [64].

During fertilization, the eggs are also in contact with saliva. As early as 1934, Jecklin suggested that salivary secretions could protect the eggs during spawning [54]. A recent study of the transcriptome and proteome of *Sepia officinalis* posterior salivary gland seems to confirm this

hypothesis. In addition to enzymes and toxins such as cephalotoxins and CRISPs (Cysteine Rich Secreted Proteins), cuttlefish saliva contains many immune effectors like α-macroglobulin, lysozyme, Bactericidal/Permeability-Increasing proteins (BPIs), and Lipopolysaccharide-Binding Proteins (LBPs) [65]. These salivary proteins very likely play a role in gamete protection or/and in improving fertilization.

After 2 or 3 minutes in the oral cavity, the eggs are deposited by the female's arms on a natural structure like marine eelgrass (*Zostera marina*) or an artificial one like a rope.

10. The cuttlefish egg case

During its development, the embryo is only secured by its egg case. The morphological evolution of the egg and its capsule from laying to hatching occurs in three phases during which the capsule undergoes major changes (**Figure 12**). The different steps described below correspond to embryonic development [66] first defined the different embryonic stages by performing a morphological study of the cuttlefish embryo during its development. The telolecithal egg presents a meroblastic discoidal cleavage (stages 1–9) associating blastomeres in central position and blastocones on its fringe. During epibolic gastrulation (stages 10–15), blastocones disappear under the ectoderm plate following the peripheral ring of blastula cells that will form the ring-shaped endo-mesoderm. At the end of gastrulation, the vitelline syncytium and extra-embryonic ectoderm completely surround the yolk and internalize the vegetal pole to form the yolk sac. The cleavage period corresponds to the first phase (P1) of egg evolution. A few hours after laying, the egg cell is covered with a lamina propria and surrounded by a thick gelatinous capsule (1.4 mm, ±0.6 mm). In contact with seawater, the gelatinous and fluid capsule polymerize. This reduces the volume of the egg by about 30% (**Figure 12**) and its thickness by 50%.

After 15 days of incubation and following polymerization (**Figure 13A**), capsule thickness is down to 614 microns (±150 microns) (**Figure 13B**), and the outer and inner layers can be distinguished. Polymerization of the capsule proteins helps tighten the layers of coiled outer and inner envelopes, highlighting an increasing melanin gradient from the inner layers to the outer layers. The egg is then tightly wrapped by a hardened, strong yet elastic capsule. These morphological characteristics of the capsule define the second phase of egg evolution (P2), which lasts from the 7th day to the end of the first month and corresponds to gastrulation and the beginning of organogenesis. The embryo develops within the limits of a disk located at the animal pole, at the surface, or above the yolk mass (**Figure 12**), while the capsule size and thickness remain unchanged. The initiation of organogenesis marks the beginning of the last phase (P3) that ends with hatching. The embryo in early organogenesis does not yet fill the perivitelline space. However, the capsule has become permeable to let in water and solutes. Thus, the accumulation of fluids in the perivitelline space causes the capsule to stretch, and its thickness continues to decrease (437.9 (±104) μm). Organogenesis corresponds to 2/3 of the development period, and it follows after the closure of the yolk sac and ends with hatching and can be divided into three phases (**Figure 12**). (1) During discoid or early organogenesis

(stages 15–20), the embryo forms a disk at the animal pole. The different embryonic territories build up above the yolk mass. (2) The second phase corresponds to an extension phase (stages 20–23). The brachial crown tightens on the yolk mass; the embryo straightens into the anteroposterior axis. Its rear end corresponding to the mantle gradually moves apart, leaving the brachial crown, mouth, and eyes toward the yolk. (3) The final growth phase (stage 23 to hatching) begins once the organs are found in their final topology.

After 72 days of incubation, a few days before hatching, the embryo completes its growth and has assimilated much of the yolk reserves. It now fills most of the available space in the egg and is surrounded by a large amount of perivitelline fluid (about 1 ml), stretching the capsule to its maximum (**Figure 13D**).

