D 2015

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

A QUALITATIVE AND QUANTITATIVE STEREOLOGICAL STUDY OF THE MICROSCOPIC MORPHOLOGY OF THE NERVOUS GANGLIA OF THE BIVALVE PEPPERY FURROW SHELL (*SCROBICULARIA PLANA*)

SUKANLAYA TANTIWISAWARUJI
TESE DE DOUTORAMENTO APRESENTADA AO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
DA UNIVERSIDADE DO PORTO EM
CIÊNCIAS BIOMÉDICAS

SUKANLAYA TANTIWISAWARUJI

A qualitative and quantitative stereological study of the microscopic morphology of the nervous ganglia of the bivalve peppery furrow shell (*Scrobicularia plana*)

Tese de candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Eduardo Jorge Sousa da Rocha

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

Coorientador: Uthaiwan Kovitvadhi

Categoria – Professor Associado

Afiliação – Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand

Coorientador: Maria João Tomé da Rocha

Categoria – Professor Auxiliar

Afiliação – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal

OBJECTIVES

The microscopic morphology of bivalves is still poorly studied, particularly in quantitative terms, and that of peppery furrow shell (*Scrobicularia plana*) is no exception. With this scenario in mind, the general aim of the Thesis was to enrich the knowledge of the bivalves' central nervous system, reviewing the state of art and unveiling new qualitative and quantitative cytological and histological data in *S. plana*, namely using technical approaches that were either never or seldom used in the neuroscience of bivalves. Based on these broad objectives, the specific aims were:

To better understand the microscopic anatomy of the central nervous system, by conducting an unprecedented three-dimensional (3D) computer assisted reconstruction of *S. plana* ganglia, while estimating for the first time their total volumes and surface areas in the 3D models, and the relative volumes of cortex and medulla. Within this scope, we wanted to start studying whether sexual differences exist regarding ganglion size and its internal composition, in view of the key roles of the neurosecretory neurons in governing gonadal maturation, particularly in females.

To examine the general histology and cytology of the neural cells in *S. plana*, registering its main aspects and looking after any still undescribed features, while trying a first identification of potentially neurosecretory neurons — *viz*. those putatively producing serotonin and dopamine — comparatively across the cerebral, pedal and visceral ganglia, and considering the gonadic sex.

To start studying, with design-based stereological methods, theorized structural differences between the various nervous ganglia types, specifically connected with intrinsic features of those elements, like their different body locations and distinct functions, and in view of the gender and gonadal maturation staging (comparing adults with maturing *vs*. spent gonads). The expectable neural cell targets would be those identified by the previous histological and ultrastructural study.

To work further on the possible effects of the animal's gender in the structure of the central nervous system, and, in parallel, to start exploring the hypothesis that if adult bivalves, including *S. plana*, continue to grow during its lifespan, then its nervous ganglia may continue to develop with age — at least until senescence, if it happens — and eventually gains in cellularity and other morphofunctional changes plausible occur. Implementing design-based stereological strategies, one aim was thus to search for changes in ganglion size and in number of distinct neural elements.

To make the first experimental assay for start testing the hypothesis that if both the nervous ganglia signalling and oestrogens play a modelling role in the bivalves' gonadal maturation and spawning — and all indicates that such impacts are not equal ways in females and males — then waterborne exposure to xenoestrogens can cause morphofunctional impacts in the central nervous system. This aim makes even more sense if the works proposed for other goals generate evidences supporting the hypothesis that sex may shape the structure of the *S. plana* central nervous system.

Pursuing the cited goals will expand the knowledge of the bivalves' nervous system, in *S. plana* especially, and has potential of introducing this species as a valuable model in neuroscience.

