UNIVERSITÉ DU QUÉBEC

CONTRIBUTION À L'AMÉLIORATION DES CONNAISSANCES SUR LA PHYSIOLOGIE DE *MYA ARENARIA* (MOLLUSQUE BIVALVE) : DESCRIPTION DU SYSTÈME NERVEUX, DES STRUCTURES FONCTIONNELLES DE LA GONADE ET DE LEURS INTERACTIONS

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RÉSUMÉ

Mya arenaria, le bivalve utilisé pour ce travail, est d'intérêt économique et fait partie des espèces sentinelles couramment employées en écotoxicologie. La mye est un organisme filtreur et sédentaire qui bioaccumule les contaminants au-dessus des taux retrouvés dans le milieu, et donc, constitue un excellent indicateur de la contamination ambiante. Un déficit d'information concernant la physiologie de la mye rend l'interprétation des données écotoxicologiques de plus en plus problématique. Notre thématique de recherche tend à répondre à ce besoin d'information. Considérant que la reproduction des bivalves semble être contrôlée par les neurosécrétions ganglionnaires et les stéroïdes, nous nous sommes intéressés à l'étude du système nerveux, du système reproducteur et à leurs interactions.

Le premier objectif de cette recherche était de mieux connaître la physiologie et la composition cellulaire du système nerveux et de la masse viscérale de *Mya arenaria*. Le système nerveux présente un plan de symétrie sagittal. Il consiste en trois paires de ganglions : les cérébroïdes situés au niveau de l'oesophage, les viscéraux situés sur la face ventrale du muscle adducteur postérieur et les pédieux situés à la base du pied. Les ganglions pédieux et viscéraux sont fusionnés, tandis que les ganglions cérébroïdes sont réunis dorsalement au moyen de la commissure cérébrale. Les ganglions cérébroïdes sont connectés aux pédieux et aux viscéraux, respectivement, par les connectifs cérébro-pédieux et cérébro-viscéraux. Notre étude a aussi démontré l'existence d'un rapprochement des connectifs cérébro-viscéraux du côté antérieur du muscle rétracteur postérieur, d'un tronc nerveux émanant du connectif cérébro-viscéral et innervant le muscle rétracteur postérieur et de plusieurs troncs nerveux dérivant des connectifs cérébro-viscéraux et innervant la gonade. La masse viscérale présente une organisation générale semblable à celles des autres bivalves, et est composée des systèmes digestif, musculaire, nerveux et reproducteur. Les deux systèmes principaux, digestif et reproducteur, sont étroitement entrelacés bien que tout à fait distincts l'un de l'autre, ce qui optimise le potentiel de transfert des nutriments vers les gamètes en développement. L'étude histologique de la masse viscérale a permis de caractériser le développement et la composition cellulaire des alvéoles gonadiques. Elles se composent de cellules somatiques de réserve, strictement nutritives ("cellules folliculaires" Coe & Turner [1938]), et des cellules de la lignée germinale. De plus, chez le mâle nous avons mis en évidence, par immunohistochimie, la présence de cellules somatiques de soutien intratubulaires. Ces cellules, appelées chez les vertébrés « cellules de Sertoli », sont uniformément distribuées à l'intérieur des tubules mâles en développement.

La sérotonine (5-hydroxytryptamine ou 5-HT) joue un rôle central dans plusieurs processus physiologiques chez les mollusques marins, particulièrement au niveau de la reproduction. Nos travaux ont montré la présence de grandes quantités de cellules sérotoninergiques à l'intérieur du système nerveux de *Mya arenaria* (ganglions cérébraux, viscéraux et pédieux), ce qui confirme son rôle de premier plan comme neurotransmetteur. Au sein des ganglions viscéraux, les corps cellulaires immunoréactifs sont regroupés en nodules, appelés glomérules, circonscrits au niveau des racines des nerfs branchiaux. Notre étude montre également l'existence de fibres sérotoninergiques dans la gonade et les branchies. La présence de cellules sérotoninergiques dans les ganglions et de fibres sérotoninergiques au niveau des branchies et à la périphérie des alvéoles gonadiques confirme la relation existant entre le système nerveux et les tissus périphériques. Ces résultats plaident en faveur d'une implication de la 5-HT dans le contrôle de certaines fonctions physiologiques, telles que la respiration et la reproduction.

Récemment, les recherches sur les perturbateurs endocriniens et la régulation de la reproduction se sont intéressées à l'étude des variations des taux d'hormones stéroïdiennes (17β-oestradiol, testostérone et progestérone) en fonction de la maturité gonadique. Nos travaux montrent que les niveaux de progestérone dans la glande digestive sont trois fois supérieurs à ceux dans la gonade. Les niveaux élevés de progestérone dans la glande digestive et la similitude des profils entre la glande digestive et la gonade suggèrent une synthèse et/ou un stockage dans la glande digestive. Nos travaux ont aussi montré que les profils des hormones stéroïdiennes (17β-oestradiol et testostérone) mesurés dans la gonade de la mye pouvaient fortement varier d'une étude à l'autre, ce qui soulève de nombreuses questions. De telles variations ont déjà été rapportées dans la littérature et peuvent s'expliquer de deux manières : une source exogène de stéroïdes et/ou des variations interannuelles d'activité métabolique.

De nouvelles perspectives de recherche, aussi bien au plan de la description de la gamétogénèse en microscopie électronique, qu'au plan des techniques de traçage moléculaire neuroanatomique de la 5-HT ou de dosage des enzymes clés intervenant dans la stéroidogenèse, peuvent être proposées pour améliorer les connaissances sur la physiologie de *Mya arenaria*.

ABSTRACT

The soft-shell clam *Mya arenaria* is of economic interest and an ecologically important bivalve. The clam is a sentinel species largely used in ecotoxicology. It is a sedentary and filter-feeding bivalve which accumulates pollutants above levels found in the environment. This species is an excellent indicator of environmental contamination. However, information is lacking about its reproductive physiology which make difficult the analysis of ecotoxicological data. The objective of our research sought information on the relationship between ganglia neurosecretions and the concentration of steroids both in gonad and in the digestive gland.

The first goal of this investigation was to describe the physiology and the cell composition of the nervous system and the visceral mass of *Mya arenaria.* The nervous system follows the typical pelecypod plan. It is formed by three pairs of ganglia: the cerebral ganglia lying on both sides of the oesophagus, the visceral ganglion located on the ventral side of the adductor muscle and the pedal ganglion which is located at the base of the foot. The two symmetric visceral and pedal ganglia are fused at the midline, whereas the cerebral ganglia are connected by the cerebral commissure. Each cerebral ganglion is connected to the pedal and visceral ganglia by connective nerves. Our study showed the presence of a link between the cerebrovisceral connectives at the anterior side of the posterior adductor muscle, and of gonadal and posterior foot retractor muscle innervations appearing to originate from the ramification of the cerebrovisceral connectives. The visceral mass of *Mya arenaria* has a general organization similar to those of other bivalves and is composed of digestive, muscular, nervous and reproductive systems. The digestive and reproductive systems are intertwined and closely associated. Gonadal development around the intestine optimizes the potential transfer of nutrients to the developing gametes. The histological study of the visceral mass provides general information on the cell composition and the development of gonadic alveoli. Alveoli consist of storage somatic ("cellules folliculaires" Coe & Turner [1938]) and germinal cells. In males, alveoli are also filled with intratubular supporting somatic cells, called "Sertoli cells," in vertebrates. A large number of somatic cells was detected by immunohistochemistry uniformly distributed in male tubules.

Serotonin (5-hydroxytryptamine or 5-HT) C10H12N2O plays a central role in several physiological processes in marine molluscs, especially in reproduction. We demonstrated that the nervous system of *Mya arenaria* contains relatively large amounts of serotonin immunoreactive cells, supporting the hypothesis that 5-HT plays a role as a eurotransmitter. In the visceral ganglia, serotonin-immunoreactive cell bodies appeared to be wholly restricted to tightly clustered populations, called glomeruli. These two glomeruli were located symmetrically at the root of the branchial nerves. Our study also showed the presence of numerous 5-HT nerve fibers of various diameters in the gonad and gills of *Mya arenaria*. The presence of gills and gonadal 5-HT immunoreactive connectives and serotonin-immunoreactive cells in the cortex ganglia confirm the presence of a pathway between the nervous system and peripheral tissues. These results indicate a role of 5-HT in the control of physiological functions such as respiration and reproduction.

Recently, research on endocrine disruption and on gametogenesis regulation focussed on variations of sex steroid levels (17β-oestradiol, testosterone and progesterone) in relation to gonadic maturity. Our study showed that the progesterone level in clam digestive gland was three times higher than in gonad. The high levels of progesterone in the digestive gland and the similarity of the steroid profile between the digestive gland and gonad suggest that, in *Mya arenaria*, the digestive gland may synthesize and/or accumulate this steroid. Our work showed interannual variations in the gonadal steroid profiles (17β-oestradiol and testosterone). Such steroid variations have been previously reported in the literature and can be explained by interannual changes in metabolic activity and/or by the presence of an exogenous source of steroids.

Based on our fondings, additional work using electron microscopy to describe gametogenesis, coupled with steroid metabolism enzyme level measurements and tracking 5-HT via neuroanatomical techniques, would be of benefit to further improve physiological knowledge in *Mya arenaria*.

LISTE DES ABRÉVIATIONS

17β-HSD	**17β-H**ydroxy**S**teroid **D**ehydrogenase / **17β-H**ydroxy**S**téroïde **D**éshydrogénase
3β-HSD	**3β-H**ydroxy**S**teroid **D**ehydrogenase / **3β-H**ydroxy**S**téroïde **D**éshydrogénase
5-HIAA	**5-H**ydroxy**I**ndole**A**cetic **A**cid (acide 5-hydroxy-indolylacétique)
5-HT	**5-H**ydroxy**T**ryptamine (serotonin / sérotonine)
5-HTP	**5-H**ydroxy**T**rypto**P**han / **5-H**ydroxy**T**rypto**P**hane
AMPc	**A**dénosine **M**ono**P**hosphate **c**yclique
ASD	**A**nti**S**erum **D**iluent
CPG (GCP)	**C**erebro**P**leural **G**anglia (**G**anglions **C**érébro-**P**leuraux)
DAB	**D**i**A**mino**B**enzidine
DGDF	**D**igestive gland, **G**onad, **D**igestive tract and **F**oot (glande digestive, gonade, tractus digestif et pied)
DHEA	**De**Hydro**E**pi**A**ndrosterone / **Dé**Hydro**É**pi**A**ndrostérone
E_1	**E**strone
E_2	**E**stradiol-17β / 17β-o**E**stradiol
ELISA	**E**nzyme **L**inked **I**mmuno**S**orbent **A**ssay
GVBD	**G**erminal **V**esicle **B**reak **D**own (dissolution de la vésicule germinative)

GPCR	**G** Protein-**C**oupled **R**eceptors (récepteurs couplés à des protéines G)
HPLC	**H**igh **P**erformance **L**iquid **C**hromatography (Chromatographie en phase Liquide à Haute Pression)
HSD	**H**ydroxy**S**teroid **D**ehydrogenase / **H**ydroxy**S**téroïde **D**éshydrogénase
IP3	**I**nositol **tri-P**hosphate
NADPH	**N**icotinamide **A**dénine **D**inucléotide **P**hosphate
NSC (CNS)	**N**euro**S**ecretory **C**ells (**C**ellules **N**euro**S**écrétrices)
P	**P**rogestrone / **P**rogestérone
PAP	**P**eroxidase **A**nti-**P**eroxidase / **P**eroxydase **A**nti-**P**eroxydase
PBS	**P**hosphate **B**uffered **S**aline (tampon phopsphate salin)
PG (GP)	**P**edal **G**anglia (**G**anglions **P**édieux)
PGs	**P**rosta**G**landin / **P**rosta**G**landine**s**
T	**T**estosterone / **T**estostérone
Trp	**Tr**ypto**p**han / **Tr**ypto**p**hane
TX-100	**T**riton **X-100**
VG (GV)	**V**isceral **G**anglia (**G**anglions **V**iscéraux)

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INTRODUCTION GÉNÉRALE

Depuis le début de l'ère industrielle, de très grandes quantités de polluants ont été introduites dans l'écosystème marin du Saint-Laurent, soit directement à partir des effluents municipaux et industriels, soit indirectement par le ruissellement et les retombées atmosphériques (Gobeil & Cossa, 1993). Depuis 20 ans, une baisse significative des apports directs en contaminants est observée. Malgré tout, les concentrations actuelles retrouvées dans le milieu sont toujours supérieures aux concentrations préindustrielles (Loring, 1975; Smith & Loring, 1981; Gobeil & Cossa, 1993; Alaee, 2003).

Au cours des 20 dernières années, la communauté scientifique internationale s'est particulièrement intéressée à l'impact des polluants d'origine anthropique sur l'environnement. Une exposition à de faibles concentrations perturbe le fonctionnement naturel des organismes, notamment la reproduction. Ces dérèglements s'observent, chez les poissons et les bivalves, par une diminution du nombre de spermatozoïdes produits, par la présence de malformations génitales, ainsi que par des changements de sexe (masculinisation ou féminisation) et du sexe ratio au sein des populations affectées (Depledge & Billinghurst, 1999; Blaise et *al.*, 2003; Gagné et *al.*, 2006).

Les mollusques bivalves, à l'exemple de *Mya arenaria*, sont des organismes filtreurs et sédentaires, qui bioaccumulent les contaminants au-dessus des taux retrouvés dans le

milieu, et donc, constituent d'excellents indicateurs de la contamination des eaux marines (Ramade, 1992). Dans le domaine marin, ils sont couramment utilisés comme organismes sentinelles dans les études écotoxicologiques évaluant les effets de la contamination ambiante (Pellerin-Massicotte, 1997; Morcillo et *al.*, 1997; Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2002). Les effets cumulatifs non létaux de l'exposition chronique aux polluants conduisent à des dérèglements du système neuroendocrinien, du système immunitaire et de l'appareil reproducteur. L'insuffisance d'information sur la physiologie des bivalves, ainsi que des facteurs et processus physiques et chimiques impliqués, rend l'interprétation des données écotoxicologiques souvent problématique. Il devient donc indispensable d'approfondir les connaissances de base sur la physiologie des organismes sentinelles.

Mya arenaria, l'espèce utilisée pour ce travail, fait partie des espèces sentinelles couramment utilisées en écotoxicologie. À l'heure actuelle, peu de choses sont connues sur sa physiologie. L'anatomie et le fonctionnement internes ressemblent, à différents niveaux, à celui des autres bivalves (Vlès, 1909; Potts, 1993), mais plusieurs de ses organes n'ont jamais encore été correctement décrits. Entre autres, citons le cas du système nerveux qui fut étudié, avec les outils et les connaissances de l'époque, par Vlès (1909). La gonade, et plus précisément l'évolution de la gamétogenèse, a fait l'objet de plusieurs études histologiques (Battle, 1932; Coe & Turner, 1938; Rogers, 1959; Shaw, 1962; Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002). Seule l'étude de Coe & Turner (1938) décrit les différents types cellulaires présents dans les alvéoles gonadiques. Récemment, les recherches sur la reproduction se sont orientées vers l'étude des hormones stéroïdiennes (Siah et *al.*, 2002, 2003; Gauthier-Clerc et *al.*, 2006) et des enzymes impliquées dans la

stéroïdogenèse (Hathaway, 1965; Mori et *al.*, 1965a, 1965b; De Longcamp et *al.*, 1970, 1974; Varaksina & Varaksin, 1988; Matsumoto et *al.*, 1997; Morcillo et *al.*, 1999; Le Curieux-Belfond et *al.*, 2001), mais l'interprétation de ces résultats amène de nombreuses questions.

Chez les invertébrés, et plus précisément chez les bivalves, la reproduction serait contrôlée par les neurosécrétions ganglionnaires et les hormones stéroïdiennes (Motavkine & Varaskine, 1989). Les travaux effectués dans le cadre de cette thèse s'inscrivent dans cette thématique de recherche. L'objectif général de cette recherche est d'améliorer les connaissances sur la physiologie et les relations existant entre le système nerveux et le système reproducteur, chez *Mya arenaria* (mollusque bivalve endobenthique). L'exposé de cette étude s'articule en six (6) chapitres :

Dans le **premier chapitre** de cette thèse, une revue des connaissances concernant la physiologie de *Mya arenaria*, le système nerveux neuroendocrinien des mollusques bivalves ainsi que l'implication des neurosécrétions et des hormones stéroïdiennes dans le contrôle de la reproduction est présentée.

Dans le **second chapitre** (en anglais et sous forme d'article), la description du système nerveux et la localisation de la sérotonine (5-HT) dans celui-ci ont été abordées.

Dans le **troisième chapitre** (en anglais et sous forme d'article), un test *in vivo* de stimulation de la ponte par la 5-HT et sa localisation par immunohistochimie dans la gonade ont été étudiés afin de mieux comprendre l'implication de la 5-HT dans le contrôle de la gamétogenèse.

Dans le **quatrième chapitre** (en anglais et sous forme d'article), la description physiologique de la masse viscérale, la composition cellulaire des alvéoles gonadiques et le développement de la gamétogenèse ont été ré-évalués avec les techniques d'aujourd'hui.

Dans le **cinquième chapitre** (en anglais et sous forme d'article), les variations des taux de progestérone, testostérone et 17β-oestradiol en fonction de la maturité de la gonade ont été mesurées afin d'approfondir nos connaissances sur l'implication des hormones stéroïdiennes dans le contrôle de la reproduction.

Enfin, dans le **sixième et dernier chapitre**, une discussion générale traite des principaux résultats, de la contribution de ce travail à l'acquisition de nouvelles connaissances et offre des perspectives de recherche sur le sujet traité.

CHAPITRE 1 : SYNTHÈSE BIBLIOGRAPHIQUE

1.1 *Mya arenaria* mollusque bivalve

Mya arenaria (Linnaeus 1758), mollusque bivalve appartenant à l'ordre des Eulamellibranches et au sous-ordre des Hétérodontes, fait partie de la famille des Myidae (Potts, 1993). *Mya arenaria* est une espèce pélécypode, endobenthique, sédentaire, suspensivore et microphage comme la moule. Elle se nourrit de petites particules en suspension (plantes et animaux microscopiques) situées juste au-dessus du sédiment à la hauteur de son siphon. *Mya arenaria* peut filtrer chaque jour jusqu'à 54 litres d'eau (Karsten, 1985). Ses noms vernaculaires les plus utilisés sont : la coque, la clanque, le Bec de jar, la pisseuse, le bedjar ou encore la mye des sables. Cette espèce d'intérêt économique, faisant l'objet de pêche à pied artisale et commerciale, est présente sur toutes les côtes de l'hémisphère nord (Abbott et *al.*, 1982) entre les latitudes 30° et 35°. Sa distribution s'étend tout au long de la côte Est du continent américain (au nord-ouest de l'océan Atlantique) du labrador méridional jusqu'à la Floride (Lubinsky, 1980).

Dans l'estuaire maritime du Saint-Laurent, *Mya arenaria* fait partie de la communauté boréo-atlantique à *Macoma baltica* (L.) (Desrosiers & Brêthes, 1984). Cet organisme endobenthique se retrouve dans les zones intertidale et subtidale, jusqu'à 200

mètres de profondeur. Il s'enfouit principalement dans les sédiments sableux, vaseux et marno-sableux riches en matière organique et quitte rarement son terrier lorsque sa taille est supérieure à 5 cm. La forte abondance de *Mya arenaria* dans l'estuaire du Saint-Laurent et dans le Fjord du Saguenay démontre ainsi une tolérance élevée aux variations de salinité (Gauthier-Clerc et *al.*, 2002).

1.1.1 Anatomie de *Mya arenaria*

Mya arenaria possède une coquille bivalve, allongée et elliptique, de couleur blanchâtre et noirâtre, pouvant atteindre 12-15 centimètres de longueur pour les plus grands spécimens. Sur l'extérieur de la coquille, des lignes concentriques appelées « stries de croissance » se distinguent. Lorsque *Mya arenaria* est enfouie dans le sédiment, un long siphon contractile s'étend de la partie postérieure de l'animal jusqu'à la surface (environ 20 cm et même 40 cm pour les grands spécimens). Le siphon est composé de deux siphons soudés, un inhalant (pompant l'eau) et un exhalant (rejetant les particules indésirables). Le pied est petit et musculeux. Il s'étend vers l'extérieur depuis une ouverture située à l'extrémité antérieure de l'animal (Fig. 1.1).

L'anatomie interne de la mye (Fig. 1.2) ressemble, à différents niveaux, à celle des autres bivalves (Vlès, 1909; Potts, 1993). Le corps est logé entre deux valves qui s'écartent et se rapprochent par l'intermédiaire de deux muscles adducteurs (antérieur et postérieur). Le manteau sécrète la coquille et il constitue une mince couche entre les valves. *Mya arenaria* a un système circulatoire ouvert. Le sang est collecté dans un sinus ventral

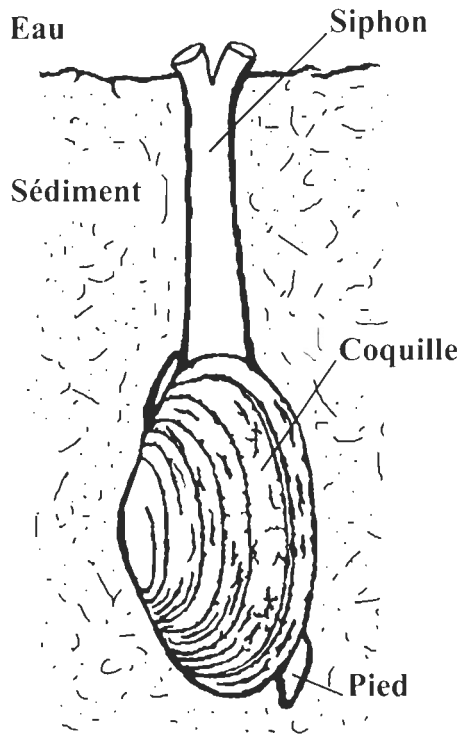

Figure 1.1: *Mya arenaria* L., mollusque bivalve endobenthique.

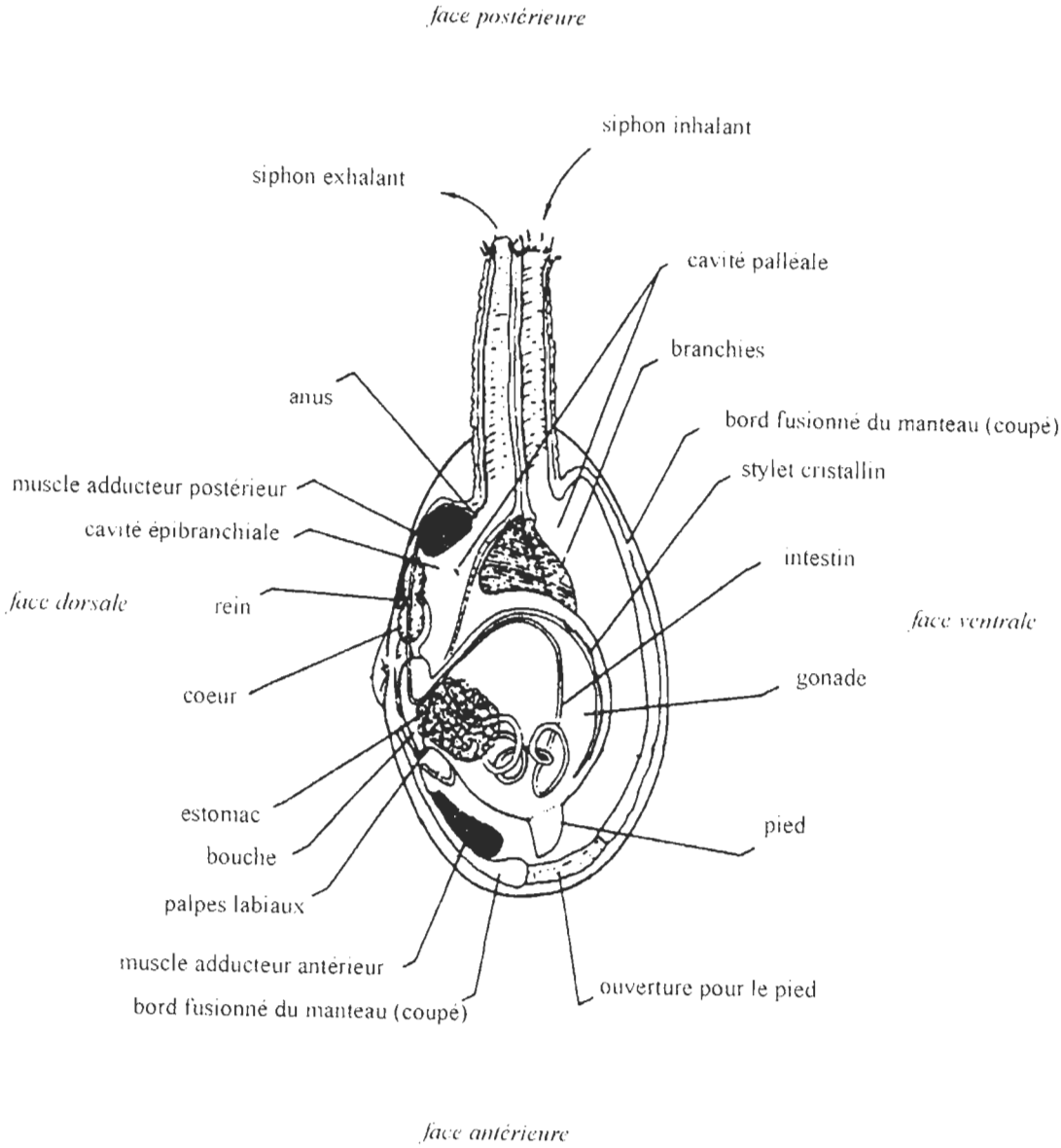

Figure 1.2: Anatomie interne de *Mya arenaria*, mollusque bivalve (Modifié de Hanks, 1963).

et gagne les reins (situés dans la cavité péricardique) où il est filtré. Par la suite, le sang pénètre dans les branchies par les veines afférentes puis en ressort par les veines efférentes qui le conduisent au cœur. Le cœur, formé de deux oreillettes et d'un ventricule, propulse le sang dans l'aorte antérieure et postérieure pour rejoindre les diverses régions de l'organisme. L'alimentation est basée sur la filtration des particules contenues dans l'eau. Les particules sont filtrées par l'action de cils placés sur les branchies. Les cils acheminent la nourriture vers le sillon digestif, puis antérieurement, vers les palpes labiaux et la bouche situés au-dessus et à l'avant du pied. La nourriture est ensuite transportée dans l'œsophage, dans l'estomac puis dans l'intestin. L'estomac est entouré par la glande digestive et est pourvu d'un cæcum postérieur dans lequel se trouve le stylet cristallin. L'intestin sillionne à travers la gonade et se poursuit par un rectum rectiligne qui traverse le péricarde et le ventricule cardiaque (Vlès, 1909). L'anus est situé au-dessus du muscle adducteur postérieur à proximité du siphon exhalant. Le système reproducteur (la gonade) se situe entre la glande digestive et le pied et s'agence autour de l'intestin. Le système reproducteur est constitué d'une paire de glandes (formées d'acinis très ramifiés) plus ou moins imbriquées l'une dans l'autre sur la ligne médiane, mais chacune garde son indépendance (Vlès, 1909). Les conduits gonadiques (gonoductes) s'ouvrent dans la cavité du manteau (Vlès, 1909; Stickney, 1963). Le système nerveux des mollusques bivalves est simple et consiste en trois paires de ganglions reliées entre elles : les ganglions cérébroïdes, les ganglions viscéraux et les ganglions pédieux. Chez *Mya arenaria*, seule l'étude de Vlès (1909) décrit, avec les outils et les connaissances de l'époque, l'ensemble du système nerveux. Il comprend trois paires de ganglions principaux : les ganglions cérébroïdes situés

au niveau de l'oesophage, les ganglions viscéraux situés sur la face ventrale du muscle adducteur postérieur et les ganglions pédieux situés à la base du pied. Les ganglions cérébroïdes sont connectés aux pédieux et aux viscéraux, respectivement, par les connectifs cérébro-pédieux et cérébro-viscéraux.

1.1.2 Biologie et physiologie de la reproduction de *Mya arenaria*

Mya arenaria est une espèce gonochorique (à sexes séparés) et itéropare (plusieurs périodes de reproduction possibles la même année et sur plusieurs années) (Coe & Turner, 1938). Dans l'hémisphère nord, la mye atteint sa maturité sexuelle lorsque sa taille est comprise entre 25 et 38 mm (Hanks, 1963). À l'intérieur de l'estuaire du Saint-Laurent et lorsque les conditions du milieu sont favorables, *Mya arenaria* présente une reproduction biannuelle (Belding, 1930; Roseberry et *al.*, 1991; Gauthier-Clerc et *al.*, 2002). La première gamétogenèse s'initie durant l'hiver et se termine par une ponte printanière. La seconde gamétogenèse s'amorce au début de l'été et s'achève parfois lors de la ponte automnale (Gauthier-Clerc et *al.*, 2002). La gamétogenèse de *Mya arenaria* (les différentes spécificités du développement de la gonade) a fait l'objet de différentes études histologiques (Coe & Turner, 1938; Rogers, 1959; Shaw, 1962; Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002). De ces études, six (6) stades de maturation ont été déterminés chez la femelle et cinq (5) chez le mâle (Tableau 1.1) (Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002). Les travaux de Coe & Turner (1938) ont montré que, chez *Mya arenaria*, la gonade n'est constituée que de deux types cellulaires : les cellules folliculaires strictement

Tableau 1.1 Classification et description des six (6) stades de maturité de la gonade femelle et des cinq (5) stades de la gonade mâle chez *Mya arenaria* selon les caractéristiques histologiques (Modifié de Coe & Turner, 1938; Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002).

Stades	Ordre		Descriptions
	Femelle	Mâle	
Indifférencié	1er	1er	Les cellules folliculaires, riches en inclusions, colonisent les alvéoles. Chez les femelles et les mâles, de petites cellules germinales (chez la femelle de petits ovocytes à noyau rond et chez le mâle de nombreuses spermatogonies) sont visibles au niveau de la membrane basale des alvéoles.
Pré-vitellogenèse	2ième		Le processus de l'ovogenèse s'initie. Les ovocytes sont plus nombreux et plus gros qu'au stade précédent. Le diamètre moyen des ovocytes est inférieur à 20 micromètres.
Vitellogenèse	3ième		Le nombre de cellules folliculaires et d'inclusions diminue, ce qui favorise une augmentation de la lumière alvéolaire. À ce stade, deux types d'ovocytes sont différentiables (des ovocytes sphériques et solidaires de la paroi alvéolaire et des ovocytes plus allongés et faiblement rattachés à cette même paroi), ce qui révèle différents degrés d'avancement. Le diamètre moyen des ovocytes est compris entre 20 et 40 micromètres.
Post-vitellogenèse	4ième		À ce stade, des ovocytes sphériques et libres sont visibles dans la lumière de l'alvéole. Il est possible de rencontrer des ovocytes à des stades de développement plus précoce au sein d'une même alvéole. Le diamètre moyen des ovocytes est supérieur à 40 micromètres.

Tableau 1.1 Classification et description des six (6) stades de maturité de la gonade femelle et des cinq (5) stades de la gonade mâle chez *Mya arenaria* selon les caractéristiques histologiques (Modifié de Coe & Turner, 1938; Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002) (suite).

Stades	Ordre		Descriptions
	Femelle	Mâle	
Développement		2ième	Le processus de spermatogenèse s'initie. Les spermatogonies se différencient en spermatocytes (primaires et secondaires), puis en spermatozoïdes de façon centripète de la membrane basale vers la lumière de l'alvéole. L'abondance des cellules folliculaires diminue progressivement avec la prolifération et la différenciation des spermatogonies.
Mûr		3ième	La lumière des alvéoles est occupée par des spermatozoïdes en position radiale (la queue dirigée vers la lumière de l'alvéole). À ce stade, il ne reste que très peu de cellules folliculaires dans les alvéoles.
Ponte	5ième	4ième	Lors de la ponte, les cellules germinales matures (spermatozoïdes et ovocytes libres) sont libérées vers l'extérieur des alvéoles. Les cellules folliculaires réapparaissent (au niveau de la membrane basale) afin de combler l'espace laissé libre par l'expulsion des gamètes.
Passé	6ième	5ième	Quelques cellules germinales (ovocytes chez la femelle et spermatozoïdes chez le mâle) subsistent à l'intérieur des alvéoles et seront rapidement lysées. Chez la femelle, les cellules folliculaires, riches en inclusions nutritives, recouvrent la membrane basale. Chez le mâle, les cellules folliculaires prolifèrent et recolonisent complètement les alvéoles.

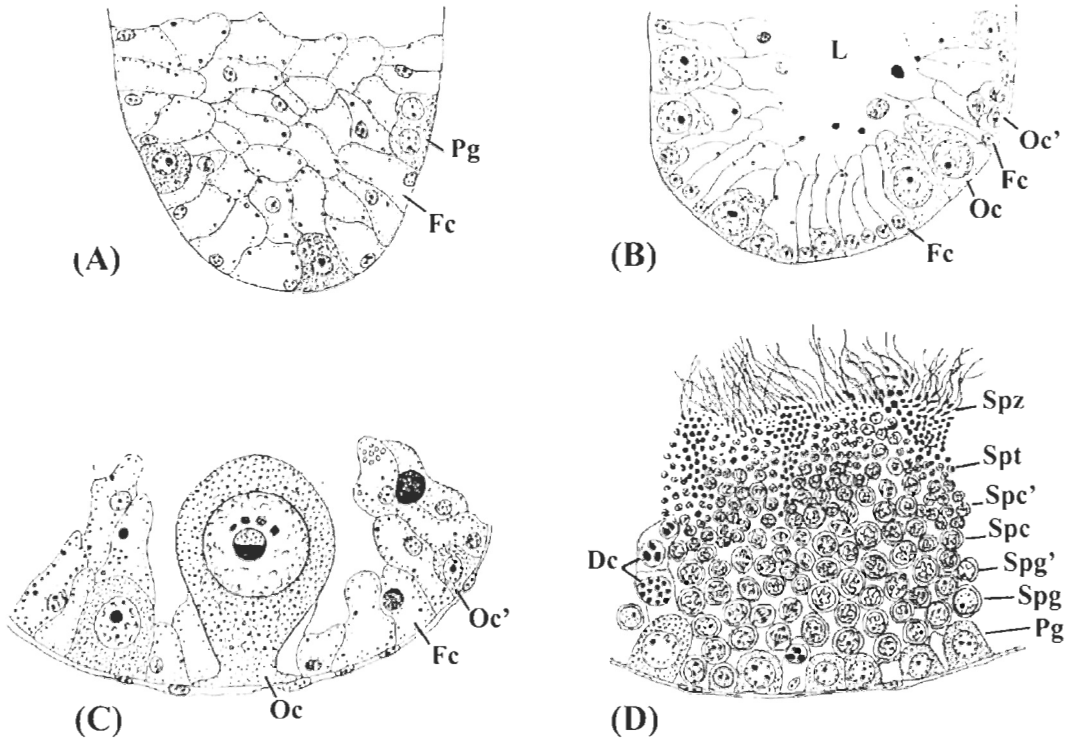

Figure 1.3: Schémas histologiques de la gonade de *Mya arenaria*: (A) Aspect d'une alvéole indifférenciée chez un individu juvénile; (B) Aspect d'une alvéole femelle au stade pré-vitellogenèse; (C) Aspect d'une alvéole femelle au stade vitellogenèse; (D) Aspect d'une alvéole mâle en développement (Modifié de Coe & Turner, 1938).

Légendes : Fc : Cellules folliculaires; Pg : cellules germinales primaires; Oc : Ovocytes; Oc' : petits ovocytes; Spg et Spg' : spermatogonies primaires et secondaires; Spc et Spc' : spermatocytes primaires et secondaires; Spt : spermatides; Spz : spermatozoides; Dc : inclusions cellulaires atypiques.

nutritives et les cellules de la lignée germinale (Fig. 1.3). Au stade juvénile, les alvéoles de la gonad sont constituées principalement de cellules folliculaires. Celles-ci sont composées d'un petit noyau caractéristique, d'une couche mince de cytoplasme et d'une grande vacuole centrale. Les gonies primaires, desquelles dériveront l'ensemble des futurs gamètes, sont dispersées, en faible quantité, le long de la membrane basale des alvéoles et sont identifiables par leurs imposants noyaux (Figs 1.3b-d). Durant la différenciation sexuelle, les cellules germinales se multiplient et se différencient en ovogonie chez la femelle et en spermatogonies chez le mâle.

1.2 Contrôle de la reproduction chez les mollusques bivalves

1.2.1 Contrôle neuroendocrinien

1.2.1.1 Système nerveux neuroendocrinien et cellules neurosécrétrices

Des travaux effectués chez les bivalves ont montré que le système reproducteur est soumis à une régulation neurohormonale du système nerveux neurosécréteur (Motavkine & Varaskine, 1989). Chez les bivalves, le système nerveux neuroendocrinien est formé de trois paires de ganglions : les ganglions cérébroïdes, les ganglions viscéraux et les ganglions pédieux. Quatre (4) types de cellules neurosécrétrices (CNS) : *a1, a2, a3* et *a4*, ont été clairement identifiés dans les ganglions de *Mytilus edulis* (Illanes-Bücher, 1979; Illanes-Bücher & Lubet, 1980) et de *Perna perna* (Benomar et *al.*, 2003). Les CNS sont

localisées dans la zone corticale antérodorsale des ganglions cérébroïdes, et dans le cortex dorsal des ganglions viscéraux et pédieux. Les plus abondantes sont les cellules de type *a1*. Elles représentent plus de 75 % de la totalité de CNS (Lubet & Mathieu, 1990; Mathieu, 1991). Chez *Mytilus edulis*, l'ablation des ganglions viscéraux et cérébraux au début de la gamétogenèse provoque un retard important de la maturité sexuelle. Au contraire, une ablation durant la fin de gamétogenèse semblerait hâter la maturation des gamètes (Lubet, 1965). Chez la même espèce, Illanes-Bucher & Lubet (1980) ont mis en évidence une corrélation significative entre le cycle de reproduction et le nombre de CNS de type *a1*. Le nombre de CNS *a1* augmente en automne lors de la reprise de la gamétogenèse et est maximum lorsque la gonade est mûre. Les facteurs provenant des ganglions cérébroïdes et viscéraux provoquent la mitose goniale, la méiose chez le mâle, la vitellogenèse et le maintien des tissus de réserve, mais ne semblent ni sexualisés, ni spécifiques au sexe (Lubet & Mathieu, 1978, 1982, 1990; Mathieu & Lubet, 1980; Lubet et *al.,* 1986, 1987; Mathieu et *al.,* 1988). Les facteurs responsables de l'activité mitogénique n'ont pas encore été identifiés, mais leurs poids moléculaires sont inférieurs à 50 000 Da chez *Limax maximus* (Melrose et *al.*, 1983) et à 5 000 Da chez *Mytilus edulis* (Mathieu et *al.*, 1988), ce qui sous-entend que ces facteurs sont des peptides.

1.2.1.2 Les amines biogènes

Les amines biogènes, présentes dans les tissus neuronaux et non neuronaux de la plupart des invertébrés, sont connues pour agir en tant que neurohormones, neuromodulateurs et neurotransmetteurs (Roeder, 1999) et sont largement étudiées. La présence des catécholamines : dopamine (Sweeney, 1963; Stefano & Catapane, 1980; Smith, 1982; Matsutani & Nomura, 1984), noradrénaline [norépinéphrine] (Stefano & Catapane, 1977, 1980; Stefano et *al.,* 1978; Osada et *al.*, 1987), et des indolamines, telles que la sérotonine (Stefano & Catapane, 1980; Smith, 1982; Matsutani & Nomura, 1984) ont été rapportées chez les bivalves comme le montrent les tableaux 1.2 et 1.3. Les amines biogènes sont synthétisées dès les premières étapes de la vie et sont largement impliquées dans le développement larvaire (Cann-Moissan et *al.*, 2002). Chez les individus adultes, les bioamines commandent de nombreuses fonctions physiologiques, telles que l'activité ciliaire (Catapane et *al.*, 1978; Smith, 1982), l'activité du cœur (Painter & Greenberg, 1982; Croll et *al.*, 1995), l'activité des muscles adducteurs (Salanki & Hiripi, 1970; Salanki et *al.*, 1974) et du muscle rétracteur du byssus (York & Twarog, 1973). Stefano & Catapane (1977, 1980) et Osada & Nomura (1989b), respectivement chez *Mytilus edulis* et *Crassostrea gigas*, ont corrélé les variations de concentration des catécholamines et des indolamines dans les ganglions et dans la gonade avec le cycle reproducteur. Ces résultats suggèrent une régulation de la gamétogenèse par les bioamines. Cependant, cette implication reste hypothétique et n'a jamais encore été clairement démontrée.

Tableau 1.2 Les catécholamines chez les mollusques bivalves : dopamine (fond blanc) et noradrénaline [norépinéphrine] (fond gris).