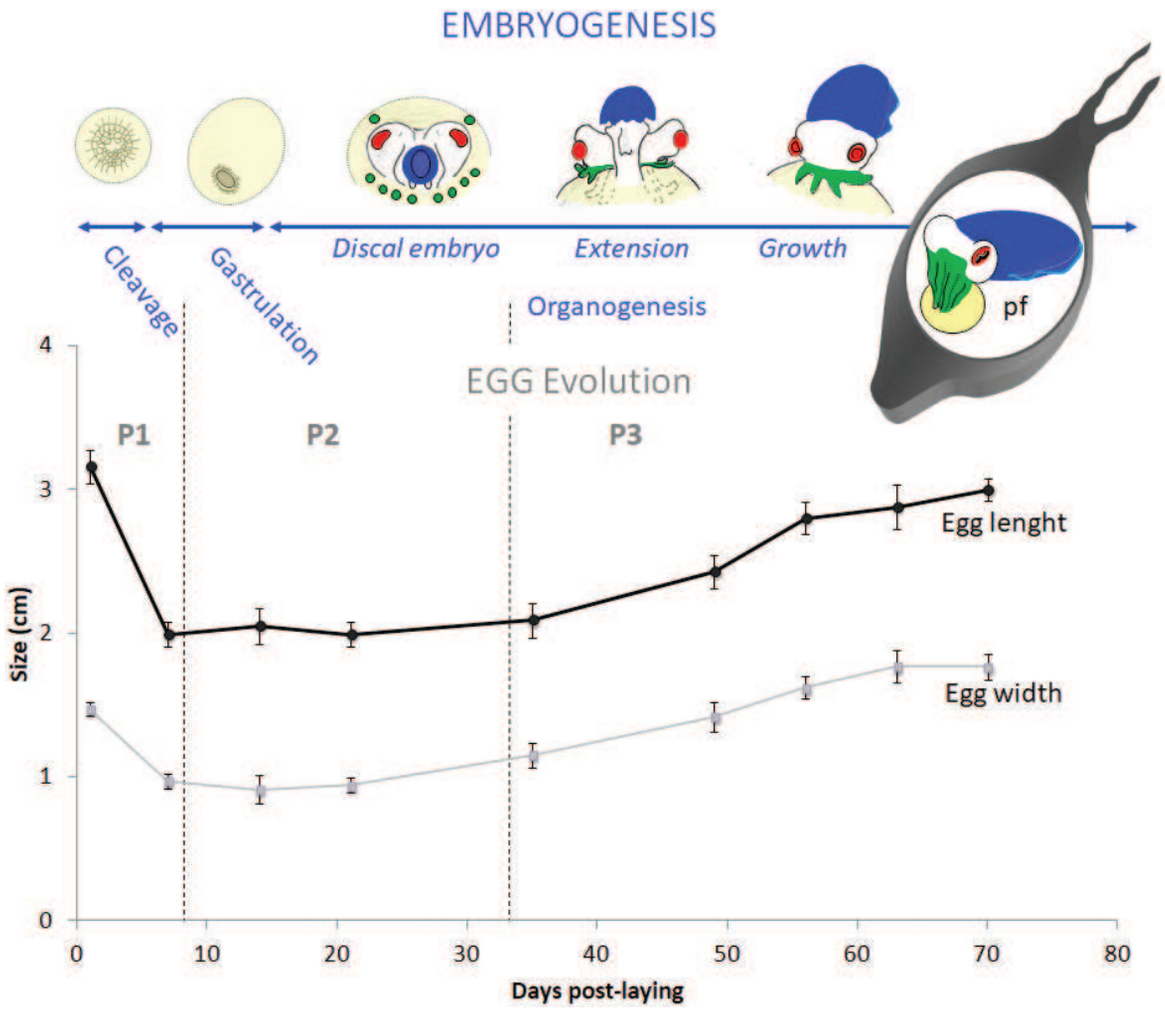

Figure 12. Evolution of *Sepia officinalis* egg size during embryogenesis at 16°C. Evolution phases of the egg case: P1, polymerization of the egg case; P2, stabilization of the egg case; P3, thinning and delamination of the egg case. Illustration of different stages of embryogenesis during cleavage, gastrulation and organogenesis. Yellow: vitellus, red: future eyes, blue: future mantle and shell, green: future arms; pf, perivitelline fluid.