ABSTRACT

This research was carried out on the bivalve peppery furrow shell, *Scrobicularia plana*, which already well-recognized as one of the important species in environmental monitoring and other types of researches. *S. plana* has its natural habitat in the intertidal soft sediment along the Atlantic coast from Europe to Africa. Its nervous system is poorly studied, and thus we aimed here to improve such lack of knowledge. A qualitative study was done to examine the general histology of the nervous ganglia, including the neural cell types at both light and electron microscopy. For light microscopy, animals were measured, anesthetized, dissected and fixed in 10% buffered formalin. They were routinely processed for paraffin embedding, and sectioned for varied purposes along the sagittal plane, using a fully motorized microtome. An immunohistochemical survey was also made for identifying neurons that could contain serotonin and dopamine, as a first step to gather the knowledge about the presence and role of neuroendocrine neurons in *S. plana*. For transmission electron microscopy, dissected ganglia were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, all buffered, and routinely processed for epoxy embedding. As to serotonergic and dopaminergic cell bodies and neurites, they were identified in all the ganglia, in adults of both sexes and in immature animals (with undefined sex). Both quantitative and qualitative methods were conducted to study three dimensional (3D) features of the ganglia, taking the 3D models to find out differences between ganglia types and if the biometric parameters can be correlated with the ganglionic volumes and surface areas, which was the case for some parameters. Stereology was applied later one, using the Cavalieri's principle for estimating the ganglion volume and the optical disector-fractionator method for estimating cellularity (numbers of neurons, glia and pigmented cells); namely investigating the influence of the sex and gonad maturation on the quantitative structural parameters. The statistical approach relied on multi-way analysis of variance. In summary, the main findings were as follows: (1) 3D-reconstruction shows that each type of ganglia has a peculiar 3D-shape, and data suggest a slight left-right asymmetry as to the cerebral ganglia shape. Regarding total surfaces, correlations exist for the cerebral and visceral ganglia, but it is the visceral that consistently shows strong positive correlations with each biometric parameter. Despite the differences in volume/surface among ganglia, the volume ratio of cortex *vs.* medulla is fairly stable (≈ 1.5), suggesting a functional optimum. In this first approach it seemed that no major differences exist between sexes; (2) Histological analysis using light and transmission electron microscopic analysis shows that each ganglion has perineurium (outermost layer), outer cortex, and inner medulla. The neurons (smaller or

larger) are typically unipolar, gliocytes are elongated, roundish or triangular, and there are pigmented cells. Generally, glial cells are much smaller than neurons, having higher nucleus to cytoplasm ratio; (3) Unbiased stereology analyses were conducted in three studies. Firstly, for investigating eventual influences of the sex and gonadal maturation status of animals that did not differ in size. Quantitative parameters were estimated in the nervous ganglia and their cells, in males, females, and undifferentiated specimens. Overall, there was a tendency for the ganglionic volume to be greater in females, followed by males, and undifferentiated animals. As for the type of ganglia, the two cerebral ones are similar in size, but the volumes increased significantly towards the pedal ganglia, which is greater than the cerebral and much smaller than the visceral. The size differences between all ganglia types are independent of the gender and of the gonad maturation status at the time. As for the relative volumes (V_V) of the cortex and medulla, the cortex is ≈ 60% and the medulla ≈ 40% of the all ganglia. As for the number neural cells, there were no significant differences among gender, but significant difference were found among ganglia types. The visceral ganglion has the highest number of cells (≈ 68000) and the cerebral ganglia have the lowest (≈ 12000). A second stereological study was on hypothesize impacts of age on the nervous ganglia in mature males *vs.* females. Considering that size is a proxy of age, the animals were split into two-size classes, that we named "Small" (age: 2+ years) and "Big" (age: 3+ years). We disclosed interganglionic, sex-related and size-related significant effects upon the ganglionic volumes, relative volumes of cortex and medulla, and total numbers of neurons, glial cells, and pigmented cells. The effect of size (age) was consistently marked, and statistically significant, with the older specimens having approximately twice as bigger ganglia (regardless of its type and of the animals' sex), that contained significantly more neural cells of all categories. The increase in cellularity took place if considering the entire ganglia, or the cortex and medulla separately. Data support our hypothesis that neurogenesis continues to occur in adult *S. plana*, irrespective of the animals' sex. In this vein, *S. plana* can become a stimulating model for neurogenesis and age-related studies. Lastly, in view that nervous system signalling and sex-steroids both influence bivalve reproduction, acute exposure to ethinylestradiol (EE_2) in water at two nominal concentrations (0.05 and 5 µg/L) was set up, to start determining whether or not endocrine modulation and/or disruption of the nervous system occurs in *S. plana*. Even though our preliminary data did not reveal significant impacts, either in the ganglion volume or in cellularity, the study served as a "kick off" for further tests, for instance using longer exposures, selecting other organic targets, and expanding the technical portfolio, *viz.* via stereological estimators of cell volume changes.