Espèces	Références	Tissus	Dosages	T
Aequipecten irradians	Sweeney, 1963	GCP+GV+GP	74 µg / g	SF
Anodonta piscinalis	Dahl et *al.*, 1966	GCP GV GP	11,6 µg / g 19,2 µg / g 47,1 µg / g	HF
Argopecten purpuratus	Martinez et *al.*, 1996	gonade GCP GV	0,2 - 2,3 ng / g* 32 - 42 ng / g* 58 - 82 ng / g*	SF
		gonade GCP GV	0,15 -1,20 ng / g* 24 - 31 ng / g* 25 - 42 ng / g*	SF
Clinocardium nuttallii	Smith, 1982	palpes branchies GP GV	1,50 ± 0,23 nmol / mg de prot. 1,40 ± 0,38 nmol / mg de prot. 0,42 ± 0,07 nmol / PG 0,18 ± 0,03 nmol / PG	H
Crassostrea gigas	Osada et *al.*, 1987	branchies gonade	3187,37 ± 986,50 ng / g 972,99 ± 638,95 ng / g	H
		branchies gonade	313,00 ± 103,95 ng / g 306,67 ± 114,40 ng / g	H
	Osada & Nomura, 1989b	branchies gonade	1,1 - 1,9 µg / g* 0 - 150 ng / g*	H
		branchies gonade	100 - 225 µg / g* 10 - 230 ng / g*	H

Tableau 1.2 Les catécholamines chez les mollusques bivalves : dopamine (fond blanc) et noradrénaline [norépinéphrine] (fond gris) (suite).

Espèces	Références	Tissus	Dosages	T
Ensis directus	Sweeney, 1963	GCP+GV+GP	37 µg / g	SF
Macoma nasuta	Smith, 1982	palpes branchies GP GV	0,56 ± 0,09 nmol / mg de prot. 0,07 ± 0,04 nmol / mg de prot. 0,12 ± 0,01 nmol / PG 0,21 ± 0,04 nmol / PG	H
Mercenaria mercenaria	Sweeney, 1963	GCP+GV+GP	261 µg / g	SF
Modiolus demissus	Malanga et *al.*, 1972	branchies	0,417 µg / g	SF
		branchies	0,030 µg / g	SF
Modiolus modiolus	Sweeney, 1963	GCP+GV+GP	85 µg / g	SF
	Malanga et *al.*, 1972	branchies	0,307 µg / g	SF
		branchies	0,032 µg / g	SF
Mya arenaria	Sweeney, 1963	GCP+GV+GP	96 µg / g	SF
Mytilus californianus	Smith, 1982	palpes branchies GCP GP GV	0,74 ± 0,16 nmol / mg de prot. 0,06 ± 0,01 nmol / mg de prot. 0,06 ± 0,02 nmol / PG 0,60 ± 0,01 nmol / PG 0,69 ± 0,04 nmol / PG	H
	Smith, 1987	GCP GP GV	24,7± 3,4 pmol / PG 31,7 ± 2,7 pmol / PG 83,4 ± 1,1 pmol / PG	H

Tableau 1.2 Les catécholamines chez les mollusques bivalves : dopamine (fond blanc) et noradrénaline [norépinéphrine] (fond gris) (suite).

Espèces	Références	Tissus	Dosages	T
Mytilus edulis	Sweeney, 1963	GCP+GV+GP	35 µg / g	SF
	Malanga et *al.*, 1972	branchies	0,419 µg / g	SF
		branchies	0,029 µg / g	SF
	Stefano & Aiello, 1975	GCP GV	Loc + Loc +++	HF
	Stefano & Catapane, 1977	GCP+GV+GP	37,25 ± 0,60 µg / g	SF
		GCP+GV+GP	3,57 ± 0,52 µg / g	SF
		GCP+GV+GP	14,80 ± 1,20 - 36,30 ± 0,80 µg / g	SF
	Stefano & Catapane, 1980	palpes branchies GCP+GV+GP GCP GP GV	ND ND 1,50 ± 0,10 - 3,60 ± 0,10 µg / g 0,93 ± 0,10 µg / g 1,79 ± 0,14 µg / g 1,58 ± 0,16 µg / g	SF
	Smith, 1982	palpes branchies GCP GP	0,53 ± 0,150 nmol / mg de prot. 0,11 ± 0,030 nmol / mg de prot. 0,03 ± 0,003 nmol / PG 0,03 ± 0,003 nmol / PG	H

Tableau 1.2 Les catécholamines chez les mollusques bivalves : dopamine (fond blanc) et noradrénaline [norépinéphrine] (fond gris) (suite).

Espèces	Références	Tissus	Dosages	T
Mytilus edulis	Osada et *al.*, 1987	branchies GCP + GP GV	3108,58 ng / g 9648,53 ng / g 58591,84 ng / g	H
		branchies GCP + GP GV	23,64 ng / g 1635,67 ng / g 9580,55 ng / g	H
Patinopecten yessoensis	Osada et *al.*, 1987	branchies gonade	6014,74 ± 1030,68 ng / g 404,86 ± 28,49 ng / g	H
		branchies gonade	755,38 ± 124,91 ng / g 26,71 ± 4,29 ng / g	H
Pecten maximus	Paulet et *al.*, 1993	GCP GV	150-300 ng / ganglion* 30-90 ng / ganglion*	H
		GCP GV	150-550 ng / ganglion* 30-60 ng / ganglion*	H
Placopecten magellanicus	Pani & Croll, 1995	GCP+GV+GP coeur gonade pied	1400 pg / mg 850 pg / mg* 400 pg / mg* 550 pg / mg*	H
		GCP+GV+GP cœur branchies gonade	1000 pg / mg 700 pg / mg 600 pg / mg 550 pg / mg*	H

Tableau 1.2 Les catécholamines chez les mollusques bivalves : dopamine (fond blanc) et noradrénaline [norépinéphrine] (fond gris) (suite).

Espèces	Références	Tissus	Dosages	T
Sphaerium sulcatum	Sweeney, 1968	animal	5,2 ± 1,6 ng / animal	SF
		animal	3,5 ± 1,2 ng / animal	SF
Spisula solidissima	Sweeney, 1963	GCP+GV+GP	26 µg / g	SF
Tresus capax	Smith, 1982	palpes	15,20 ± 2,90 nmol / mg de prot.	H
		branchies	4,20 ± 0,27 nmol / mg de prot.	
		GCP	0,39 ± 0,08 nmol / PG	
		GP	1,06 ± 0,11 nmol / PG	
		GV	1,40 ± 0,07 nmol / PG	

Légende : PG: paire de ganglions; GCP: ganglions cérébro-pleuraux; GV: ganglions viscéraux; GP: ganglions pédieux; T: techniques; prot.: protéines; SF: spectrofluorométrie; HF: histochimie en fluorescence; H: HPLC; *: données prises graphiquement dans les articles.

Tableau 1.3 La 5-HT chez les mollusques bivalves.

Espèces	Références	Tissus	Dosages	T
Anadonta cataracta	Welsh & Moorhead, 1960	GCP+GV+GP	1,3 µg / g	SF
Anodonta cygnea	Salanki et *al.*, 1974	GCP+GV+GP	33,7 ± 3,8 - 42,2 ± 4,8 µg / g	SF
Anodonta piscinalis	Dahl et *al.*, 1966	GCP GV GP	58,35 µg / g 29,73 µg / g 62,50 µg / g	HF
Argopecten purpuratus	Martinez et *al.*, 1996	gonade GCP GV	0,5 - 2,1 ng / g* 74 - 108 ng / g* 50 - 54 ng / g*	SF
Artica islandica	Welsh & Moorhead, 1960	GCP+GV+GP	20 µg / g	SF
Clinocardium nuttallii	Smith, 1982	palpes branchies GP GV	0,26 ± 0,24 nmol / mg de prot. 0,09 ± 0,03 nmol / mg de prot. 0,22 ± 0,01 nmol / PG 0,24 ± 0,04 nmol / PG	H
Dreissena polymorpha	Ram et *al.*, 1992	gonade	Loc +++	I
Ensis directus	Welsh & Moorhead, 1960	GCP+GV+GP	21 - 39 µg / g	SF
Ligumia subrostrata	Dietz et *al.*, 1981	branchies	2,26 ± 0,18 µg / g	SF
Macoma nasuta	Smith, 1982	palpes branchies GP GV	0,34 ± 0,04 nmol / mg de prot. 0,65 ± 0,17 nmol / mg de prot. 0,20 ± 0,06 nmol / PG 0,15± 0,004 nmol / PG	H
Mya arenaria	Welsh & Moorhead, 1960	GCP+GV+GP	22 µg / g	SF

Tableau 1.3 La 5-HT chez les mollusques bivalves (suite).

Espèces	Références	Tissus	Dosages	T
Mytilus californianus	Smith, 1982	palpes branchies GCP GP GV	0,57± 0,47 nmol / mg de prot. 0,10 ± 0,01 nmol / mg de prot. 0,09 ± 0,02 nmol / PG 0,22 ± 0,05 nmol / PG 0,41 ± 0,07 nmol / PG	H
	Smith, 1987	GCP GP GV	42,1 ± 4,3 pmol / PG 5,9 ± 6,7 pmol / PG 1484,0 ± 22,4 pmol / PG	H
Mytilus edulis	Welsh & Moorhead, 1960	GCP GP GV	15 µg / g 15 µg / g 10 µg / g	SF
	York & Twarog, 1973	GP MRB	21,10 ± 2,50 µg / g 0,66 ± 0,13 µg / g	F
	Stefano & Aiello, 1975	GCP GP	Loc ++ Loc ++	HF
	Stefano et *al.*, 1976	GV	123 ± 12 - 263 ± 85 ng / PG	SF
	Stephano & Catapane, 1977	GCP+GV+GP	25,1 ± 2,71 - 57,28 ± 2,49 µg / g	SF
	Stephano & Catapane, 1980	GCP+GV+GP	26,3 ± 1,3 - 69,9 ± 0,8 µg / g	SF
	Smith, 1982	palpes branchies GP GV	0,23 ± 0,18 nmol / mg de prot. 0,11 ± 0,03 nmol / mg de prot. 0,04 ± 0,01 nmol / PG 0,06 ± 0,003 nmol / PG	H

Tableau 1.3 La 5-HT chez les mollusques bivalves (suite).

Espèces	Références	Tissus	Dosages	T
Mytilus galloprovincialis	Vitellaro-Zuccarello et *al.*,1988	GV	Loc ++	C
	Vitellaro-Zuccarello et *al.*,1991	GV GCP	Loc ++ Loc ++	I
Mytilus sp.	De Biasi et *al.*, 1984	GP	Loc ++	HF
Patinopecten yessoensis	Matsutani & Nomura, 1986b	gonade GCP GV GP	Loc ++ Loc ++ Loc ++ Loc --	I
Pecten magellanicus	Welsh & Moorhead, 1960	GP	36 µg / g	SF
Pecten maximus	Paulet et *al.*, 1993	GCP GV	150 - 550 ng / ganglion* 200 - 460 ng / ganglion*	H
Placopecten magellanicus	Croll et *al.*, 1995	pied MA gonade GCP+GV+GP GCP + GV manteau branchies cœur palpes	2509 ± 391 pg / mg 363 ± 207 pg / mg et Loc - - 791 ± 408 pg / mg et Loc ++ 1483 ± 828 pg /m g Loc ++ 280 ± 123 pg / mg et Loc ++ 202 ± 177 pg / mg et Loc ++ 183± 113 pg / mg et Loc ++ 63 ± 37 pg / mg et Loc ++	I et H
	Pani & Croll, 1995	pied GCP+GV+GP gonad	2700 pg / mg 1150 pg / mg 1000 pg / mg	H

Tableau 1.3 La 5-HT chez les mollusques bivalves (suite).

Espèces	Références	Tissus	Dosages	T
Sphaerium sulcatum	Sweeney, 1968	animal	13,4 ± 2,5 ng / animal	SF
Spisula solidissima	Welsh & Moorhead, 1960	GCP+GV+GP	8,0 - 14,3 µg / g	SF
	Masseau et *al.*, 2002	gonade	Loc +++ 200 - 900 ng / g	I H
Tapes philippinarum	Campioni et *al.*, 1997	gonade GCP GV GP	Loc +++ Loc +++ Loc + Loc +	I
Tresus capax	Smith, 1982	palpes branchies GCP GP GV	1,80 ± 0,32 nmol / mg de prot. 0,93 ± 0,14 nmol / mg de prot. 0,70 ± 0,11 nmol / PG 0,39 ± 0,06 nmol / PG 0,48 ± 0,06 nmol / PG	H
Venus mercenaria	Welsh & Moorhead, 1960	GCP+GV+GP	40 µg / g	SF

Légende : PG: paire de ganglions; GCP: ganglions cérébro-pleuraux; GV: ganglions viscéraux; GP: ganglions pédieux; MA: muscle adducteur; MRB: muscle rétracteur du byssus; T: techniques; prot.: protéines; SF: spectrofluorométrie; HF: histochimie en fluorescence; H: HPLC; I: immunohistochimie; Loc: localisation; C: immunocoloration à l'or colloïdal; *: données prises graphiquement dans les articles.

1.2.1.3 Implication des catécholamines dans le contrôle de la reproduction

Chez les mollusques bivalves, la présence de catécholamines (dopamine, noradrénaline [norépinéphrine]) a été établie dans les branchies, les palpes labiaux, le muscle adducteur, le système nerveux et la gonade de nombreuses espèces (Tableau 1.2). Chez *Mya arenaria*, parmi les catécholamines, seule la dopamine a été quantifiée. Les ganglions cérébro-pleuraux, viscéraux et pédieux contiennent, réunis, 96 µg de dopamine par gramme (g) de tissu (Sweeney, 1963). Chez *Crassostrea gigas*, *Patinopecten yessoensis* et *Mytilus edulis*, les variations annuelles des taux de dopamine (Osada et *al.*, 1987; Paulet et *al.*, 1993) et de noradrénaline [norépinéphrine] sont corrélées au cycle reproducteur (Osada et *al.*, 1987; Osada & Nomura, 1989b). Les concentrations de noradrénaline [norépinéphrine] et de dopamine augmentent durant le développement de la gonade, diminuent durant la période active de ponte (période de développement maximum des gonies) (Osada et *al.*, 1987; Osada & Nomura, 1989a). Ces résultats suggèrent que les catécholamines, et plus précisément la dopamine, sont impliquées dans la reproduction et dans la ponte (Osada et *al.*, 1987).

L'augmentation des cellules neurosécrétrices (CNS) dopaminergiques lors de la ponte induirait la libération des ovocytes chez de nombreux mollusques, par stimulation des mécanismes sérotoninergiques via les mécanismes dopaminergiques (Matsutani & Nomura, 1986a). Expérimentalement, la dopamine ne stimule la ponte qu'à fortes concentrations (Matsutani & Nomura, 1987) ce qui démontre que sa fonction dans la gamétogenèse reste à préciser.

1.2.1.4 Implication de la sérotonine (5-HT) dans le contrôle de la reproduction

La sérotonine (5-hydroxytryptamine / 5-HT) $C_{10}H_{12}N_2O$ est présente chez de nombreux invertébrés, de l'hydrozoaire à l'échinoderme en passant par les mollusques (Welsh & Moorhead, 1960; Uemura et *al.,* 1987; Fujii & Takeda, 1988; Roeder, 1999). Chez les mollusques bivalves, la présence de 5-HT a été démontrée dans les branchies, les palpes labiaux, le système nerveux et la gonade de nombreuses espèces (Tableau 1.3). Chez *Mya arenaria*, 22 µg de 5-HT/g de tissu sont contenus dans les ganglions cérébro-pleuraux, viscéraux et pédieux, homogénéisés ensemble (Welsh & Moorhead, 1960). Le rôle de la 5-HT (comme neurotransmetteur), dans de nombreuses fonctions physiologiques, est largement documenté : activité du cœur (Painter & Greenberg, 1982; Croll et *al.*, 1995), mouvement du manteau et du siphon (Ram et *al.*, 1999), activité du muscle adducteur (Croll et *al.*, 1995; Martinez et *al.*, 1996) et dans l'activité ciliaire (Gosselin, 1961; Stefano & Aiello, 1975; Stefano et *al.*, 1977; Malanga & Poll, 1979; Smith, 1982; Scheide & Dietz, 1983; Croll et *al.*, 1995). L'injection de 5-HT et de dopamine, respectivement, augmente et diminue l'activité ciliaire chez *Mytilus edulis* (Stefano et *al.,* 1977). La concentration de 5-HT dans certains tissus serait un indicateur de l'activité globale de l'animal (Fujii & Takeda, 1988). Par ailleurs, la 5-HT induit la métamorphose chez les larves du gastéropode *Ilyanassa obsoleta* (Couper & Leise, 1996), contrôle la sécrétion de la gonadotrophine chez certains vertébrés (Pinilla et *al.*, 1994) et a un effet antidépresseur pour le genre humain (Gardier et *al.*, 2001). Le rôle de la 5-HT (comme neurohormone) dans la reproduction des bivalves est mal connu. Selon Stefano &

Catapane (1977), la concentration de 5-HT dans les ganglions nerveux de la moule subit des variations saisonnières que l'on peut corréler avec le cycle sexuel : les valeurs les plus élevées sont mesurées en été, période de repos sexuel, et diminuent progressivement en automne, hiver et début du printemps tandis que la gamétogenèse s'intensifie (Lenoir & Mathieu, 1986). Les résultats obtenus chez plusieurs espèces établissent que la 5-HT entraîne l'émission des gamètes (Tableau 1.4), la parturition (Fong & Warner, 1995; Fong et *al.*, 1996, 1998), stimule la mobilité des spermatozoïdes (Kadam & Koide, 1990; Kadam et *al.*, 1991) et le développement des oocytes bloqués en prophase-I de méiose (Tableau 1.5).

L'un des rôles reconnus de la 5-HT dans la reproduction est l'induction de la ponte. *In vitro*, elle déclenche la libération des spermatozoïdes et des ovocytes chez de nombreuses espèces (Tableau 1.4), notamment chez *Crassostrea virginica, Tridacna gigas* ou encore *Patinopecten yessoensis* (Matsutani & Nomura, 1982; Gibbons & Castagna, 1984; Braley, 1985; Tanaka & Murakoshi, 1985; Hirai et *al.*, 1988). La présence de fibres nerveuses sérotoninergiques le long de l'épithélium germinal et autour des gonoductes (Matsutani & Nomura, 1984; Ram et *al.*, 1992; Paulet et *al.*, 1993; Croll et *al.*, 1995; Campioni et *al.*, 1997; Masseau et *al.*, 2002), respectivement chez *Patinopecten yessoensis, Dreissena polymorpha, Pecten maximus, Tapes philippinarum, Spisula solidissima* et *Placopecten magellanicus,* confirme que la libération de la 5-HT, par ces fibres, serait responsable de l'induction de la ponte (effets myotropes sur les tubules). Matsutani & Nomura (1984) proposent que l'innervation sérotoninergique dans la gonade de

Tableau 1.4 Induction de la ponte chez les mollusques bivalves par la 5-HT.

Espèces	Références	CT	I	RM	RF	T
Amusium pleuronectes	Belda & Del Norte, 1988	2mM	G 0,4ml	Ponte	Ponte	26-28°C
Arctica islandica	Gibbons & Castagna, 1984	2mM	M 0,4ml	Ponte	Ponte	15-16°C
Argopecten irradians		2mM	G 0,4ml	Ponte	Ponte	20-21°C
Argopecten purpuratus	Bariles & Gaete, 1991	0,02µM-20mM	G 0,4ml	Ponte	Pas de ponte	14-18°C
Chlamys asperrima	O'connor & Heasman, 1995	1µM-10mM	G 0,5ml	Ponte	Ponte	15°C
		1mM	M 0,05 ml	Ponte	Ponte	
Chlamys varia	Louro et *al.*, 2003	0,2mM	M 0,2ml	Ponte	Ponte	12-14°C
Crassostrea gigas	Osanai, 1985	1 mM	MV 0,5ml		Ponte	24-30°C
Crassostrea virginica	Gibbons & Castagna, 1984	2 mM	G 0,4ml	Ponte	Ponte	25°C
Dreissena polymorpha	Ram et *al.*, 1993	1mM	G 0,1 ml	Ponte	Ponte	20-25°C
		1mM	EA	Ponte	Ponte	
	Fong et *al.*, 1993, 1994a	0,1-1mM	EA	Ponte	Ponte	12°C
Geukensia demissa	Gibbons & Castagna, 1984	2mM	M 0,4ml	Ponte	Ponte	28°C
Hippopus hippopus	Braley, 1985	2mM	G 1,5-3ml	Ponte		27,8-30,5°C
Hippopus porcellanus	Alcazar et *al.*,1987	1mM	G 2ml	Ponte	Ponte	30°C

Tableau 1.4 Induction de la ponte chez les mollusques bivalves par la 5-HT (suite).

Espèces	Références	CT	I	RM	RF	T
Katelysia scalarina	Kent et *al.*,1998	2-15mM	M 30-100µl	Ponte	Pas de ponte	25°C
Mercenaria mercenaria	Gibbons & Castagna, 1984	2mM	M 0,4ml	Ponte	Ponte	28-29°C
	Gibbons & Castagna, 1985	0,02-20mM	M 0,4ml	Ponte	Ponte	
Patinopecten yessoensis	Matsutani & Nomura, 1982	0,02µM-2mM	G 0,4ml	Ponte	Pas de ponte	6,7-10,5°C
	Matsutani & Nomura, 1986a	0,2mM	G 0,4ml	Ponte	Ponte	
	Matsutani & Nomura, 1987	0,1µM-1mM	PG		RO	10°C
Pecten albicans	Tanaka & Murakoshi, 1985	0,025-2,5mM	G 0,5ml	Ponte	Pas de ponte	
Pecten ziczac	Vélez et *al.*,1990	0,1-6mM	G + M 0,4ml	Ponte	Pas de ponte	20°C
Placuna placenta	Madrones-Ladja, 1997	2mM	G 0,5ml	Ponte	Ponte	30 C°
Spisula sachalinensis	Hirai et *al.*, 1984a, 1984b	20µM et +	G 0,5ml	Ponte	Ponte	
	Hirai et *al.*, 1988	0,2µM-2mM	G 0,4ml	Ponte	Ponte	
Spisula solidissima	Gibbons & Castagna, 1984	2mM	G 0,4ml	Ponte	Ponte	19°C
	Hirai et *al.*, 1984a, 1984b	20µM et +	G 0,5ml	Ponte	Ponte	
	Hirai et *al.*, 1988	0,2µM-2mM	G 0,4ml	Ponte	Ponte	

Tableau 1.4 Induction de la ponte chez les mollusques bivalves par la 5-HT (suite).

Espèces	Références	CT	I	RM	RF	T
Tridacna crocea	Braley, 1985	2mM	G 0,5-2ml	Ponte	Ponte	27,8-30,5°C
Tridacna derasa	Braley, 1985	2mM	G 1,5ml	Ponte	Ponte	27,8-30,5 C°
Tridacna gigas	Braley, 1985	2mM	G 1ml et +	Ponte	Ponte	27,8-30,5°C
	Crawford et *al.*, 1986	1mM	G 1-2 ml	Ponte	Ponte	19-25°C
Tridacna maxima	Braley, 1985	2mM	G 1-5ml	Ponte	Ponte	27,8-30,5°C
Tridacna squamosa	Braley, 1985	2mM	G 0,5-1ml	Ponte		27,8-30,5°C

Légende : CT: concentrations testées; RM: résultat chez le mâle; RF: résultat chez la femelle; T: température expérimentale; G: injection intragonadique; M: injection dans le muscle adducteur; MV: injection dans la masse viscérale; RO: relarguage d'ovocytes; PG: stimulation sur des pièces de gonade; EA: application externe de la 5-HT.

Tableau 1.5 Induction de la GVBD chez les mollusques bivalves par la 5-HT.

Espèces	Références
Crassostrea gigas	Osanai, 1985
	Osanai & Kuraishi, 1988
	Kyozuka et *al.*, 1997
Dreissena polymorpha	Fong et *al.*, 1994b
Hiatella flaccida	Deguchi & Osanai, 1995
	Togo et *al.*, 1993
Mytilus edulis	Osanai & Kuraishi, 1988
Ruditapes decussatus	Hamida et *al.*, 2004
Ruditapes philippinarium	Osanai & Kuraishi, 1988
	Guerrier et *al.*, 1993
	Gobet et *al.*, 1994
Spisula sachalinensis	Hirai et *al.*, 1988
	Hirai et *al.*, 1984a, 1984b
	Varaksin et *al.*, 1992
Spisula solidissima	Hirai et *al.*, 1988
	Hirai et *al.*, 1984a, 1984b
	Toraya et *al.*, 1987
	Kadam & Koide, 1989a
	Krantic et *al.*, 1991, 1993b

Patinopecten yessoensis serait modulée par les ganglions viscéraux. À l'inverse, les résultats chez les espèces *Tapes philippinarum* (Campioni et *al.*, 1997) et *Placopecten magellanicus* (Croll et *al.*, 1995) établissent que les CNS des ganglions cérébro-pleuraux (GCP) ont une plus forte immunoréactivité envers la 5-HT que les CNS des ganglions viscéraux (GV), attestant ainsi d'une application possible des GCP dans le contrôle de la ponte. Chez *Argopecten purpuratus*, une diminution du taux de 5-HT dans les GCP durant la ponte, aucunement rencontrée dans les GV (Martinez et *al.*, 1996), renforce l'idée d'un contrôle par les GCP de la ponte chez les bivalves. Il est important de souligner, malgré l'ensemble de ses résultats, qu'aucune étude n'a établi avec certitude l'existence d'une relation entre l'une des paires de ganglions et l'innervation sérotoninergique de la gonade.

Chez les bivalves, les ovocytes expulsés lors de la ponte (avant fécondation) sont bloqués soit au stade de première prophase (Prophase-I) ou soit au stade de première métaphase (Métaphase-I). La dissolution de la vésicule germinale (GVBD) des ovocytes bloqués en prophase-I ou la formation du premier globule polaire pour ceux bloqués en métaphase-I, sont les signes de la reprise de la méiose. La GVBD permet aux ovocytes bloqués en prophase-I d'atteindre le stade de métaphase-I nécessaire à une fécondation future (Osanai, 1985). La reprise de la méiose (la GVBD) dépend d'une augmentation de la concentration intracellulaire en Ca^{2+}, résultant d'une libération de ces ions à partir des stocks intracellulaires (Abdelmajid et *al.*, 1993; Colas & Dubé, 1998). Chez de nombreux bivalves, la GVBD peut être provoquée par l'ajout de 5-HT dans le milieu (Tableau 1.5). Les ovocytes de *Mytilus edulis* y sont insensibles (Osanai & Kuraishi, 1988). Chez *Crassostrea gigas* et *Ruditapes philippinarum,* la stimulation de la GVBD est possible

indépendamment du taux de calcium présent dans le milieu (Osanai & Kuraishi, 1988; Guerrier et *al.,* 1993; Leclerc et *al.,* 2000). La 5-HT, en se fixant sur son récepteur, serait responsable indirectement du flux de Ca^{2+} intracellulaire accompagnant la GVBD (Guerrier et *al.,* 1993; Deguchi & Osanai, 1995). Chez *Spisula solidissima*, la 5-HT stimule la mobilité des spermatozoïdes immobilisés par un traitement au froid (Kadam & Koide, 1990). Ces résultats sous-entendent que les gamètes (ovocytes et spermatozoïdes) de certaines espèces possèdent, sur leurs membranes plasmiques, des récepteurs sensibles à la 5-HT (Matsutani & Nomura, 1987; Krantic et *al.,* 1993a; Gobet et *al.*, 1994).

Par une caractérisation pharmacologique (utilisation d'agonistes et d'antagonistes), la présence de récepteurs de types $5\text{-}HT_1$, $5\text{-}HT_2$, $5\text{-}HT_3$ et $5\text{-}HT_5$ sur les membranes plasmiques des ovocytes et des spermatozoïdes est confirmée chez *Crassostrea gigas, Patinopecten yessoensis, Ruditapes philippinarum* et *Spisula solidissima* (Tableau 1.6) (Kadam & Koide, 1989b, 1990; Bandivdekar et *al.*, 1989, 1991, 1992; Kadam et *al.*, 1991; Krantic et *al.*, 1991, 1993b; Gobet et *al.*, 1994; Osada et *al.*, 1998). Les résultats de la caractérisation pharmacologique n'étant pas toujours clairs, l'analyse du génome est et a été nécessaire afin de déterminer l'homologie des récepteurs sérotoninergiques de mollusques avec ceux de mammifères, et ainsi définir leur degré d'évolution. Jusqu'ici, chez les mollusques, les gènes de cinq récepteurs ont été clonés et leurs séquences d'acides aminés en ont été déduites : deux chez l'escargot *Lymnaea stagnalis* (Sugamori et *al.*, 1993; Gerhardt et *al.*, 1996) et trois chez *Aplysia californica* (Li et *al.*, 1995; Angers et *al.*, 1998). Le premier récepteur cloné et identifié le fut chez *Lymnaea stagnalis.* Ce récepteur

Tableau 1.6 Caractérisation génétique et pharmacologique des récepteurs sérotoninergiques présents dans la gonade des mollusques.

Espèces	Références	Analyses	Profils pharmacologiques : Agonistes/Antagonistes	Récepteurs
Aplysia californica	Li et *al.*, 1995	Clonage		Ap5-HT_{B1} et Ap5-HT_{B2}
	Angers et *al.*, 1998	Clonage	*Ag.* : 5-CT > PAPP > 5-HT > 8-OH-DPAT *Antag.* : Methiothepin > Methysergide > Clozapine > Metergoline > Yohimbine > Mesulergine > Ketanserin > NAN-190	5-HT_{ap1}
Crassostrea gigas	Kyozuka et *al.*, 1997	GVBD	*Ag.* : 5-HT > α-methyl-5HT >> 8-OH-DPAT = TFMPP = mCPBG *Antag.* : Propranolol = Cyproheptadine > Metoclopramide > Mianserin	Présence d'un seul type de récepteur sur la MP-Ov.
	Osada et *al.*, 1998	[3H]5HT: MP-Ov.	*Ag.* : 8-OH-DPAT > 5-HT > α-methyl-5HT *Antag.* : Metoclopramide > Retanserin > Methiothepin	5-HT_1
Dreissena polymorpha	Fong et *al.*, 1993	Ponte	*Ag.* : 5-HT > 8-OH-DPAT > TFMPP > 2-methyl-5HT > α-methyl-5HT *Antag.* : Cyproheptadine > Mianserin > NAN-190 > Propranolol = Ketanserin	5-HT_1/5-HT_2 nouveau type de récepteur non décrit

Tableau 1.6 Caractérisation génétique et pharmacologique des récepteurs sérotoninergiques présents dans la gonade des mollusques (suite).

Espèces	Références	Analyses	Profils pharmacologiques : Agonistes/Antagonistes	Récepteurs
Lymnaea stagnalis	Sugamori et *al.*, 1993	Clonage	*Ag.* : 5-CT > 8-OH-DPAT > 5-HT *Antag.* : Methiothepin > LSD > Clozapine > Ergotamine > Methysergide > Metergoline > Ketanserin	5-HT_{lym}
	Gerhardt et *al.*, 1996	Clonage	*Ag.* : 5-HT *Antag.* : Metergoline > Ritanserin > Mianserin > Methysergide > Clozapine > m-chlorophényl-pipérazine > Yohimbine > Ketanserine > Spiperone	5-HT_{2lym}
Patinopecten yessoensis	Osada et *al.*, 1998	[3H]5HT: MP-Ov.	*Ag.* : 8-OH-DPAT > 5-HT > α-methyl-5HT *Antag.* : Metoclopramide > Retanserin > Methiothepin	$5\text{-HT}_1/5\text{-HT}_2$
Ruditapes philippinarum	Gobet et *al.*, 1994	GVBD	*Ag.* : 5-HT > 8-OH-DPAT = TFMPP *Antag.* : Ritanserin > Mianserin > MDL 72222 = Methoclopramide > Spiperone	Présence d'un seul de récepteur sur la MP-Ov.
	Fong et *al.*, 1997	GVBD	*Ag.* : 5-HT > α-methyl-5HT > 8-OH-DPAT > TFMPP > 1-phenyl biguanide *Antag.* : Cyproheptadine > Mianserin = Metoclopramide > Propranolol	5-HT_2 nouveau type de récepteur non décrit

Tableau 1.6 Caractérisation génétique et pharmacologique des récepteurs sérotoninergiques présents dans la gonade des mollusques (suite).

Espèces	Références	Analyses	Profils pharmacologiques : Agonistes/Antagonistes	Récepteurs
Sphaerium transversum	Fong et *al.*, 1996	Part.	*Ag.* : α-methyl-5HT > 5-HT >>> TFMPP = 8-OH-DPAT *Antag.* : Cyproheptadine > Mianserin >>> Propranolol = 1-phenyl biguanide = 1-(1-naphthyl) piperazine = Oxymetazoline	5-HT_1/5-HT_2
Spisula solidissima	Kadam & Koide, 1989b	GVBD	*Ag.* : 5-HT > 8-OH-DPAT >>> 5-MT = 2-methyl-5-HT = 5-hydroxyindole-3-acetic acid = RU 24969 *Antag.* : Mianserin >>> Ketanserin = Metergoline	5-HT_{1A}
	Kadam & Koide, 1990	MO Sp.	*Ag.* : 5-HT > 8-OH-DPAT > 2-methyl-5-HT > 5-MT > RU 24969 = 5-HIAA *Antag.* : Ketanserin = Mianserin = Metergoline	5-HT_3
	Bandivdekar et *al.*, 1989, 1991	[3H]5HT: MP-Ov.	*Ag.* : 5-HT > 5-CT > 8-OH-DPAT > 2-methyl-5HT > α-methyl-5HT *Antag.* : ICS 205-930 > Mianserin > Methysergide > BMY 7378 > Ketanserin > Quizapine	5-HT_1/5-HT_3
	Kadam et *al.*, 1991	GVBD	*Ag.* : 8-OH-DPAT > α-methyl-5HT >>> PAPP = m-CPP-HCL = 5-CT = CGS 12066 = 1-phenyl-biguanide = 2-Methyl-5HT *Antag.* : Mianserin > Ketanserin > GR 38320F > Methysergide > Propranolol > BMY 7378 > Quizapine >>> ICS 205-930 = MDL 72222	5-HT_{1A}/ 5-HT_2

Tableau 1.6 Caractérisation génétique et pharmacologique des récepteurs sérotoninergiques présents dans la gonade des mollusques (suite).

Espèces	Références	Analyses	Profils pharmacologiques : Agonistes/Antagonistes	Récepteurs
Spisula solidissima	Kadam et *al.*, 1991	MO Sp.	*Ag.* : 8-OH-DPAT = α-methyl-5HT > 5-CT > 2-methyl-5HT >>> PAPP = m-CPP-HCL = CGS 12066 = 1-phenyl biguanide *Antag.* : Mianserin > ICS 205-930 = GR 38320F > Ketanserin >>>> Methysergide = Propranolol = BMY 7378 = Quizapine = MDL 72222	$5\text{-}HT_{1A}$/ $5\text{-}HT_2/5\text{-}HT_3$
	Bandivdekar et *al.*, 1992	[3H]5HT: MP-Sp.	*Ag.* : 2-methyl-5HT > 8-OH-DPAT > 5-HT > 5-CT > α-methyl-5HT *Antag.* : ICS 205-930 > BMY 7378 > Mianserin > Methysergide >>> 1-phenyl biguanide = Ketanserin	$5\text{-}HT_1/5\text{-}HT3$
	Krantic et *al.*, 1991	GVBD	*Ag.* : 5-HT > 8-OH-DPAT > 2-methyl-5HT = TFMPP = Methysergide *Antag.* : Ritanserin > ICS 205-930 > Ketanserin > Propranolol > Mianserin > Metoclopramide = MDL 72222 = Spiperone	Présence de récepteur sur les ovocytes
	Krantic et *al.*, 1993a	GVBD	*Ag.* : 5-HT > 8-OH-DPAT > TFMPP *Antag.* : Mianserin = Metoclopramide = Ritanserin > Propranolol > MDL 72222	Présence de récepteur sur la MP-Ov.
		[3H]5HT: MP-Ov.	*Ag.* : 8-OH-DPAT > 5-HT > TFMPP *Antag.* : Mianserin = Metoclopramide = Ritanserin = MDL 72222 > Propranolol = ICS 205-930 = Imipramine	

Tableau 1.6 Caractérisation génétique et pharmacologique des récepteurs sérotoninergiques présents dans la gonade des mollusques (suite).

Espèces	Références	Analyses	Profils pharmacologiques : Agonistes/Antagonistes	Récepteurs
Spisula solidissima	Krantic et *al.*, 1993b	[3H]5HT: MP-Ov.	*Ag.* : 8-OH-DPAT > 5-HT > TFMPP *Antag.* : Mianserin = Metoclopramide = Ritanserin = MDL 72222 > Propranolol = ICS 205930 = Imipramine	5-HT_5

Légende : Part. = parturition; MO Sp. = mobilité des spermatozoïdes; MP-Ov. = membrane plasmique des ovocytes; MP-Sp. = membrane plasmique des spermatozoïdes; GVBD = disparition des vésicules germinales; Ag. = agoniste; Antag. = antagoniste; 5-CT = 5-carboxamidotryptamine; PAPP = *p*-aminophenethyl-*m*-trifluoromethylphenyl piperazine; 5-HT = sérotonine; 8-OH-DPAT = (±)-8-hydroxy-2-(dipropylamino)tetralin; TFMPP = 1-(a,a,a-trifluoro-m-tolyl)piperazine; mCPBG =1-(*m*-chlorophenyl)biguanide; 5-HIAA = 5-hydroxyindole-3-acetic acid; 5-MT = 5-methoxytryptamine; m-CPP-HCL = m-chlorophénylpipérazine hydrochloride.

nommé 5-HT_{lym} a une forte homologie avec la famille des récepteurs de mammifère de type 5-HT_1 (53%) (Sugamori et *al.*, 1993). 5-HT_{lym} est exprimé spécifiquement dans certaines CNS et dans le cœur. En revanche, le second récepteur sérotoninergique cloné chez *Lymnaea* est structurellement et fonctionnellement semblable au récepteur de mammifère de type 5-HT_2 (46-49%) (Gerhardt et *al.*, 1996). Ce récepteur nommé 5-HT_{2lym} est exprimé principalement dans les tissus périphériques, à savoir le cœur, l'œsophage, la glande salivaire et le spermoviducte. Chez *Aplysia californica*, Li et *al.* (1995) ont cloné deux types de récepteurs appelés Ap5-HT_{B1} et Ap5-HT_{B2}. Les séquences de Ap5-HT_{B1} et Ap5-HT_{B2} sont analogues à 90 %, mais leurs zones d'expression diffèrent : Ap5-HT_{B1} est exprimé au niveau du système reproducteur et Ap5-HT_{B2} au niveau du système nerveux. Leur classification en terme de similarité avec les récepteurs de vertébrés est problématique. Les mécanismes de transduction classent Ap5-HT_{B1} et Ap5-HT_{B2} parmi les récepteurs de type 5-HT_2, tandis que leur structure moléculaire ne s'y apparente pas. Le troisième récepteur sérotoninergique cloné, nommé 5-HT_{ap1}, a une forte homologie avec la famille des récepteurs de mammifère de type 5-HT_1 (51,8% pour 5-HT_{1a} humain). 5-HT_{ap1} est exprimé dans de nombreux tissus, en particulier dans les branchies, le cœur, le spermoviducte, le rein, l'ovotestis et le CNS, démontrant ainsi l'implication de la 5-HT dans la régulation de nombreuses fonctions physiologiques (Angers et *al.*, 1998).