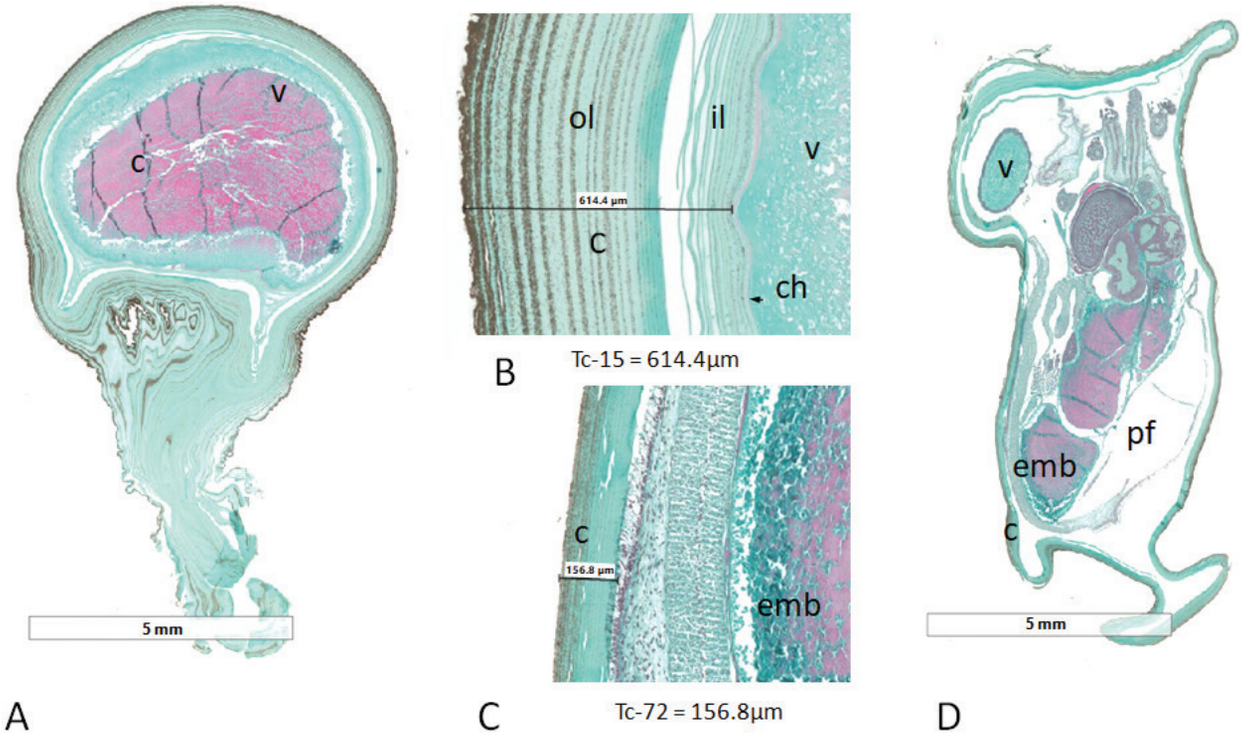

Figure 13. Longitudinal sections of the egg after 15 days (A) and 72 days (D). ANG stained in Prenant-Gabe triple staining. Magnification of the egg case including capsule thickness after 15 days (B) and 72 days (C). C, capsule or egg case; ch, chorion; emb, embryo; il, inner layer; pf, perivitelline fluid; ol, outer layer; Ct, capsule thickness; v, vitellus. (photo credits: V. Cornet).

At this stage, the embryo's features are similar to the adult's; the embryo enters a linear growth phase. All essential elements of the brachial device, the nervous system, the palleal, and visceral parts are now in place Organogenesis ends with the transfer of the outer yolk sac to the inner yolk sac, enabling faster assimilation of energy resources.

The outer and inner capsule envelopes have now completely merged, and the outermost layers of the capsule including melanin appear to be delaminated (**Figure 13C**). Thus, at the time of hatching, the capsule has undergone significant changes: it has become extremely thin (156.8 (±110) microns) and friable, so that it will break easily and release the juvenile.

At the time of hatching (Stage 30), 75–80 days after egg-laying and at 16°C, the release of enzymes by the Hoyle organ located on the end of the dorsal mantle facilitate the emergence of the juvenile [67]. Hatching is also facilitated by the thinning of the capsule.

11. Egg case composition

The capsule of *Sepia officinalis* eggs has a specific black color (**Figure 14A**). Only females belonging to the Sepiidae family include melanin granules into the egg capsule. Melanin is secreted by the ink bag and is integrated into egg case via secretions from the main nidamental gland (**Figure 11A**). Other compounds of the ink such as proteins may well also integrate the capsule.