RESUMO

Este estudo usou como modelo o bivalve lambujinha, *Scrobicularia plana*, reconhecida como uma espécie importante em monitorização ambiental e em outros tipos de pesquisas. A *S. plana* tem como habitat natural o sedimento macio intertidal, ao longo da costa atlântica da Europa e até ao norte de África. O seu sistema nervoso é pouco estudado e, assim, procurou-se nesta Tese contribuir para minorar tais falhas de conhecimento. Foi executado um estudo qualitativo sobre a histologia geral dos gânglios nervosos, incluindo sobre os tipos de células neurais, tanto em microscopia de campo claro como microscopia eletrónica de transmissão. Para a primeira, os animais foram medidos, anestesiados, dissecados e fixados em formalina tamponada a 10%. As peças foram processadas de forma rotineira para inclusão em parafina, cortando-se o animal segundo o plano sagital, utilizando-se micrótomos motorizados. Entre outros, fez-se um estudo imuno-histoquímico para se identificarem neurónios que pudessem possuir serotonina e dopamina, como um primeiro passo para aumentar o conhecimento sobre a presença e papel de neurónios neuroendócrinos em *S. plana*. Para microscopia eletrónica, gânglios isolados foram fixados em glutaraldeído a 2,5%, pós-fixados em tetróxido de ósmio 1%, ambos tamponados, e processados rotineiramente para inclusão em resina epóxi. Quanto aos somata e neurites serotoninérgicos e dopaminérgicos, eles foram identificados em todos os gânglios, em adultos de ambos os sexos e em animais imaturos (*i.e.*, com sexo indefinido). Metodologias qualitativas e quantitativas permitiram estudar características tridimensionais (3D) dos gânglios, tendo os modelos 3D permitido elucidar diferenças entre os vários tipos de gânglios e se os parâmetros biométricos eram correlacionáveis com os volumes ganglionares e áreas de superfície; o que foi o caso para alguns parâmetros. Depois, foi usada estereologia, através do princípio de Cavalieri, para estimar o volume ganglionar, e do método "optical disector-fractionator" para estimar a celularidade (números de neurónios, de células gliais e de pigmentadas); estudando-se a influência do sexo e da maturação da gónada nos parâmetros estruturais quantitativos. A abordagem estatística baseou-se em análise de variância múltipla. Em resumo, os principais resultados foram os seguintes: (1) a reconstrução 3D mostrou que cada tipo de gânglio tem formas particulares e que os dados sugerem uma ligeira assimetria esquerda-direita na forma dos gânglios cerebrais. Em relação a áreas de superfície, existem correlações para gânglios cerebral e visceral, mas é este que mostra consistentemente fortes correlações, positivas, com cada parâmetro biométrico do animal. Apesar das diferenças de volume/superfície entre gânglios, a proporção volume do córtex *vs*. medula é bastante estável (≈ 1,5), sugerindo um ótimo funcional. Nesta primeira abordagem, não surgiram diferenças assinaláveis entre sexos; (2) A análise histológica e ultraestrutural mostrou que cada gânglio tem perineuro (um invólucro), córtex (externo) e medula (interna). Há neurónios (pequenos