1.2.1.5 Synthèse de la sérotonine et récepteurs sérotoninergiques

La sérotonine est une amine biogène hydroxy-indolique synthétisée à partir du L-tryptophane (Trp), acide aminé essentiel de structure indolique (Fig. 1.4). La synthèse a lieu dans le compartiment cérébral où la demi-vie de la 5-HT est de quelques minutes), dans les neurones sérotoninergiques et également dans le compartiment extra cérébral (où la demi-vie de la 5-HT est d'une dizaine d'heures): chez les vertébrés, cette dernière a lieu essentiellement dans les cellules entérochromaffines du tractus digestif et également dans les plaquettes. La transformation du Trp en 5-HT comporte deux étapes : (i) l'hydroxylation du Trp en 5-hydroxytryptophane (5-HTP) sous l'influence de la tryptophane hydroxylase qui est l'étape limitante de la synthèse; (ii) la décarboxylation du 5-HTP en 5-HT sous l'influence de la décarboxylase des acides aminés L-aromatiques. L'abondance de 5-HTP, chez *Placopecten magellanicus,* suggère que la voie de synthèse de la 5-HT chez les bivalves est semblable à celle établie chez les

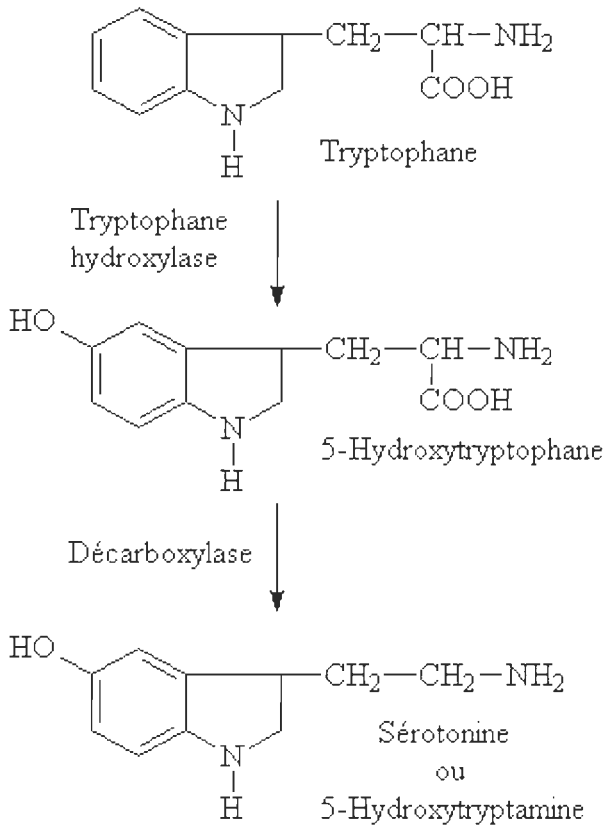

Figure 1.4: Biosynthèse de la sérotonine.

mammifères (Pani & Croll, 1998) et d'autres mollusques (Eisenstadt et *al.*, 1973; Osborne, 1973; Osborne & Neuhoff, 1974). Par la suite, la 5-HT est stockée d'une part dans les neurones sérotoninergiques, d'autre part dans les cellules entérochromaffines. Chez les mammifères, les plaquettes sanguines sont capables de capter activement et passivement la 5-HT. Au niveau de la synapse, la libération de 5-H, par les neurones sérotoninergiques activés, dans la fente synaptique conduit à modifier l'activité des neurones ou cellules cibles. C'est en se fixant à ces récepteurs spécifiques, que le neurotransmetteur transmet l'information de l'élément présynaptique à l'élément postsynaptique. Ces récepteurs sont des protéines ou des complexes protéiques qui déclencheront toute une cascade d'évènements conduisant à la stimulation (ou l'inhibition) d'un système fonctionnel cible. Les récepteurs sérotoninergiques sont classés en sept (7) familles : de $5HT_1$ à $5HT_7$. Certaines familles renferment plusieurs sous types : 5-HT_1 (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}), 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, 5-HT_7 (Bradley et *al.,* 1986; Peroutka, 1986, 1988; Hoyer & Neijt, 1988). Deux types de récepteurs sont mis en oeuvre dans le fonctionnement du système sérotoninergique: d'une part les récepteurs de type canaux ioniques (5-HT_3) qui dépolarisent rapidement la cellule en permettant le passage des cations; d'autre part, les récepteurs de type GPCR (récepteurs couplés à des protéines G) qui modulent les activités cellulaires via la production d'un second messager, parmi lesquels l'adénosine monophosphate cyclique (AMPc), le phosphate d'inositol (IP3) et le diacylglycerol. Les récepteurs de type canaux ioniques sont constitués de cinq (5) unités protéiques organisées en canal. Les cinq (5) unités (deux unités α, une unité β, une unité δ et une unité γ) traversent complètement la membrane cellulaire. Les récepteurs sérotoninergiques

proprement dits sont situés sur les deux (2) unités α de ces canaux. Les récepteurs sérotoninergiques de type GPCR sont tous construits sur le modèle de la bactériorhodopsine : protéine monocaténaire à sept (7) domaines transmembranaires réunis par des boucles intra et extracellulaires; la 3ème boucle intracellulaire joue un rôle essentiel dans le couplage avec les protéines G correspondantes. Chez les mollusques, les récepteurs clonés chez *Lymnaea stagnalis* (5-HT_{lym} et 5-HT_{2lym}) et ceux clonés chez *Aplysia californica* (Ap5-HT_{B1}, Ap5-HT_{B2} et 5-HT_{ap1}) comptent sept (7) régions hydrophobes représentant les sept (7) domaines transmembranaires des récepteurs sérotoninergiques (Sugamori et *al.*, 1993; Li et *al.*, 1995; Gerhardt et *al.*, 1996; Angers et *al.*, 1998). Chez les mollusques *Lymnaea stagnalis* et *Aplysia californica*, les récepteurs 5-HT_{lym} et 5-HT_{ap1} sont identifiés comme GPCR (Sugamori et *al.*, 1993; Angers et *al.*, 1998). Pour maintenir une certaine efficacité fonctionnelle au niveau synaptique, il existe trois (3) mécanismes d'inactivation du neurotransmetteur : la diffusion passive, la dégradation enzymatique et la recapture. La dégradation enzymatique de la 5-HT se compose de deux étapes : (i) l'oxydation de la sérotonine par une mono-amine-oxydase en 5-hydroxy-indolacétaldéhyde; (ii) puis une réduction en 5-HIAA (acide 5-hydroxy-indolylacétique) par une aldéhyde-réductase. Le mécanisme le plus efficace et le plus économique demeure sans aucun doute la recapture de la 5-HT par des transporteurs spécifiques situés sur la membrane cellulaire des terminaisons nerveuses.

1.2.2 Contrôle stéroïdien

1.2.2.1 La voie de biosynthèse des hormones stéroïdiennes

La première étape de la biosynthèse des hormones stéroïdiennes, ou stéroïdogenèse, est la conversion du cholestérol (esters de cholestérol) en prégnénolone (Andrew et *al.*, 1998) par le cytochrome $P450_{scc}$, et ce, après sa mobilisation à partir des gouttelettes lipidiques libres (Jarzebski, 1985). Chez *Mytilus edulis* et *Mya arenaria,* le cholestérol, principal précurseur des stéroïdes, est le composant le plus abondant des stérols, et donc aisément biodisponible (Jarzebski, 1985). À partir de la prégnènolone, il existe deux voies de métabolisation des androgènes et des œstrogènes. La première voie est la conversion de la prégnènolone en progestérone par la 3β-hydroxystéroïde déshydrogénase-isomérase (3β-HSD), subséquemment transformée en 17β-estradiol (E_2) en trois étapes : (i) la conversion de la progestérone en 17α-hydroxyprogestérone par la 17α-hydrolase; (ii) la conversion de la 17α-hydroxyprogestérone en estrone (E_1) par la 17, 20 lyase; (iii) pour finir, la conversion de E1 en 17β-estradiol (ou E_2) par la 17β-hydroxystéroïde déshydrogénase-isomérase (17β-HSD). La seconde voie est la transformation de la prégnènolone en androstènedione (prégnènolone → 17α-hydroxyprégnènolone par la 17α-hydrolase, 17α-hydroxyprégnènolone → androstènedione par la 17, 20 lyase), suivie de la conversion en testostérone par la 17β-HSD. Pour finir, la testostérone peut être convertie en E_2 par la cytochrome P450 aromatase. Certaine des étapes de la stéroïdogenèse sont catalysées par les cytochromes P450 stéroïdogéniques (C_{27scc}, C_{21scc}, 21-, 17α-,

11β-hydroxylase et l'aromatase). Ce sont des protéines possédant un groupement hème et un protoporphyrine comme co-facteur. La réaction catalysée par le cytochrome P450 est l'hydroxylation des substrats lipophiles en présence du nicotinamide adénine dinucléotide phosphate (NADPH) et d'une molécule d'oxygène. Les enzymes de la stéroïdogenèse (3β-HSD, 17β-HSD, HSD-isomérase, réductase, lyase, aromatase) ont déjà été observées chez les mollusques bivalves dans de nombreux tissus, dont la gonade, la glande digestive, le muscle adducteur, le manteau, les intestins et les branchies (Hathaway, 1965; Mori et *al.*, 1965a, 1965b, 1966; De Longcamp et *al.*, 1970, 1974; Varaksina & Varaksin, 1988; Matsumoto et *al.*, 1997; Morcillo et *al.*, 1999; Le Curieux-Belfond et *al.*, 2001). Osada et *al.* (2004) suggère même que les oestrogènes soient synthétisés par l'aromatase P450 dans des cellules spécifiques (cellules estrogéniques).

1.2.2.2 Implication des hormones stéroïdiennes dans le contrôle de la reproduction

Comme le montre la figure 1.5, les hormones stéroïdiennes (17β-oestradiol, testostérone, androstènedione, oestrone et progestérone) et les enzymes clés intervenant dans la stéroidogenèse, en particulier la 3β-HSD isomérase, la 17, 20 lyase, l'aromatase et la 17β-HSD, ont été identifiés chez de nombreux bivalves, comme *Crassostrea gigas* (Mori, 1980), *Mytilus edulis* (Reis-Henriques et *al.*, 1990; Reis-Henriques & Coimbra, 1990), *Patinopecten yessoensis* (Matsumoto et *al.*, 1997) et *Mya arenaria* (Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2002, 2003). Les progestagènes, les androgènes et les œstrogènes commanderaient le stockage énergétique et réguleraient la maturation sexuelle (Mori, 1969,

1980; Reis-Henriques et *al.,* 1990; Reis-Henriques & Coimbra, 1990; Matsumoto et *al.,* 1997; Gauthier-Clerc et *al.*, 2002). Des injections d'E_2 dans la gonade stimulent la glycolyse, accélèrent la maturation sexuelle (De Longcamp et *al.,* 1974) et augmentent la respiration et la consommation en oxygène (O_2). Chez *Crassostrea gigas,* l'E_2 active le développement de la gonade femelle et le diamètre des ovocytes primaires (Li et *al.*, 1998) et est considérée comme responsable de la maturation sexuelle (Mori, 1969). Chez les mâles, elle déclenche la mobilité des spermatozoïdes lors de la spermatogenèse (Mori et *al.*, 1969; Mori, 1969, 1980). L'E_2 serait aussi impliquée dans l'induction et le contrôle de la vitellogenèse chez *Crassostrea gigas* et *Patinopecten yessoensis* (Matsumoto et *al.*, 1997; Li et *al.*, 1998). Li et *al.* (1998) ont établi qu'une exposition des ovocytes à l'E_2 active la synthèse de protéines de réserve de type vitellines, par stimulation oestrogénique du récepteur spécifique (Blaise et *al.*, 1999).

Les concentrations de progestérone (Reis-Henriques et Coimbra, 1990; Siah et *al.*, 2002), de testostérone (Gauthier-Clerc et *al.,* 2006) et d'E_2 (Matsumoto et *al.,* 1997; Gauthier-Clerc et *al.,* 2006) dans la gonade de certain mollusque présenteraient des variations corrélable avec le cycle sexuel, donc l'état de maturité gonadique. Les profils des taux de progestérone dans les gonades mâles et femelles sont quasi identiques chez *Mya arenaria* et de *Mytilus edulis* (Reis-Henriques & Coimbra, 1990; Siah et *al.*, 2002), tandis que ceux de la 17β-oestradiol sont différents chez *Crassostrea gigas* et *Patinopecten yessoensis* (Matsumoto et *al.,* 1997). Les mâles ont des concentrations d'E_2 plus faibles que les femelles (Matsumoto et *al.,* 1997). Reis-Henriques & Coimbra (1990) ont retrouvé de fortes concentrations de progestérone au cours de la ponte chez les mâles et les femelles, ce

Références	Espèces
[a] Botticelli et *al.*, 1961	*Pecten hericius*
[b] Hathaway, 1965	*Crassostrea gigas*
[c] Mori et *al.*, 1965a	*Crassostrea gigas*
[d] Mori et *al.*, 1965b	*Crassostrea gigas*
[e] Mori et *al.*, 1966	*Crassostrea gigas*
[f] Idler et *al.*, 1969	*Pecten magellanicus*
[g] Saliot & Barbier, 1971	*Pecten maximus*
[h] De Longcamp et *al.*, 1970, 1974	*Mytilus edulis*
[i] Mori, 1980	*Crassostrea gigas*
[j] Varaksina & Varaksin, 1988	*Crenomytilus grayanus*
	Patinopecten yessoensis
[k] Reis-Henriques et *al.*, 1990	*Mytilus edulis*
[l] Reis-Henriques & Coimbra, 1990	*Mytilus edulis*
[m] Matsumoto et *al.*, 1997	*Crassostrea gigas*
	Patinopecten yessoensis
[n] Morcillo et *al.*, 1998a, 1998b	*Ruditapes decussata*
[o] Morcillo et *al.*, 1999	*Mytilus galloprovincialis*
[p] Morcillo & Porte, 2000	*Ruditapes decussata*
[q] Le Curieux-Belfond et *al.*, 2001	*Crassostrea gigas*
[r] Siah et *al.*, 2002, 2003	*Mya arenaria*
[s] Zhu et *al.*, 2003	*Mytilus edulis*
[t] Osada et *al.*, 2004	*Patinopecten yessoensis*
[u] Le Curieux-Belfond et *al.*, 2005	*Crassostrea gigas*
[v] Gauthier-Clerc et *al.*, 2006	*Mya arenaria*

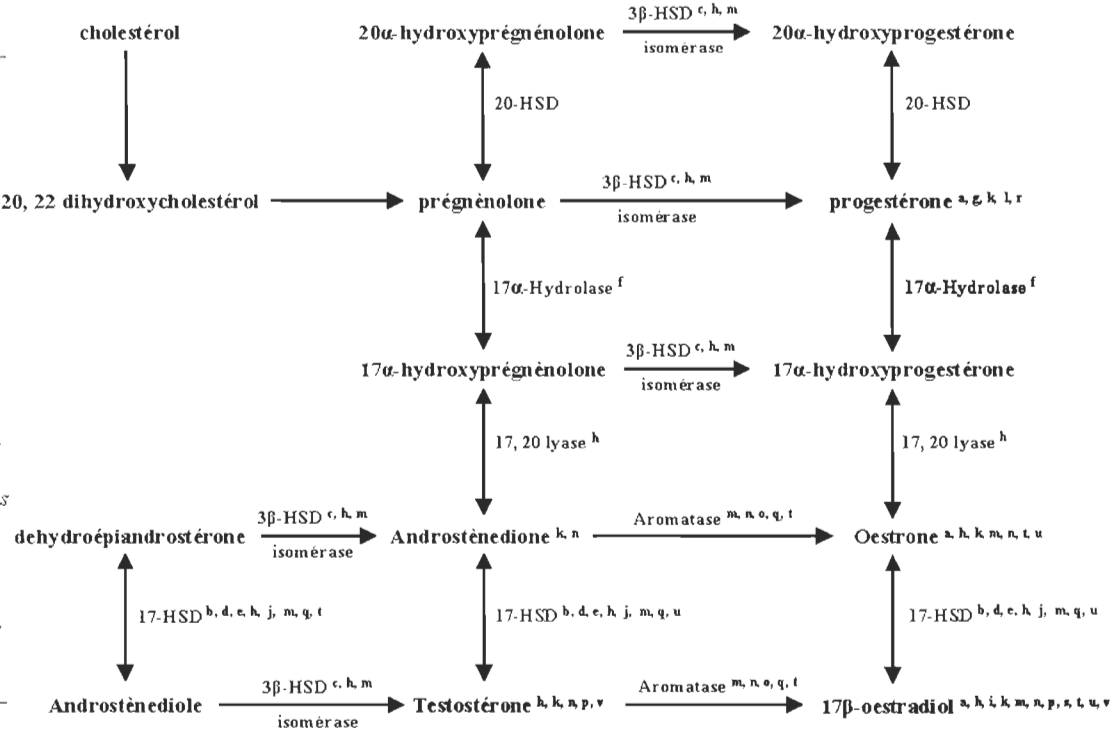

Figure 1.5: La stéroïdogenèse chez les mollusques bivalves : activités enzymatiques et stéroïdes impliquées.

qui indiquerait une implication de cette hormone dans la libération des gamètes matures. La présence dans la gonade des bivalves de plusieurs enzymes impliquées dans la stéroidogenèse (Fig. 1.5), particulièrement la 17β-HSD, renforcent l'hypothèse d'une implication des hormones stéroïdiennes dans la reproduction. Citons le cas de l'activité de la 17β-HSD qui augmente dans la gonade de *Patinopecten yessoensis, Crenomytilus grayanus* (Varaksina & Varaksin, 1988; Matsumoto et *al.,* 1997) et *Crassostrea gigas* (Mori et *al.,* 1966; Matsumoto et a*l.,* 1997) durant la gamétogenèse.

Actuellement, l'origine des hormones stéroïdiennes est un sujet de discussion parmi la communauté scientifique (Swevers et *al.,* 1991). Chez les mollusques bivalves, considérant que les enzymes de la stéroïdogenèse ont déjà été observées et que les concentrations d'hormones stéroïdiennes dans la gonade présentent des variations corrélables avec le cycle sexuel, un bon nombre d'études laisse sous-entendre une source endogène et un rôle physiologique des stéroïdes dans le contrôle de la reproduction. Les stéroïdes, produits naturellement par des vertébrés, et les composés stéroïdogéniques (phytoestrogène, phytoandrogène, etc.) peuvent être présents dans l'eau, le sédiment et la nourriture (Langston et *al.,* 2005). Le Curieux-Belfond et *al.* (2005) ont étudié, *in vivo*, la bioaccumulation et le métabolisme de la 17β-oestradiol chez l'huître *Crassostrea gigas*. Lorsque la 17β-oestradiol dissoute dans l'eau de mer est injectée dans le muscle adducteur de l'huître, elle est rapidement acheminée, puis accumulée par la gonade, les branchies, le manteau, les palpes labiaux et la glande digestive (après 48h, la concentration peut augmenter jusqu'à 31 fois) (Le Curieux-Belfond et *al.*, 2005). Chez les bivalves, le cytochrome P450 est une enzyme clé dans le métabolisme oxydatif de divers substrats,

xénobiotiques et endogènes. Le Curieux-Belfond et *al.* (2005) proposent de considérer la 17β-oestradiol comme un contaminant potentiel en eau de mer et, conséquemment, sa bioaccumulation et sa transformation en oestrone par la 17β-HSD pourraient être d'excellents biomarqueurs des perturbateurs endocriniens. Le manque d'informations sur l'origine endogène des stéroïdes n'exclut pas une fonction biologique de ces molécules, mais néanmoins, soulève des questions sur leur rôle endocrine, principalement dans le contrôle de la reproduction (Couch et *al.*, 1987; Janer et *al.*, 2004). Le problème n'est pas le manque de conversion des enzymes de la stéroïdogenèse, mais plutôt, la nécessité de démontrer que ces enzymes sont spécifiques, soit à la stéroidogenèse (biosynthèse des hormones stéroïdiennes) et/ou soit à la désintoxication (dégradation des stéroïdes exogènes accumulés par l'organisme).

1.2.3 Rôle des prostaglandines dans la reproduction

Les prostaglandines (PGs : PGF2α, PGE2 et 6-keto-F1α) et les dérivés actifs des acides gras poly-insaturés sont présents chez de nombreux mollusques (Stanley-Samuelson, 1987), dont les bivalves *Patinopecten yessoensis*, *Mytilus edulis* et *Crassostrea virginica* et *C. gigas* (Nomura & Ogata, 1976; Ogata et *al.*, 1978; Ono et *al.*, 1982; Ruggeri & Thoroughgood, 1985; Osada et *al.*, 1989). La présence de PGs a été démontrée dans le manteau, les branchies, le pied, la glande digestive et la gonade de *Mytilus edulis*, ainsi que dans les branchies et le pied de *Patinopecten yessoensis* (Nomura & Ogata, 1976). Plusieurs types de PGs ont été détectés chez les bivalves : de type F2α dans la gonade de

Crassostrea gigas (Ono et *al.*, 1982), de type F2α, PGE2 et 6-keto-F1α chez *Mytilus edulis* et *Crassostrea virginica* (Ruggeri & Thoroughgood, 1985) et de type F2α, E2, D2 et 6-keto-F1α dans le système nerveux, les branchies, la gonade et l'hémolymphe de *Patinopecten yessoensis* (Osada et *al.*, 1989). À l'exception de la 6-keto-F1α, les taux des trois autres PGs retrouvés chez *Patinopecten yessoensis* sont quatre (4) fois plus importants dans les gonades femelles durant la période de ponte (Osada et *al.,* 1989). La composition quantitative et qualitative des acides gras, donc des PGs, change considérablement au cours du cycle reproducteur dans de nombreux tissus, incluant la gonade (Pollero et *al.*, 1979; Zandee et *al.*, 1980; Piretti et *al.*, 1987, 1988). Une augmentation des taux de PGs au cours la période de reproduction a été démontrée : de type F2α dans les follicules ovariens de *Crassostrea gigas* et *Patinopecten yessoensis* (Ono et *al.*, 1982; Osada & Nomura, 1990) et de type F2α et E2 dans l'hémolymphe de *Patinopecten yessoensis* (Osada & Nomura, 1990). À l'inverse, chez l'espèce hermaphrodite *Argopecten purpuratus*, les taux de PGs F2α et E2 diminuent dans les deux parties (mâle et femelle) de la gonade lors de la gamétogenèse (Martinez et *al.*, 1999). Chez le gastéropode *Helisoma duryi*, l'injection dans la gonade de PGs de type E2 augmente le nombre et la masse des ovocytes produits (Kunigelis & Saleuddin, 1986). Ces résultats sous-entendent que les PGs F2α et E2 sont impliquées dans le contrôle de la gamétogenèse (Martinez et *al.*, 1999), en étant des indicateurs de la maturité gonadique (Ono et *al.*, 1982; Osada & Nomura, 1990).

Chez les mollusques, plusieurs études suggèrent une implication des PGs dans la libération des gamètes matures (Kikuchi & Uki, 1974; Uki & Kikuchi, 1974; Morse et *al.*, 1977; Tanaka, 1978; Matsutani & Nomura; 1986a; Madrones-Ladja, 1997). Chez

Patinopecten yessoensis, les taux de PGs F2α, E2 et D2 diminuent dans la gonade femelle et augmentent dans la gonade mâle durant le ponte (Osada et *al.,* 1989). La ponte peut être déclenchée par une irradiation UV de l'eau de mer, chez *Haliotis discus hannai*, *Haliotis rufescens* et *Nordotis gigantea* (Kikuchi & Uki, 1974), *Placuna placenta* (Madrones-Ladja, 1997), *Patinopecten yessoensis* (Uki & Kikuchi, 1974; Matsutani & Nomura, 1986a) ou par une simple addition de peroxyde d'hydrogène au milieu, chez *Nordotis gigantea* et *Haliotis rufescens* (Morse et *al.*, 1977; Tanaka, 1978). L'oxygène actif dérivé du peroxyde d'hydrogène et de l'irradiation UV agit de concert avec les PGs endopéroxyde synthétases. Une inhibition de la ponte par l'aspirine et l'indométacine, via l'inhibition de la cyclooxygénase (PGs synthétases), confirme que les PGs sont indispensables à la reproduction, mais n'ont aucun effet direct sur la ponte (Matsutani & Nomura, 1986a). Il est admis que les PGs F2α et E2 pourraient être responsables, respectivement, de l'inhibition ou de l'activation de l'action de la sérotonine (Matsutani & Nomura, 1987) et de la dopamine (Osada et *al.,* 1987) sur la ponte. Des hauts taux de PG F2α dans l'ovaire préviendraient la ponte jusqu'à entière maturation. Par la suite, une augmentation de la dopamine ou de la sérotonine induite par des stimuli extérieurs (Osada et *al.*, 1987; Matsutani & Nomura, 1987) inhiberait la production des PG F2α, et donc, supprimerait l'inhibition de la ponte produite par cette même prostaglandine. Les résultats de Matsutani & Nomura (1987) confirment cette hypothèse en démontrant que la PG F2α réduit de façon significative l'action de la 5-HT sur la ponte.

1.2.4 Régulation croisée

Étant donné que le rôle des hormones stéroïdiennes, des neurosécrétions et des prostaglandines (PGs) dans le contrôle de la reproduction est progressivement élucidé, la recherche se tourne à présent vers une compréhension globale des mécanismes régulant la gamétogenèse. Chez les bivalves, plusieurs études présentent des résultats suggérant l'existence d'une régulation complexe de la gamétogenèse par les neurosécrétions ganglionnaires, les hormones stéroïdiennes et les PGs. La sérotonine endogène serait régulée par la dopamine au niveau de CNS (Stefano et *al.*, 1976; Stefano & Catapane, 1977). Une diminution du taux de dopamine correspondrait à une augmentation du taux de sérotonine, tandis qu'une augmentation de la dopamine correspondrait à une diminution de la sérotonine (Pani & Croll, 1998). Les résultats de nombreuses études démontrent clairement l'implication de la 5-HT dans l'émission des gamètes et dans la GVBD (Tableaux 1.4 et 1.5). Une préincubation avec de la dopamine réduit significativement l'action de la 5HT sur la ponte, et suggère des rôles opposés de ces deux hormones au cours de la reproduction (Fong et *al.*, 1993). Selon Osada & Nomura (1989b), les œstrogènes réguleraient la synthèse de certaines neurosécrétions, comme les monoamines, ces dernières exerçant, à leurs tours, une action sur la stéroïdogenèse. Un prétraitement à la 17β-oestradiol (E_2) inhibe l'augmentation des catécholamines dans la gonade de *Patinopecten yessoensis* (Osada & Nomura, 1989a, 1990). L'E_2 est aussi impliquée indirectement dans la GVBD et l'émission des gamètes stimulée par une injection de 5-HT. L'E_2 augmente la synthèse de l'ARN messager des récepteurs sérotoninergiques dans les

ovocytes de *Patinopecten yessoensis* et de *Crassostrea gigas*. Une augmentation du nombre de récepteurs sérotoninergiques sur la membrane plasmique des ovocytes modifie la sensibilité de la cellule vis-à-vis de la 5-HT (Osada et *al.*, 1998). À l'inverse, selon Osada et *al.* (1992), l'émission des gamètes, induite par la sérotonine, serait inhibée par la 17β-oestradiol (E_2) via la synthèse des PGs. Un prétraitement à l'œstradiol neutralise l'augmentation des PGs F2α et E2 (Osada & Nomura, 1989a, 1990). Leur concentration dans la gonade constituerait des indicateurs de la maturité gonadique (Ono et *al.*, 1982; Osada & Nomura, 1989a). Matsunami & Nomura (1987) ont établi, in vitro, l'existence d'une régulation endogène des effets de la 5-HT par les PGs (PGs F2α et E2, respectivement, inhibiteur et activateur). L'ensemble de ces résultats confirme la complexité des interactions entre les hormones stéroïdiennes, les neurosécrétions et les prostaglandines dans la régulation de la reproduction. Les travaux effectués dans le cadre de cette thèse s'inscrivent dans cette thématique de recherche et ont pour objectif d'approfondir les connaissances sur la physiologie et les relations existant entre le système nerveux et le système reproducteur, chez *Mya arenaria*.

CHAPITRE 2 : STUDIES OF THE NERVOUS SYSTEM OF *MYA ARENARIA* (MOLLUSCA: BIVALVIA): ANATOMICAL STUDY AND IMMUNOHISTOCHEMICAL LOCALIZATION OF SEROTONIN-LIKE IMMUNOREACTIVE CELLS IN CEREBRAL, VISCERAL AND PEDAL GANGLIA.

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2.1 Abstract

The nervous system in bivalves is involved in many regulation processes like growth and reproduction. The nervous system of the soft-shell clam *Mya arenaria* follows the typical pelecypod plan. It is formed by three pairs of ganglia (cerebral, visceral and pedal), with each cerebral ganglion connected to the pedal and visceral ganglia by connective nerves. The two symmetric visceral and pedal ganglia are fused at the midline. Serotonin (5-hydroxytryptamine or 5-HT) $C_{10}H_{12}N_2O$ plays a central role in several physiological processes in marine molluscs, especially reproduction. 5-HT acts as a neurohormone to modulate spawning, parturition and meiosis by re-initiating prophase in arrested oocytes. In *Mya arenaria*, the nervous system appears to be the primary source of 5-HT for peripheral tissues like gonad. In this study, immunohistochemistry detected large numbers of serotonin-immunoreactive cells in the cortices of the cerebral and pedal ganglia. In the visceral ganglia, serotonin-immunoreactive cell bodies appeared to be wholly restricted to tightly clustered populations. These two glomeruli were localized symmetrically at the roots of the branchial nerves. Such gonadal innervation appears to originate from the ramification of the cerebrovisceral connectives. The presence in the cerebral ganglia cortex and the absence in the visceral ganglia cortex of serotonin-immunoreactive cells suggest that gonadal serotoninergic innervation could be modulated by cerebral ganglia.

2.2 Key words

Serotonin, 5-hydroxytryptamine, marine bivalves, *Mya arenaria*, nervous system, immunohistochemistry.

2.3 Introduction

The nervous system in molluscan bivalves is composed of three pairs of ganglia: cerebral, pedal and visceral. Ganglia have been described in *Aulacomya atra atra* (Zaixso, 2003), *Mytilus edulis* and *M. galloprovinciallis* (List, 1902; Makman & Stefano, 1984), in *Perna perna* (Benomar et *al.*, 2003), in *Sphaerium sulcatum* (Sweeney, 1968) and in *Patinopecten yessoensis* (Matsutani & Nomura, 1984). In bivalves, neurosecretory cells (NSCs) are located in the three ganglia and are described as four different types (*a1, a2, a3* and *a4*), and clearly identified in the ganglia of *Mytilus edulis* (Illanes-Bücher, 1979; Illanes-Bücher & Lubet, 1980) and *Perna perna* (Benomar et *al.*, 2003). A correlation between the reproductive cycle and the number of active neurosecretory cells has been proposed by several authors (Lubet, 1955, 1959; Lubet et *al.*, 1986). The number of *a1* active NSCs increases during the spawning period and rises again during gonadal tubule reorganization (Illanes-Bücher & Lubet, 1980).

Serotonin (5-hydroxytryptamine or 5-HT), belonging to the biogenic monoamines, is a neurotransmitter, a neuromodulator and a neurohormone in invertebrates (Walker, 1984; Roeder, 1999). Serotonin is present in gills of *Mytilus edulis* (Stefano & Aiello, 1975; Malanga & Poll, 1979), in gills and labial palps of *M. californianus, Tresus capax, Clinocardium nuttalli* and *Macoma nasuta* (Smith, 1982), and in the primitive nervous systems of *Mytilus edulis, M. galloprovincialis* (Stefano & Aiello, 1975; De Biasi et *al.*, 1984; Vitellaro-Zuccarello et *al.*, 1988, 1991), *Anodonta piscinalis* (Dahl et *al.*, 1966), *Patinopecten yessoensis* (Matsutani & Nomura, 1984, 1986), *Venus verrucosa*

(Siniscalchi et *al.*, 2004) and *Tapes philippinarum* (Campioni et *al.*, 1997). In molluscs, 5-HT functions as a neurotransmitter in numerous physiological functions: heart function (Painter & Greenberg, 1982; Croll et *al.*, 1995), muscle adductor activity (Croll et *al.*, 1995; Martinez et *al.*, 1996), gill and siphon movement (Ram et *al.*, 1999) and ciliary activity (Gosselin, 1961; Stefano & Aiello, 1975; Malanga & Poll, 1979; Croll et *al.*, 1995). In addition to its physiological effects, 5-HT is also known to play a major role in controlling reproduction in bivalve molluscs. Serotonin-like immunoreactivity has been observed in the gonads of *Patinopecten yessoensis* (Matsutani & Nomura, 1984, 1986), *Dreissena polymorpha* (Ram et *al.*, 1992), *Pecten maximus* (Paulet et *al.*, 1993), *Tapes philippinarum* (Campioni et *al.*, 1997), *Spisula solidissima* (Masseau et *al.*, 2002), *Venus verrucosa* (Siniscalchi et *al.*, 2004) and *Placopecten magellanicus* (Croll et *al.*, 1995). Receptor binding studies also suggest that bivalve gametes possess receptors with high affinity for 5HT (Bandivdekar et *al.*, 1992; Krantic et *al.*, 1993). Furthermore, in vitro application of 5-HT to mollusc gonad modulates meiosis reinitiation of prophase-arrested oocytes, as evidenced by germinal vesicle breakdown in *Crassostrea gigas* (Osanai, 1985; Osanai & Kuraishi, 1988; Kyozuka et *al.*, 1997), *Spisula solidissima* (Hirai et *al.*, 1988; Krantic et *al.*, 1991), *S. sachalinensis* (Hirai et *al.*, 1988; Varaksin et *al.*, 1992), and *Tapes philippinarum* (Osanai & Kuraishi, 1988; Guerrier et *al.*, 1993; Gobet et *al.*, 1994), parturition in *Sphaerium transversum* (Fong & Warner, 1995) and spawning in *Argopecten irradians* and *A. purpuratus* (Gibbons & Castagna, 1984; Bariles & Gaete, 1991), *Crassostrea gigas* (Gibbons & Castagna, 1984; Osanai, 1985), *Patinopecten yessoensis* (Matsutani & Nomura, 1982, 1986, 1987), *Pecten albicans* (Tanaka & Murakoshi, 1985),

Spisula solidissima and *S. sachalinensis* (Gibbons & Castagna, 1984; Hirai et *al.*, 1988), *Tridacna* sp., *Hippopus hippopus* (Braley, 1985), *Arctica islandica, Geukensia demissa, Mercenaria mercenaria* (Gibbons & Castagna, 1984) and *Venus verrucosa* (Siniscalchi et *al.*, 2004).

In the present study, we used the soft-shell clam *Mya arenaria* L. (Karsten, 1985), an endobenthic and sedentary pelecypod, as the animal model. This intertidal marine bivalve is found in coastal marine and estuarine regions of the Northern Hemisphere (Abbott, 1968; Potts, 1993) and is an economically and ecologically important component of the *Macoma baltica* tidal community of the St. Lawrence lower estuary ecosystem (Desrosiers & Brethes, 1984; Wallace, 1997; Department of Fisheries and Oceans Canada, 1998). In *Mya arenaria,* 5-HT has been quantified in the nervous system (Welsh & Moorhead, 1960) and serotonin-like immunoreactivity has been observed in gonads and gills (Garnerot et *al.*, 2006). Garnerot et *al.* (2006) also observed 5-HT-induced spawning movements in ripe clams and in both sexes of *Mya arenaria,* while only a few males released sperm. Thus, a better knowledge of serotonin distribution in the nervous system is essential to understand the neurophysiological mechanisms controlling gametogenesis and spawning in this bivalve. The aim of this investigation was to describe the nervous system and to ascertain the existence and features of serotonin (5-HT) immunoreactive neurons and nerve fibers in the cerebral, visceral and pedal ganglia of *Mya arenaria*.

2.4 Material and methods

2.4.1 Chemicals and reagents

Xylene, Triton X-100 (TX-100), 3,3'-diaminobenzidine tetrahydrochloride (DAB) and serotonin (5-hydroxytryptamine creatinine sulfate salt), rabbit polyclonal anti-serotonin and anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat were obtained from Sigma Chemical Co.

2.4.2 Clam collection

Clams were collected from April to July 2006 at Metis Beach (48°40' 44"; 68°02' 17") on the southern coast of the St. Lawrence Estuary (Quebec, Canada). Organisms (n = 86) were collected at low tide, brought back to the laboratory and kept at 4°C. All animals used were superior to 65 mm in shell length. Bivalves (n = 36) were dissected on the same day and pedal, visceral and cerebral ganglia were prepared for immunohistochemistry and histochemistry. A portion of the collected bivalves (n = 50) was placed in a 65-L glass aquarium (containing 1/3 sediment and 2/3 water) with continuous seawater flow before being used in the description of the nervous system.

2.4.3 Anatomical description of the nervous system

Live specimens of *Mya arenaria* were dissected under a binocular microscope for the anatomical description of the nervous system. First, cerebral (CG), visceral (VG) and pedal (PG) ganglia were quickly located. In the second stage, inter-ganglion connective tissues and commissures were found and cleared from each ganglion. Finally, to establish a precise mapping of the nervous system (localization of nerves along connective tissues), the nervous system was lightly stained with neutral red (1%) dye, a biological stain used on living cells.

2.4.4 Light microscopy

2.4.4.1 Paraffin embedding and sectioning

Cerebral, visceral and pedal ganglia were quickly removed from live specimens of *Mya arenaria* under a binocular microscope and placed in Davidson's fixative overnight. Fixed tissue was embedded in paraffin according to standard methods and cut to a thickness of 3 μm using a Zeiss microtome.

2.4.4.2 Serotonin (5-HT) immunohistochemistry

All operations were performed at room temperature unless otherwise stated. Paraffin-embedded sections were deparaffinized by incubating slices twice for 10 min each time in xylene, treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity for 30 min, and then rehydrated for 5 min in 100% (v/v) ethanol, 5 min in 95% ethanol, 5 min in 70% ethanol, 5 min in ddH_2O and, finally, twice for 5 min in Tris buffer 1 (Tris 1, 50 mM Tris, 150 mM NaCl, 0.25% w/v gelatin, 0.5% v/v Triton X 100, pH 7.4). Sections were incubated overnight in a humidity chamber at 4°C with primary monoclonal antiserum (rabbit polyclonal anti-5HT) diluted 1:500 in Tris 1. Sections were incubated for 2 hours with secondary antiserum (goat anti-rabbit antiserum) diluted 1:100 in Tris buffer 2 (Tris 2, 50mM Tris, 150 mM NaCl, pH 7.4) and then washed twice in Tris 1 for 5 min each time and twice for 5 min each as above. Staining was viewed by incubation with DAB/chromogen for at least 30 min in 0.05 M Tris-HCl buffer (pH 7.6) at room temperature. Final peroxidase reactions were performed by adding H_2O_2 to the DAB reaction solution. Slides were then rinsed twice for 5 min each with Tris 2, dehydrated and counterstained by incubation for 5 min in 70% ethanol, 5 min in 95% ethanol, immersed quickly in light green solution (0.2%) and dipped twice, for 5 min each time, in 100% ethanol.

2.4.4.3 Mount and photography

After dehydration, sections were incubated twice for 5 min in xylene, mounted with Cytoseal (VWR Scientific) and cover-slipped. All slides were observed with an Olympus BX41 light microscope. Images were captured by an Evolution VF camera using Image-Pro Plus 5.0.2 software.

2.5 Results

The nervous system of *Mya arenaria* follows the typical pelecypod plan and is formed by three pairs of ganglia, each cerebral ganglion being connected to the pedal and visceral ganglia by connective nerves (Fig. 2.1). In *Mya arenaria,* the two symmetric visceral and pedal ganglia are fused at the midline. The cerebral ganglia (average diameter 0.8 mm) lying on either side of the oesophagus are connected by the cerebral commissure. The visceral ganglion (average diameter 1 mm), located on the ventral side of the adductor muscle, is the largest of the central ganglia. The cerebrovisceral connective tissue crosses through the digestive gland and gonad and connects the cerebral and visceral ganglia. Moreover, the existence of gonadal and posterior foot retractor muscle connectives branching from the cerebrovisceral connective tissues has been demonstrated. The pedal ganglion (average diameter 0.6 mm) is located at the base of the foot. The cerebropedal connective tissue skirts along the anterior foot retractor muscle to connect the cerebral and pedal ganglia. A histological examination of the ganglia of *Mya arenaria* shows that they

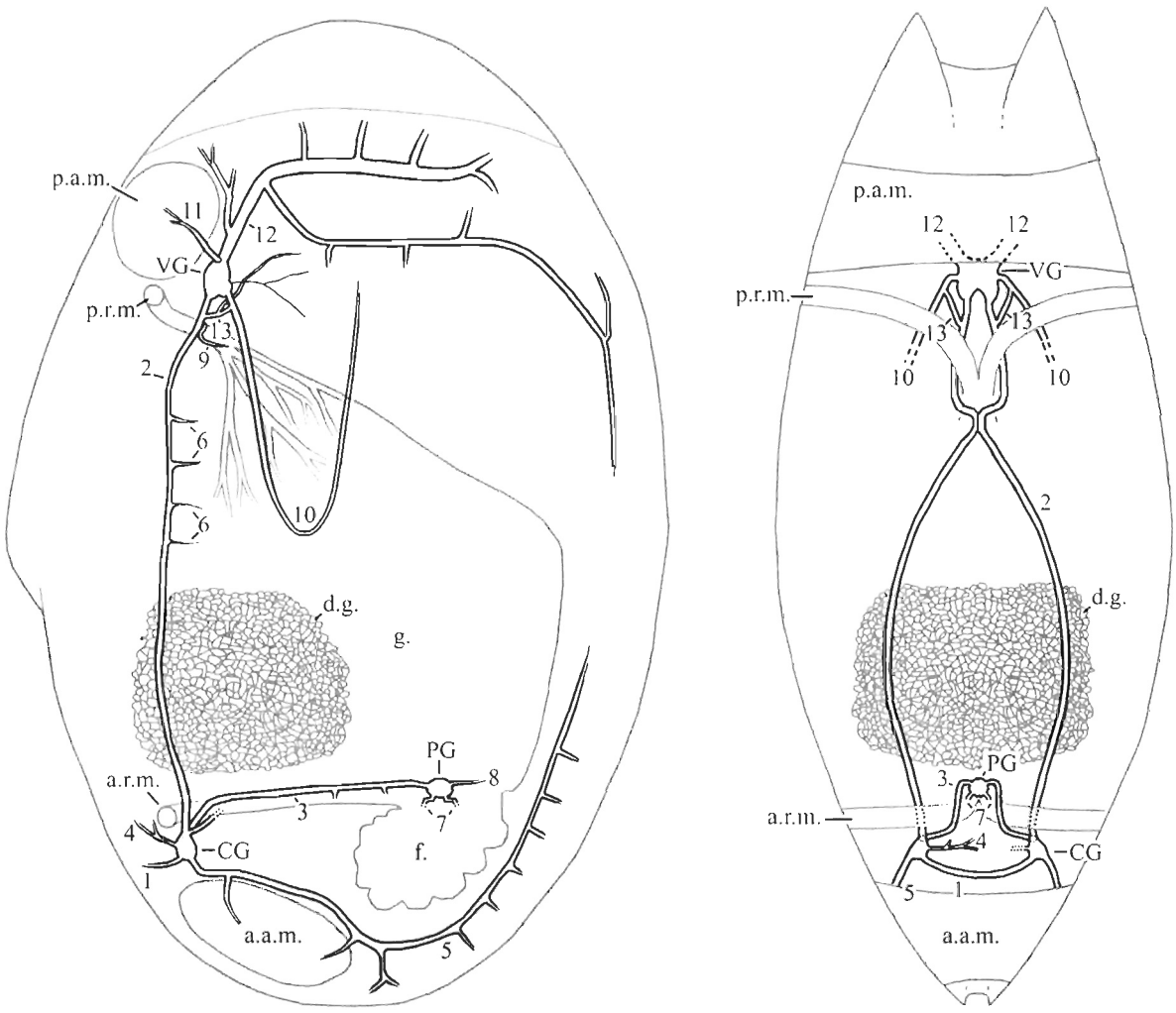

Figure 2.1 Schematic diagram of the nervous system of soft-shell clam *Mya arenaria.* Legend : (CG) cerebral ganglion, (PG) pedal ganglion, (VG) visceral ganglion, (1) cerebral commissure, (2) cerebropedal connective, (3) cerebrovisceral connective, (4) buccal nerve, (5) anterior pallial and adductor nerve, (6) gonadal nerve, (7) pedal nerve, (8) hyaline style nerve, (9) posterior foot retractor nerve, (10) branchial nerve, (11) posterior adductor nerve, (12) siphon and posterior pallial nerve, (13) cerebrobranchial nerve (f.) foot, (d.g.) digestive gland, (g.) gonad, (a.r.m.) anterior foot retractor muscle, (a.a.m.) anterior adductor muscle, (p.r.m.) posterior foot retractor muscle, (p.a.m.) posterior adductor muscle.

follow a general organization similar to that of other bivalves. These neural ganglia consist of a perineurium (a connective-tissue sheath) covering the ganglionic cortex. The cortex contains several cellular bodies, which send their processes into the central neuropil (Fig. 2.2).