Structural analysis of the egg capsule by photonic microscopy reveals a lamellar structure of the inner and outer envelopes (**Figure 13B**), with successive spirally wound layers. The outer envelope contains melanin deposits gathered in layers that become increasingly intense. Observations of the outer envelope by Transmission Electron Microscopy showed the presence of melanin deposits and revealed the occurrence of isolated or grouped structures whose size ranged between 0.4 and 1 μm, corresponding to bacterial structures (**Figure 13B** and **D**). These bacteria probably come from the accessory nidamental gland. The egg case ultrastructure shows a narrow mesh composed of glycoproteins and polysaccharides.

SepECP 1 and SepECP2 are cationic, cysteine-rich protein of 71 and 74 kDa, respectively (**Figure 15**). These two proteins were characterized as the main constituents of the cuttlefish egg case [16]. SepECPs are only secreted by females, mainly by the MNG and also by the oviduct gland. These two proteins are highly cationic, with 73 positively charged residues for ECP1 and 43 for ECP2. They exhibit bacteriostatic activity against a few pathogenic GRAM- bacteria from the Vibrio genus. Their bacteriostatic activity could explain the occurrence of

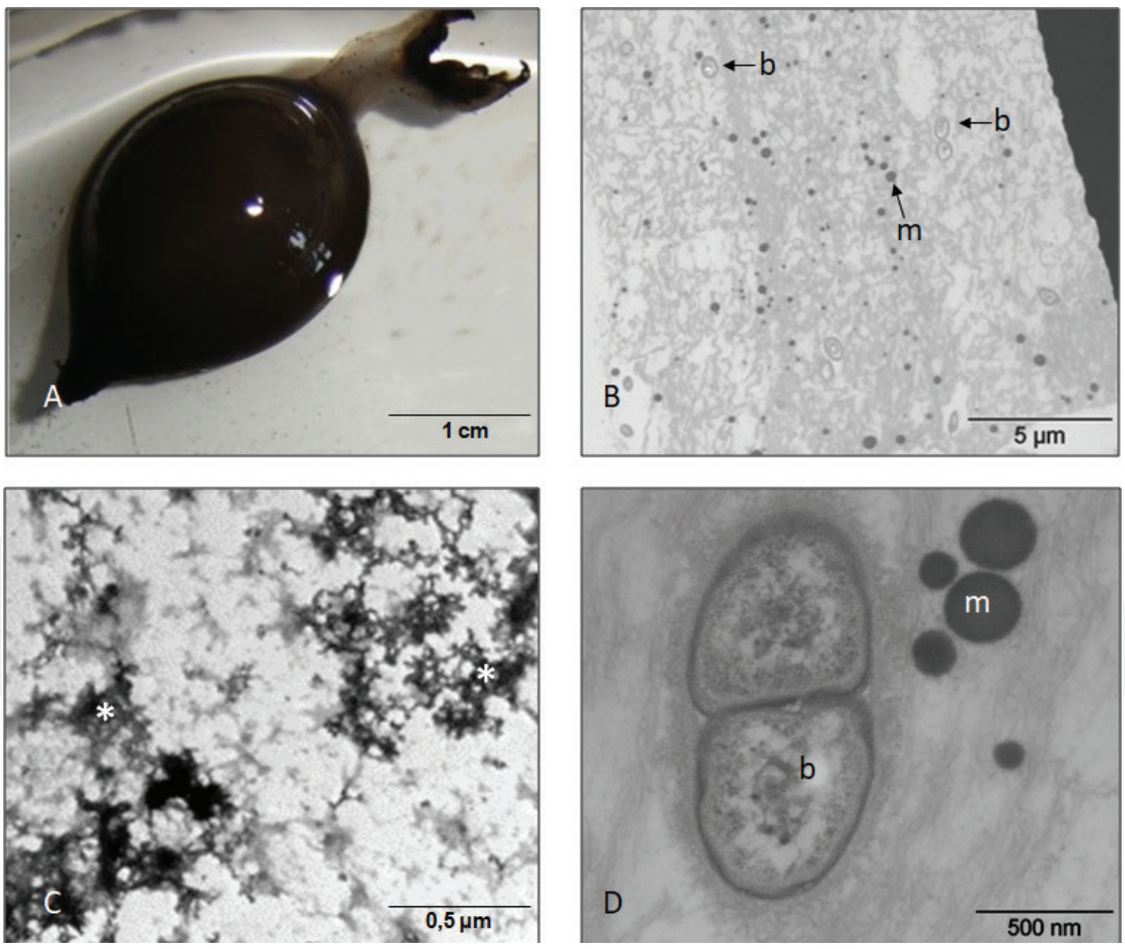

Figure 14. Photographs of the *Sepia officinalis* egg case and its components. (A) Freshly laid egg. (B and C) thin sections of the outer layer of the egg case in TEM. (D) Dividing bacteria and melanin granules. (C) Observation in TEM of SepECPs extracted from the egg case. White asterisks correspond to the protein network; b, bacteria; m, melanin. (Photo credits: C. Zatylny-Gaudin, V. Cornet, D. Goux).