ou grandes), tipicamente unipolares, células gliais alongadas, arredondadas ou triangulares, e células pigmentadas. Regra geral as células gliais são muito menores do que os neurónios, tendo uma razão núcleo citoplasma mais elevada; (3) A análise estereológica foi realizada em três estudos. Em primeiro lugar para a investigação de eventuais efeitos do sexo do animal e da maturação da gónada; em animais de dimensões similares. Os parâmetros quantitativos foram estimados nos gânglios nervosos e nas suas células, em machos, fêmeas e exemplares indiferenciados. Em geral, houve uma tendência para o volume ganglionar ser algo maior em fêmeas, seguindo-se machos e os indiferenciados. Quanto ao tipo de gânglio, os cerebrais são semelhantes em tamanho, mas os volumes aumentam significativamente no gânglio pedálico, que é maior do que os cerebrais e muito menor do que o visceral. As diferenças de tamanho entre todos os tipos de gânglios são independentes do sexo e estado de maturação da gónada; na fase estudada. Quanto aos volumes relativos (V_V) do córtex e da medula, o córtex é ≈ 60% e a medula≈ 40% d o volume ganglionar. Quanto ao número de células neurais, não houve diferenças significativas entre os sexos, mas foi encontrada diferença significativa entre tipos de gânglios. O gânglio visceral tem o maior número de células (≈ 68000) e gânglios cerebrais têm o menor (≈ 12000). Um segundo estudo estereológico foi feito para elucidar a hipótese da idade poder associar-se a impactos nos gânglios nervosos, em animais maturos de ambos os sexos. Sabendo-se que o tamanho do bivalve é um "proxy" da sua idade, os animais foram repartidos em duas classes, designadas por "Small" (idade: 2+ anos) e "Big" (idade: 3+ anos). Apuraram-se efeitos significativos, dependentes to tipo de gânglio, do sexo e da idade, nos volumes ganglionares, volumes relativos do córtex e medula, número total de neurónios, de células gliais e de células pigmentadas. O efeito da dimensão (idade) foi claro, e sublinhado na estatística, com os espécimes mais velhos a terem gânglios sensivelmente duas vezes maiores (independentemente do seu tipo e sexo dos animais), contendo mais células neurais de todas as categorias. O aumento da celularidade ocorreu no gânglio no seu todo, e no córtex e medula analisados separadamente. Os dados apoiam a nossa hipótese de que a neurogénese continua a ocorrer em adultos de *S. plana*, independentemente do sexo dos animais. Nesse sentido, a *S. plana* pode ser um modelo aliciante para estudar-se neurogénese e aspetos do envelhecimento. Por fim, dado que sistema nervoso e esteroides sexuais influem a reprodução de bivalves, fez-se um ensaio de exposição aguda a etinilestradiol, em duas concentrações nominais (0,05 e 5 µg/L). Iniciou-se assim a pesquisa para saber se há modulação/disrupção endócrina do sistema nervoso em *S. plana*. Embora os dados preliminares não demostrem impactos, pelo menos no volume do gânglio e na celularidade, o estudo foi um "pontapé de saída" para mais ensaios, *e.g.*, com exposições longas, seleção de outros alvos biológicos, e expansão abordagens, *viz*. via estimadores estereológicos de alterações do volume celular.

AUTHOR STATEMENT

As listed below, the doctoral Thesis contains two Chapters which are works that are published or accepted for publication in international journals with peer-review. Other Chapters are formatted with the aim of future submissions. Moreover, some data from the works that conducted to the present Thesis were presented as scientific communications in national and international congresses. All the works that contributed to this Thesis were made by the candidate in close cooperation and co-authorship with supervisors and other researchers. In all included works, the candidate made substantial contributions to conception, design, acquisition of data, and also to their analysis and interpretation. The candidate drafted every thesis Chapter and approved its final version.

Peer-reviewed articles

1. Tantiwisawaruji, S., Rocha, E., Kovitvadhi, U. & Rocha, M.J. (2014) The bivalve nervous system and its relevance for the physiology of reproduction. *Indian Journal of Anatomy* 3, 227-242. (Chapter I.)
2. Tantiwisawaruji, S., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2015) Qualitative and quantitative insights into the 3D-microanatomy of the nervous ganglia of *Scrobicularia plana* (Bivalvia, Tellinoidea, Semelidae). *Molluscan Research* (*article in press*). (Chapter III.)