The nervous system appears to be the primary source of 5-HT for the peripheral tissues of *Mya arenaria*. Immunohistochemical analyses detected large numbers of serotonin-immunoreactive cells in the cortices of the cerebral, visceral and pedal ganglia (Fig. 2.3), but these cells were not uniformly distributed in the bivalve's nervous system. More serotonin-immunoreactive cells were detected in the cerebral ganglia than in the fused pedal and visceral ganglia. In the cerebral ganglia, serotonin-immunoreactive neurons are scattered throughout the cortex (Fig. 2.3c) and arranged around the nerves' starting zones (Fig. 2.2). By contrast, serotonin-immunoreactive cells were symmetrically distributed in the pedal and visceral ganglia cortex (Figs 2.2 and 2.3a). In the visceral ganglia, serotonin-immunoreactive cell bodies appeared to be wholly restricted to tightly clustered populations, called glomeruli. These two glomeruli were located symmetrically at the roots of the branchial nerves (Figs 2.3e-f). The pedal, cerebral and visceral ganglia also contained numerous immunoreactive fibers throughout their neuropilar regions and in the emanating trunks.

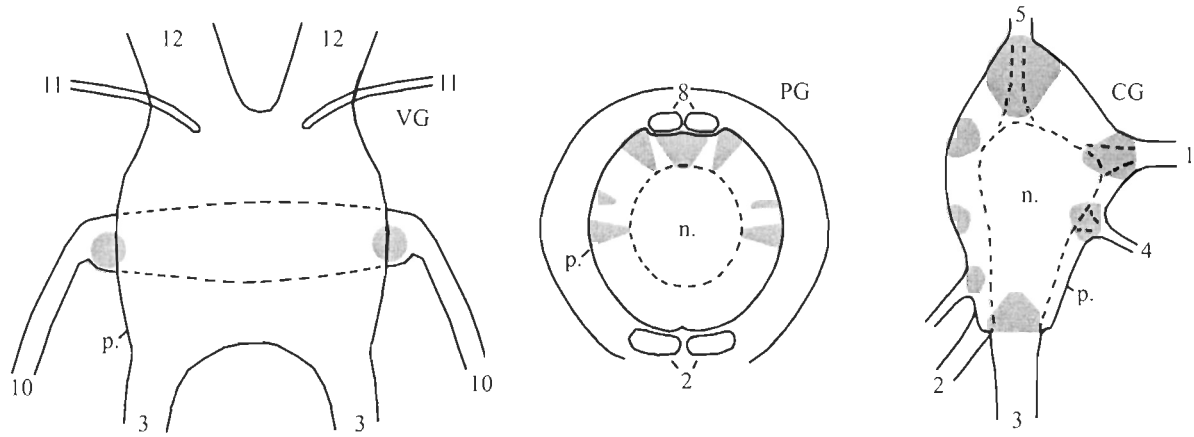

Figure 2.2 Diagram of serotonin-like immunoreactivity in the nervous system of *Mya arenaria.* The schematic map of cerebral, pedal and visceral ganglia indicates the serotonin-positive cells most consistently observed in each: (CG) cerebral ganglion, (PG) pedal ganglion, (VG) visceral ganglion, (1) cerebral commissure, (2) cerebropedal connective, (3) cerebrovisceral connective, (4) buccal nerve, (5) anterior pallial and adductor nerves, (8) hyaline style nerve, (10) branchial nerve, (11) posterior adductor nerve, (12) siphon and posterior pallial nerves, (n.) neuropil, (p.) perineurium.

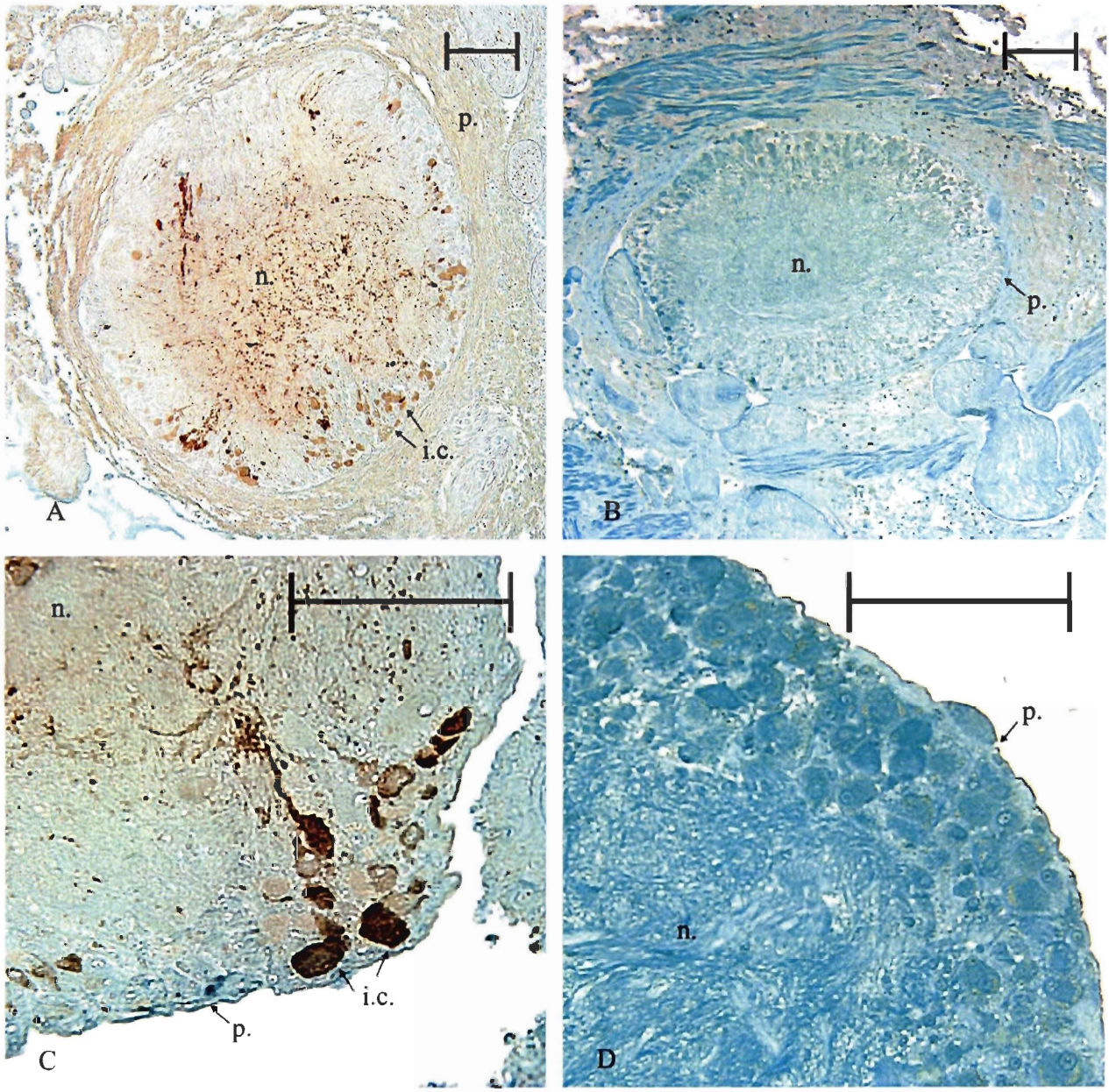

Figure 2.3 Suite →

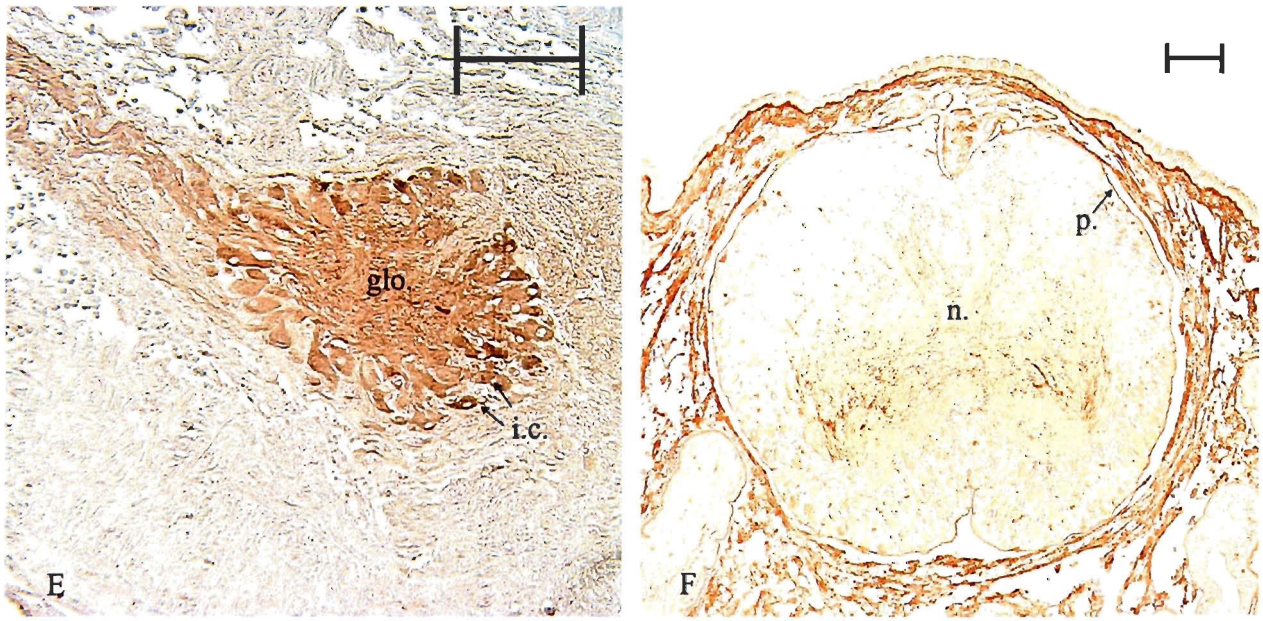

Figure 2.3 Serotonin (5-HT) immunohistochemical localization in the nervous system of *Mya arenaria*. (A) 5-HT immunohistochemistry-stained sections of pedal ganglia. (B) Negative control of immunohistochemistry-stained sections of pedal ganglia. (C) 5-HT immunohistochemistry-stained sections of cerebral ganglia. (D) Negative control of immunohistochemistry-stained sections of cerebral ganglia. (E) Immunohistochemistry-stained section of accessory ganglia in visceral ganglia. (F) Immunohistochemistry-stained section of visceral ganglia (glo. = glomerulus, p. = perineurium, n. = neuropil, i.c. = 5-HT immunoreactive cells). Scale bars = 100 μm in A-F. Paraffin sections were prepared at 3-μm thick.

2.6 Discussion

In *Mya arenaria*, the morphological and anatomical study of the nervous system and the histological examination of the ganglia demonstrate a general similarity of organization with other bivalves (List, 1902; Makman & Stefano, 1984; Matsutani & Nomura, 1984; Benomar et *al.*, 2003; Zaixso, 2003). The nervous system follows the typical pelecypod plan of three pairs of ganglia: a pair of cerebral ganglia and two symmetric visceral and pedal ganglia fused at the midline. The nervous system of this species differs from that of the Mytilidae family, with the exception of *Aulacomya atra atra* (Zaixso, 2003), by the absence of a common trunk to the cerebrovisceral and cerebropedal connectives. This variation is also observed in *Sphaerium sulcatum* (Sweeney, 1968).

In this study, using paraffin sections and immunological techniques, we demonstrated that the nervous system of *Mya arenaria* contains relatively large amounts of serotonin (5-HT), supporting the hypothesis that 5-HT plays a role as a neurotransmitter. These results are confirmed by a previous biochemical study (Welsh & Moorehead, 1960), which demonstrated very high levels of 5-HT (22 µg/g) in the nervous system of *Mya arenaria*. The nervous system contains numerous immunoreactive cell bodies, particularly in the cerebral and pedal ganglia (Fig. 2.2), and appears to be a major source of monoamines for physiological actions in this animal. The same tendency has been reported in *Anodonta piscinalis* (Dahl et *al.*, 1966), *Sphaerium sulcatum* (Sweeney, 1968), *Mytilus edulis* (Stefano & Aiello, 1975), *Patinopecten yessoensis* (Matsutani & Nomura, 1984),

Pecten maximus (Paulet et *al.*, 1993), *Placopecten magellanicus* (Croll et *al.*, 1995) and *Tapes philippinarum* (Campioni et *al.*, 1997).

In *Mya arenaria*, the visceral ganglia contain only 5-HT positive neurons in two tightly clustered populations (called glomeruli) that are very rich in immunoreactive fibers, as already reported in *Venus verrucosa* (Siniscalchi et *al.*, 2004). Specific serotonergic actions in the nervous system are difficult to determine on the basis of evidence presented in this study. The location of 5-HT immunoreactive cells in glomeruli of the visceral ganglia at the roots of the branchial nerves (Fig. 2.2), and the presence of 5-HT immunoreactive fibers in gills (Garnerot et *al.*, 2006), suggest the implication of serotonin in peripheral neurotransmission, like respiration and nutrition.

The neuropil of the three ganglia exhibited intense serotonin-immunoreactivity (Figs 2.3a-f). The neuropilar regions within the visceral ganglia of *Mya arenaria* contain large numbers of immunoreactive fibers. Similarly, 5-HT-containing fibers are widely distributed in the ganglia of *Patinopecten yessoensis* (Masutani & Nomura, 1984, 1986) and *Placopecten magellanicus* (Croll et *al.*, 1995). The presence of 5-HT immunoreactive fibers in the visceral neuropil can depend on an extensive axonal branching of visceral ganglia neurons (restricted almost entirely to the accessory ganglia) or, more probably, the visceral ganglia may receive serotonergic nerve fibers from the cerebral ganglia. This second hypothesis is supported by: (1) the presence of a large amount of immunoreactive fibers in the cerebrovisceral connective; (2) the absence of a common trunk to the cerebrovisceral and cerebropedal connectives (the cerebropedal connective is fixed directly

to the cerebral ganglia); and (3) the results of Stefano & Aiello (1975) on axonal transport, showing that 5-HT is transported from the cerebral ganglia to the visceral ganglia.

In addition to this physiological effect like neurotransmitter, 5-HT is known to play a major reproductive role in bivalve molluscs. Serotonin-like immunoreactivity has also been observed in the gonads of many species (Matsutani & Nomura, 1984, 1986; Ram et *al.*, 1992; Paulet et *al.*, 1993; Croll et *al.*, 1995; Campioni et *al.*, 1997; Masseau et *al.*, 2002; Siniscalchi et *al.*, 2004). Garnerot et *al.* ([2006] "chapter 3") found numerous 5-HT nerve fibers of various diameters around germinal tubule in the gonad of *Mya arenaria.* During spawning, a quantitative change in 5-HT level was shown in cerebropedal ganglia of *Argopecten purpuratus*, but not in its visceral ganglia (Martinez et *al.*, 1996). In contrast, Paulet et *al.* (1993) observed a small number of serotonin-immunoreactive cells in the accessory lobes of the visceral ganglia, which send nerves directly to the gonad, in *Pecten maximus*. Matsutani & Nomura (1984) also suggested that serotoninergic innervation in the gonad of *Patinopecten yessoensis* can be modulated by visceral ganglia. However, these authors observed that the gonadal nerves derived from two sources: the cerebrovisceral connectives and the visceral ganglia, which send some nerve bundles directly into the gonad. In the present study, we have determined that such gonadal innervation appears to originate from the ramification of the cerebrovisceral connectives (Fig. 2.1), as has already been reported in *Mya arenaria* (Stickney, 1963) and *Venus verrucosa* (Siniscalchi et *al.*, 2004). Stickney (1963) suggested that cerebrovisceral commissures pass directly under the terminal gonoducts and that two emanating trunks from each of these commissures follow the gonoduct from the vesicle deep into the gonad. The present anatomical description

showed that, in *Mya arenaria*, several gonadal connectives branching from the cerebrovisceral connectives go into the reproductive system (Fig. 2.1). In *Argopecten purpuratus*, a quantitative change in 5-HT level was demonstrated in cerebropedal ganglia, but not in visceral ganglia (Martinez et *al.*, 1996). In contrast, Matsutani & Nomura (1984) suggested that serotoninergic innervation in the gonad can be modulated by visceral ganglia in *Patinopecten yessoensis*. The presence in the cerebral ganglia cortex (around the cerebrovisceral connective starting zones) and the absence in the visceral ganglia cortex of serotonin-immunoreactive cells in *Mya arenaria* suggest that gonadal serotoninergic innervation can be modulated by the cerebral ganglia. However, further investigation is required to establish how many serotonergic neurons direct their axons to the gonads and to determine the location of their somata.

In conclusion, the presence of 5-HT immunoreactive nerve fibers and cells in the cortex and neuropil of ganglia, in the connectives, nerves, gonads and gills ("chapter 3" Garnerot et *al.* [2006]) of *Mya arenaria* indicates that 5-HT is involved in both peripheral and central neurotransmission. To study the relationship between reproduction and the nervous system, it is necessary to understand the precise origin of gonadal serotoninergic innervation. Further immunohistochemical studies associated with tracing neuroanatomical techniques may help in understanding the synaptic circuitry of *Mya arenaria* ganglia in peripheral tissues.

2.7 Acknowledgments

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CHAPITRE 3 : IMMUNOHISTOCHEMICAL LOCALIZATION OF SEROTONIN (5-HYDROXYTRYPTAMINE) IN THE GONAD AND DIGESTIVE GLAND OF *MYA ARENARIA* (MOLLUSCA: BIVALVIA)

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3.1 Abstract

Serotonin (5-hydroxytryptamine or 5-HT) $C_{10}H_{12}N_2O$ plays a central role in several physiological processes in marine molluscs, especially in reproduction. 5-HT acts as a neurohormone to modulate spawning, parturition and meiosis by reinitiating prophase in arrested oocytes. Preliminary experiments using 10^{-5} M 5-HT dissolved in aquarium water showed that 5-HT induced spawning movements in ripe clams and in both sexes of *Mya arenaria* while only a few males released sperm. The occurrence of serotoninergic fibers was demonstrated by PAP immunohistochemical reaction in the gonad of both sexes during gametogenesis. In an organism infected by the trematode parasite *Prosorhynchus squamatus*, we demonstrated that serotoninergic innervation completely disappeared around the gonad's tubules. Although the gonad and digestive gland are intertwined, no serotoninergic innervations were found in the digestive gland. These findings suggest, for the first time to our knowledge, that serotonin might be involved in the regulation of gametogenesis in the soft shell clam.

3.2 Key words

Serotonin, 5-hydroxytryptamine, marine bivalves, *Mya arenaria*, gametogenesis, gonad, digestive gland, immunohistochemistry, *Prosorhynchus squamatus*.

3.3 Introduction

Serotonin (5-hydroxytryptamine or 5-HT), a biogenic monoamine, is a neurotransmitter, a neuromodulator and a neurohormone in invertebrates (Walker, 1984; Roeder, 1999). Serotonin is present in gills of *Mytilus edulis* (Stefano & Aiello, 1975; Malanga & Poll, 1979), in gills and labial palps of *M. californianus, Tresus capax, Clinocardium nuttallii* and *Macoma nasuta* (Smith, 1982), in the gonad of *Patinopecten yessoensis* (Matsutani & Nomura, 1984) and in the nervous system of *Mytilus edulis, M. galloprovinvialis* (Stefano & Aiello, 1975; De Biasi et *al.*, 1984; Vitellaro-Zuccarello et *al.*, 1988, 1991), *Patinopecten yessoensis* (Matsutani & Nomura, 1984), and *Tapes philippinarum* (Campioni et *al.*, 1997).

In mollusc gonad, 5-HT acts as a neurohormone to modulate spawning, parturition (Fong & Warner, 1995), and meiosis reinitiation of prophase-arrested oocytes, as evidenced by germinal vesicle breakdown (GVBD). Intragonadal injection of 5-HT induced spawning in *Pecten albicans* (Tanaka & Murakoshi, 1985), *Patinopecten yessoensis* (Matsutani & Nomura, 1982, 1986, 1987), *Argopecten irradians* and *A. purpuratus* (Gibbons & Castagna, 1984; Bariles & Gaete, 1991), *Spisula solidissima* and *S. sachalinensis* (Gibbons & Castagna, 1984; Hirai et *al.*, 1988), *Crassostrea gigas* (Gibbons & Castagna, 1984; Osanai, 1985), *Tridacna sp.*, *Hippopus hippopus* (Braley, 1985), *Arctica islandica, Geukensia demissa* and *Mercenaria mercenaria* (Gibbons & Castagna, 1984). *In vitro* experiments conducted with isolated oocytes in *Spisula solidissima* (Hirai et *al.*, 1988; Krantic et *al.*, 1991), *Spisula sachalinensis* (Hirai et *al.*, 1988; Varaksin et *al.*, 1992),

Crassostrea gigas (Osanai, 1985; Osanai & Kuraishi, 1988; Kyozuka et *al.*, 1997), and *Tapes philippinarum* (Osanai & Kuraishi, 1988; Guerrier et *al.*, 1993; Gobet et *al.*, 1994) provided direct evidence of 5-HT induction of GVBD. In the marine shrimp *Sicyonia ingentis* fertilization releases the oocytes from the metaphase block to undergo cell division, but this process can be artificially produced by serotonin stimulation or by high concentrations of K^+ or Mg^{2+} as the ones found in sea water (Lindsay et *al.*, 1992).

In bivalves, the reproductive cycle is controlled by interactions occurring between ganglion neurosecretions and steroids, thus showing a direct relation between ganglia and gonad (Motavkine & Varaskine, 1989). In rats, changes in testicular steroidogenesis and spermatogenesis would be under the influence of brain 5-HT neurones in adult (Das et *al.*, 1985) and 5-HT was necessary for the development of normal spermatogenesis in prepubertal (Aragon et *al.*, 2005). In bivalves, monoamine concentrations can be controlled by estrogens (Osada & Nomura, 1989). Estradiol could therefore be indirectly involved in GVBD and spawning by stimulating the synthesis of 5-HT RNA messenger receptors in oocytes (Osada et *al.*, 1998). Wang & Croll (2004) observed for their part, that injections of estradiol, testosterone, progesterone, and dehydroepiandrosterone (DHEA) all accelerated gonadal differentiation and shifted sex ratios toward more males in the sea scallop. Testosterone and estradiol both showed transient increases at the onset of vitellogenesis in female clams and during the spawning stage in both sexes (Gauthier-Clerc et *al.*, 2006). These findings indicate that these hormones could have a role as endogenous modulators of gametogenesis as neurohormones to promote sexual maturation. Serotonin and dopamine are also implicated in bivalves in many physiological processes as immunocompetence

(Kream et *al.*, 1980). Recently, a link between immunosuppression and spawning was observed in *Mytilus edulis* L. (Cartier et *al.*, 2004). Immunological modulation by steroid hormones was already recognised in fish (Watanuki et *al.*, 2002). These findings thus suggested that there could be a close link between immunocompetence, gametogenesis and neuroendocrine regulation.

In the present study, we used the soft-shell clam *Mya arenaria* L. (Linne 1758), an endobenthic and sedentary pelecypod as animal model. There is therefore a need to understand the role of nervous systems and 5-HT in the reproductive cycle. Experiments were conducted to determine whether serotonin could be involved in gametogenesis control and whether it could induce spawning in *Mya arenaria.*

3.4 Material and methods

3.4.1 Chemicals and reagents

Type XIV protease, Triton X-100 (TX-100), bovine serum albumin (BSA), 3,3'-diaminobenzidine tetrahydrochloride and serotonin (5-Hydroxytryptamine creatinine sulfate salt) were obtained from Sigma Chemical Co. Paraformaldehyde was obtained from Baker. Rabbit polyclonal antibody against serotonin, goat IgG (whole molecule) antibody against rabbit antibody and rabbit peroxidase anti-peroxidase (rabbit PAP) were purchased from Sigma Chemical Co.

3.4.2 Clam collection

Clams were collected from April to October 2004 at Metis Beach (48°40' 44"; 68°02' 17") on the southern coast of the St. Lawrence Estuary (Quebec, Canada). Environmental contamination at Metis Beach is considered minimal when compared to that reported for all other sites along the St. Lawrence Lower Estuary (Lebeuf et *al.*, 1995). Organisms (n = 206) were collected at low tide, brought back to the laboratory and kept at 4°C. All animals used were superior at 65 mm in shell length. The same day, bivalves (n = 176) were dissected and tissue specimens were prepared and stored at -80°C until analysis. During the breeding season, a part of bivalves collected (n = 30) were placed in a 65-l glass aquarium (containing 1/3 of sediment and 2/3 of water) with continuous seawater flow for acclimatization. In *Mya arenaria,* the digestive gland, gonad, digestive tract and foot (DGDF) are intertwined. To maintain the integrity of the specimen, these four tissues were dissected as only one piece and named as DGDF.

3.4.3 Spawning induction

Clams (n = 40) placed for acclimatization were used for the serotonin (5-HT) spawning induction. As early as the clams collected at Metis Beach reached the ripe stage, the experiment of spawning induction was initiated. After having cut off continuous seawater flow, serotonin was dissolved in the aquarium water at a concentration of 10-5 M to determine whether serotonin could be involved in gametogenesis control and whether it

could induce spawning in *Mya arenaria*. This 5-HT concentration is similar to the concentration used to induce spawning in other species of bivalves (Matsutani & Nomura, 1987; Hirai et *al.*, 1988; Bariles & Gaete, 1991). The external application of 5-HT made it possible to stimulate clams without extracting them from the sediment. Clams were assessed for spawning every minute over a 3-hour period.

3.4.4 Tissue preparation

Tissue specimens (n = 176) were treated with 0.5% type XIV protease for 10 minutes and then washed in normal saline (0.15 M NaCl). Specimens were fixed in 4% paraformaldehyde at 4°C for 4 hours, rinsed twice for 30 minutes each time with cacodylate buffer (0.2 M cacodylic acid [Na salt] in 0.3 M NaCl, pH 7.5), washed again, twice, for 30 minutes each time with 4% Triton X-100 in phosphate buffered saline (PBS, 50mM Na2HPO4, 140 mM NaCl, pH 7.2), then submerged in 30% sucrose overnight. After incubation in sucrose, preparations were embedded in Cryomatrix (Thermo Shandon), frozen and stored until staining at -80°C. Sections (7 µm thick) were prepared at -18°C using a Shandon cryotome (Thermo Electron Corporation, Pittsburgh).

3.4.5 Histology

Sections were stained with Lee's methylene blue/basic fuchsin stain. DGDF organization and gametogenesis stages of both sexes were determined using a light

microscope (Olympus BX41). Five maturation stages were determined for the males (indifferent, development 1, development 2, spawning and spent) and six for the females (indifferent, pre-vitellogenic, vitellogenic, post-vitellogenic, spawning and spent) (Coe & Turner, 1938; Brousseau, 1976; Roseberry et *al.*, 1991; Potts, 1993; Gauthier-Clerc et *al.*, 2002). For immunohistochemistry, three samples were selected at each gametogenic stage (n = 33). One organism parasitized by a castrator trematode *Prosorhynchus squamatus* was also studied (n = 1).

3.4.6 Serotonin (5-HT) immunohistochemistry

Tissue sections were washed, rinsed and incubated at 4°C. The tissue sections were then washed in PBS (twice, 30 minutes), and rinsed in PBS containing 4% Triton X-100 (twice, 30 minutes). To eliminate endogenous peroxidase activity, the samples were treated with 3% hydrogen peroxide for 10 min. Finally the tissue sections were immersed in an antiserum diluent's (ASD consisting of 0.5% Triton X-100, 1% bovine serum, in PBS) for 1 hour. Sections were incubated for 72 hours in primary antiserum (rabbit polyclonal anti-5HT) diluted 1:10,000 in ASD. Negative controls were included using the same procedure, but omitting the primary antiserum. After rinsing in PBS-X (0.5% TX-100 in PBS) for 6 hours, the sections were exposed for 72 hours to goat anti-rabbit antiserum diluted 1:400 in ASD. After rinsing in PBS-X (0.5% TX-100 in PBS) for 6 hours, sections were incubated with rabbit PAP for 24 hours at a final dilution of 1/200. Sections were then incubated at least 2 hours in 0.05 M Tris-HCl buffer (pH 7.6) with diaminobenzidine

at room temperature. Final peroxidase reactions were performed by adding H_2O_2 to DAB reaction solution, followed by rinsing with deionised water. After this step, all slides were mounted and cover-slipped using Geltol mounting media (Thermo Electron Corporation, Pittsburgh). All slides were observed with an Olympus BX41 light microscope at 400X magnification. Images were captured by an Evolution VF camera using Image-Pro Plus 5.0.2 software.

3.5 Results

Preliminary experiments showed that serotonin (10^{-5} M) induced spawning movements in ripe clams and in both sexes of *Mya arenaria*. Spawning movements began after 128 ± 43 min of exposure for males and 132 ± 25 min for females. During this external application of 5-HT, only 6 of 18 males released sperm. Lee's methylene blue-basic fuchsine-stained section showed the histological aspect in the stage 2 male gonad (Fig. 3.1a), in the post-vitellogenic female gonad (Fig. 3.1b), in the digestive gland (Fig. 3.2a) and in the gonad parasitized by *Prosorhynchus squamatus* (Fig. 3.3a). Immunohistochemistry showed the presence of 5-HT in the gonad (stage 2 male gonad: Fig. 3.1c; post-vitellogenic female gonad: Fig. 3.1d) and the gills (Fig. 3.2b) for all gametogenic stages of *Mya arenaria* in both sexes when compared to the negative control (Figs 3.1e, 3.1f). Serotonin-immunoreactive fibers were clearly visible around germinal tubules for both sexes and all gametogenic stages. Numerous 5-HT nerve fibers of various diameters were found. 5-HT innervation was also found in the muscular fibers of gonadal

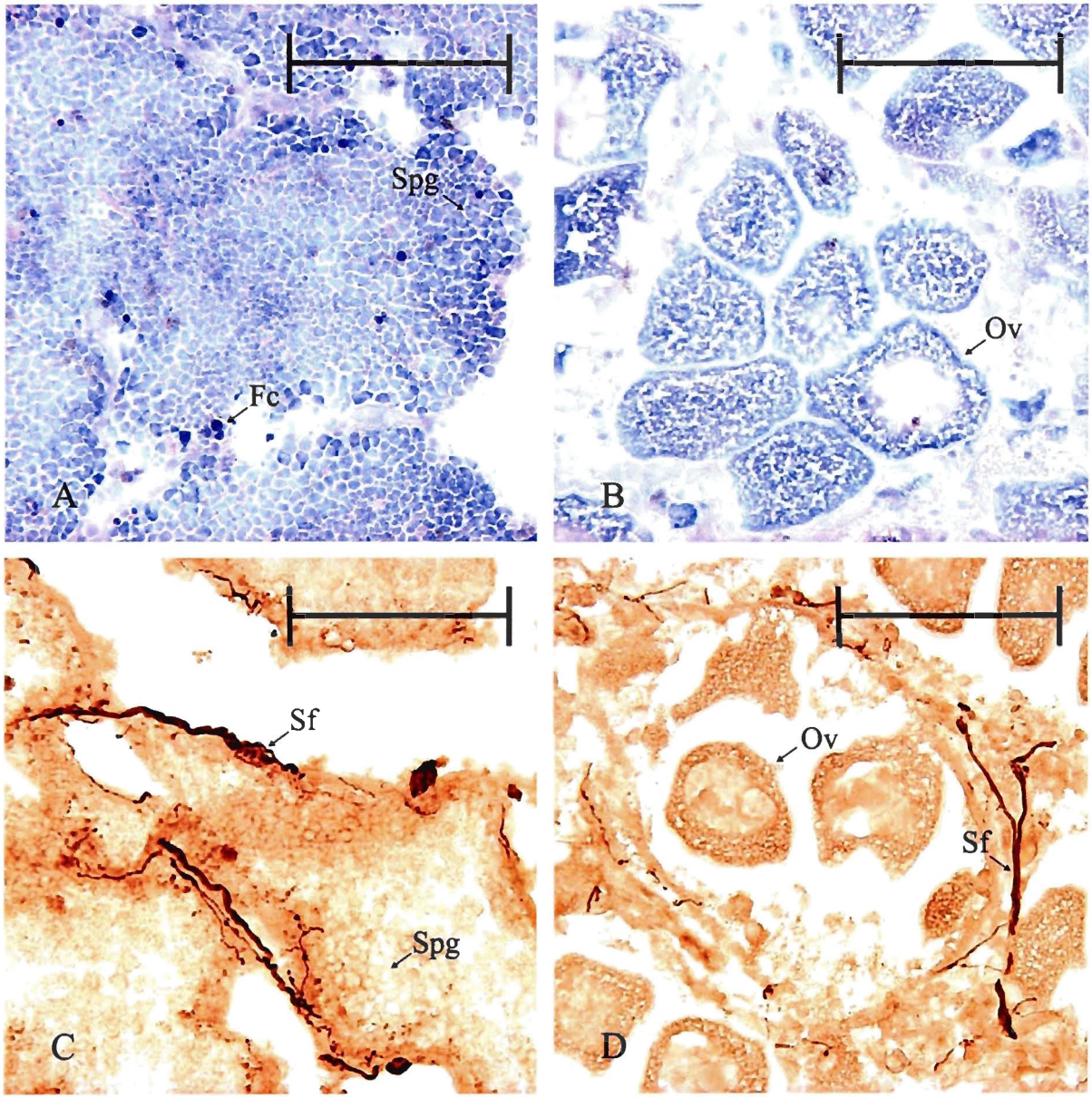

Figure 3.1 Suite →

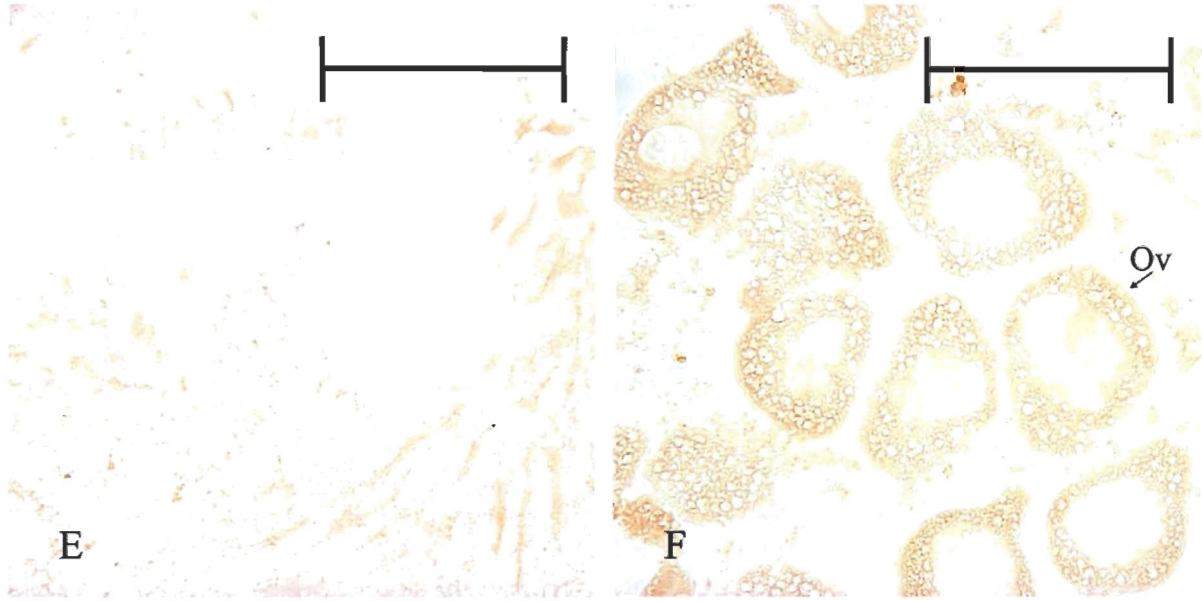

Figure 3.1: Histology and serotonin (5-HT) immunohistochemistry localization in the gonad of *Mya arenaria*. (A) Histological aspect of the male gonad, Lee's methylene blue-basic fuchsin stained section of stage 2 of development (*Spg*: spermatogonia; *Fc*: follicular cell). (B) Histological aspect of the female gonad, Lee's methylene blue-basic fuchsin-stained section of post-vitellogenic stage (*Ov*: Ovocyte). (C) 5-HT immunohistochemistry stained section of male, stage 2 of development (*Sf* : serotonin fibers). (D) 5-HT immunohistochemistry stained section of female, post-vitellogenic stage (*Ov*: Ovocyte; *Sf*: serotonin fibers). Negative controls of 5-HT immunohistochemistry stained section (E) of male gonad in stage 2 male of development and (F) post-vitellogenic female gonad stage (*Ov*: Ovocyte). Scale bars =100 μm in A-F. Cryotome sections were prepared at 7μm thick.

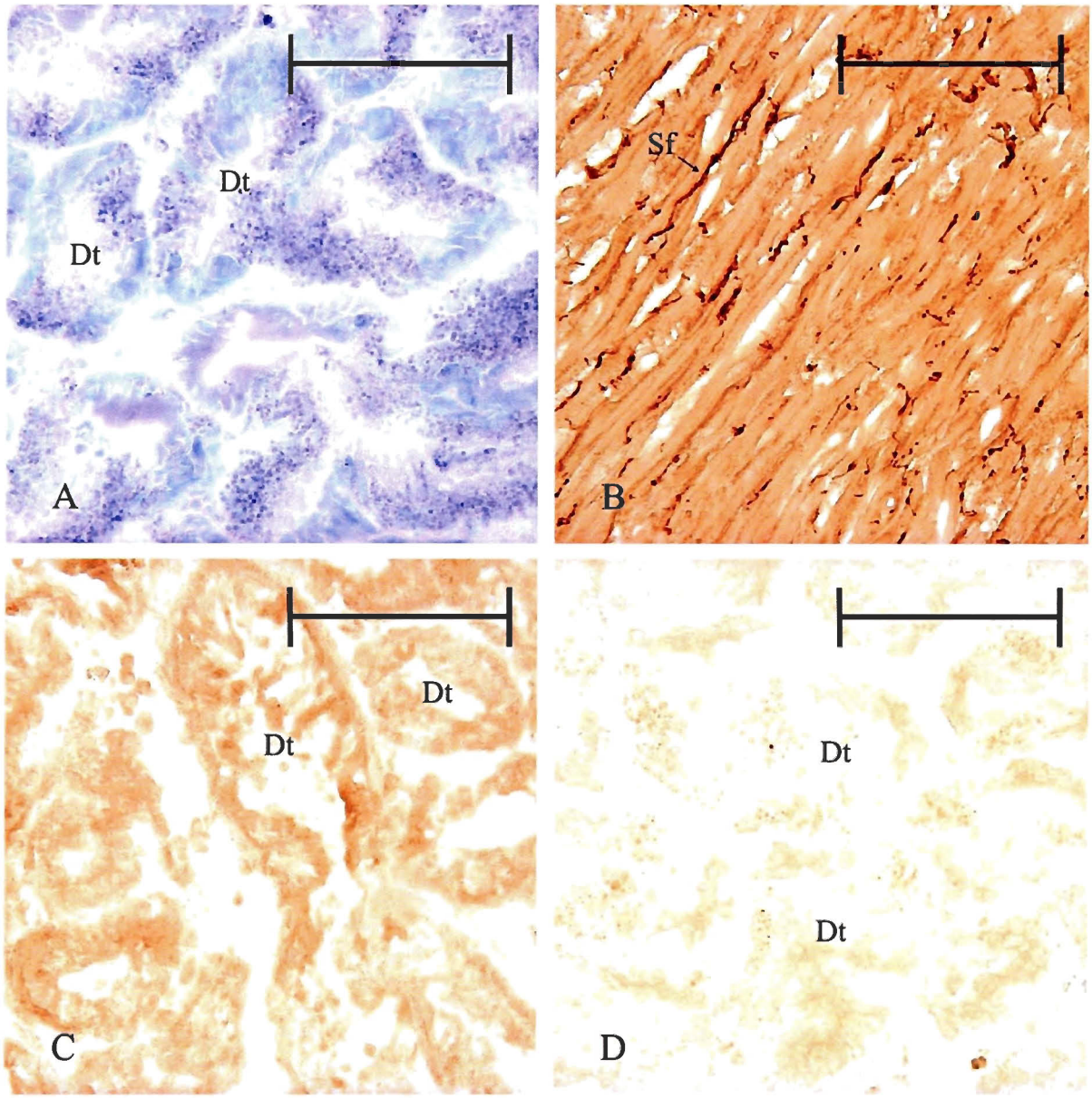

Figure 3.2: Histology and immunohistochemistry localization of 5-HT in the digestive gland and the gills. (A) Histological aspect of digestive gland, Lee's methylene blue-basic fuchsin stained section through the digestive gland (*Dt*: digestive tubule). (B) 5-HT immunohistochemistry stained section of gills, fiber innervations (*Sf* : serotoninergic fibers). (C) 5-HT immunohistochemistry stained section of digestive gland (*Dt*: digestive tubule). (D) Negative control of 5-HT immunohistochemistry stained section of digestive gland (*Dt*: digestive tubule). Scale bars = 100 µm in A-D. Cryotome sections were prepared at 7 µm thick.