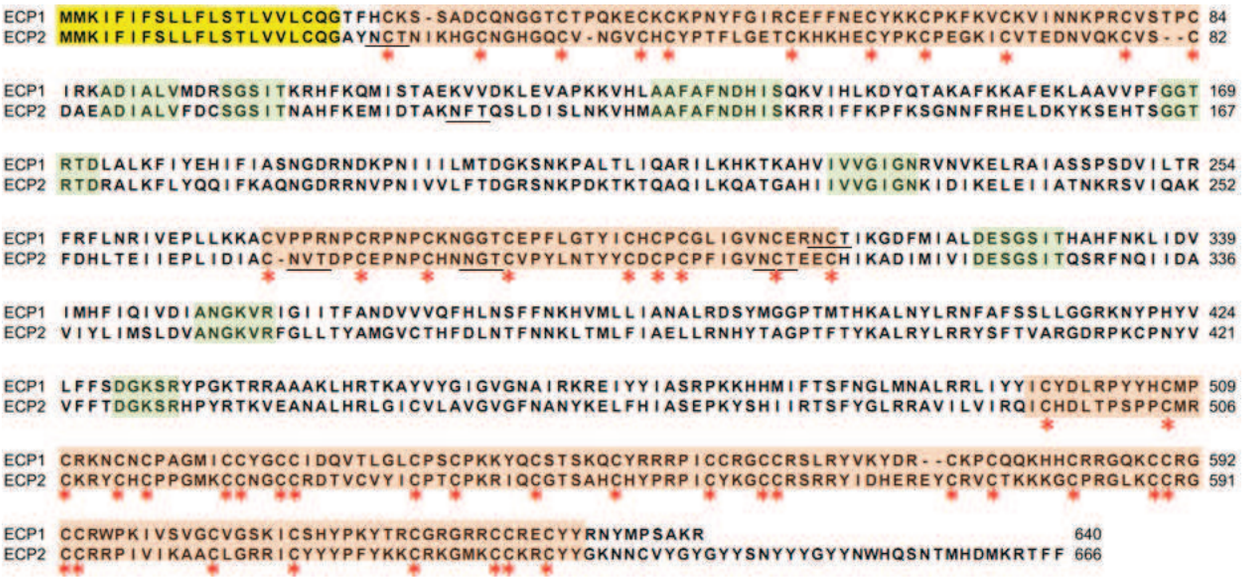

Figure 15. Amino acid alignments of the SepECP precursors. Yellow: signal peptide; orange: conserved cysteine domains; green: conserved motifs up to five amino acids. Red asterisks indicate conserved cysteines, and underlined sequences correspond to potential glycosylation sites.

bacteria in the egg case, corresponding to potential bacterial symbionts. The two SepECPs display 48 conserved cysteines grouped in three cysteine domains (**Figure 15**). These cysteines could be implied in intramolecular and intermolecular disulfide bonds involved in the formation of heterodimers. SepECPS are indeed involved in the formation of a network (**Figure 14C**) or dense matrix protecting the embryo against mechanical shocks and microbial infection during its development. No infection or biofilm is observed on cuttlefish eggs under controlled conditions or in natural environments. The capsule seems very effective: both antifouling and antibacterial coatings prevent pathogenic bacteria from proliferating [63].

During embryo development, the egg case becomes increasingly thin, but it retains elasticity to allow for embryonic growth. SepECPs are probably cleaved during the last phase (P3) to allow for hatching. During this phase, when the capsule seems more fragile, the embryo keeps developing without being affected by pathogens. When they are degraded, highly cationic SepECPs probably generate antibacterial cationic peptides. Last of all, a role of the perivitelline fluid in embryo protection should not be ruled out.

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