Peer-reviewed proceeding

1. Tantiwisawaruji, S., Silva, A., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2015) A stereological estimation of the nervous ganglia volumes and number of neurons in the peppery furrow shell *Scrobicularia plana* (da Costa, 1778). *Microscopy and Microanalysis* 21, 99-100.

Presentations in congresses

Oral presentation

1. Tantiwisawaruji, S., Silva, A., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2014) Do body size and sex shape the neural ganglia volume and cellularity in bivalves? A study using as model organism the adult peppery furrow shell (*Scrobicularia plana*). *International Zoological Congress of "Grigore Antipa" Museum 2014*, Bucharest, Romania (19-22 November 2014).

Poster presentations

2. Tantiwisawaruji, S., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2012) "The nervous system of the peppery furrow shell *Scrobicularia plana* (da Costa, 1778): unveiling morphological features by computer-assisted 3D reconstruction". *International Meeting on Biology and Conservation of Freshwater Bivalves 2012*, Bragança, Portugal (4–7 September).
3. Tantiwisawaruji, S., Lopes, C., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2013) Structural characterization of neurons and glial cells of the bivalve *Scrobicularia plana*. *World Congress Malacology 2013*, Azores, Portugal (21-28 July).
4. Tantiwisawaruji, S., Silva, A., Kovitvadhi, U., Pardal, M.A., Rocha, M.J. & Rocha, E. (2014) A stereological estimation of the nervous ganglia volumes and number of neurons in the peppery furrow shell *Scrobicularia plana* (da Costa, 1778). *International Conference on Microscopy and Microanalysis, XLVIII Congress of the Portuguese Microscopy Society 2014 (INCOMAM'14)*, Porto, Portugal (6-7 November).

DEDICATION OF THE THESIS

This doctoral dissertation is dedicated to my parents, all my family members, truly great friends and beloved teachers for their endless love, support and encouragement. It is also crucial to state that this work is dedicated to my beloved son and husband, who have been always by my side, no matter how far we were from each other and how long that the destiny would made us apart — they have always been in my heart and soul. For five years, we have been away from each other but all their true love and care have never changed, and that is a tremendous support for my accomplishment.

Time is flying and another year just ended. Have you achieved your dreams?
"If you feel it is right, do it.
It's your life and nobody else's.
Make decisions that please you.
Let nobody put you down.
If you have a dream, act on it and it will probably come true."

Katie and Judy Griffler

ACKNOWLEDGEMENTS

First of all, I would like to express my sincerest gratitude to my supervisor, Professor Eduardo Rocha, for giving me a precious opportunity to learn valuable scientific skills, with a friendly team, working in a research laboratory environment. His expertise, excellent supervision, patience and kindness have been pivotal for me to complete this Thesis. I also would like to convey my grateful thanks to my co-supervisor Professor Maria João Rocha, for her kind assistance, encouragement, generosity, and critical corrections. I am also deeply in debt to co-supervisor Professor Uthaiwan Kovitvadhi for understanding, inspiring and supporting me to continue studying with an open mind, and also for her critical suggestions about the work.

I am extremely thankful to Professor Dr. Miguel Ângelo Pardal, of the Centre for Functional Ecology, Faculty of Science and Technology, University of Coimbra for support in the field collections of the animals, and for sharing his professional advice and experience.

My special appreciation and thanks go also to Professor Rogério Monteiro, former Head of the Laboratory of Histology and Embryology, of the ICBAS, which authorized my hosting in that unit. His friendship and caring attitude had made my life more tolerable during the first two years in Porto.

My grateful thanks are due to King Mongkut's University of Technology Thonburi (KMUTT), Ministry of Science and Technology of Thailand, for the scholarship conceded during my doctoral study. I would like to mention the collaboration of the Office of the Civil Service Commission and Royal Thai Embassy in Lisbon, for the scholarship transfer and for kindly dealing with all the administrative issues. It is an enormous pleasure to acknowledge Fernanda Malhão, Célia Lopes and Helena Galante for vital supporting, encouraging, a day-to-day teaching and caring, discussing the many technical issues, and giving critical feedback during these five years. I owe my deep gratitude to Ana Silva too, who was always helpful and that made me never feeling alone. I cannot forget a great beautiful friendship and motivation of my true friend, Catarina Cruzeiro, who always warmed my heart and indeed my better half. Many thanks for your kind help!