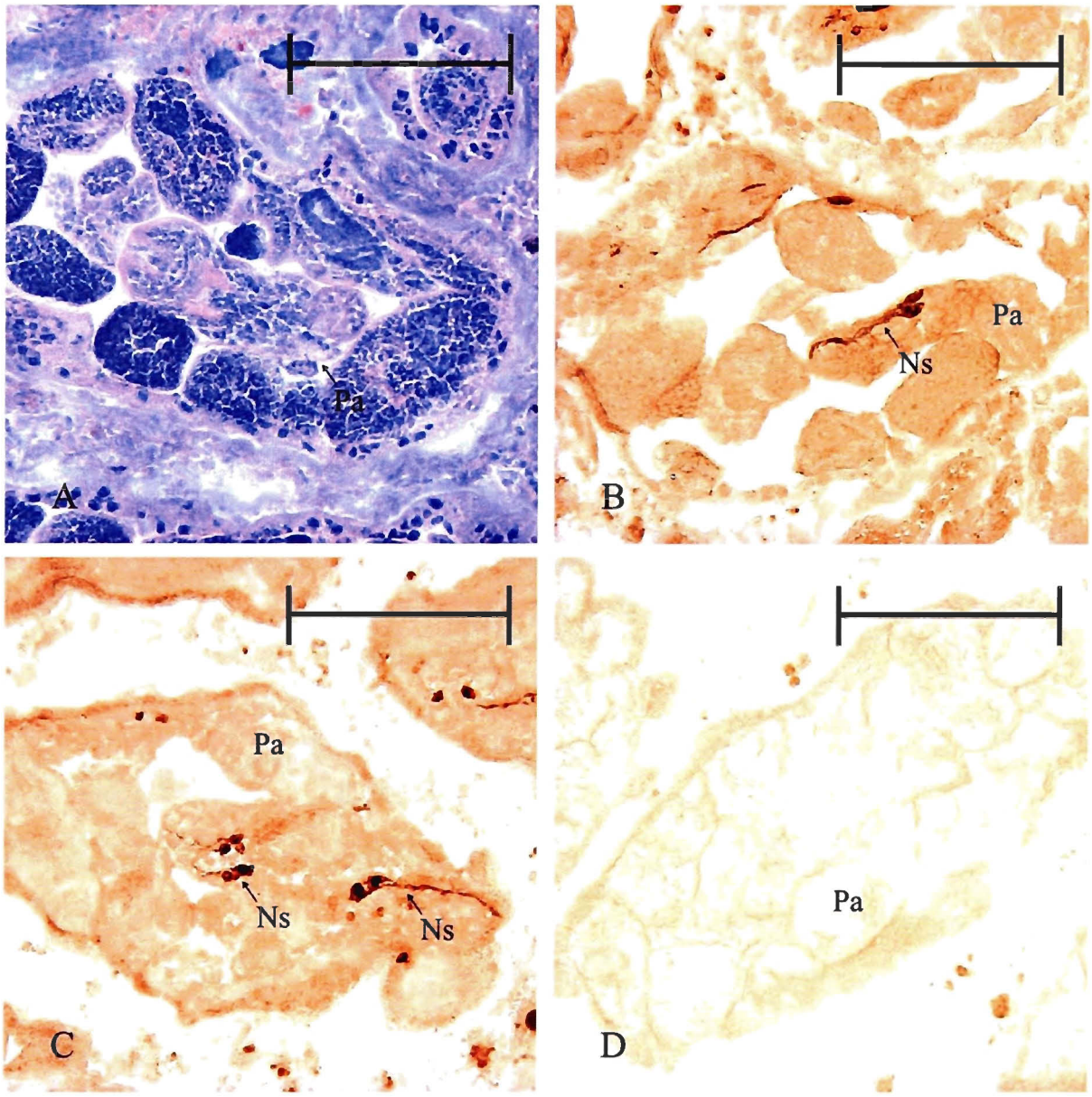

Figure 3.3: Histology and 5-HT immunohistochemistry localization in the gonad parasitized by *Prosorhynchus squamatus*. (A) Histological aspect of the female gonad parasitized with *Prosorhynchus s.*, blue-basic fuchsin-stained section (*Pa*: Parasite). (B-C) 5-HT immunohistochemistry stained section of parasitized female (*Pa*: Parasite; *Ns*: nervous system). (D) Negative control of 5-HT immunohistochemistry stained section of parasitized female (*Pa*: Parasite; *Ns*: nervous system). Scale bars =100 µm in A-F. Cryotome sections were prepared at 7µm thick.

external epithelium. In the digestive gland, no serotoninergic fibers were found around the digestive tubules (Fig. 3.2c) when compared to the negative control (Fig. 3.2d). Only a few 5-HT nerve fibers were located in the muscles around the digestive system. In the gonad parasitized by *Prosorhynchus squamatus*, serotonin-immunoreactive fibers around the tubules and host genital tubules disappeared. 5-HT staining was clearly visible inside the parasite (Figs 3.3b–c) when compared to the negative control (Fig. 3.3d).

3.6 Discussion

Recent studies have suggested that bivalve's reproduction is regulated by the neuroendocrine system, but little information is available on the relationship between ganglia and gonads (Motavkine & Varaskine, 1989). Many studies in bivalve have showed that serotonin is present and has physiological effects on muscles (York & Twarog, 1973), tonic relaxation of smooth muscles (Gies, 1986), siphon activity (Ram et *al.,* 1993) and ciliated tissues (Stefano et *al.*, 1977; Smith, 1982).

Experiments comparing injected versus externally applied 5-HT showed no significant difference in the frequency of spawning in *Dreissena polymorpha* (Ram et *al.*, 1993). In the present study, experiments with external application of 5-HT were initiated to determine if serotonin could be involved in regulating gametogenesis and can induce spawning. 5-HT dissolved in aquarium seawater stimulated spawning movements in ripe *Mya arenaria* of both sexes, but few males released sperm. These results suggested that two different mechanisms might be involved for controlling the release of sperm and oocytes in

the gonad. In the scallop *Argopecten purpuratus*, the serotoninergic route would conduct information for male spawning, while catecholamines would be involved in the release of oocytes (Martinez et *al.*, 1996). Similar observations were made in zebra mussels where treatment with serotonin uptake-inhibitors reduced spawning in males and blocked the fertilization of oocytes (Hardege et *al.*, 1997; Gagné & Blaise, 2003).

Another aim of this study was to investigate serotoninergic innervation in the gonad of *Mya arenaria.* Fixation and staining immunohistochemical PAP protocols preserved and defined immunoreactive 5-HT in the DGDF with a high degree of resolution. The only problem encountered was a partial loss of gametes for five maturation stages (male spawning and spent; female post-vitellogenic, spawning and spent). During gametogenesis, the rapid increase of germ cells numbers and, in the same time, the decrease of germ cell connections with germinal tubule, support the partial loss of less fixed gametes during tissue sections immersions. The present immunohistochemical PAP method enabled us to show the presence of serotoninergic fibers around germinal tubules and in the muscular fibers of gonad external epithelium. Serotoninergic fibers were present throughout the gametogenic stages. An earlier investigation using a histochemical fluorescence technique indicated the presence of serotonin in serotoninergic nerve, in the muscle under the epithelium around the gonad, inside the gonad and along the gonoduct of *Patinopecten yessoensis* (Matsutani & Nomura, 1984). These results are in agreement with our findings and confirmed that ganglia and gonad are connected by nerve fibers. Therefore, 5-HT released from these nerves may be responsible for the induction of spawning and GVBD in many marine bivalves (Matsutani & Nomura, 1987; Hirai et *al.*,

1988; Krantic et *al.*, 1991). Spawning induction and GVBD by serotonin could indicate that 5-HT receptors are present in the gonad and on the oocyte surface of these marine bivalves, but this remains to be demonstrated. $5HT_5$ receptors were shown on oocytes of *Spisula solidissima,* (Krantic et *al.*, 1993), mixed $5HT_1/5HT_2$ receptors in *Patinopecten yessoensis* as well as $5\text{-}HT_1$ receptors in *Crassostrea gigas* (Osada et *al.*, 1998). In the present study, numerous 5-HT nerve fibers of various diameters were found, but no relationship could be made with cerebral, pedal or visceral ganglia. In *M. arenaria*, the pedal ganglia located at the junction between the gonad and the foot and/or the cerebral ganglia located between the digestive gland and the mantle could be responsible for controlling gametogenesis. In *Argopecten purpuratus*, a quantitative change in 5-HT level was shown in cerebropedal ganglia, but not in visceral ganglia (Martinez et *al.*, 1996). In contrast, Matsutani & Nomura (1984) suggested that serotoninergic innervation in the gonad could be modulated by the visceral ganglia in *Patinopecten yessoensis*. Differences between these two results may be explained by physiological differences between these species, like the reproductive biology: *A. purpuratus* is gonochoristic like *Mya arenaria* and *P. yessoensis* is hermaphroditic. In *M. arenaria* digestive gland, no serotoninergic fibers were found around the digestive tubules. These results suggested that serotoninergic innervation does not control clam energy storage or utilization of energy reserves in the digestive gland. 5-HT would thus only control muscular activity by axons located in the muscle around the digestive system.

When the mussels *Mytilus edulis* and *M. galloprovincialis* are infected by the trematode parasite *Prosorhynchus squamatus*, the organism is invariably castrated (Coustau et *al.*, 1990) and the action of the parasite appears at a very early stage of reproduction (Matthews, 1973). In the present study and in *Mytilus edulis* (Coustau et *al.*, 1990), host genital follicles were not observed in the parasitized organisms, but were replaced by sporocysts at different stages of development. The exact molecular mechanisms involved in the castration by *P. squamatus* are yet unclear. Coustau et *al.* (1993) suggested that the parasite has an endogenous mechanism blocking gametogenesis, like the inhibition of gonad mitotic activity. In the present study, we showed that serotoninergic innervations around the tubules completely disappeared in the parasitized organism. These results suggested that *P. squamatus* could modify the gonadic structure while blocking the proliferation of nerve fibers and, consequently, the neuroendocrine stimulation of gonadal mitosis initiating gametogenesis (Mathieu et *al.*, 1988). In the present study, we also showed that the nervous system of *P. squamatus* is localized in the anterior region of the parasite, as also shown by Matthews (1973). This study is, to our knowledge, the first report on the role of serotonin in *Mya arenaria* spawning regulation and on the presence of serotoninergic innervations around gonad tubules in healthy and parasitized gonads.

In conclusion, ganglion fibers, through serotonin, could play a significant role in gametogenesis regulation in *Mya arenaria.* Further work is under way to study the role of 5-HT in gametogenesis and determine which ganglion pairs control gametogenesis as a way of understanding the action of the castrator trematode *Prosorhynchus squamatus* on nerve fibers.

3.7 Acknowledgments

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CHAPITRE 4 : ANATOMICAL STUDY OF THE VISCERAL MASS AND NEW KNOWLEDGE OF GAMETOGENESIS IN THE SOFT-SHELL CLAM (*MYA ARENARIA*): HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL CELL IDENTIFICATION

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4.1 Abstract

The soft shell clam *Mya arenaria* (Linnaeus, 1758) an endobenthic and sedentary pelecypod, is an economical and ecologically important bivalve. The present study aimed to describe the anatomical structure of the visceral mass. Histology confirmed the presence of the four systems intertwined and closely associated within the visceral mass.

(1) The digestive system occupies a large portion of the visceral mass and is composed of two pairs of labial palps, a mouth, a short eosophagus, a stomach, a hyaline style, a digestive tract, a digestive gland and an anus.

(2) Three principal components of the muscle system were identified: a wedge-shaped foot, two pairs (anterior and posterior) of foot retractor muscle and muscular fibers passing through the reproductive system. The totality of the muscular fibers, which envelop and cross over the visceral mass, maintains the integrity of the visceral mass and enable foot movements. Using staining and actin immunohistochemistry, large numbers of muscle fibers were detected into the walls of the digestive tract/stomach and blood vessel.

(3) Several components of the nervous system were identified in the visceral mass: the pedal ganglia, the cerebropedal connectives connect the cerebral and pedal ganglia, the cerebrovisceral connectives connect the cerebral and visceral ganglia. Moreover, gonadal and posterior foot retractor muscle connectives branching from the cerebro-visceral connectives have been clearly demonstrated. Using immunohistochemistry, the anti-α-tubulin stained the presence of numerous nerve fibers inside the visceral mass: in the

neuropil of the pedal ganglia, around germinal tubules of both sexes and inside gonadal muscle fibers.

(4) The gonad (the reproductive system) consisting of highly ramified tubules bearing numerous alveoli. Tubules merge into gonoducts and one fused genital vesicle. The genital aperture is located on either side of the posterior dorsal apex of the visceral mass. Alveoli consist of a basement membrane surrounding two cellular types: storage cells (Coe & Turner's "follicle cells" [1938]) and germinal cells. In males, α-tubulin-immunoreactive cells, called "supporting somatic cells", were scattered throughout the alveoli between the storage and germinal cells and are arranged in radial columns oriented toward the tubule centre. In females, using immunohistochemistry, α-tubulin-immunoreactive zones were focused in the foot of developing ova. In the present study, we have also clarified the pattern of gametogenic development and each gametogenic stage in both sexes of *Mya arenaria* : indifferent, development, ripe (post-vitellogenic for females), spawning and spent. These standardized stages will facilitate the comparison of results in future studies.

4.2 Key words

Actin, α-tubulin, marine bivalves, *Mya arenaria*, gametogenesis, gonad, digestive gland, foot, histology, immunohistochemistry.

4.3 Introduction

The soft-shell clam *Mya arenaria* (Linnaeus, 1758) an endobenthic and sedentary pelecypod, is of economic interest and an ecologically important bivalve (Wallace, 1997; Department of Fisheries and Oceans Canada, 1998). Due to poor detoxification processes and high uptake of chemicals, this species has the potential to accumulate contaminants from both the water and sediment compartments and is considered, in ecotoxicology, as a good bioindicator of environmental quality (Pellerin-Massicotte et *al.*, 1993; Blaise et *al.*, 1999; Gagné et *al.*, 2002). This intertidal marine bivalve is found in coastal marine and estuarine regions in the Northern Hemisphere (Abbott, 1968; Potts, 1993) and is a component of the *Macoma baltica* tidal community of the St. Lawrence lower estuary ecosystem (Desrosiers & Brethes, 1984).

In the Bivalvia, the digestive and reproductive systems (digestive gland, gonad, digestive tract) are intertwined and closely associated, either within the visceral mass (as in *Mya arenaria* [Vlès, 1909]), or more distinctly separated from the visceral mass, as in *Crassostrea virginica* and *Pecten maximus* (Galtsoff, 1964; Beninger & Le Pennec, 1991).

Though Vlès (1909) was the first to describe in some detail the anatomy of *Mya arenaria*; this author devoted very little time to the reproductive organs. Coe & Turner (1938) described the reproductive systems more precisely from a developmental and gametogenic viewpoint. Because *Mya arenaria* differs from many marine bivalves in this responsiveness to artificial spawning, Stickney (1963) described and compared with other

species the general morphology of the reproductive system and ciliated and non-ciliated epithelial tissues in the terminal gonoduct. He found that the morphology of the reproductive system is similar in both sexes and consists of paired alveolar gonads (consisting of highly ramified tubules bearing numerous terminal and lateral alveoli) connected by paired gonoducts to a common vesicle. From the vesicle, a pair of short terminal gonoducts leads to the genital apertures, opening into the dorsal pallial cavity (Vlès, 1909; Stickney, 1963). The histological structure of the alveolar gonads has been described by Coe & Turner (1938). Alveoli consist of a basement membrane surrounding two cellular types: storage cells (Coe & Turner's "follicle cells" [1938]) and germinal cells. The undifferentiated gonads consist of cylindrical, vacuolated and transparent masses of storage cells, with scattered germinal cells along the basement membrane of the gonoducts between the storage cells. From these few germinal cells all the future gametes will be derived. In marine bivalves, the histological and ultrastructural characteristics of gametogenesis have recently begun to be elucidated and other cellular types were characterized both inside (intra-acinal) and outside (extra-acinal) the acinus (Pipe, 1987a, 1987b; Dorange & Le Pennec, 1989; Eckelbarger & Davis, 1996; Osada et *al.*, 2004). In *Mytilus edulis,* Pipe (1987a) showed the presence in the male alveoli of Sertoli cells. It would appear that nutrients are channelled principally through the Sertoli cells directly toward the developing gametes. In *Pecten maximus,* Dorange & Le Pennec (1989) observed the ultrastructure of auxiliary cells, closely associated with developing oocytes. In *Patinopecten yessoensis,* Osada et *al.* (2004) suggested that estrogen may be synthesized

in the estrogenic cells, distributed along the inside of the acinar wall of the testis and along the outside of the acinar wall in the ovary.

Studies on sexual maturation of bivalves have shown that the cycle and frequency of reproductive activity was influenced by geographical distribution, and thus by environmental factors like food availability, ambient temperature (Bayne, 1974; Lubet, 1976; Ruiz et *al.*, 1992; Mathieu, 1994), diseases like gonadal neoplasm (Barber, 1996; Van Beneden et *al.*, 1998) and pollutants (Morcillo & Porte, 2000; Gauthier-Clerc et *al.*, 2002). Coe & Turner (1938) and Rogers (1959) described, respectively, changes that occur in gonadal development, but little was known about the complete yearly cycle. Shaw (1962) was the first to describe the seasonal cycle of gonadal development in female *Mya arenaria.* In the St. Lawrence lower estuary, data on sexual maturation in *Mya arenaria* showed a bimodal reproductive pattern: the first spawning period normally occurs at the beginning of the summer (between May and June) with a second gametogenesis in autumn (Roseberry et *al.*, 1991; Tremblay, 1992; Gauthier-Clerc et *al.*, 2002). Similar observations have been reported on the North Atlantic coast from Chesapeake Bay to the Gulf of Maine (Battle, 1932; Shaw, 1962; Brousseau, 1978). This bimodal reproductive pattern is closely linked to the availability of nutrients and may depend on the location of the population (Potts, 1993) and on environmental events (Gauthier-Clerc et *al.*, 2002).

From an economic and ecotoxicological point of view, there is a need to better understand bivalve reproductive systems and their relationship to the visceral mass and

other tissues or organs that are intertwined or closely associated. A detailed study of the morphology and histology of the reproductive system in the soft-shell clam *Mya arenaria* is required. The present study sought to describe: (1) the anatomy of the visceral mass, (2) the cellular composition of the reproductive system, and (3) the cellular changes that occur in the gonad during gametogenesis.

4.4 Material and methods

4.4.1 Chemicals and reagents

Xylene, Triton X-100 (TX-100), 3,3'-diaminobenzidine tetrahydrochloride (DAB), rabbit polyclonal anti-actin, mousse monoclonal anti-tubulin, anti-mouse IgG (whole molecule) peroxidase antibody produced in goat and anti-rabbit IgG (whole molecule) peroxidase antibody produced in goat were obtained from Sigma Chemical Co.

4.4.2 Clam collection

Clams were collected from April to July 2006 at Metis Beach (48°40' 44"; 68°02' 17") on the southern coast of the St. Lawrence Estuary (Quebec, Canada). Organisms (n = 86) were collected at low tide, brought back to the laboratory and kept at 4°C. All animals used were superior to 65 mm in shell length. Bivalves (n = 36) were dissected the same day and tissue specimens were prepared for immunohistochemical and

histochemical analyses. In *Mya arenaria,* the digestive gland, gonad, digestive tract and foot (DGDF) are intertwined. To maintain the integrity of the specimen, these four tissues were dissected as a single unit and labelled DGDF.

4.4.3 Anatomical description of the visceral mass

Live specimens of *Mya arenaria* were dissected for the anatomical description of the visceral mass under a binocular microscope. The digestive system, muscle, nervous system and reproductive system were first quickly located. Then, to establish a precise description of the various organs, the visceral mass was lightly stained with neutral red (1%) dye, a biological stain used on living cells.

4.4.4 Light microscopy

4.4.4.1 Paraffin embedding and sectioning

DGDF were rapidly removed from live specimens of *Mya arenaria* and placed in Davidson's fixative overnight. Fixed tissue was embedded in paraffin according to standard methods and cut to a thickness of 3 μm using a Zeiss microtome.

4.4.4.2 Histological staining

Paraffin-embedded sections were deparaffinized according to standard methods. Sections were stained with Prenant-Gabe's trichrome (Gabe, 1968).

4.4.4.3 Immunohistochemistry actin and α-tubulin

All operations were performed at room temperature unless otherwise stated. Paraffin-embedded sections were deparaffinized by incubating twice for 10 min each time in xylene. To eliminate endogenous peroxidase activity, the samples were treated with 3% hydrogen peroxide in methanol for 30 min. After rinsing in 100% (v/v) ethanol for 5 min, the sections were rehydrated with decreasing ethanol concentrations (95%, 70% for 5 min each) and placed in distilled water for 5 min. Finally the tissue sections were immersed twice for 5 min each time in Tris buffer 1 (Tris 1, 50 mM Tris, 150 mM NaCl, 0.25% w/v gelatin, 0.5% v/v TX100, pH 7.4). Sections were incubated overnight in a humidity chamber at 4°C with primary monoclonal antiserum (rabbit polyclonal anti-actin and mouse monoclonal anti-tubulin) diluted 1:500 in Tris 1. Sections were incubated for 2 hours with secondary antiserum (goat anti-rabbit anti-serum or goat anti-mouse anti-serum) diluted 1:100 in Tris buffer 2 (Tris 2, 50mM Tris, 150 mM NaCl, pH 7.4) and then washed in Tris 1 twice for 5 min each time and twice for 5 min as above. Staining was viewed by incubation with 3,3-diaminobenzidine/chromogen at least 30 min in 0.05 M Tris-HCl buffer (pH 7.6) at room temperature. Final peroxidase reactions were performed by adding H_2O_2 to DAB

reaction solution. Slides were then rinsed well twice for 5 min with Tris 2, dehydrated and counterstained by incubation for 5 min in 70% ethanol, 5 min in 95% ethanol, quickly immersed in light green solution (0.2%), and twice in 100% ethanol for 5 min each time.

4.4.4.4 Mounting of sections and photography

After dehydration, sections were incubated twice for 5 min in xylene, mounted with Cytoseal (VWR Scientific) and cover-slipped. All slides were observed with an Olympus BX41 light microscope at 400X magnification. Images were captured by an Evolution VF camera using Image-Pro Plus 5.0.2 software.

4.5 Results

4.5.1 Anatomical description of the visceral mass

The visceral mass of *Mya arenaria* is similar in both sexes and is composed of digestive system, muscular system, nervous system and reproductive system (Fig. 4.1).

The digestive system occupies a large portion of the visceral mass and is composed of two pairs of labial palps, a mouth, a short eosophagus, a stomach, a crystalline caecum/hyaline style, a digestive tract, a digestive gland and an anus (Fig. 4.1). After the mouth, an opening is found between two pairs of long and triangular labial palps then the short oesophagus leads into the large stomach. Oesophagus and stomach are surrounded by

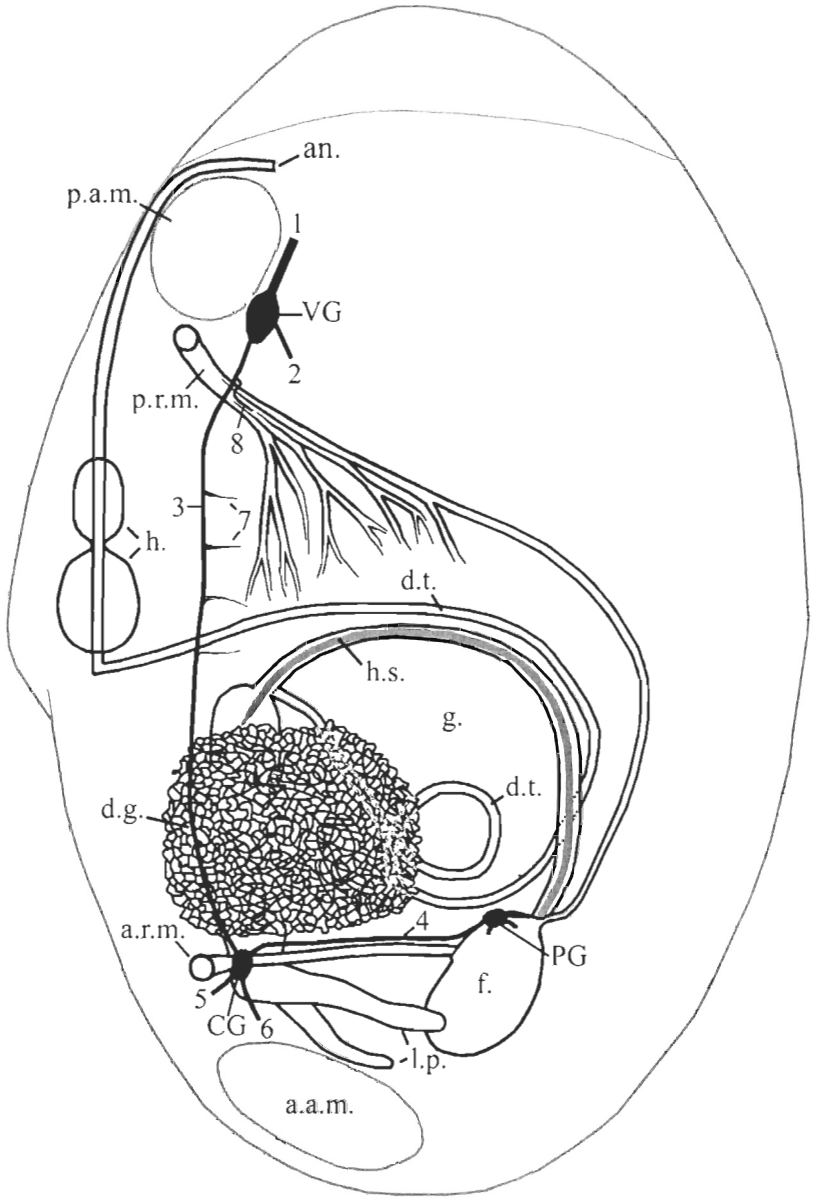


Figure 4.1: Schematic diagram of visceral mass of the clam *Mya arenaria*. Legend : (h.) heart, (h.s.) caecum/hyaline style, (f.) foot, (d.t.) digestive tract, (d.g.) digestive gland, (an.) anus, (l.p.) labial palps, (g.) gonad, (a.r.m.) anterior foot retractor muscle, (a.a.m.) anterior adductor muscle, (p.r.m.) posterior foot retractor muscle, (p.a.m.) posterior adductor muscle, (CG) cerebral ganglion, (PG) pedal ganglion, (VG) visceral ganglion, (1) siphon and posterior pallial nerve, (2) branchial nerve, (3) cerebrovisceral connective, (4) cerebropedal connective, (5) cerebral commissure, (6) anterior pallial and adductor nerve, (7) gonadal nerve, (8) posterior foot retractor nerve.

the digestive gland. The digestive gland is located inside of the posterior dorsal visceral mass and occupies a good part of the visceral mass (Fig. 4.1). Two ducts, the hyaline caecum and the digestive tract, enter in the ventral posterior wall of the stomach. In *Mya arenaria*, there is no communication between hyaline caecum and the digestive tract. The crystalline caecum extends dorso-ventrally in the visceral mass while forming a large U handle and fixes/finishes at the foot base (Fig. 4.1). The digestive tract leaves the floor of the ventral posterior region of the stomach and extends dorso-ventrally into the visceral mass. When the digestive tract starts, the intestine is relatively broad. Thence, this tract decreases gradually in diameter, makes loops, and then at the foot level, it extends posteriorly in writing a large U handle, passing slightly on the right from the stylet. Here, it goes posteriorly through the anterior wall of the pericardial cavity, penetrates the ventricle, and terminates in the anus. The anus is located between the posterior adductor muscle and the dorsal siphon (Fig. 4.1).

In the visceral mass of *Mya arenaria*, three principal components of the muscle system were identified: a foot and two pairs (anterior and posterior) of foot retractor muscles (Fig. 4.1). The foot is small, wedge-shaped, inflatable and thin and has no byssal gland. The extrinsic pedal musculature consists of bilateral pairs of thin anterior and posterior pedal retractor muscles (Fig. 4.1). Each retractor muscle is attached to the shell valves by a dorsal round insertion slightly separated from the adductor muscle scar. Both anterior and posterior retractor muscles, the right and left muscles fuse rapidly into a single bundle then converge on the sagittal typical pelecypod plane towards the foot. Each

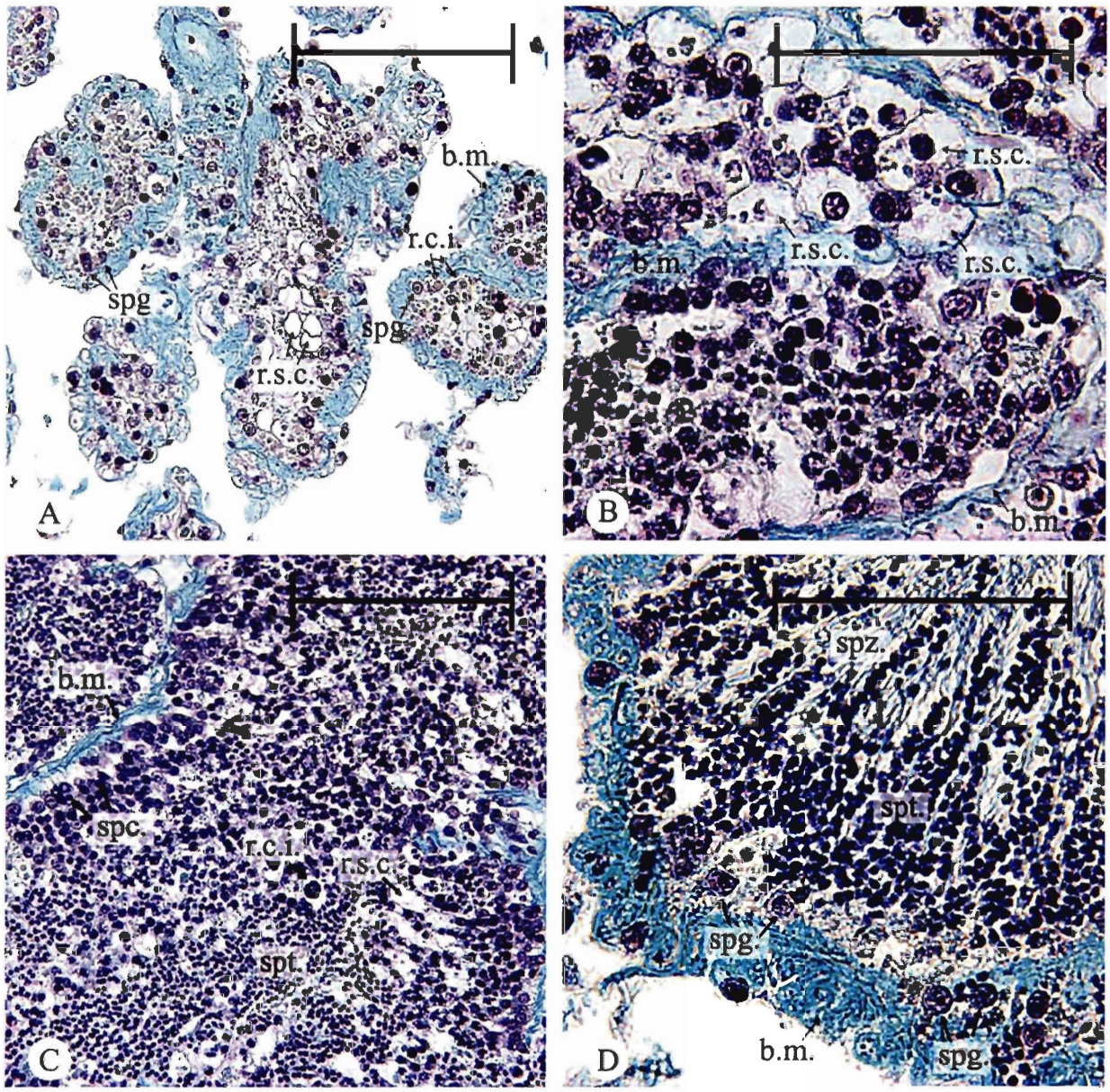
posterior retractor muscle extends further fanwise fibres anteriorly, which is enveloping the visceral mass ventrally before entering into the foot.

Several components of the nervous system were also identified in the visceral mass of *Mya arenaria* (Fig. 4.1). The pedal ganglia, fused at the midline, are located at the base of the foot. The cerebropedal connective skirts along the anterior foot retractor muscle to connect the cerebral and pedal ganglia. The cerebrovisceral connective crosses through the digestive gland and gonad and connects the cerebral and visceral ganglia. Gonadal and posterior foot retractor muscle connectives branching from the cerebrovisceral connectives have been clearly demonstrated.

The gonad occupies a large portion of the visceral mass and remains isolated from other systems (Fig. 4.1). The gonad consists of highly ramified tubules bearing numerous alveoli. Tubules merge into gonoducts and one fused genital vesicle. The genital aperture is located on either side of the posterior dorsal apex of the visceral mass, ventral to the cerebrovisceral commissure and anterior to the foot posterior retractor muscle.

4.5.2 Histology and immunohistochemistry of the visceral mass

Using Prenant-Gabe's trichrome, histology confirmed the presence of a single fused genital vesicle and the high branching tubules and alveoli. Five morphological criteria were used to characterize each gametogenic stage in male (Figs 4.2a–h) and female (Figs 4.3a–h) clams: indifferent, development, ripe, spawning and spent.



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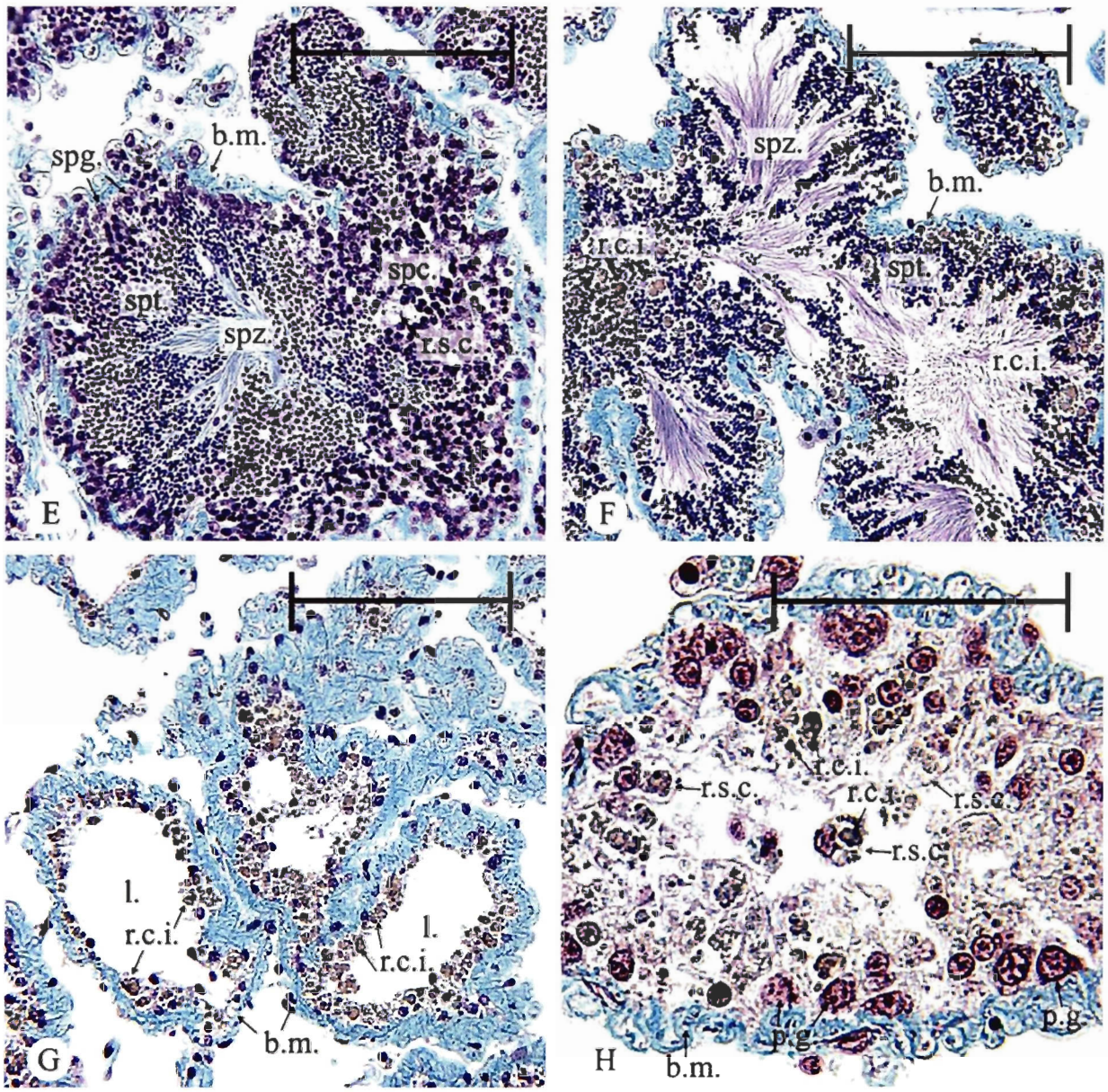
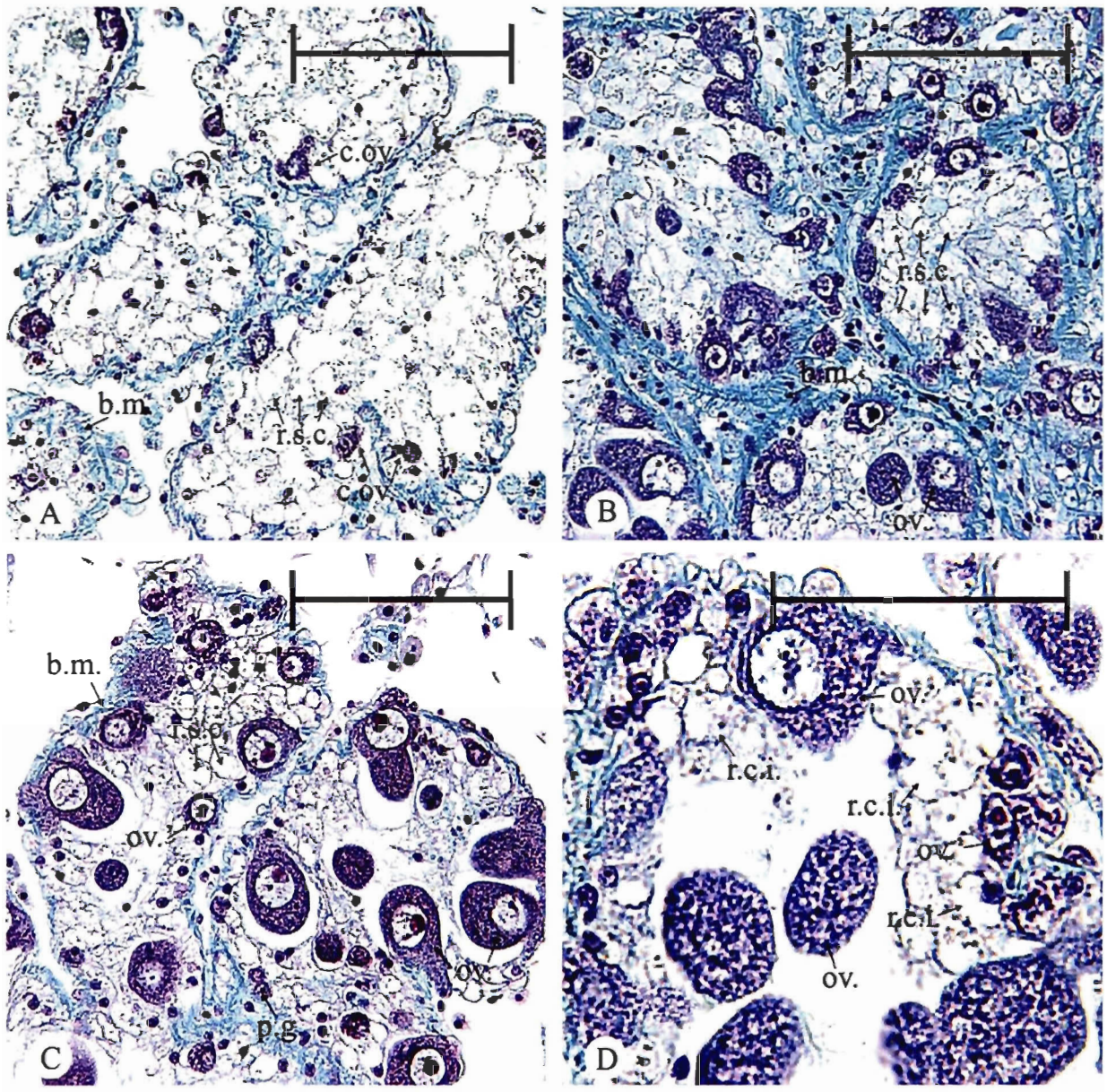


Figure 4.2: Histology of gametogenesis in male gonad of *Mya arenaria.* Prenant-Gabe's trichrome-stained section of (A) indifferent, (B)-(C) development 1, (D) development 2, (E) ripe, (F)-(H) spent stages. (b.m. = basal membrane, l. = lumen, r.s.c. = storage somatic cells, p.g. = undifferentiated gonia, spg. = spermatogonia, spc. = spermatocytes, spt. = spermatids, spz. = spermatozoa, r.c.i. = storage somatic cells inclusions). Scale bars = 100 μm in A, C and E–G and scale bars = 50 μm in B, D and H. Paraffin sections were prepared at 3-μm thick.



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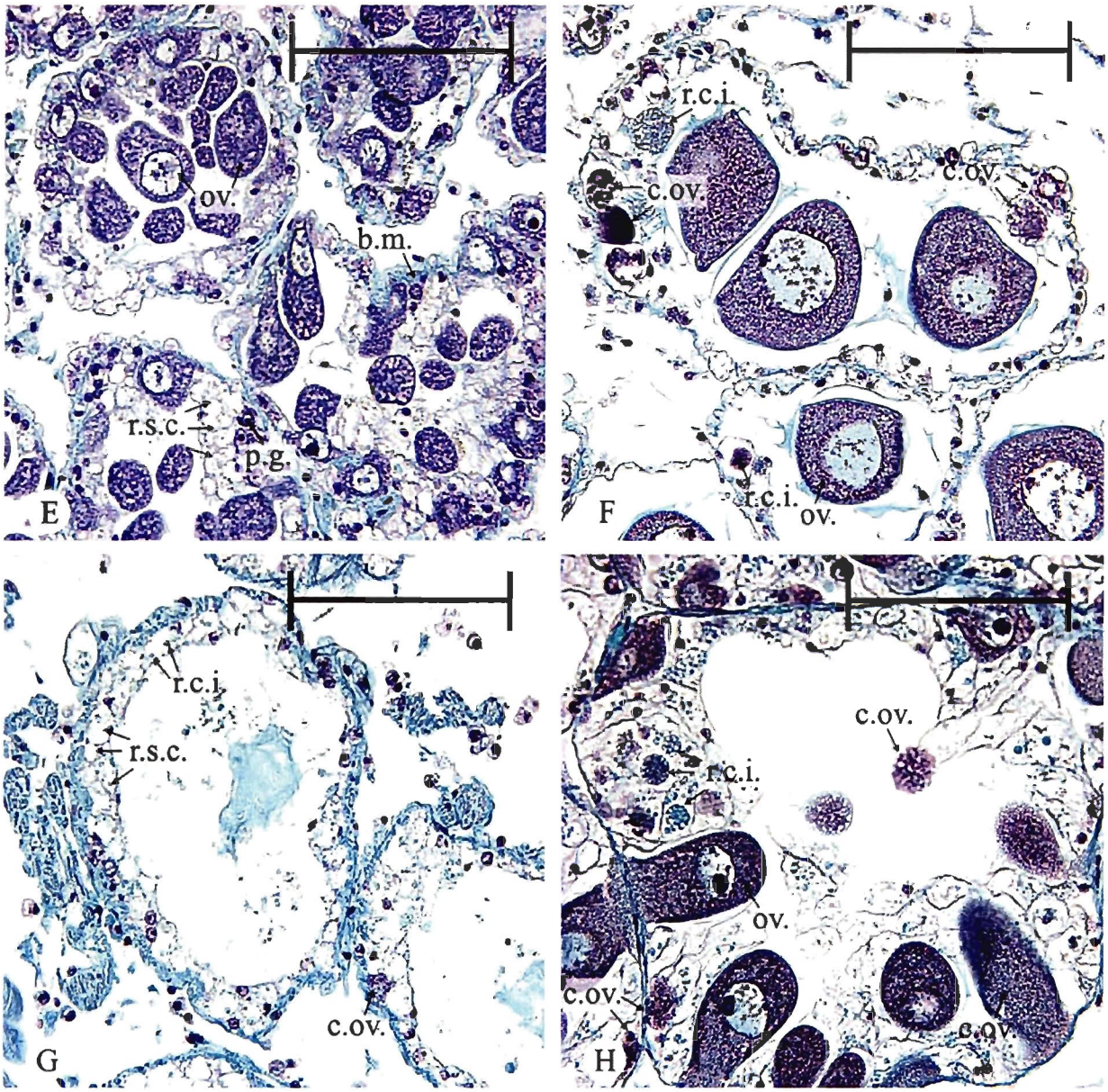


Figure 4.3: Histology of gametogenesis in female gonad of *Mya arenaria.* Prenant-Gabe's trichrome stained section of (A) indifferent, (B) pre-vitellogenic, (C)-(D) vitellogenic, (E) post-vitellogenic, (F) spawning, (G)-(H) spent stages. (b.m. = basal membrane, l. = lumen, r.s.c. = storage somatic cells, p.g. = undifferentiated gonia, r.c.i. = storage somatic cells inclusions, ov. = ovocytes, ov.' = smaller ovocytes, c.ov. = cytolisis ovocytes). Scale bars = 100 μm in A–C and E–H and scale bars = 50 μm in D. Paraffin sections were prepared at 3-μm thick.

The indifferent stage corresponds to sexual repose and a period of energy build-up. Examination of the indifferent stage in both sexes showed that each alveolus is constituted by a wall and vacuolated storage cells (storage cells called follicular cells by Coe & Turner, 1938) (Figs 4.2a, 4.3a). The walls consist of undifferentiated germinal epithelium having a sometimes shrunken appearance. The tubules present are filled with storage cells having few inclusions. Even at these early stages during which inclusions are present, it is possible to differentiate male from female clams: in females the granules are much smaller, uniform in size and much more numerous (Figs 4.2a, 4.3a).

The development stages are described by a specific gametogenic activity, a gonia multiplication and an increase in gonadal mass. In males, during stage 1 development (Fig. 4.2b), the primary spermatocytes proliferate starting from the spermatogonies existing at the basal membrane and force their way between storage cells to the centre of the alveoli (centripetal differentiation). At this stage, spermatocytes predominate in the tubules while storage cells and cell inclusions progressively disappear. During stage 2 development (Fig. 4.2c), the spermatids differentiated starting from the spermatocytes nearing the centre of the alveoli. At this stage, spermatocytes and spermatids predominate in the tubules and few storage cells aggregate at the basal membrane. In females, the pre-vitellogenic stage is characterized by the appearance of oogonia and small developing oocytes (primary ovocytes) attached to the basal membrane and growing between the storage cells nearing the centres of the alveoli (Fig. 4.3b). The female vitellogenic stage is defined by the occurrence of the central lumen, an increase in oocyte numbers and a decrease in storage cell abundance (Figs 4.3c–d). Storage cell numbers drop because they undergo cytolysis.

Primary ovocytes are attached to basal membranes and grow between the storage cells, half-grown oocytes are still attached to alveolar walls by slender stalks and a few ova have reached maturity, broken loose and appeared in the lumen (Figs 4.3c–d). Female gonads begin to fill in.

Ripe stages are described by a well-developed gonad, an increase in mature gametes in the tubules, and the elongated appearance of the wall. In males, the ripe stage is characterized by radial columns and the appearance of spermatozoa that are only visible at the centre of the tubule (Figs 4.2d–e). During the post-vitellogenic stage (female ripe stage), each alveolus contains a large number of post-vitellogenic, spherical, mature ova that appear to be loose within the ovarian central lumen or attached to the alveolar walls by slender stalks (Fig. 4.3e).

Spawning stages are characterized by the release of gametes and by the loss of size and colour in the gonad. In males, spermatozoa still occupy a substantial portion of the central tubules. Spermatozoa are arranged in more or less radial columns with their tails oriented towards the centre. Female aveoli are packed with free oocytes in the central lumen and some stalked oocytes appear on alveolar walls.

The spent stage corresponds to an empty gonad due to sexual repose and/or to the final period of gamete release. Spawning is finished, tubules have a shrunken appearance and contain numerous phagocytes or even pycnotic gametes. Reabsorption of the gametes and reinvasion of the tubules by storage cells follow. In males, the tubules are collapsed and the storage cells increase in number (Figs 4.2g–h). Degenerating spermatozoa and

spermatids occupy the tubule less and less and numerous granular inclusions appear on the storage cells (Figs 4.2f–h). In females, each tubule now consists of a single row of cells (Fig. 4.3g) and occasionally contains residual gametes in the central lumen (Fig. 4.3f). Unspent ova are at their largest at this stage (Fig. 4.3f) and will degenerate (oocyte atresia) and undergo cytolysis (Figs 4.3f, 4.3h). Reabsorption products are collected within the storage cells as spherical granular inclusions (Fig. 4.3h). Even at these later stages, during which inclusions are present, it is possible to differentiate male from female clams. The granule forms are similar to those found in the indifferent stages in males. Contrary, the granules are large and round in females (Figs 4.2h, 4.3h).

Large numbers of α-tubulin-immunoreactive cells were detected by immunohistochemical staining in both sexes of *Mya arenaria*. In females, α-tubulin-immunoreactive zones were focused in the foot (before slender stalks) of developing ova (Fig. 4.4a). In males, α-tubulin-immunoreactive cells were scattered throughout the tubule between the storage and germinal cells. These supporting somatic cells are uniformly distributed in male alveoli and are arranged in radial columns oriented toward the tubule centre (Fig. 4.4b). Actin-immunoreactive cells were clearly visible in the acinal walls at all gametogenic stages for both sexes (Figs 4.4c–d) compared to the negative controls.

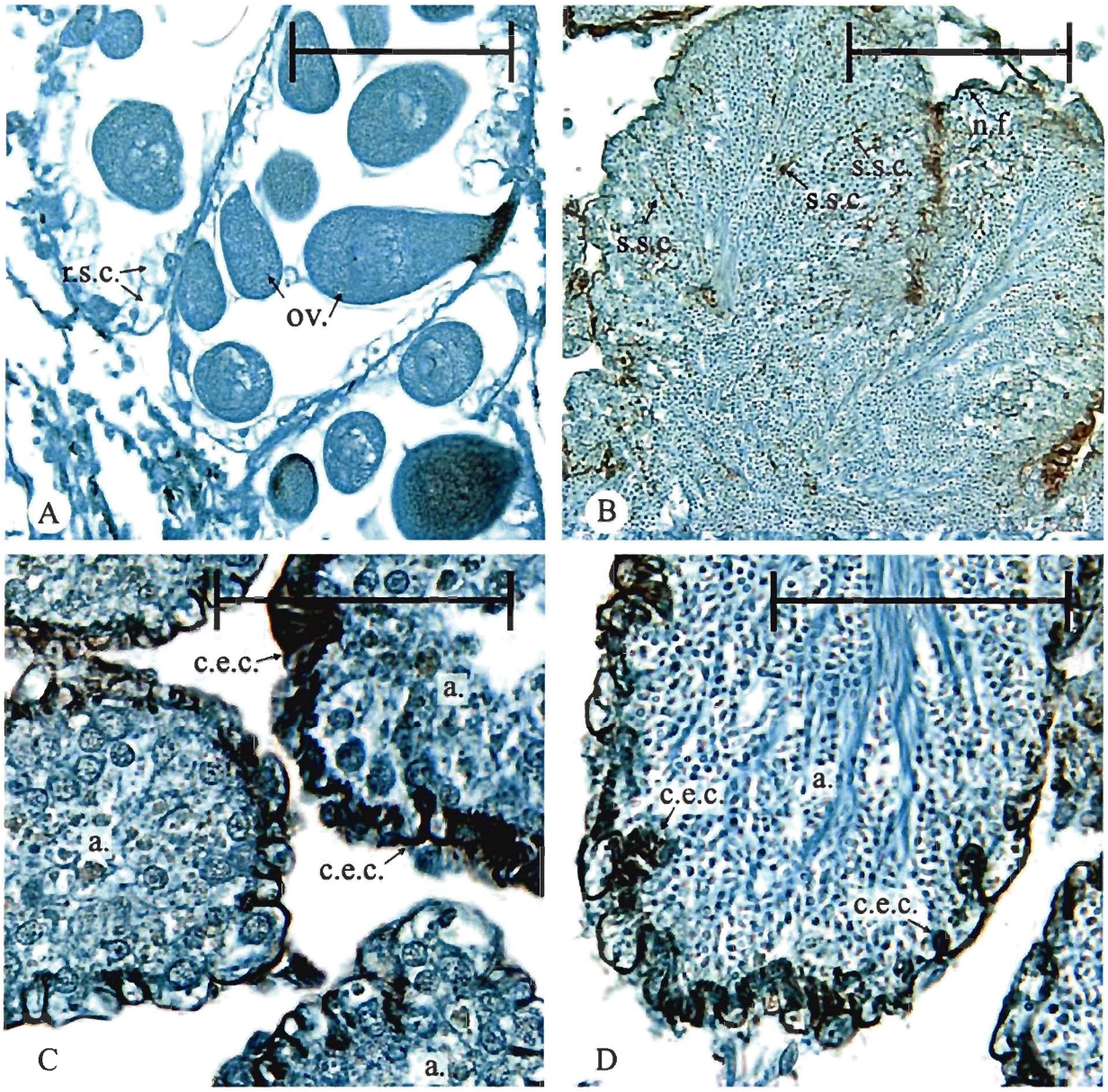
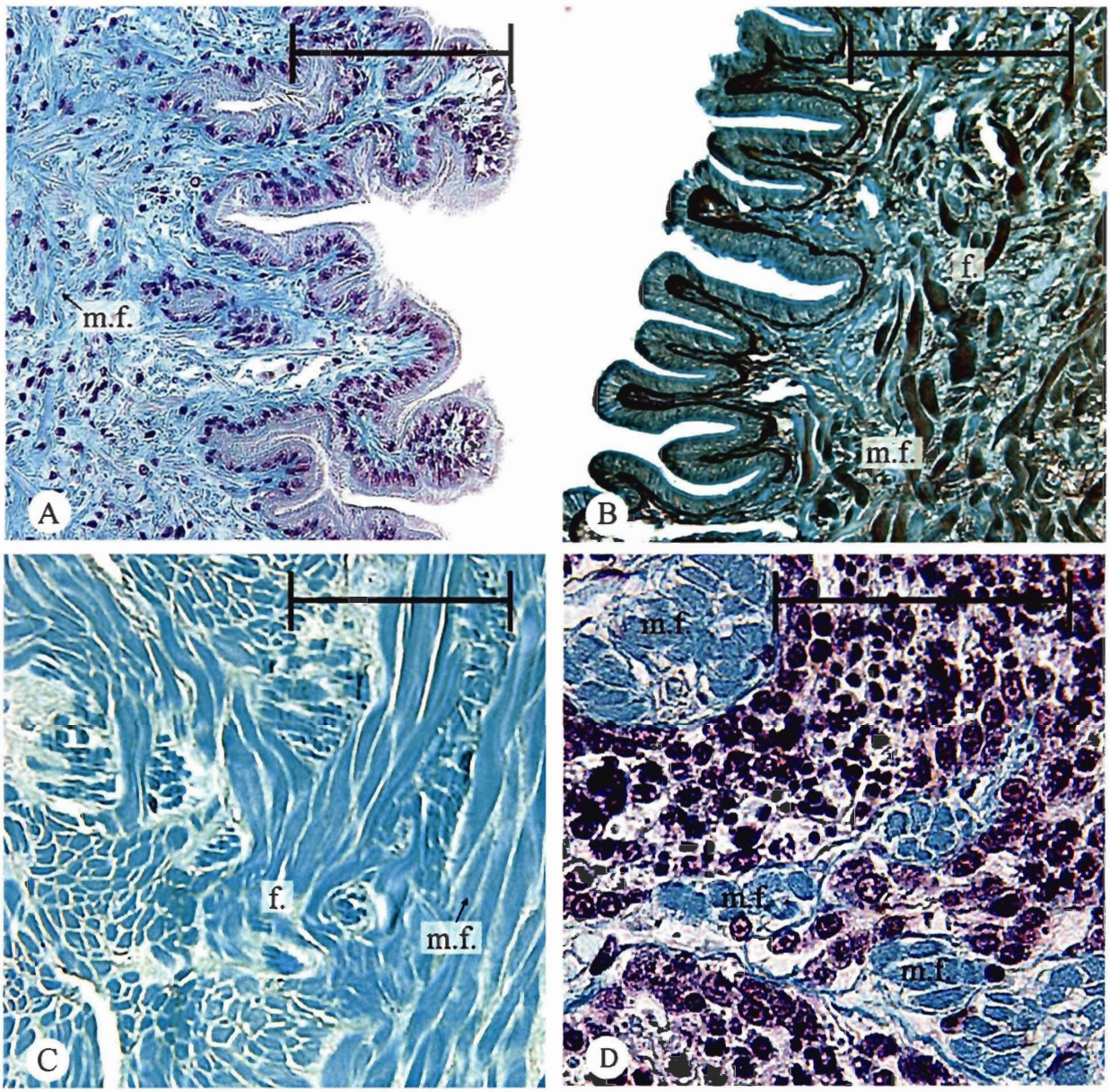


Figure 4.4: Actine and α-tubulin immunohistochemistry localization in the gonad of *Mya arenaria*: α-tubulin immunohistochemistry localization of female gonad at vitellogenic stage (A) and in male gonad at ripe stage (B); actin immunohistochemistry localization in male gonad at indifferent stage (C) and at spawning stage (D); (a. = alveoli, r.s.c = storage somatic cells, s.s.c. = supporting somatic cells, ov. = Ovocyte, n.f. = nervous fibers, c.e.c. = contractile epithelial cells). Scale bars = 100 μm in A–B and scale bars = 50 μm in C–D. Paraffin sections were prepared at 3-μm thick.

Using Prenant-Gabe's trichrome and actin immunohistochemistry, large numbers of muscle fibers were detected inside the foot (Figs 4.5a–c), passing through the reproductive system, (sometimes through the digestive gland) (Figs 4.5d-e), into the walls of the digestive tract (Figs 4.5g-h) and stomach (Figs 4.5i-k), and into the walls of the blood vessel (Figs 4.5l-n). The foot is composed of interlaced/interwoven muscle fibers. The walls of the digestive tract consist of a circular inner muscular layer and an outer lengthwise muscular layer.

Using Prenant-Gabe's trichrome, a histological analysis corroborated the presence of several components of the nervous system: pedal ganglia, cerebropedal and cerebrovisceral connectives. The organization of the pedal ganglion of *Mya arenaria* is generally similar to that of other bivalves. Pedal ganglia are composed of a perineurium (a connective-tissue sheath) covering the ganglionic cortex. The cortex contains several cellular bodies that send their processes into the central neuropil (Figs 4.6a–b), when compared to the negative control (Fig. 4.6c). Anti-α-tubulin and anti-actin immunohistochemistry coloured the nervous system structures differently (Figs 4.6a–b). First, the anti-α-tubulin stained the presence of numerous nerve fibers when compared to the negative control (Fig. 4.6c). α-tubulin-immunoreactive fibers were clearly visible in the pedal ganglia, mainly in the central neuropil (Fig. 4.6a) and around germinal tubules for all gametogenic stages in both sexes (Figs 4.6e and 4.6g). By contrast, the anti-actin stained the perineurium of the pedal ganglia (Fig. 4.6b) and connectives (Fig. 4.6d) when compared to the negative control (Fig. 4.6c).



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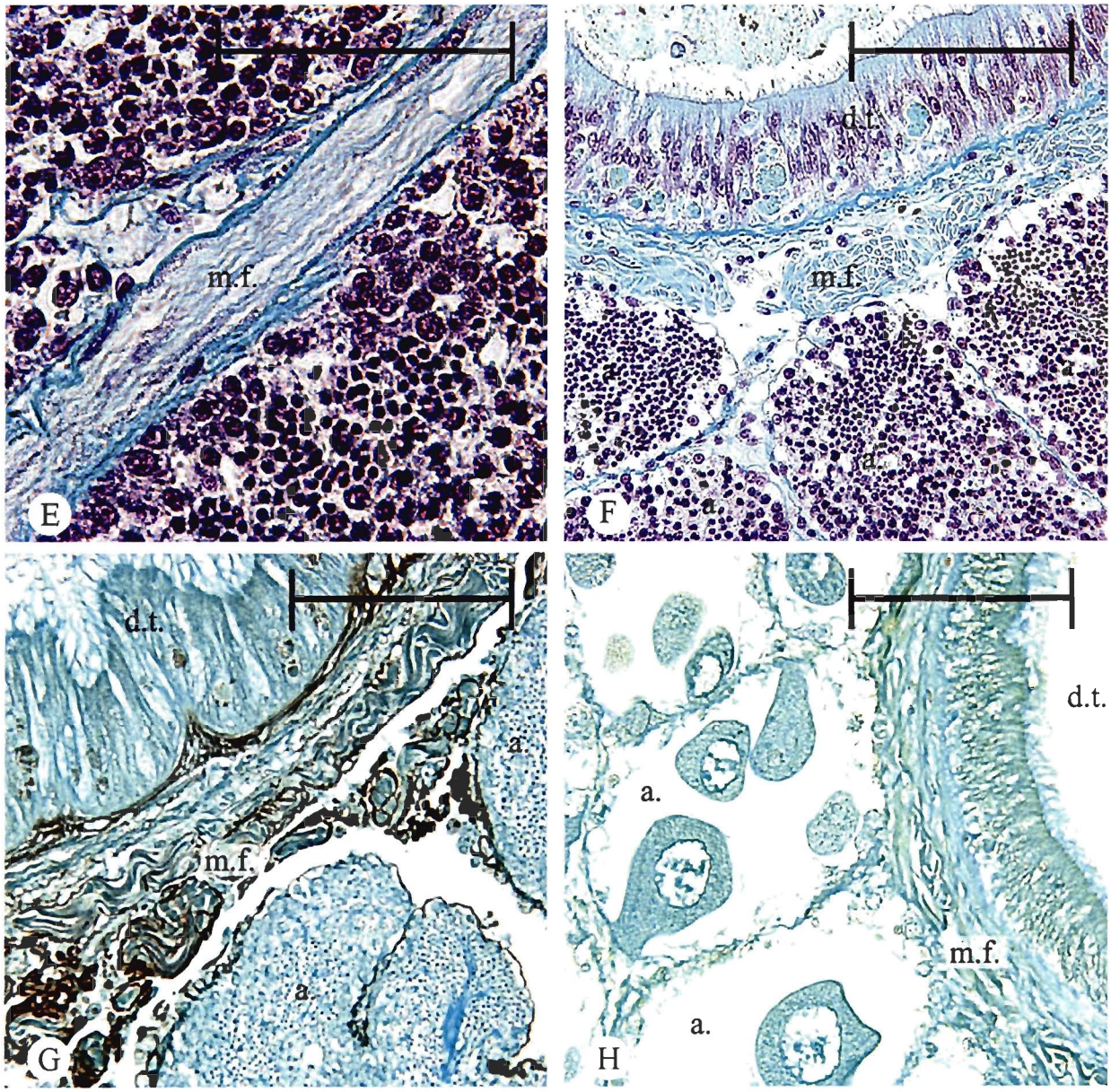

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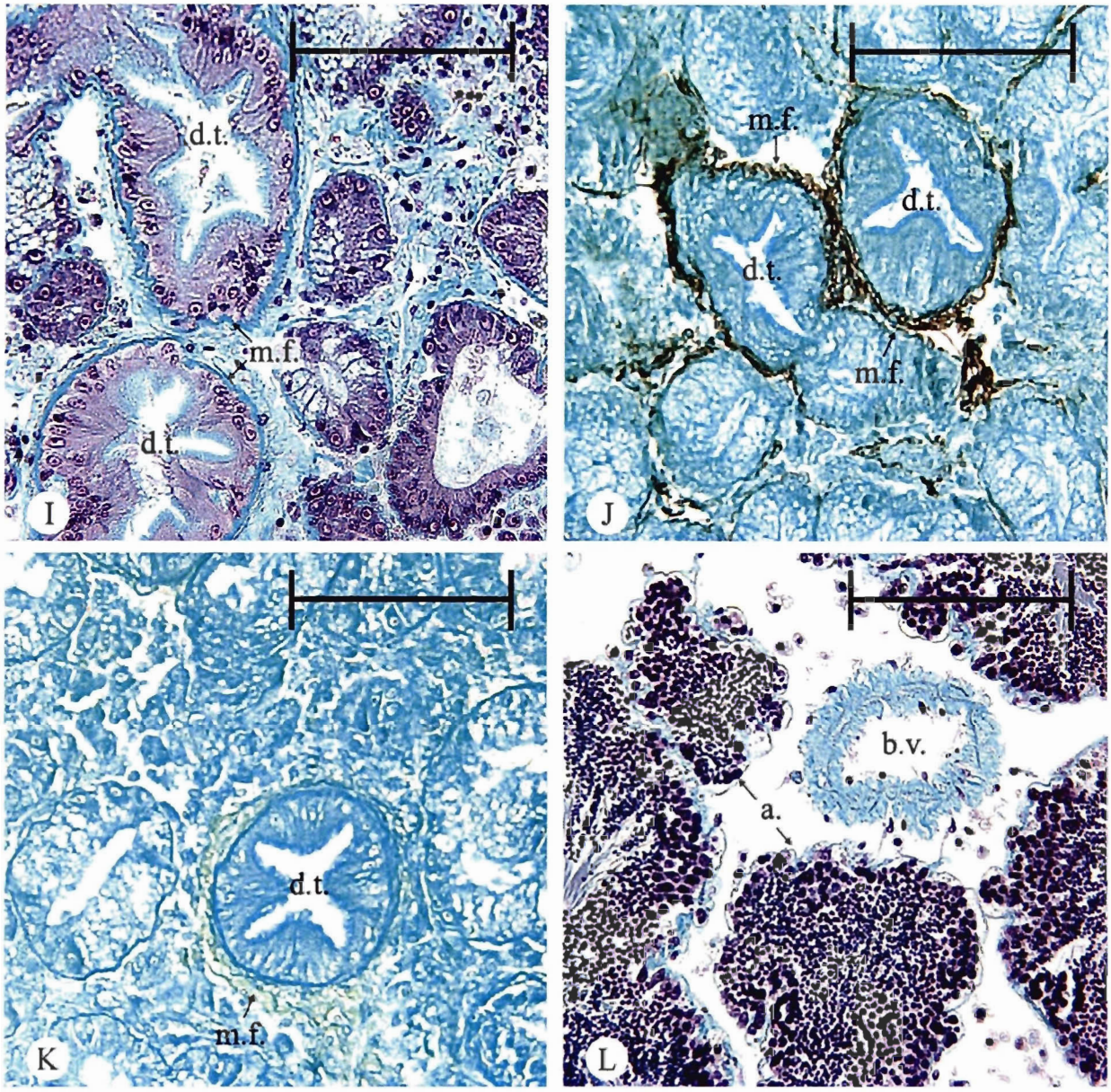

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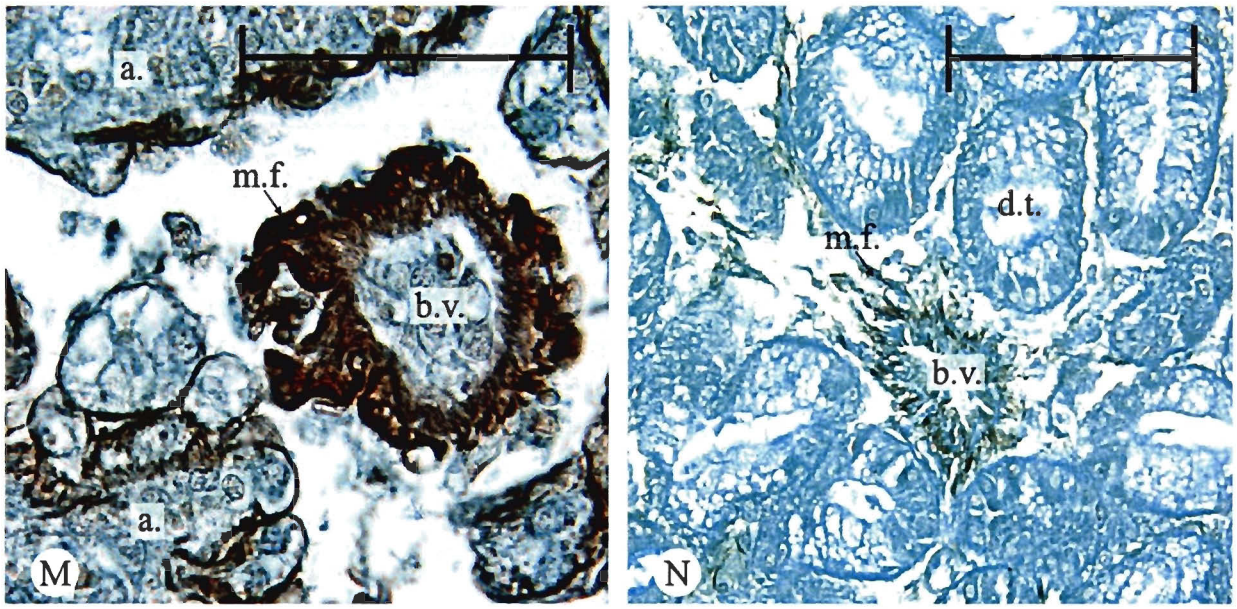
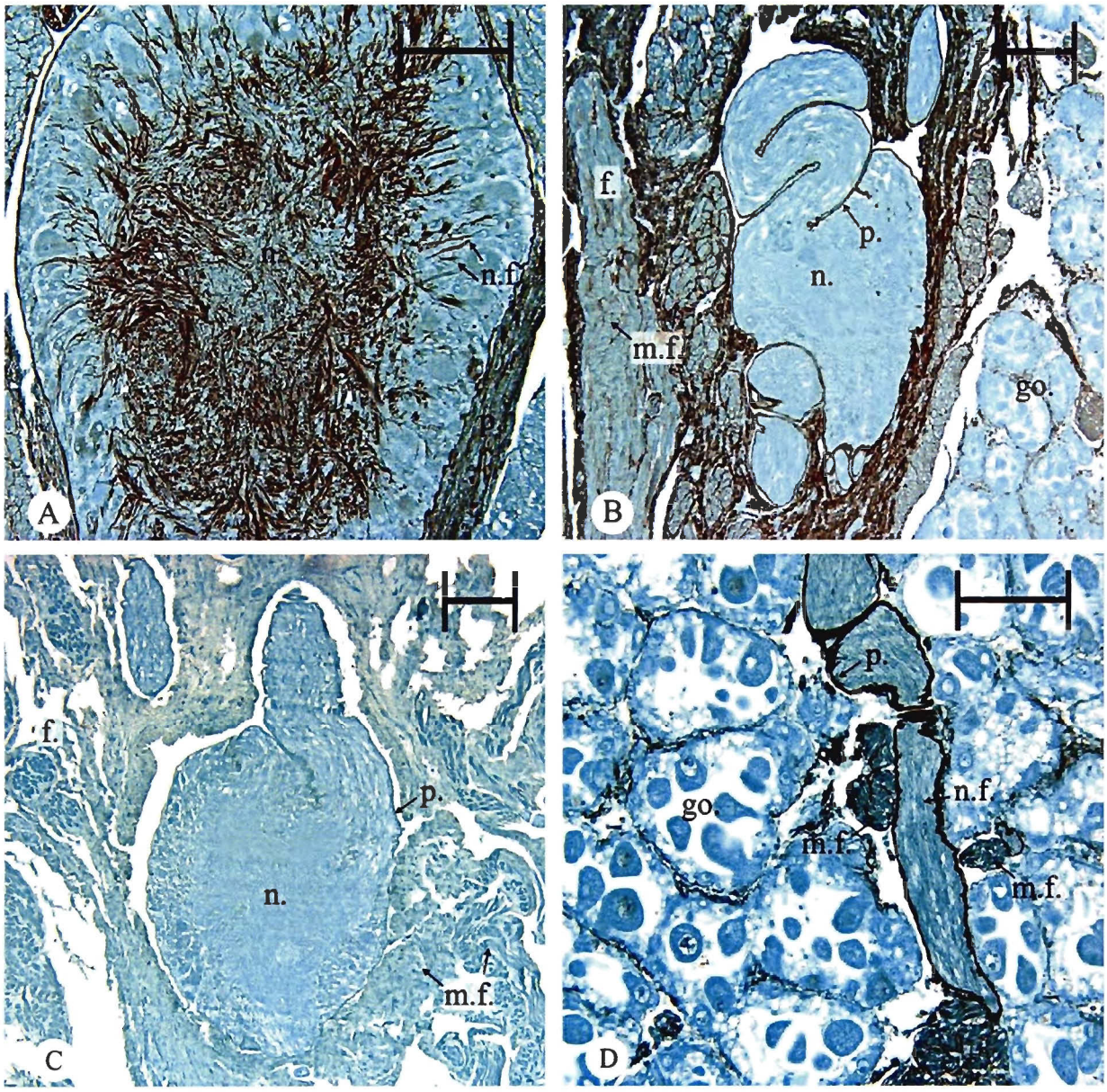


Figure 4.5: Muscular fibre localization in the DGDF of *Mya arenaria*. (A) Prenant-Gabe's trichrome, (B) actin immunohistochemistry stained sections of foot. (C) Negative control of immunohistochemistry stained sections of foot. (D)-(E) Histological aspect of the male gonad, Prenant-Gabe's trichrome-stained section of stage 1 development. (F) Prenant-Gabe's trichrome and (G) actin immunohistochemistry stained sections of digestive tract. (H) Negative control of immunohistochemistry stained sections of digestive tract. (I) Prenant-Gabe's trichrome, (J) actin immunohistochemistry stained sections of digestive gland. (K) Negative controle of immunohistochemistry stained sections of digestive gland. (L) Prenant-Gabe's trichrome, (M) actin immunohistochemistry stained sections of gonad and blood vessel. (N) Negative control of immunohistochemistry stained sections of gonad and blood vessel. (m.f. = muscular fibers, f. = foot, a. = alvoli, d.t. = digestive tubule, b.v. = blood vessel). Scale bars = 100 μm in A-C, F-L and N, and scale bars = 50 μm in D-E and M. Paraffin sections were prepared at 3-μm thick.



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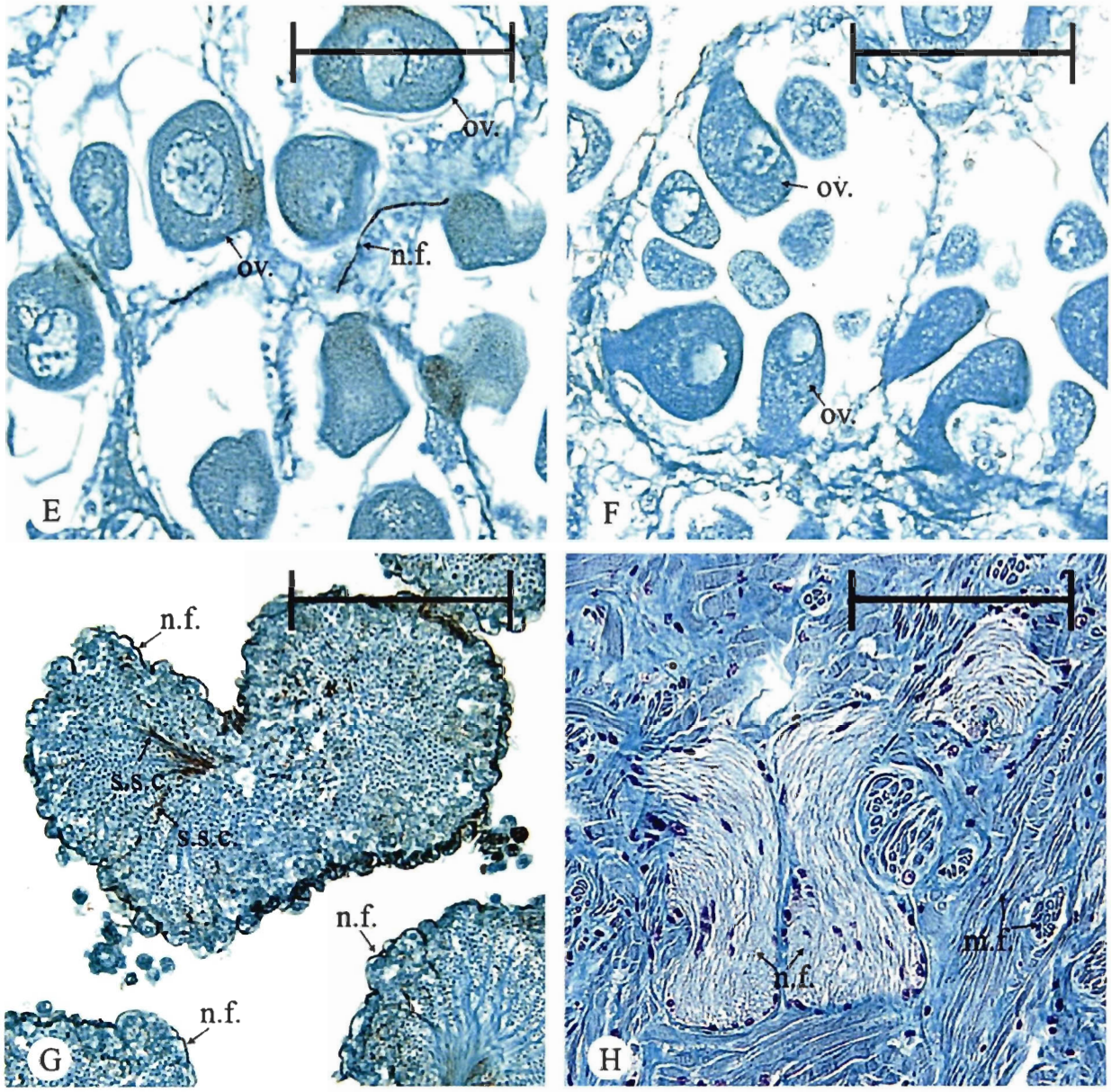

Figure 4.6: Nervous fibre localization in the DGDF of *Mya arenaria.* (A) α-tubulin and (B) actin immunohistochemistry-stained section of pedal ganglia. (C) Negative control of immunohistochemistry-stained section of pedal ganglia. (D) actin and (E) α-tubulin immunohistochemistry-stained section of female gonad at vitellogenic stage. (F) Negative control of immunohistochemistry-stained section of female gonad at vitellogenic stage. (G) α-tubulin immunohistochemistry-stained section of male gonad at stage 2 development. (H) Histological aspect in the foot, Prenant-Gabe's trichrome-stained section. (p. = perineurium, n. = neuropil, n.f. = nervous fibers, m.f. = muscular fibers, go. = gonad, f. = foot, ov. = ovocyte, s.s.c. = supporting somatic cells). Scale bars =100 μm. Paraffin sections were prepared at 3-μm thick.

4.6 Discussion

This study of the functional and histological morphology of visceral mass broadens our current understanding of the lifestyle of the Myidae and defines new anatomical features that may be useful for any future discussion.

The morphological and anatomical study showed that the general organization of the visceral mass of *Mya arenaria* is similar to that of other bivalves. The digestive system, muscular system, nervous system and reproductive system are intertwined and closely associated within the visceral mass.

The primary characteristic of the visceral mass is that the two main systems, digestive and reproductive, though physically close, are quite distinct. According to Vlès (1909) and Stickney (1963), the gonads always remain isolated from other systems. The reproductive system of *Mya arenaria* follows the typical pelecypod plan and is formed by a pair of organs. This system is composed of a pair of genital apertures, one genital vesicle, a pair of gonoducts and highly ramified tubules bearing numerous alveoli. In most specimens, the gonoducts are joined together in a common vesicle (Vlès, 1909; Stickney, 1963), as observed in the present study.

Gametogenesis depends on the transfer of nutrients and energy reserves accumulated by storage cells, which are acquired almost exclusively by other tissues or organs and transferred to the gonad. In *Mya arenaria*, the digestive systems were described for the first time by Vlès (1909). Inside the digestive system, the mouth, eosophagus, stomach and

digestive gland never intermingle with the highly ramified gonadal tubules and alveoli. Only the crystalline caecum and digestive tract twist through the gonad. Bivalves present a seasonal cycle of storage and mobilization of energy reserves that correlate with the annual reproductive cycle and with food availability (Gabbott, 1975; Bayne et *al.*, 1982; Ruiz et *al.*, 1992; Mathieu & Lubet, 1993). The digestive gland stores nutrients that are then dispatched throughout the organism, either to provide energy for active metabolism or to build up reserves for further energy requirements like gametogenesis (Berthelin et *al.*, 2000). Gonadal development around the intestine optimizes the potential transfer of nutrients to developing gametes. The cytological and enzymatic equipment of the intestinal epithelium (Le Pennec et *al.,* 1991) suggests that the intestine is well adapted for both a digestive and a transfer function. Beninger et *al.* (2003) showed a pathway of nutrient transfer from the intestines and, more generally, the digestive system, to developing oocytes in Bivalvia. The scallop's intestinal cells may themselves move nutrients from the lumen to the basal lamina, with hemocytes subsequently acting as transport vectors to the surrounding gonadal tissue. Gonadic tubules would then support the transport of energy directly amidst the gametogenic cells. Both sides of the crystalline caecum are filled with a long hyaline style. In the stomach, the crystalline style has a central role in abrading food particles, a consequence of style rotation, and in secreting a variety of extracellular enzymes (Nelson, 1918; Purchon, 1971; Morton, 1970, 1973).

In the present study, we showed that the muscular system of the visceral mass is constituted of a wedge-shaped foot and extrinsic pedal musculature consisting of two anterior and posterior bilateral pairs of pedal retractor muscles. It has two functions in

Mya arenaria, an endobenthic pelecypod. The first is to enable foot deployment for burrowing (Moore, 1969). Bivalves move downward into the substrate by extending the foot into the sediment, anchoring the foot by expanding its tip, and pulling the shell downward toward the anchor by muscular action (Pojeta, 1987). In clams each retractor muscle is attached to the shell valves by a dorsal round insertion and fuses rapidly into a single bundle that then converges toward the foot. This configuration of foot and extrinsic muscle enables the foot to spread out, stimulated by the nervous system and the pedal ganglia. The second function is to maintain the integrity of the visceral mass and to prevent compaction and spoilage of tissues. The morphological study showed that each posterior retractor muscle extends more fanwise fibres anteriorly, enveloping the visceral mass ventrally. Moreover, using Prenant-Gabe's trichrome and actin immunohistochemistry, several muscle fibers of all sizes connecting walls transversely were detected in the reproductive system, and sometimes the digestive gland, in the interacinal spaces. These observations about numerous bands of muscle fibers passing through the gonad are similar to the findings of Stickney (1963). These results suggest that the totality of the muscular fibers, which envelop and cross over the visceral mass, maintain the integrity of the visceral mass, during the foot movements.