My special thanks to Professor Tânia Madureira and Professor Paula Silva, for their precious friendship, hospitality and unconditional help. There are no words to express my gratitude for you girls.

My thanks go also for Professor Marta Santos, Professor Ricardo Marcos, and Dr. Susana Galante for their kindness and friendship. I also thank the internship students of the

Laboratory of Histology and Embryology (at ICBAS) and of the Histomorphology, Physiopathology and Applied Toxicology Group (at CIIMAR), for their assistance.

I owe a special note of gratitude to Professor Alexandre Lobo da Cunha (namely as supervisor of the Electron Microscopy Unit, ICBAS), Elsa Oliveira, Ângela Alves and Sónia Rocha for their assistance and sharing their expertise in electron microscopic techniques.

I have shared the good and hard times with many Thai friends in Portugal. Thank you for being with me to preserve both of those times of our lives, especially Dr. Ouayporn Tangthonchai, Dr. Pornchai Julamat and Dr. Chommanard Sumngern. I would especially like to thank my good friends Dr. Piyathip Tinnaworn, Dr. Suradet Buta-chon, and Dr. Ratchanee Tang-on, not only for their willingness to help and share their personal experiences with me but also for their emotional support which is very much appreciated. I truly appreciate for the support from the great friends Dr. Suthee Ploisawatchai, Dr. Banyat Lekprasert, Rungtiva Kapol and Praphanit Suangtho. Also, my very special thanks go to Pattareeya Yawanopas Guimarães, and her husband, Emanuel Guimarães for their inestimable help for the whole time of my stay in Portugal.

It is an honor for me to express my gratitude to Professor Anake Kijjoa and Professor Madalena Pinto for their friendship, guidance and unconditional support. To the working team of Chemistry Laboratory (ICBAS), Júlia Bessa, Sónia Santos, Isabel Silva, and students who always showed the interest, kind concern and availability to support.

I would especially like to thank everyone not mentioned here who, in one way or another, always gave me assistance and friendship towards my studies.

Last but not least, word of dedication goes to my parents, all my family members, truly great friends, as well as my beloved teachers, who have been by my side, no matter how far they are. Their emotional support, encouragement, and endless love stand by me through every moment of my life and that is the marvelous support allowing my accomplishments.

CONTENTS

OBJECTIVES……...…. I

ABSTRACT……...…. III

RESUMO……. V

AUTHOR STATEMENT……..... VII

DEDICATION OF THE THESIS.……..... IX

ACKNOWLEDGEMENTS.……...……. XI

Chapter 1 Introduction — The Bivalve Nervous System And Its Relevance For The Physiology Of Reproduction..... 3

Chapter 2 The Peppery Furrow Shell (*Scrobicularia Plana* (da Costa, 1778)) — An Overview ……...…..... 21

Chapter 3 Qualitative And Quantitative Insights Into The 3D-Microanatomy Of The Nervous Ganglia Of The Peppery Furrow Shell *Scrobicularia Plana* (Bivalvia, Tellinoidea, Semelidae)……..... 41

Chapter 4 Overview Of The Neurocytology Of Ganglia And Identification Of Putative Serotonin- and Dopamine-secreting Neurons In the Bivalve Peppery Furrow Shell (*Scrobicularia Plana*)…….……... 65

Chapter 5 A Stereological Study Of The Volumes And Cellularity Of the Nervous Ganglia Of Males, Females And Undifferentiated Adults Of The Peppery Furrow Shell (*Scrobicularia Plana*)....…….... 95

Chapter 6 Impacts Of Age In The Nervous Ganglia Volume And Cellularity In Two Adult Size-Classes Of The Bivalve Peppery Furrow Shell *Scrobicularia Plana*……..…… 131

Chapter 7 Do Estrogens Influence The Bivalve Nervous Ganglia Size And Cellularity? A Study On The Pedal Ganglia Of The Peppery Furrow Shell *Scrobicularia Plana* Acutely Exposed To Ethinylestradiol……..……...… 169