Histological techniques and an assessment of gamete development provide general information on gonadal evolution. For example, seasonal variations in the gametogenic stages have been tracked by histological examination, which also allows for the identification of any phenomenon liable to affect reproductive activity in bivalves (Bayne, 1974; Brousseau, 1978; Roseberry et *al.*, 1991; Tremblay, 1992; Paulet et *al.*, 1997;

Gauthier-Clerc et *al.*, 2002). Several researchers have used recognized histological features generally recovered in all bivalves to define gametogenic stages of *Mya arenaria.* Unfortunately, stage determination is often subjective; standardized stage numbers would facilitate the comparison of results. The present study completes those from Siah et *al.* (2002, 2003) and Gauthier-Clerc et *al.* (2006) who have used a similar scale, respectively, five and six maturation stages were determined for male and female gonads during microscopic examinations. In the present study, we have delimited a clearly defined pattern of gametogenic development and each gametogenic stage in both sexes of *Mya arenaria* : indifferent, development, ripe (post-vitellogenic for females), spawning and spent. The development stage can be further divided into development 1 and 2 for males and pre-vitellogenic and vitellogenic for females.

In *Mya arenaria*, the acini is a simple structure containing only germinal cells with associated storage cells within a thin germinal epithelium. Actin-immunoreactive cells were clearly visible in the acinal wall at all gametogenic stages in both sexes. During the indifferent stage, the wall has a shrunken appearance due to the presence of contractile epithelial cells inside the germinal epithelium. The spent stage corresponds to an empty gonad, actin in the myoepithellial cells promoting shrinkage of the tubule. The myoepithelial cells that partially surround the acini might contract during spawning to force mature eggs through the gonoducts, but they do not present an effective barrier to the movement of molecules (Eckerbarger & Davis, 1996).

Storage cells are intragonadal and may concentrate nutrient reserves in *Mya arenaria* (Coe & Turner, 1938), *Paphia staminea* (Quayle, 1943) *Macoma baltica* (Caddy, 1967) and *Crassostrea gigas* (Eckerbarger & Davis, 1996), or in the connective tissue of the gonad of *Crassostrea gigas* (Loosanoff, 1965, Berthelin et *al.*, 2000) and *Ostrea edulis* (Loosanoff, 1963). Recent studies have also pointed to the involvement of such cells in oogenesis (labelled "trophocytes" or "auxiliary cells" [Motavkine & Varaksine, 1983; De Gaulejac et *al.*, 1995]), in *Patinopecten yessoensis, Crenomytilus grayana* and *Pinna nobilis*, and in *Mytilus edulis* and *Crassostrea gigas* (Eckerbarger & Davis, 1996), where they are called "follicular cells" (Pipe, 1987a). In the Pectinidae, adductor muscle cells that are unspecialized storage cells are implicated in the storage of protein reserves (Lubet et *al.*, 1986; Epp et *al.*, 1988). In the Veneridae, both specialized and non-specialized cells are used in the storage and release of nutrients (Mathieu & Lubet, 1993). In some families like the Mytilidae, reserves are retained only in specialized storage cells. In *Mytilus edulis,* two types of storage cells have been described: vesicular connective tissue cells (VCT) and adipogranular cells (ADG) (Pipe, 1987a; Lenoir, 1989). The ADG cells accumulate lipids and proteins and VCT cells or glycogen cells are filled with a single large-membraned vesicle containing mainly glycogen.

In *Mya arenaria,* gonadal histology showed that storage cells are of variable volume and dimension. These cells were called follicular cells by Coe & Turner (1938). During gametogenesis, a decrease in storage cell numbers from the onset of vitellogenesis to the ripe stages was observed. Throughout the indifferent stage, a period of energy accumulation and sexual rest, each alveolus is filled by a wall of storage cells. Then storage cells are

replaced by a proliferation of germinal cells. Previous studies have suggested that, in molluscs, energy reserves contained in storage cells could be mobilized during periods of reproductive activity, to fuel the development of gonads and production of gametes (Coe & Turner, 1938; Pipe, 1987a, 1987b; Dorange & Le Pennec, 1989; Eckelbarger, 1994; De Gaulejac et *al.*, 1995). In females, several storage cells are associated with each oocyte, at least during early oogenesis. In *Pecten maximus,* however, only a single cell is associated wich each oocyte (Dorange & Le Pennec, 1989). In the present study, the presence of dense inclusions in the storage cells and strong storage cellular variations during the early developmental stages suggests that these cells nourish the rapidly growing ova and intense proliferation of spermatogonia. Coe & Turner (1938) stated that inclusions were reserve nutritive substances derived in part from the cytolysis of degenerating ovocytes and in part from cytoplasmic activities. These inclusions consisted of small globules of a lipoidal nature and larger globules of albuminous composition (Coe & Turner, 1938).

Using immunohistochemistry, α-tubulin-immunoreactive cells were detected between storage and germinal cells in the gonad of male *Mya arenaria*. α-tubulin has been shown to be a good marker for supporting-cell localization (Mathieu, Kellner-Cousin & Heude, pers. comm.). These supporting cells, called "Sertoli cells," are uniformly distributed in male tubules and arranged in radial columns oriented toward the tubule centre. We called these new cells supporting cells because Sertoli function was not yet described in marine bivalves. In the blue mussel *M. edulis*, storage cells in the female and supporting cells in the male are the somatic support for gamete development (Pipe, 1987a). Their principal functions appear to be the regulation of material passing into the developing germ cells.

During early gonadal development, storage cells and supporting cells are characterized by the presence of numerous lipid inclusions; however, during gametogenesis, the lipids are depleted (Pipe, 1987b). Following spawning, the well-developed lysosomal system is evident in the Sertoli cells and appears to be engaged in intracellular digestion of phagocytosed waste sperm and residual cytoplasm (Pipe, 1987a). In *Mytilus edulis,* storage cells appear to endocytose the resorption of the products of gamete degeneration (Pipe, 1987a).

Oocyte evolution has already been described for species such as *Patinopecten yessoensis*, *Crenomytilus grayan*a and *Pecten maximus* (Motavkine & Varaksine, 1983), *Mytilus edulis* (Pipe, 1987b; Lubet et *al.*, 1987) and *Crassostrea virginica* (Eckerbarger & Davis, 1996). In *Mya arenaria*, the apparition of oogonia and young oocytes along internal wall of the acini characterize the beginning of gametogenesis activity. These ovocytes are attached to basal membrane and grow between the storage cells. Thereafter, vitellogenic oocytes gradually fill the acini, whereas the storage cells decrease and detach from the apical zone of the oocytes when they become pedunculated. The germinal vesicle of the oocytes migrates apically and a prominent nucleolus persists. Using mmunohistochemistry, α-tubulin-immunoreactive localization was detected in the slender stalks of the oocyte peduncule; α-tubulin is responsible for oocyte peduncule formation in bivalves. Contact between the "auxiliary cell" and the developing oocyte would be maintained by a desmosome-like junction in *Pecten maximus* (Dorange & Le Pennec, 1989). Upon reaching maturity, oocytes separate from the acinus wall and migrate into the lumen, where they remain until spawning.

In spent females, every tubule consists of a single row of cells and occasionally contains residual gametes in the central lumen. Unspent ova are largest at this stage. These observations are similar to the findings of Lango-Reynoso et *al.* (2000) in *Crassostrea gigas*. After spawning, resting ovocytes continue to grow before degenerating. The process of oocytic degeneration is a commonly observed phenomenon in bivalves (Motavkine & Varaksine, 1983; Lubet et *al.*, 1987; Pipe, 1987b; Dorange & Le Pennec, 1989). In some species, the cells implicated in the oocytic lysis seem to be macrophagic haemocytes (Auffret, 1985; Dorange & Le Pennec, 1989), and likely play a role in the resorption of lysitic material. Lubet et *al.* (1987) and Pipe (1987b) suggested that epithelial cells of gonoducts could resorb this material. In *Pinna nobilis*, De Gaulejac et *al.* (1995) concluded that the ovarian "auxiliary cells" are responsible for phagocytosis and intracellular digestion of product originating from the degenerating oocyte. During the spent stage in *Mya arenaria*, the presence in the storage cells of specific sex inclusions, granular in males and spherical in females, suggests that storage cells are responsible for phagocytosis of products originating from the degenerating gonia. Coe & Turner (1938) stated that inclusions were reserve nutritive substances derived in part from the cytolysis of degenerating gonia. The gradual disappearance of inclusions during sexual repose, corresponding to the indifferent stage, also suggests that storage cells could be responsible for resorption of this material.

The anatomical study of the visceral mass in *Mya arenaria* showed the presence of several components of the nervous system: pedal ganglia at the base of the foot, cerebropedal and cerebrovisceral connective tissues cross through the digestive gland and

gonad to connect the cerebral and visceral or pedal ganglia. Moreover, the presence of the posterior foot retractor muscle connectives branching from the cerebrovisceral connectives has been clearly demonstrated. The pedal ganglia and the posterior foot retractor muscle connectives control the muscle contractions, respectively, of the foot and the posterior foot retractor muscle. In *Mya arenaria*, Garnerot et *al.* (2006) found numerous 5-HT nerve fibers of various diameters around germinal tubules, but no relationship could be established with cerebral, pedal or visceral ganglia. We have determined that such gonadal innervation appears to originate from the ramification of the cerebrovisceral connectives, as already reported in *Mya arenaria* (Stickney, 1963) and *Venus verrucosa* (Siniscalchi et *al.*, 2004). The present anatomical description suggests that seroninergic innervation could be modulated by cerebral and/or visceral ganglia. However, further investigation of this issue is required to establish the origin of gonadal seroninergic innervation. For neuroanatomical studies in invertebrates, antibodies directed against 5-HT and α-tubulin are an accepted standard. Nerve cell axons are rich in acetylated α-tubulin and α-tubulin antibodies have been used to stain the nervous system of, for example, nematodes (Siddiqui et *al.*, 1989), molluscs (Jackson et *al.*, 1995) and echinoderms (Garcia-Arras & Viruet, 1993). Using immunohistochemistry, anti-α-tubulin stain nervous fibers inside the visceral mass: in neuropile of the pedal ganglia, around germinal tubules of both sexes and inside gonadal muscule fibers. This confirms the 5-HT results ("chapter 3" Garnerot et *al.* [2006]), which show that α-tubulin acts as a marker for nerve fibers and would be sufficient to establish the origin of gonadal seroninergic innervation or to localize nerve fibers inside tissues.

Serotonin (5-hydroxytryptamine or 5-HT) plays a central role in several physiological processes, especially reproduction, in marine molluscs. Many studies on bivalves have confirmed the presence of serotonin and its physiological effects on muscles (York & Twarog, 1973), siphon activity (Ram et *al.*, 1993), tonic relaxation of smooth muscles (Gies, 1986), and ciliated tissues (Stefano et *al.*, 1977; Smith, 1982). 5-HT also acts as a neurohormone to modulate spawning (Matsutani & Nomura, 1982; Gibbons & Castagna, 1984; Braley, 1985; Bariles & Gaete, 1991; Garnerot et *al.*, 2006), parturition (Fong & Warner, 1995), and meiosis by reinitiating prophase in arrested oocytes (Hirai et *al.*, 1988; Krantic et *al.*, 1991; Varaksin et *al.*, 1992). In *Mya arenaria*, the external application of 5-HT induced spawning movements, like siphon activity, in ripe clams of both sexes, while only a few males released sperm (Garnerot et *al.*, 2006). Garnerot et *al.* (2006) also suggested that two different mechanisms might be involved in controlling the release of sperm and oocytes in the gonad. In the scallop *Argopecten purpuratus*, the serotoninergic route would conduct information for male spawning, while catecholamines would be involved in the release of oocytes (Martinez et *al.*, 1996). An overall knowledge of clam physiology enables us to understand the discrepancy in the responses of both sexes. Sperm release is probably an effect of 5-HT action on the tonic relaxation of visceral mass muscle system, the tonic relaxation of myoepithelial cells, and spermatozoan mobility. The addition of 5-HT to cold-immobilized *Spisula solidissima* sperm instantaneously reinitiated spermatozoan mobility (Kadam & Koide, 1990; Kadam et *al.*, 1991). Thereafter, when 5-HT action is sufficiently reduced, the visceral muscular system can return to its initial

state while contracting. The muscular tensing of visceral mass and the increased inner pressure supports partial sperm release into the palleal cavity before release in water.

The observations presented here show that further work using electron microscopy would be useful to fully describe the ultrastructural features underlying spermatogenesis and oogenesis in *Mya arenaria,* to compare and distinguish differences with other species.

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CHAPITRE 5 : RELATIONSHIP BETWEEN LEVELS OF SEXUAL STEROIDS (ESTRADIOL-17B, PROGESTERONE AND TESTOSTERONE), LIPIDS AND GAMETOGENESIS IN MALE AND FEMALE *MYA ARENARIA* (MOLLUSCA: BIVALVIA)

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5.1 Abstract

In marine bivalves, the fluctuations in sex steroids that correlate with the cycle of sexual maturation suggest their possible implication in regulating gametogenesis. Progesterone, estradiol-17β and testosterone were recently characterized in *Mya arenaria*, concentrations of these steroids being reported during a complete gametogenic cycle. In this study, we determined levels of lipids, estradiol-17β, testosterone and progesterone in female and male *M. arenaria* during the reproductive cycle. Both steroids were measured by ELISA in gonads and digestive glands. In females, no significant variations were observed in gonadal lipid levels. During gametogenesis, lipid levels in the digestive gland fell significantly between the indifferent and vitellogenic stages and increased between the vitellogenic and spent stages. Lipids stored in the digestive gland can be mobilized to the gonad to be used as a source of energy and a substrate for oocyte growth. In males, by contrast, lipid concentrations in both tissues remained stable during gametogenesis. These results suggest that energy support of the digestive gland might not be the same in both sexes. Progesterone levels in the digestive glands of clams were three times higher than in their gonads. This finding, and the similar profile of progesterone in digestive glands and gonads, suggests that, in *Mya arenaria*, the digestive gland could play a key role in steroidal metabolism. Estradiol-17β and testosterone profiles in gonad showed interannuel variations in hormone levels. These variations in sex steroid levels can be explained by interannual trophic conditions and/or by the still unknown role played by steroids in physiological processes other than gametogenesis. Further studies are therefore needed to

portray the pathway of steroidal synthesis in clam gonad and other tissues, as a means of understanding the involvement of steroids in gametogenesis and, ultimately, in other physiological processes.

5.2 Key words

Estradiol-17β, progesterone, testosterone, marine bivalves, *Mya arenaria*, gametogenesis, gonad, digestive gland, ELISA.

5.3 Introduction

Steroidogenesis and sex steroids play a major role in the reproductive cycle of vertebrates. In invertebrates and particularly in bivalves, a direct relationship has been established between ganglia and gonad (Motavkine & Varaskine, 1989), thus indicating that the reproductive cycle can be controlled by ganglion neurosecretions. The presence of sex steroids (in particular, estradiol-17β, testosterone, androstenedione, estrone and progesterone) and many enzymes involved in steroidogenesis (Desmolase, 3β-hydroxysteroid dehydrogenase isomerase, 17β-hydroxysteroid dehydrogenase, $C_{17,\ 20}$ lyase, 5α-reductase, P450 aromatase) has been reported in bivalves such as *Crassostrea gigas* (Hathaway, 1965; Mori et *al.*, 1965a, 1965b; Mori, 1980; Matsumoto et *al.*, 1997; Le Curieux-Belfond et *al.*, 2001), *Crenomytilus grayanus* (Varaksina & Varaksin,1988), *Mya arenaria* (Siah et *al.*, 2002, 2003; Gauthier-Clerc et *al.*, 2006), *Mytilus edulis* and *M. galloprovincialis* (De Longcamp et *al.*, 1970, 1974; Reis-Henriques et *al.*, 1990; Reis-Henriques & Coimbra, 1990; Morcillo et *al.*, 1999), *Patinopecten yessoensis* (Varaksina & Varaksin, 1988; Matsumoto et *al.*, 1997; Osada et *al.*, 2004), *Pecten hericius, P. magellanicus* and *P. maximus* (Botticelli et *al.*, 1961; Idler et *al.*, 1969; Saliot & Barbier, 1971) and *Ruditapes decussata* (Morcillo et *al.*, 1998a, 1998b; Morcillo & Porte, 2000). The presence of sex steroids and steroidogenic enzymes in the reproductive organs of marine bivalves opens up a new field of research into the role of sex steroids in the bivalve reproductive cycle and supports a possible parallel with the well-known hormone-regulating system of vertebrates.

Recent studies on marine bivalves found variations in sex steroid levels that were well correlated with gametogenesis (Mori, 1969, 1980; Reis-Henriques et *al.,* 1990; Reis-Henriques & Coimbra, 1990; Matsumoto et *al.,* 1997; Gauthier-Clerc et *al.*, 2002, 2006; Siah et *al.*, 2002; Osada et *al.*, 2004). When injected into bivalves, estradiol (E2), testosterone (T), progesterone (P) and dehydroepiandrosterone (DHEA) accelerated gonadal differentiation (Wang & Croll, 2004). Varaksina & Varaksin (1991) and Varaksina et *al.* (1992) also reported that injections of E2, T and P stimulated both spermatogenesis and oogenesis in adult *Mizuhopecten yessoensis* scallops and led to increased gonad weight and oocyte diameter. Earlier studies have also hinted at the role played by sex steroids in molluscan gender determination. In the clam *Mulinia lateralis*, Moss (1989) reported that administration of methyltestosterone accelerates sexual maturation, increases spawning frequency and changes the sex ratio from 0.8 to 1.6 (M/F). E2, T, P and DHEA injections into the adductor muscle also tipped the sex ratios in favour of males in *P. magellanicus* (Wang & Croll, 2004). In contrast, E2 injections during the early stages of sexual maturation appeared to induce sexual reversal (from male to female) in the oyster *Crassostrea gigas* (Mori et *al.*, 1969). Osada et *al.* (2004) suggested that estrogen may be synthesized in the estrogenic cells through aromatization by P450 aromatase and that estrogen may play a physiological role in spermatogenesis. Estradiol-17β-induced vitellogenin and vitellogenin-like protein synthesis have been demonstrated in the clam *Mya arenaria* (Blaise et *al.*, 1999), in the Japanese scallop *Patinopecten yessoensis* (Osada et *al.*, 2003) and the Pacific oyster *Crassostrea gigas* (Li et *al.*, 1998). In the scallop *P. yessoensis*, estradiol-17β (E2) may regulate levels of catecholamines (Osada & Nomura,

1989) and prostaglandins (Osada & Nomura, 1990). E2 might be involved in spawning and increase the number of egg release induced by serotonin (Osada et *al.*, 1992). E2 is indirectly involved in spawning by stimulating the synthesis of 5-HT RNA messenger receptors in oocytes (Osada et *al.*, 1998). Together, these results suggest that sex steroids may play an important role in marine bivalve gametogenesis (oogenesis and spermatogenesis).

The present study used the soft-shell clam *Mya arenaria* L. (Karsten, 1985), an endobenthic and sedentary pelecypod. Progesterone, estradiol-17β and testosterone were recently characterized in *Mya arenaria* (Siah et *al.*, 2002, 2003; Pelletier, 2006, pers. comm.). During a complete gametogenic cycle, progesterone (P), estradiol-17β (E_2) and testosterone (T) concentrations were reported in the mussel *Mytilus edulis* for P (Reis-Henriques & Coimbra, 1990), in the Japanese scallop *Patinopecten yessoensis* for E_2 (Osada et *al.*, 2004) and in the clam *Mya arenaria* for P (Siah et *al.*, 2002), E2 and T (Gauthier-Clerc et *al.*, 2006). Gauthier-Clerc et *al.* (2006) were the first to describe a steroid profile in digestive gland and gonad in relation to each stage of gametogenic development. In *Mya arenaria,* the digestive system (oesophagus, stomach, crystalline cecum, digestive tract and digestive gland) and gonad are intertwined inside the visceral mass. In this paper, we report on the concentrations of lipids and three sex steroids (estradiol-17β, testosterone and progesterone) in the gonad and digestive gland relative to each stage of gonadal development. The purpose of this study was to discuss the correlation between the results obtained for these two tissues. Lipid and steroid profiles are necessary

to better interpret their presence and involvement in the reproductive cycle of *Mya arenaria.*

5.4 Material and methods

5.4.1 Clam collection

Clams were collected from April to October 2004 at Metis Beach (48°40' 44"; 68°02' 17") on the southern coast of the St. Lawrence lower estuary (Quebec, Canada). Environmental contamination at Metis Beach is considered minimal when compared to that reported for all other sites along the St. Lawrence lower estuary (Lebeuf et *al.*, 1995). Clams (n = 176) were collected at low tide, brought back to the laboratory and kept at 4°C. Bivalves were dissected on the same day and tissue specimens were prepared and stored at -80°C until analysis.

5.4.2 Histology

Tissue specimens were washed in normal saline (0.15 M NaCl) and fixed in 4% paraformaldehyde at 4°C for 4 hours, rinsed twice for 30 minutes with phosphate-buffered saline (PBS, 50mM Na2HPO4, 140 mM NaCl, pH 7.2), then submerged in 30% sucrose overnight at 4°C. After incubation in sucrose, preparations were embedded in Cryomatrix (Thermo Shandon), frozen and stored until staining at -80°C. Sections (7-µm thick) were

prepared at 18°C using a Shandon cryotome (Thermo Electron Corp., PA). Sections were stained with Lee's methylene blue-basic fuchsin stain. Gonadal organization and gametogenic stages of both sexes were determined using a light microscope (Olympus BX41). Five maturation stages were determined for males (indifferent, development 1, development 2, spawning and spent) and six for females (indifferent, pre-vitellogenic, vitellogenic, post-vitellogenic, spawning and spent) (Coe & Turner, 1938; Brousseau, 1976; Roseberry et *al.*, 1991; Potts, 1993; Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2003).

5.4.3 Steroids and lipids analysis

5.4.3.1 Lipids

The lipid concentration in the gonad and digestive gland of *Mya arenaria* (n = 4–11 at each stage for both sexes) was measured using the sulfo-phospho-vanillin reaction method described by Frings et *al.* (1972) using olive oil as the standard. Tissues were homogenized in phosphate buffer (100 mM Na_2HPO_4, pH 7). Lipid concentrations were expressed as the means ± standard error to the mean (SEM).

5.4.3.2 Steroids extractions and assays

Steroid extractions and assays were performed as described in Siah et *al.* (2003) and Gauthier-Clerc et *al.* (2006). Briefly, frozen gonad and digestive gland tissues (n = 3-5 at

each stage for both sexes) were separately thawed and homogenized in nanopure water (1:5 v:v) using 15 to 20 up-and-down strokes of a glass-teflon homogenizer and then sonicated for 30 sec. 400 µL of a pre-warmed HCl buffer (25 mM) were added to 500 µL of the homogenate and incubated at 40°C for 15 min. After incubation, 1.25 mL of phosphate buffer (70 mM Na_2HPO_4, pH 7.4) was added. Thereafter, the organic phase containing steroids was extracted four times with 7 mL dichloromethane, evaporated under nitrogen gas at room temperature and dissolved in 400 µL enzyme immunoassay buffer (EIA) before quantification with the ELISA kit (Cayman Chemical Co. Ann Arbor, MI). All samples and standards were prepared and analyzed in duplicate. All steroid concentrations presented in the text and in the figures are expressed as the means ± standard error to the mean (SEM).

5.4.4 Stastistics

A one-way analysis of variance was performed to examine differences between the means of lipids and of each steroid hormone level (testosterone, progesterone and estradiol-17β). All data were statistically analyzed with SigmaStat 3.0 (Jandel Corp.). The two groups were compared by a Student *t*-test for single comparisons, in the presence of a normal distribution of data, and by a Kruskal-Wallis one-way analysis when the distribution of data was not normal. To assess multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed on the data or a Kruskal-Wallis test was used when the normality of distribution was not respected. Normal distribution and

homogeneity of variances were tested earlier on the data. For all statistical tests, individual clams were used as replicates and the results were considered significant with a probability (p) value of < 0.05.

5.5 Results

5.5.1 Variation in lipid levels in gonad and digestive gland of *Mya arenaria*

Changes in lipid levels in both male and female clams relative to each stage of gonadal development are shown in figure 5.1.

In females, lipid concentrations varied from 16.56 to 26.38 mg/g wet weight (ww) for digestive gland. The concentration of lipids decreased significantly *($t = 2.87$, $p = 0.035$)* by 37% between the indifferent and the vitellogenic stage, and increased *($t = 4.42$, $p = 0.03$)* by 30% from the vitellogenic to the post-vitellogenic/spent stages.

In males, lipid concentrations varied from 18.41 to 24.21 mg/g ww for digestive gland. The concentration of lipids increased significantly *($t = 2.33$, $p = 0.042$)* between the indifferent and the spawning/spent stages.

In both sexes, no significant variations were observed in gonadal lipid levels.

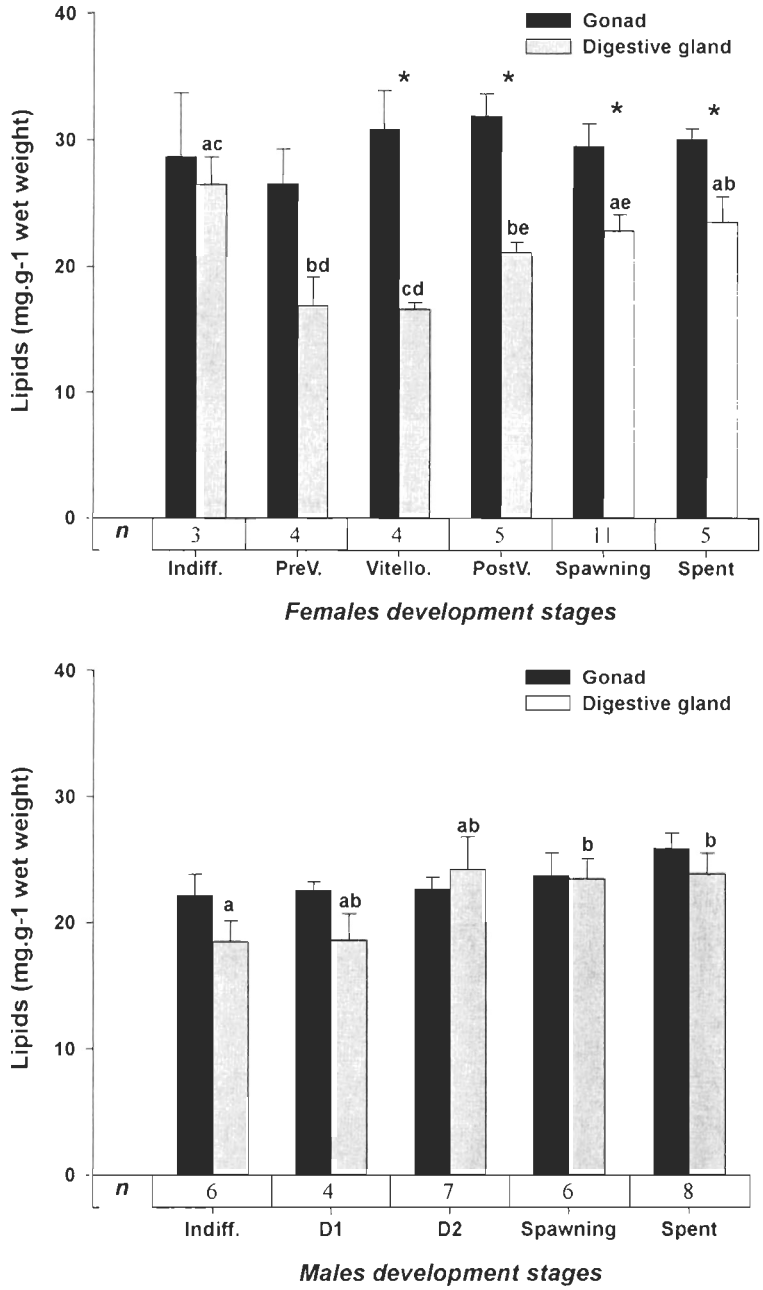

Figure 5.1: Variation in lipid concentrations in gonad and digestive gland of *Mya arenaria*. Each value of lipid concentration indicates the mean ± SEM, "a" ($p < 0.05$) is significantly different from "b" ($p < 0.05$); "*" indicates a significant difference ($p < 0.05$) between gonad and digestive gland according to the corresponding developmental stage.

5.5.1.1 Variation in 17β-estradiol, testosterone and progesterone levels in gonads and digestive glands of *Mya arenaria*

Changes in estradiol-17β (E2), testosterone (T) and progesterone (P) levels in gonads and digestive glands of both male and female *Mya arenaria* are shown in figures 5.2, 5.3 and 5.4, respectively.

In females, E2 and T levels in gonad varied, respectively, from 3.49 to 7.46 ng/g ww and from 1.20 to 2.41 ng/g ww. In digestive glands, E2 and T levels varied, respectively, from 1.06 to 3.50 ng/g ww and from 0.32 to 0.74 ng/g ww. A significant reduction (gonad: $t = 4.70$, $p = 0.005$, digestive gland: $t = 8.09$, $p = 0.015$) in E2 levels was observed from the onset of vitellogenesis to the spawning stage in both tissues (53% in gonad and 69% in digestive gland) (Fig. 5.2). Simultaneously, significant decreases were observed in T titers in gonad between the indifferent and the post-vitellogenic stage (50%: $t = 6.69$, $p = 0.001$) (Fig. 5.3). In the digestive gland, T concentrations were quite stable throughout sexual maturation, with a significant decrease between the post-vitellogenic and the spawning/spent stage (50%: $t = 3.306$, $p = 0{,}046$) (Fig. 5.3). P concentrations in gonads varied from 1.60 to 5.58 ng/g ww, with a significant decrease observed between the indifferent and the pre-vitellogenic stages (31%: $t = 3.80$, $p = 0.013$) (Fig. 5.4). In digestive gland, no significant variations in P levels were observed during female gametogenesis (Fig. 5.4).

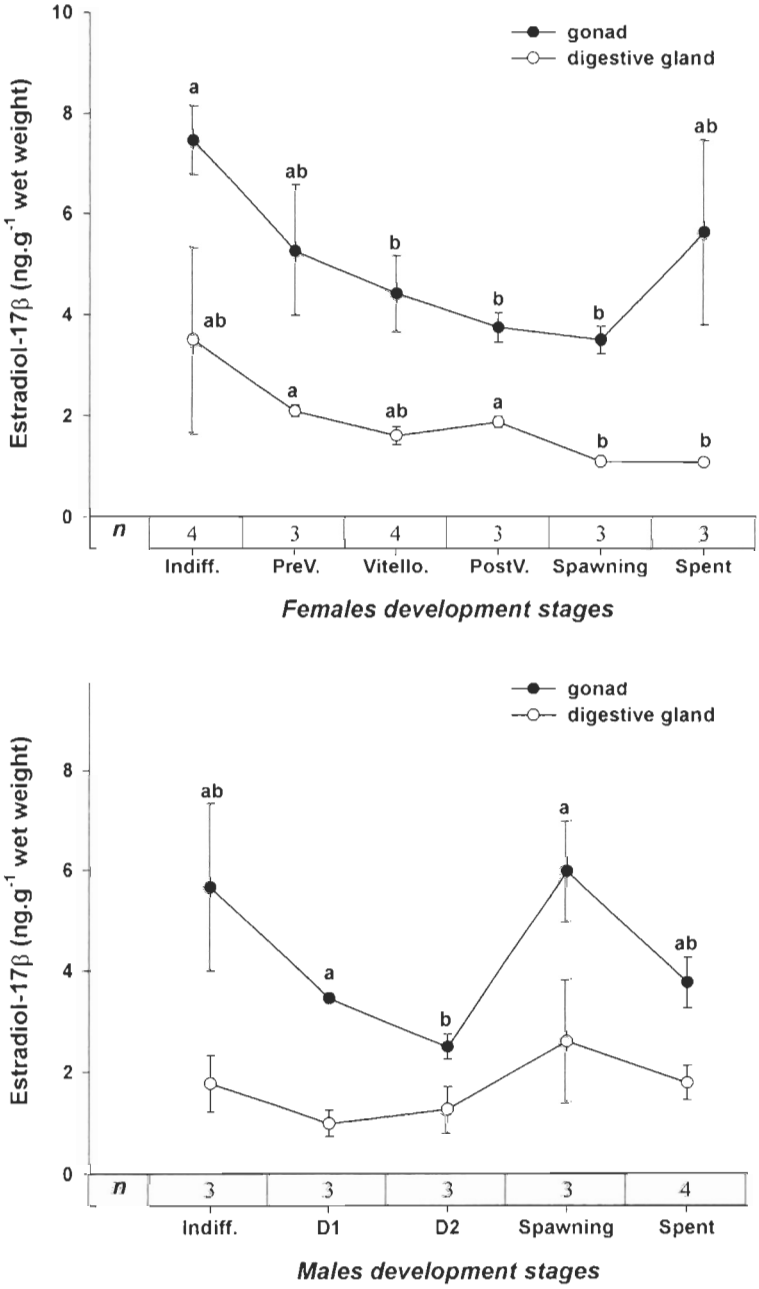

Figure 5.2: Variations in estradiol-17β levels in *Mya arenaria* gonads and digestive glands based on developmental stages in both sexes. Each value represents the mean ± SEM, "a" ($p < 0.05$) is significantly different from "b" ($p < 0.05$).

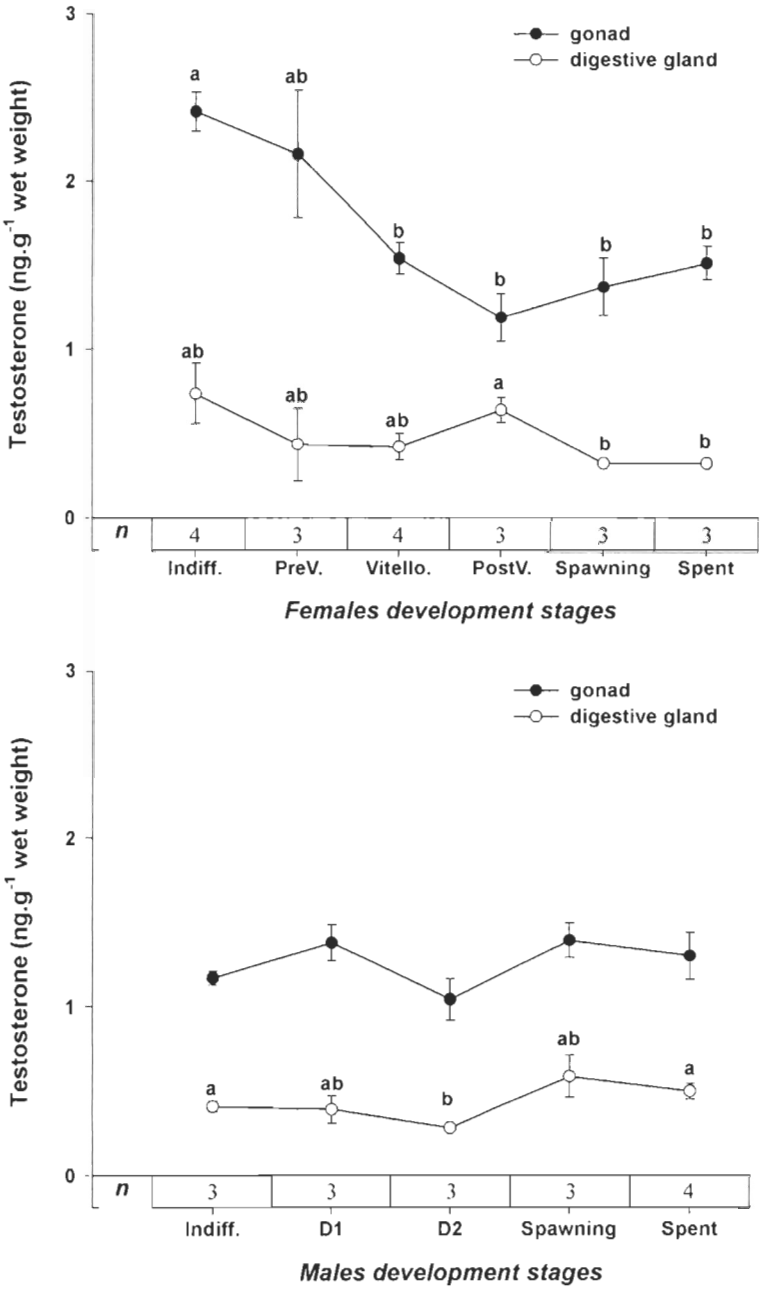

Figure 5.3: Variations in testosterone levels in *Mya arenaria* gonads and digestive glands based on developmental stages in both sexes. Each value represents the mean ± SEM, "a" ($p < 0.05$) is significantly different from "b" ($p < 0.05$).

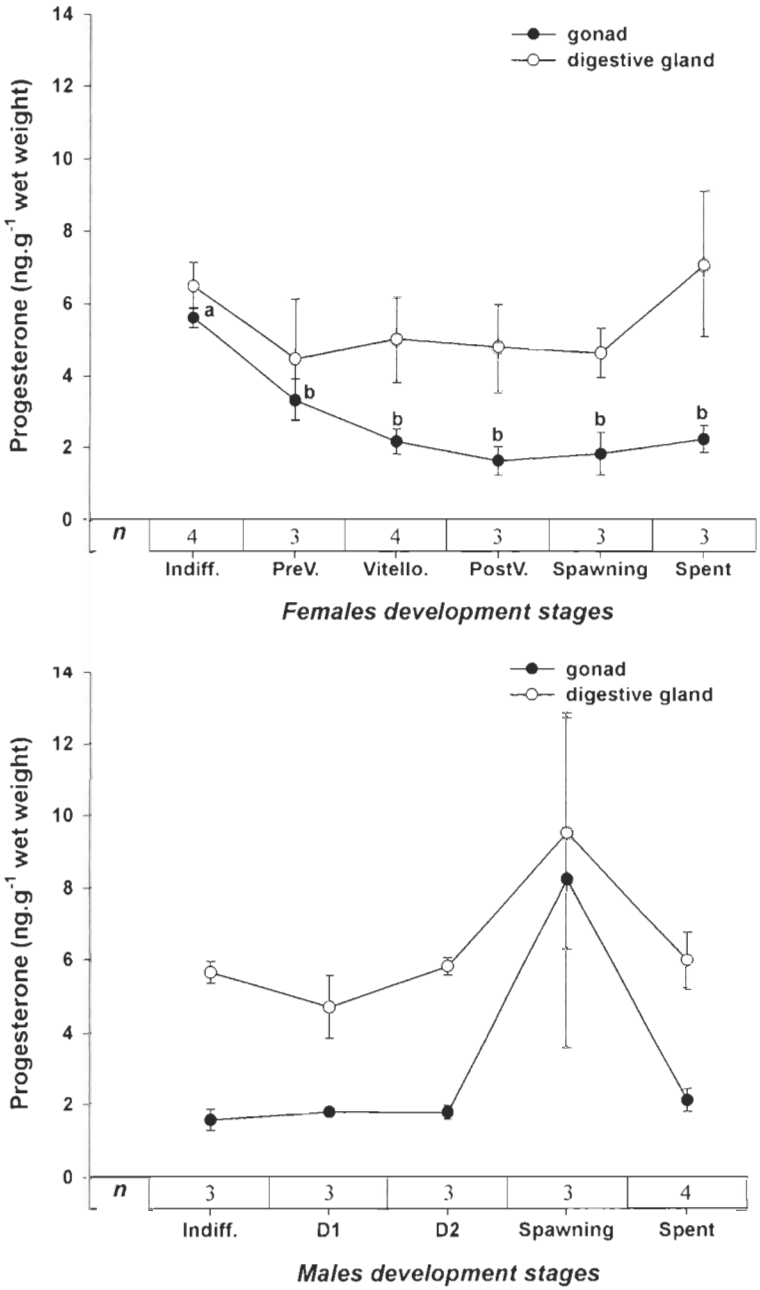

Figure 5.4: Variations in progesterone levels in *Mya arenaria* gonads and digestive glands based on development stages in both sexes. Each value represents the mean ± SEM, "a" ($p < 0.05$) is significantly different from "b" ($p < 0.05$).

In males, E2 levels in gonad varied from 2.61 to 5.97 ng/g wet weight. A significant decrease of 55% in E2 levels was observed in the gonad at the onset of gametogenesis (from the indifferent to the second developmental stage: $F = 0.268$, $p = 0.632$) (Fig. 5.2). During the spawning stage, in comparison with less advanced stages, a significant increase ($F = 1.10$, $p = 0.353$) was observed in E2 levels and the highest values were reached (5.97 g/g ww) (Fig. 5.2). After the spawning stage, the mean of the E2 levels in both tissues remained lowest at the developmental stages. No significant variations were observed in E2 levels in digestive gland or in P levels in either tissue. T concentrations in digestive glands varied from 0.28 to 0.59 g/g ww. No significant variations were observed in T levels in gonad during male gametogenesis (Fig. 5.4). In digestive gland, T levels decreased significantly between the indifferent and the second developmental stage (30%: $F = 12.22$, $p = 0.025$), then increased rapidly during the spawning and spent stages (44%: $F = 15.28$, $p = 0.011$) (Fig. 5.3).

Throughout gametogensis in both sexes, gonadal estradiol-17β levels were 2.6–2.8 times higher and testosterone concentrations were 2.7–3.2 times higher than in digestive glands (Figs 5.2 and 5.3). By contrast, gonadal progesterone levels were 2.2 to 2.7 times lower than P levels in digestive gland (Fig. 5.4).

5.6 Discussion

In the St. Lawrence lower estuary, sexual maturation in *Mya arenaria* showed a bimodal reproductive pattern, with a first reproductive period early in spring and a second

in autumn (Roseberry et *al.*, 1991; Tremblay, 1992; Gauthier-Clerc et *al.*, 2002). Similar observations have been reported on the North Atlantic coast from Chesapeake Bay to the Gulf of Maine (Battle, 1932; Shaw, 1962; Brousseau, 1978). Throughout the reproductive period, sexual maturation and energy metabolism are linked to the availability of nutrients such as algae and auspicious water temperatures (Brousseau, 1978; Roseberry et *al.*, 1991; Gauthier-Clerc et *al.*, 2002; Assoi Etchian et *al.*, 2004). Previous studies have reported monthly variations in lipid levels during a complete gametogenic cycle in the clam *Mya arenaria* from May to October (Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2002). In this study, lipid profiles of gonads and digestive glands of molluscs agree with other studies showing that energy reserves are mobilized during the reproductive period for gametes production and gonad development (Pipe, 1987). Since gonads and digestive glands are side by side and intestines twist through the gonad, lipid transfers between the digestive gland, the intestine and the gonad cannot be excluded. Clayton (1996) suggested that macromolecular lipoprotein complexes convey lipids toward tissues for storage in *Mya arenaria*. At the beginning of vitellogenesis (from the indifferent to vitellogenic stages), we observed a depletion in lipid levels in the digestive gland. Lipids stored in the digestive gland are probably mobilized to the gonad to serve in ovocyte growth. In contrast, male lipid profiles in both tissues remained similar during gametogenesis. These results therefore demonstrate that energy support of the digestive gland might be different in male and female *Mya arenaria*.

Previous studies have reported the presence in bivalves of estradiol-17β, testosterone, androstenedione, estrone and progesterone in gonads (Botticelli et *al.,* 1961; Hathaway, 1965; Saliot & Barbier, 1971; Reis-Henriques et *al.,* 1990; Reis-Henriques & Coimbra, 1990; Matsumoto et *al.,* 1997; Morcillo et *al.,* 1999; Morcillo & Porte, 2000; Siah et *al.,* 2002, 2003; Zhu et *al.*, 2003; Gauthier-Clerc et *al.*, 2006), but in many cases their endogenous origin is questioned (Swevers et *al.,* 1991; Le Curieux-Belfond et *al.*, 2005). Progesterone, estradiol-17β and testosterone were characterized and measured during a complete gametogenic cycle in *Mya arenaria* (Siah et *al.*, 2002, 2003; Gauthier-Clerc et *al.,* 2006; Pelletier, 2006 pers. comm.). The present study is the first to describe the progesterone profile in the gonad and digestive gland relative to each gonadal development stage. Gonadal progesterone levels measured in *Mytilus edulis* (5–10 ng/g of gonad) by Reis-Henriques & Coimbra (1990) and in *Mya arenaria* (1–5 ng/g of gonad) by Siah et *al.* (2002) approximate our own data (1.5–8 ng/g of gonad). However, in the present study, the progesterone level in clam digestive gland was three times higher than in gonad. The high levels of progesterone in the digestive gland and the similarity of the steroid profile between the digestive gland and gonad suggest that, in *Mya arenaria,* the digestive gland could synthesize and/or accumulate this steroid. If one accepts the hypothesis that, in bivalves, as in vertebrates, progesterone is the natural precursor to the other sex steroids, the digestive gland plays a key role in steroidogenesis and/or in steroid metabolism. Since gonad and digestive gland are side by side and the intestines twist through the gonad, steroidal transfer cannot be ruled out. Progesterone seasonal patterns being similar in *Mya arenaria* and *Mytilus edulis* of both sexes, Reis-Henriques & Coimbra (1990) and

Siah et *al.* (2002) suggest that progesterone could play the same role for males and females. In the present study, progesterone levels in females were different from males. In females, a drop in progesterone levels was observed at the onset of vitellogenesis in both tissues (only gonad results were significant), whereas no significant variations were observed in either tissue in males (Fig. 5.3). In males, a tendancy towards increased progesterone levels was observed during the spawning stage in both tissues, reinforcing the point that progesterone pattern are likely different between sexes.

The presence and levels of estradiol-17β and testosterone have been demonstrated in many bivalves, including *Mya arenaria* (Gauthier-Clerc et *al.*, 2006), *Mytilus edulis* (Reis-Henriques et *al.*, 1990; Reis-Henriques & Coimbra, 1990; Zhu et *al.*, 2003) and *Patinopecten yessoensis* (Matsumoto et *al.*, 1997; Osada et *al.*, 2004). In *Mya arenaria*, steroid concentrations reported earlier by Gauthier-Clerc et *al.* (2006) show evidence of interannual variations: gonadal estradiol-17β and testosterone levels reported in this study were, respectively, 17 and 45 times more concentrated (Table 5.1). Inter-species and interannual variations were also reported in *Mytilus edulis* and *Patinopecten yessoensis* (Table 5.1). In *M. edulis*, gonadal estradiol-17β concentrations measured by Zhu et *al.* (2003) were approximately 180 times higher than those measured by De Longcamp et *al.* (1974). In *Patinopecten yessoensis,* those measured by Osada et *al.* (2004) were 5 to 10 times higher than levels measured by Matsumoto et *al.* (1997). These sex steroids variations can be explained by changes in metabolic activity and/or sex-dependent and tissue-specific physiological parameters. The presence of many enzymes involved in steroidal metabolism have been reported in various tissues other than the gonad:

Tableau 5.1 Concentrations of estradiol-17β, testosterone and progesterone in the gonads of three marine bivalves, as reported in the literature.

Species	Authors	Bivalves collection	Methods	Progesterone*	Testosterone*	Estradiol-17β*
Mya arenaria (St. Lawrence maritime estuary)	Siah et *al.*, 2002, 2003	Anse à l'Orignal	ELISA	F : ~ 2,9 to 5,0 M : ~ 3,0 to 4,8		
		Trois-Pistoles		F : ~ 0,9 to 4,8 M : ~ 1,4 to 3,5		
		Rimouski harbor		F : ~ 0,07 to 0,41 M : ~ 0,22 to 0,40		
		Les Capucins		F : ~ 2,3 to 4,0 M : ~ 1,8 to 4,8		
	Gauthier-Clerc et *al.*, 2006	Metis Beach	ELISA		F : ~ 0,03 to 0,05 M : ~ 0,02 to 0,03	F : ~ 0,20 to 0,37 M : ~ 0,14 to 0,41
	Present study	Metis Beach	ELISA	F : 1,6 to 5,6 M : 1,5 to 8,2	F : 1,2 to 2,4 M : 1,0 to 1,4	F : 3,5 to 7,5 M : 2,5 to 6,0
Mytilus edulis	De Longcamp et *al.*, 1974	Luc sur Mer	RIA		F : 2,1(II) and 5,4 (III) M : 1,4 (II) and 43 (III)	F : 4,2 (III) M : 4,9 (III)
	Reis-Henriques & Coimbra, 1990	Lagoon of Aveiro	RIA	F: ~ 4 to 30 M: ~ 4,0 to 4,3		

Tableau 5.1 Concentrations of estradiol-17β, testosterone and progesterone in the gonads of three marine bivalves, as reported in the literature (suite).

Species	Authors	Bivalves collection	Methods	Progesterone*	Testosterone*	Estradiol-17β*
Mytilus edulis	Zhu et al., 2003	Long Island Sound	RIA HPLC			854 165 ± 54
Patinopecten yessoensis	Matsumoto et *al.*, 1997	Onagawa Bay (Miyagi prefecture)	HPLC			F: ~ 0,6 to 1,1 M: ~ 0,4 to 0,55
	Osada et *al.*, 2004		HPLC			F: ~ 1,7 to 4,8 M: ~ 1,7 to 4,7

Legend : "*" ng/g wet weigt; "F" female; "M" male; "II" stage II; "III" stage III; "~" indicates values estimated visually on figures of cited papers.

nephridium epithelia, digestive diverticulum intestine (Mori et *al.*, 1966), adductor muscle, elongated epitheloid tissues adjacent to the visceral ganglion (Mori, 1965a, 1965b), digestive glands and gills (Le Curieux-Belfond et *al.*, 2001). Janer et *al.* (2005) demonstrated tissue-specific pathways in androgen metabolism and the ubiquity of some androgen biotransformation processes in invertebrates. Steroids can be involved in controlling other physiological functions such as digestion or respiration, and steroid levels could be an indication of specific physiological activities. Sex steroid variations can also be explained by an exogenous source (Swevers et *al.*, 1991). Steroids produced naturally by vertebrates and steroid-like (phytoestrogen, phytoandrogen, etc.) compounds may be present in water, sediment and food (Langston et *al.,* 2005). Le Curieux-Belfond et *al.* (2005) have investigated, *in vivo*, the bioaccumulation and metabolism of estradiol-17β in the oyster *Crassostrea gigas*. Estradiol-17β dissolved in seawater and injected into the adductor muscle was rapidly transported to and accumulated by gonad, gills, mantle, labial palps, digestive gland (concentrated up to 31 times after 48 h) (Le Curieux-Belfond et *al.*, 2005). Sediment-bound estrogens, however, were likely considered to be a major contributor to this accumulation in benthic invertebrates, particularly where environmental concentrations are elevated (Langston et *al.,* 2005). Upstream of our sampling zone are the "Reford Gardens," a local tourist attraction that receives over 40 000 visitors every summer. Until 2006, untreated domestic wastewater was discharged directly into the St. Lawrence River. Considering the current, effluents from the Reford Gardens could have contaminated our sampling site with exogenous vertebrate steroids. In bivalves, cytochrome P450 is a key enzyme in the oxidative metabolism of a diverse array of

xenobiotic and endogenous substrate. Le Curieux-Belfond et *al.* (2005) proposed estradiol-17β as a potential contaminant in seawater; its bioaccumulation and transformation into estrone by 17β-HSD-like activity could therefore be a potential biomarker of endocrine disruption. Some physiological perturbations in marine bivalves could be linked to steroids and steroid-like contaminants as estrogenic or anti-estrogenic compounds (Gauthier-Clerc et *al.*, 2002).

In the present study, similar fluctuations between estradiol-17β and testosterone in females and between estradiol-17β and progesterone in males were also reported. Simultaneously, a peak in progesterone and estradiol-17β in both tissues was observed in males at the spawning stage. In females, by contrast, estradiol-17β and testosterone levels in gonads drop at the onset of vitellogenesis and remain quite stable throughout sexual maturation, with a slight decrease in the digestive gland after spawning. These results suggest that the enzymatic activity involved in steroid metabolism (metabollization and/or detoxification of lipophilic substances) is different in males and females. In males, the principal enzymatic activity is the conversion of progesterone into estradiol-17β by 17α-hydrolase (conversion of progesterone into 17α-hydroxyprogesterone), 17, 20-lyase (conversion of 17α-hydroxyprogesterone into estrone) and 17β-HSD (conversion of estrone into estradiol-17β). The main enzymatic activity in females is aromatization. In vertebrates, aromatase-like activity converts androstenedione into estrone and testosterone into estradiol. Osada et *al.* (2004) reported good aromatase activity in *Patinopecten yessoensis* gonads, with maximum activity detected before spawning in both sexes. In *Mya arenaria*, Gauthier-Clerc et *al.* (2006) suggested that the aromatization pathway to convert

testosterone into estradiol could be marginal in the gonad. In addition, and consistent with the findings for vertebrates, Janer et *al.* (2005) observed sexual dimorphism in androgen metabolism in invertebrates. In the context of steroidogenesis, 17α-hydrolase, 17, 20-lyase and perhaps 17β-HSD could regulate aromatase activity by controlling levels of substrates and products of these enzymes. In a detoxification context, they may prepare compounds for later conjugation and elimination.

In conclusion, we have demonstrated that energy support of the digestive gland for gametogenesis might not be identical in male and female *Mya arenaria.* The mechanism of nutrient transfer from the digestive system to the gonad acini and developing oocytes remains to be investigated. A comparison of our results and those of other studies show large interannual variations in estradiol-17β and testosterone profiles in *Mya arenaria.* Inter-species and interannual variations were also reported in other bivalves, raising many questions about the role of endocrines and the endogenous origin of steroids. It now appears necessary to check for and locate the clam enzymes involved in steroid metabolism and to show that the enzyme in question is specific to steroidogenesis.

5.7 Acknowledgments

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CHAPITRE 6 : DISCUSSION GÉNÉRALE ET PERSPECTIVES

Mya arenaria (Linnaeus 1758) est une espèce d'intérêt économique et écotoxicologique. Elle fait l'objet de pêche à pied, artisale et commerciale (Wallace, 1997; Department of Fisheries and Oceans Canada, 1998), et est utilisée à titre d'espèce sentinelle (Pellerin-Massicotte, 1997; Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2002; Blaise et *al.*, 1999). Une exposition chronique à certains polluants conduit au dérèglement du système neuroendocrinien, du système immunitaire et de l'appareil reproducteur (Blaise et *al.*, 1999, 2002; Gauthier-Clerc et *al.*, 2002; Siah et *al.*, 2003). Un déficit d'information concernant la physiologie des organismes sentinelles rend l'interprétation des résultats écotoxicologiques contraignante. Notre thématique de recherche tend à répondre à ce besoin d'information. Considérant que la reproduction des bivalves semble être contrôlée par les neurosécrétions ganglionnaires et les stéroïdes (Motavkine & Varaskine, 1989), nous nous somme intéressé à l'étude du système nerveux, du système reproducteur et à leurs interactions chez *Mya arenaria* (Mollusque bivalve). Plus précisément, nous avons étudié :

1- la physiologie et la composition cellulaire du système nerveux et de la masse viscérale chez *Mya arenaria*;

2- les relations sérotoninergiques qui existent entre le système nerveux et le système reproducteur chez *Mya arenaria*;

3- les variations du niveau des hormones stéroïdiennes (progestérone, testostérone et 17β-oestradiol) au cours du cycle reproducteur chez *Mya arenaria*.

6.1 Anatomie du système nerveux de *Mya arenaria*

Le système nerveux de *Mya arenaria* fut étudié pour la première fois en 1909 par Vlès. Pour ses descriptions, Vlès (1909) s'appuya sur le mémoire de Duvernoy (1847) décrivant le système nerveux des mollusques Acéphales. Nos travaux confirment que l'analyse morpho-anatomique du système nerveux et l'examen histologique des ganglions chez *Mya arenaria* sont analogues avec ceux des autres bivalves (List, 1902; Makman & Stefano, 1984; Matsutani & Nomura, 1984; Benomar et *al.*, 2003; Zaixso, 2003). Le système nerveux présente un plan de symétrie sagittal. Il est formé de trois (3) paires de ganglions : les ganglions cérébroïdes situés au niveau de l'œsophage; les ganglions viscéraux localisés contre la face ventrale du muscle adducteur postérieur; les ganglions pédieux placés à la base du pied. Les ganglions pédieux et viscéraux sont fusionnés, tandis que les ganglions cérébroïdes sont réunis dorsalement au moyen de la commissure cérébrale. Les ganglions pédieux et viscéraux sont connectés aux ganglions cérébroïdes à l'aide des connectifs cérébro-pédieux et cérébro-viscéraux. L'architecture interne des ganglions se compose, de l'extérieur vers l'intérieur, du périneurium, du cortex ganglionnaire et du neuropile médian.

En comparant nos résultats avec ceux de Vlès (1909), plusieurs différences ont été rencontrées.

-Notre étude montre l'existence d'un rapprochement des connectifs cérébro-viscéraux du côté antérieur du muscle rétracteur postérieur sur une courte distance d'environ 1-2mm. Au niveau de l'accolement, les deux connectifs ne sont aucunement fusionnés.

-Vlès (1909) suggère la présence, chez *Mya arenaria*, de deux paires de ganglions secondaires : les ganglions médians situés le long des connectifs cérébro-viscéraux et les ganglions siphonaux résidant à la base du siphon. Nous n'avons jamais retrouvé ces deux paires de ganglions lors de nos dissections, ce qui nous a permis de rejeter ces deux affirmations.

- Vlès (1909) sous-entend l'existence d'une jonction entre les nerfs palléaux postérieurs et antérieurs, formant de la sorte le cercle palléal. L'analyse morpho-anatomique ne nous a pas permis de confirmer cette jonction.

- En revanche, notre étude montre la présence : (1) d'un tronc nerveux émanant du connectif cérébro-viscéral et innervant le muscle rétracteur postérieur; (2) de plusieurs troncs nerveux dérivant des connectifs cérébro-viscéraux et innervant la gonade; (3) de nerfs innervant le muscle adducteur postérieur; (4) de nerfs cérébro-branchiaux; et (5) de nerfs buccaux;

- Enfin, nous avons aussi redécrit la structure du cordon nerveux palléal postérieur innervant le siphon et le manteau.

6.2 Anatomie de la masse viscérale de *Mya arenaria*

Chez *Mya arenaria*, la masse viscérale présente une organisation générale semblable à celles des autres bivalves, et est composée du système digestif, du système musculaire, du système nerveux et du système reproducteur. Les deux systèmes principaux, digestif et reproducteur, sont étroitement entrelacés bien que tout à fait distincts l'un de l'autre. Le développement de la gonade autour de l'intestin optimise le potentiel de transfert des nutriments vers les gamètes en développement. Le système musculaire est constitué du pied, des muscles rétracteurs et de muscles transversaux. L'ensemble de ce système maintient l'intégrité de la masse viscérale et le déploiement du pied. La gamétogenèse de *Mya arenaria* a fait l'objet, par le passé, de différentes études histologiques (Coe & Turner, 1938; Rogers, 1959; Shaw, 1962; Brousseau, 1976; Potts, 1993; Gauthier-Clerc et *al.*, 2002). Dans la présente étude, la description de l'évolution de la gamétogenèse, chez les mâles et les femelles, s'effectue au moyen de cinq (5) stades bien définis : indifférencié, développement, mûr, ponte et passé. Les travaux de Coe & Turner (1938) effectués chez *Mya arenaria* ont montré que les alvéoles gonadiques sont constituées de deux types cellulaires : les cellules de réserve intra-tubulaires ("cellules folliculaires") et les cellules de la lignée germinale. La composition cellulaire des alvéoles gonadiques change de manière importante tout au long de la gamétogenèse : au début de la gamétogenèse, période de repos sexuel, les alvéoles sont principalement composées des cellules de réserves. Par la suite, lors de la gamétogenèse, les cellules de réserves sont substituées par les cellules de la lignée germinale en développement. Nos travaux ont montré qu'il existe, dans les alvéoles mâles,

un autre type cellulaire : les cellules somatiques de soutien intratubulaires. Ces cellules, appelées chez les vertébrés « cellules de Sertoli », sont uniformément distribuées dans les tubules.

6.3 Localisation de la 5-HT et implication dans la ponte

La sérotonine (5-hydroxytryptamine ou 5-HT) est présente chez de nombreux bivalves (Dahl et *al.,* 1966; York & Twarog, 1973; Salanki et *al.*, 1974; Stefano et *al.*, 1976; Smith, 1982; Martinez et *al.*, 1996), dont *Mya arenaria* (Welsh & Moorhead, 1960). Elle est impliquée dans de nombreux processus physiologiques, à savoir l'activité des muscles (York & Twarog, 1973), du cœur (Painter & Greenberg, 1982; Croll et *al.*, 1995) et du siphon (Ram et *al.*, 1999), la relaxation tonique des muscles lisses (Gies, 1986) ainsi que l'activité ciliaire (Gosselin, 1961; Stefano & Aiello, 1975; Stefano et *al.,* 1977; Malanga & Poll, 1979; Smith, 1982; Scheide & Dietz, 1983; Croll et *al.*, 1995). Nos travaux ont montré la présence de grandes quantités de cellules sérotoninergiques à l'intérieur du système nerveux de *Mya arenaria* (ganglions cérébraux, viscéraux et pédieux), ce qui confirme son rôle de premier plan comme neurotransmetteur. Les cellules sérotoninergiques, dont la majorité sont localisées dans les ganglions cérébraux et pédieux, semblent être la principale source de monoamine chez *Mya arenaria*. Cette tendance est mentionnée chez d'autres bivalves (Dahl et *al.*, 1966; Sweeney, 1968; Stefano & Aiello, 1975; Matsutani & Nomura, 1984; Paulet et *al.*, 1993; Croll et *al.,* 1995; Campioni et *al.*, 1997). Au sein des ganglions viscéraux, les corps cellulaires immunoréactifs sont regroupés

en nodules, appelés glomérules, circonscrits au niveau des racines des nerfs branchiaux. Cette disposition est similaire à celle retrouvée chez *Venus verrucosa* (Siniscalchi et *al.*, 2004). Chez plusieurs espèces de bivalves, des fibres sérotoninergiques ont été observées dans la gonade (Matsutani & Nomura, 1984, 1986b; Ram et *al.*, 1992; Paulet et *al.*, 1993; Croll et *al.*, 1995; Campioni et *al.*, 1997; Masseau et *al.*, 2002; Siniscalchi et *al.*, 2004), tout comme chez *Mya arenaria* (présente étude : Garnerot et *al.*, 2006). Notre étude montre également l'existence de fibres sérotoninergiques dans les muscles et les branchies. Les présences de cellules sérotoninergiques à la base des nerfs branchiaux (dans les ganglions viscéraux), de fibres sérotoninergiques au niveau des branchies et à la périphérie des alvéoles gonadiques confirment la relation existant entre le système nerveux et les tissus périphériques. Ces résultats suggèrent une implication de la sérotonine dans le contrôle de certaines fonctions physiologiques, telles que la respiration, la nutrition et la reproduction.

Chez les bivalves, les rôles identifiés de la 5-HT dans la reproduction sont l'induction de la ponte et l'émission des gamètes, la parturition (Fong & Warner, 1995; Fong et *al.*, 1996, 1998), la stimulation de la mobilité des spermatozoïdes (Kadam & Koide, 1990; Kadam et *al.*, 1991) et le déclenchement de la reprise de la méiose chez les oocytes bloqués en prophase-I de méiose. Chez *Mya arenaria*, l'application externe de 5-HT provoque les mouvements de ponte. Par contre, seuls quelques mâles matures émettent des spermatozoïdes (présente étude : Garnerot et *al.*, 2006). Les connaissances acquises sur la physiologie de la masse viscérale nous permettent maintenant d'expliquer les différences mâle/femelle obtenues. L'application de 5-HT stimulerait les mouvements musculaires spécifiques à la ponte, mais n'induirait pas la libération des gamètes matures. L'émission

des spermatozoïdes est, sans doute, un effet indirect de la 5-HT sur la relaxation tonique du système musculaire de la masse viscérale (muscles transversaux, muscles rétracteurs du pied et cellules myoépithéliales) et sur la mobilité des spermatozoïdes. Chez *Spisula solidissima*, la 5-HT stimule la mobilité des spermatozoïdes immobilisés par un traitement au froid (Kadam & Koide, 1990; Kadam et *al.*, 1991). Lorsque l'effet de la 5-HT s'estompe, le système musculaire de la masse viscérale, regagnant son état initial, se contracte. Ce rétrécissement occasionne une augmentation de la pression dans les tubules gonadiques, supportant de la sorte la libération partielle du sperme dans la cavité palléale, puis dans le milieu.

Les résultats chez les espèces *Tapes philippinarum* (Campioni et *al.*, 1997) et *Placopecten magellanicus* (Croll et *al.*, 1995) établissent que les cellules neurosécrétrices des ganglions cérébro-pleuraux (GCP) ont une plus forte immunoréactivité envers la sérotonine (5-HT) que celles des ganglions viscéraux (GV), attestant ainsi d'une application possible des GCP dans l'innervation sérotoninergique de la gonade. Chez *Argopecten purpuratus*, une diminution du taux de 5-HT dans les GCP durant la ponte, aucunement rencontrée dans les GV (Martinez et *al.*, 1996), renforce cette idée. À l'inverse, Matsutani & Nomura (1984) suggèrent un contrôle de l'innervation sérotoninergique de la gonade par les ganglions viscéraux, chez *Patinopecten yessoensis*. Chez *Pecten maximus*, Paulet et *al.* (1993) ont montré la présence de cellules sérotoninergiques dans les lobes accessoires des ganglions viscéraux, d'ou provient l'innervation gonadique. Cependant chez ces deux espèces, l'innervation sérotoninergique de la gonade dérive des ganglions viscéraux et des connectifs cérébro-viscéraux (Matsutani & Nomura, 1984; Paulet et *al.*, 1993). Dans la

présente étude, nous avons déterminé que l'innervation sérotoninergique de la gonade de *Mya arenaria* provient des connectifs cérébro-viscéraux, comme déjà rapporté chez *Venus verrucosa* (Siniscalchi et *al.*, 2004). La présence dans le cortex des ganglions cérébraux et l'absence dans le cortex dans les ganglions viscéraux de cellules sérotoninergiques suggèrent que l'innervation gonadique chez *Mya arenaria* pourrait être modulée par les ganglions cérébraux.

6.4 Variation des taux de lipide, progestérone, testostérone et 17β-oestradiol dans la gonade et la glande digestive de *Mya arenaria*

La première étape de la biosynthèse des hormones stéroïdiennes est la conversion du principal précurseur des stéroïdes, le cholestérol, en prégnénolone (Andrew et *al.,* 1998). Chez *Mya arenaria,* le cholestérol est le composant le plus abondant des stérols, donc aisément biodisponible, et est mobilisé à partir de gouttelettes lipidiques libres (Jarzebski, 1985). Nos travaux montrent que les réserves lipidiques sont mobilisées pendant l'activité reproductrice pour le développement des gamètes et de la gonade. Chez les femelles, les lipides stockés dans la glande digestive sont rapidement transférés vers la gonade, pour y être utilisés comme source d'énergie par les ovocytes en croissance. En revanche, chez les mâles, ce transfert n'est pas observé, ce qui suggère une utilisation des réserves énergétiques différente entre les mâles et les femelles.

Chez les bivalves, les hormones stéroïdiennes seraient susceptibles de contrôler le stockage énergétique, la glycolyse, la respiration et la maturation sexuelle (Mori, 1969,

1980; De Longcamp et *al.*, 1974; Reis-Henriques et *al.*, 1990; Reis-Henriques & Coimbra, 1990; Matsumoto et *al.*, 1997; Gauthier-Clerc et *al.*, 2002). Récemment au sein de notre laboratoire, des recherches sur le contrôle de la reproduction ont montré la présence des hormones stéroïdiennes (17β-oestradiol, testostérone et progestérone) dans la gonade de *Mya arenaria* (Siah et *al.*, 2002, 2003; Pelletier, 2006, pers. comm.). Nos travaux montrent que les niveaux de progestérone gonadique sont analogues à ceux retrouvés par Siah et *al.*, (2002). Par contre, les niveaux de progestérone dans la glande digestive sont trois (3) fois supérieurs à ceux dans la gonade. Les niveaux élevés de progestérone dans la glande digestive et la similitude des profils entre la glande digestive et la gonade suggèrent une synthèse et/ou un stockage dans la glande digestive. La progestérone étant le précurseur de la synthèse des œstrogènes, ses réserves pourront par la suite être utilisées lors de la conversion de la progestérone en 17β-oestradiol. En ce qui concerne les taux de 17β-oestradiol et de testostérone intragonadiques, nos travaux montrent qu'ils sont, respectivement, 17 et 45 fois supérieures à ceux retrouvés par Gauthier-Clerc et *al.* (2006). De telles variations ont déjà été rapportées dans la littérature chez *Mytilus edulis* et *Patinopecten yessoensis* (De Longcamp et *al.*, 1974; Matsumoto et *al.*, 1997; Zhu et *al.*, 2003; Osada et *al.*, 2004) et peuvent s'expliquer de deux manières : une source exogène de stéroïdes et/ou des variations interannuelles d'activités métaboliques : (1) les stéroïdes produits naturellement par des vertébrés et les substances proches structurellement (phytoestrogène, phytoandrogène, etc.) sont retrouvés dans l'eau, le sédiment et la nourriture (Langston et *al.,* 2005), et sont rapidement bioaccumulés dans l'organisme des bivalves (Le Curieux-Belfond et *al.,* 2005). Le Curieux-Belfond et *al.* (2005) proposent de

considérer la 17β-oestradiol comme un contaminant potentiel en eau de mer et, par conséquent, sa bioaccumulation et sa transformation en oestrone par la 17β-HSD pourraient être d'excellents biomarqueurs des perturbateurs endocriniens. (2) la présence des enzymes clés de la stéroïdogenèse est rapportée dans divers tissus : gonade, muscle, branchies, glande digestive (Mori et *al.*, 1965a, 1965b, 1966; Le Curieux-Belfond et *al.*, 2001), suggérant un contrôle par les hormones stéroïdiennes de plusieurs fonctions physiologiques. Pour finir, de fortes corrélations entre les variations de 17β-oestradiol versus testostérone chez les femelles et de 17β-oestradiol versus progestérone chez le mâle suggèrent une activité métabolique (stéroïdogenése et/ou désintoxication) différente entre les deux sexes. La voie enzymatique dominante serait, chez le mâle, la conversion de la progestérone en17β-oestradiol par la 17α-Hydrolase et, chez la femelle, l'aromatisation de la testostérone en 17β-oestradiol.

6.5 Conclusions et perspectives

Chez les bivalves, la reproduction serait contrôlée par les neurosécrétions ganglionnaires et les hormones stéroïdiennes (Motavkine & Varaskine, 1989). Les travaux effectués dans le cadre de cette thèse s'inscrivent dans cette thématique de recherche et nous ont permis de compléter certaines connaissances sur l'anatomie et la la physiologie de *Mya arenaria* (mollusque bivalve endobenthique). Nous avons aussi démontré l'existence d'un lien entre le système nerveux neuroendocrinien et le système reproducteur. Cependant,

ces constats plaident en faveur de futures recherches afin de mieux comprendre les fonctions du système nerveux dans la régulation de la reproduction.

Chez *Mya arenaria,* l'étude morpho-anatomique de la masse viscérale a montré que celle-ci suit une organisation générale similaire à celles des autres bivalves. Le système reproducteur est étroitement lié aux autres systèmes, en particulier le système digestif. L'étude histologique a permis de caractériser 5 stades de développement de la gamétogénèse. Coe & Turner (1938) furent les premiers à décrire la constitution cellulaire des alvéoles gonadiques. Elles se composent des cellules somatiques de réserve, strictement nutritives, et des cellules de la lignée germinale. Durant cette recherche, nous avons mis en évidence la présence, chez le mâle, de cellules somatiques de soutien (ou cellules de Sertoli), mais leurs fonctions restent encore à être élucidées. La description de la gamétogénèse en microscopie électronique confirmerait la présence et la fonction des cellules somatiques de soutien dans la gamétogénèse.

La présence de fibres et de cellules sérotoninergiques dans les ganglions et la gonade de *Mya arenaria* indique que la 5-HT est impliquée dans la neurotransmission périphérique et centrale. Soulignons qu'aucune étude n'a encore établi, avec certitude, l'existence d'une relation entre l'une des paires de ganglions et l'innervation sérotoninergique de la gonade. Des techniques de traçage moléculaire neuroanatomique associées à des analyses immunohistochimiques contribueraient à connaître l'origine exacte de l'innervation peptidinegique de certains tissus périphériques.

Chez les bivalves, la 5-HT interviendrait, au niveau de la reproduction, sur l'induction de la ponte et de la parturition, la stimulation de la mobilité des spermatozoïdes et le développement des oocytes bloqués en prophase-I de méiose (GVBD). Nos résultats sur l'induction sérotoninergique de la ponte chez *Mya arenaria* n'étant pas sans équivoque, de futures recherches seront nécessaires. Afin de comprendre la fonction physiologique de la 5-HT dans la gonade, nous suggérerons (1) de vérifier l'action de la 5-HT sur la GVBD et la mobilité des spermatozoïdes, (2) de caractériser, localiser et quantifier les récepteurs sérotoninergiques dans la gonade, et plus précisément au niveau des gamètes.

Récemment au sein de notre laboratoire, les recherches sur les perturbateurs endocriniens et la régulation de la reproduction se sont intéressées à l'étude des variations des taux d'hormones stéroïdiennes en fonction de la maturité gonadique (Siah et *al.*, 2002, 2003; Gauthier-Clerc et *al.*, 2006). Nos travaux ont montré que les profils d'hormones stéroïdiennes mesurés dans la gonade de la mye pouvaient fortement varier d'une étude à l'autre, ce qui soulève encore de nombreuses questions. Des recherches concernant (1) les variations de l'activé des enzymes clés intervenant dans la stéroidogenèse, en particulier la 17β-HSD, (2) les variations des taux endogènes et (3) la bioaccumulation des stéroïdes en mésocosme (en circuit fermé avec contrôle de paramètres abiotiques) devraient permettre d'approfondir davantage nos connaissances sur l'implication des hormones stéroïdiennes dans le contrôle de la reproduction.

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ANNEXES

Annexe 1 : Protocole de localisation de la sérotonine par immunohistochimie

(les étapes pour d'autres molécules [actine, α-tubuline, etc,…] sont les mêmes)

1 – Technique de congélation des tissus

a - Inclusion des tissus et coupes histologiques

Étapes :

1	Incubation des morceaux de gonade dans une solution de protéase type IV à 0,5 % [Protéase diluée dans une solution de 0,45 M NaCl + 20 mM tampon phosphate; pH=7,2] → 5 – 10 min
2	Rinçage du tissu dans du PBS (tampon phosphate salin) [40 mM de Na_2HPO_4 + 140 mM de NaCl; pH=7,2]
3	Fixation du tissu paraformaldéhyde 4 % → 4 heures à 4 °C
4	2 rinçages avec du tampon cacodylate 0,2 M [0,2 M Cacodylic acide + 0,3 M NaCl ; Ph=7,5] → 30 minutes à 4 °C
5	Rinçage dans une solution de triton X-100 4% dilué dans du PBS → 30 minutes à 4 °C
6	Incubation dans une solution de sucrose 30 % → 1 nuit à 4 °C
7	Incorporation des tissus dans de la Cryomatrix
8	Congélation des tissus sur la Cryobare du Cryotome (possibilité de stockage des tissus congelés à –80 °C ou –20 °C)
9	Réaliser des coupes histologiques avec un cryotome - Coupe entre 16 à 20 µm pour les ganglions - Coupe entre 5 à 7 µm pour la gonade
10	Déposer les coupes sur des lames histologiques

b - Traitement des tissus

Étapes :

1	Séchage des lames à l'air libre (augmente la fixation des tissus sur la lame) → 30 minutes et +
2	2 rinçages des lames dans du PBS → 30 minutes
3	2 rinçages dans une solution de triton X-100 4 % dilué dans du PBS → 30 minutes
4	Incubation dans une solution d'ASD (anti Sérum diluant) [0,5% de triton X-100 + 1-2 % de sérum bovin dilué dans du PBS] **Témoins positifs* :** Incubation dans une solution d'ASD dans laquelle a été préalablement dilué le neuropeptide [10^{-4}] → 1 heure à 4 °C

Il est indispensable de s'assurer, par des **témoins positifs et négatifs,** de la fiabilité des réactions anticorps / antigènes.

*** Les témoins positifs permettent de vérifier la spécificité de l'anticorps avec son antigène**

5	Destruction de l'activité peroxydasique endogène par une solution de H_2O_2 [30 volumes] à 3% → 10 minutes à 1 heure à 4 °C
6	Incubation dans une solution d'ASD dans laquelle l'anticorps antisérotonine a été dilué (rabbit polyclonal anti-5HT) [dilution 1/10.000] **Témoins négatifs** :** Incubation dans une solution d'ASD sans anticorps → 72 heures à 4 °C

**** Les témoins négatifs permettent de mettre en évidence les marquages non spécifiques. Pour faire disparaître une partie du bruit de fond, il est possible de faire une contre coloration (voir « Technique d'inclusion dans de la paraffine »).**

7 Rinçage des lames dans une solution de triton X-100 0,5 % [0,5 % de triton X-100 dans du PBS]

→ 6 heures à 4 °C

8 Incubation dans une solution d'ASD dans laquelle l'anticorps anti-anticorps de lapin, couplé à une peroxydase, a été dilué (Anti-Rabbit IgG (whole molecule) – peroxidase antibody produced in goat) [dilution 1/400]

→ 24 heures à 4 °C

9 Rinçage des lames dans une solution de triton X-100 0,5 % [0,5 % de triton X-100 dans du PBS]

→ 6 heures à 4 °C

10 Révélation de la peroxydase (la peroxydase est couplée à l'anti-anticorps de lapin)

- Incubation dans une solution de révélation [3mg de diaminobenzidine dans 10 ml de tampon Tris-HCl 0,05 M, pH 7.6]

→ 1 à 2 heures

11 Ajout de H_2O_2 [30 volumes] dans la solution de révélation

→ quelques minutes jusqu'à coloration des lames

12 Rinçage des lames à l'eau distillée

13 Montage des lamelles avec un milieu de montage aqueux (GELTOL [Thermo Electron Corporation, Pittsburgh])

14 Observation des lames au microscope

2 – Technique d'inclusion dans de la paraffine

a - Inclusion des tissus et coupes histologiques

Étapes :

1 Fixation des tissus dans une solution de Davidson (solution fille)

→ 24 - 48 heures à 4 °C (chambre froide)

Solution de Davidson (solution mère) :

- Solution A : mélanger dan l'ordre 400 ml de glycérol + 800 ml de formol 40 % + 1200 ml d'éthanol 95 % + 1200 ml d'eau de mer filtrée [stocker à 4 °C]
- Solution B : acide acétique

Solution de Davidson (solution fille) :

→ Mélanger 9 volumes de A avec 1 volume de B

2 Déshydratation des tissus

a- Éthanol 80 %

→ 48 heures

b- Éthanol 95 %

→ 48 heures

c- Éthanol 100 %

→ 48 heures

d- Buthanol 100 %

→ 1 semaine

3 Inclusion des tissus dans la paraffine

a- Mettre les tissus dans un premier bain de paraffine liquide [60 °C]

→ 24 heures

b- Mettre les tissus dans un second bain de paraffine liquide [60 °C]

→ 24 heures

c- Inclure les tissus dans des blocs de paraffine liquide [60 °C]

d- Laisser refroidir à température ambiante puis au réfrigérateur

4 Réaliser des coupes histologiques avec un microtome

- Coupe entre 3 à 5 µm pour les ganglions et les gonades

5 Déposer les coupes sur des lames histologiques

6 Placer les lames 1 nuit à l'étuve [37 °C] (augmente la fixation des tissus sur la lame)

b - Traitement des tissus

Étapes :

1 Protocole de déparaffinage des lames

a- Xylène 100 % (dissoudre la paraffine)

→ 5 minutes

b- Xylène 100 %

→ 5 minutes

c- Éthanol 100 % (éliminer le xylène des lames)

→ 5 minutes

d- Destruction de l'activité peroxydasique endogène grâce à une solution de méthanol 100 % contenant 3 % de H_2O_2 [30 volumes]

→ 30 minutes

e- Éthanol 100 %

→ 5 minutes

2 Protocole de réhydratation des lames

a- Éthanol 95 %

→ 5 minutes

b- Éthanol 70 %

→ 5 minutes

c- H_2O distillée

→ 5 minutes

3 2 rinçages des lames dans du tampon Tris 1 [50 mM Tris, 150 mM NaCl, 0,25 % w/v gélatine, 0,5 % v/v Triton X-100, pH 7.4]

Témoins positifs* : Incubation dans deux solutions de tampon Tris 1 dans laquelle a été dilué du neuropeptide [10^{-4}]

→ 5 minutes

Il est indispensable de s'assurer, par des **témoins positifs et négatifs,** de la fiabilité des réactions anticorps / antigènes.

*** Les témoins positifs permettent de vérifier la spécificité de l'anticorps avec son antigène**

4 Incubation dans une solution de Tris 1 dans laquelle l'anticorps antisérotonine a été dilué (rabbit polyclonal anti-5HT) [dilution 1/500]

Témoins négatifs :** Incubation dans une solution de Tris 1 sans anticorps

→ toute la nuit à 4 °C

**** Les témoins négatifs permettent de mettre en évidence les marquages non spécifiques.**

5 2 rinçages des lames dans du tampon Tris 1

→ 5 minutes

6 Incubation dans une solution de Tris 2 [50 mM Tris, 150 mM NaCl, pH 7.4] dans laquelle l'anticorps anti-anticorps de lapin, couplé à une peroxydase, a été dilué (Anti-Rabbit IgG (whole molecule) – peroxidase antibody produced in goat) [dilution 1/100]

→ 2 heures à température ambiante

7 2 rinçages des lames dans du tampon Tris 1

→ 5 minutes

8 Révélation de la peroxydase (la peroxydase est couplée à l'anti-anticorps de lapin)

- Incubation dans la solution de révélation [3mg de diaminobenzidine (chromogène) dans 10 ml de tampon Tris-HCl 0,05 M, pH 7.6]

→ 30 minutes

9 - Ajout de H_2O_2 [30 volumes] dans la solution de révélation

→ quelques minutes jusqu'à coloration des lames

10 2 rinçages des lames dans du tampon Tris 2

→ 5 minutes

11 Déshydratation et contre coloration des lames

a- Éthanol 95 %

→ 5 minutes

B- Éthanol 70 % contenant 0,2 % de vert lumière (colorant)

→ quelques secondes

c- Éthanol 100 %

→ 5 minutes

d- Éthanol 100 %

→ 5 minutes

12 Montage des lames

a- Xylène 100 %

→ 5 minutes

b- Xylène 100 %

→ 5 minutes

d- Montage des lamelles sur les lames avec du Cytoseal [VWR Scientific]

13 Observation des lames au microscope