Chapter 8 Final Remarks……...……...……. 189

CHAPTER 1

INTRODUCTION — THE BIVALVE NERVOUS SYSTEM AND ITS RELEVANCE FOR THE PHYSIOLOGY OF REPRODUCTION

227
Indian Journal of Anatomy
Review Article
Volume 3 Number 4, October - December 2014

The Bivalve Nervous System And Its Relevance For The Physiology Of Reproduction

Sukanlaya Tantiwisawaruji*, Eduardo Rocha, Uthaiwan Kovitvadhi***, Maria J. Rocha******

Abstract

Bivalves are widespread invertebrates that are mostly marine and benthic, with great impacts in the aquatic systems food chains. Their soft body is laterally compressed and covered with a hard shell, often having bilateral symmetry. Strong adductor muscles help in the shell movement. Various species are used as bioindicators of environmental quality. Many, such as mussels, clams, scallops, or oysters, are heavily harvested / reared for human consumption. Bivalves availability, adaptability and simple anatomy make them attractive for both fundamental and applied research. One particular target for such studies is the nervous system. It is typically made of a central nervous system holding three types of ganglia (cerebral, pedal, visceral), organized into an outer neuron- and glia-rich cortex and an inner axon-rich medulla. Nerves interconnect the ganglia as well as these and peripheral nervous system components, made of sensorial structures such as eyes (mantle, tentacles), and osphradia (gills) and statocysts (foot); They are involved in photoreception or are mechano or chemoreceptors. Among other roles, the nervous system governs reproduction, via influences in the sexual development, gametogenesis, fertilization and spawning. Such modelling is via neurotransmitters and neurohormones, interplaying with direct / indirect impacts of biotic (*eg*, food abundance) and abiotic (*eg*, temperature, pH, salinity) factors. We know now that many pollutants can disrupt the nervous system and gonads and their poorly known interaction. Knowing the nervous system functional morphology is critical to understand such disruptions and foreseen reproductive consequences. Accordingly, this work offers a systematic overview about the bivalve nervous system and related reproductive events.

Keywords: Anatomy; Histology; Bivalves; Nervous system; Ganglia; Neurons; Glial cells; Neurocytology; Neurophysiology; Reproduction.

Author's Affiliation: *PhD Student, King Mongkut's University of Technology Thonburi (KMUTT), Bangkok 10140, Thailand. Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), 4050-313, Porto, Portugal. CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, U.Porto, 4050-123, Porto, Portugal., **Full Professor, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), 4050-313, Porto, Portugal. CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, U.Porto, 4050-123, Porto, Portugal., ***Associate Professor, Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand., ****Associate Professor, Superior Institute of Health Sciences-North (ISCS-N), 4585-116 Gandra-Paredes, Portugal. Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), 4050-313, Porto, Portugal. CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, U.Porto, 4050-123, Porto, Portugal.

Corresponding Author: Eduardo Rocha, Full Professor, Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Rua de Jorge Viterbo Ferreira n.º 228, 4050-313 Porto, Portugal.

E-mail: erocha@icbas.up.pt

Introduction

In almost all metazoans, the coordination is accomplished by two main mechanisms, hormones and nervous system signals. These two central systems interact with each other to maintain the homeostasis of animals and to respond appropriate information to the environmental stimulus [1-2]. In addition to these basic vital functions, the nervous systems of higher organisms are able to perceive and react to a greater range of environmental stimuli in intricate and varied way including responsible for feeling, thinking, and learning [3]. In vertebrates there are more complicated components of the nervous system. Anatomically, there are two systems: the central nervous system (CNS) and the peripheral nervous system (PNS). CNS consists of brain and spinal cord. The PNS comprises the somatic and

autonomic nervous systems. Somatic afferents carry sensory information from the skin, muscle, and joints to the CNS, while motor efferent nerves innervate skeletal muscle to cause the movement contraction. [4] The autonomic nervous system can be thought of as a motor system for visceral organs, because it projects to these organs to innervate and control the function of smooth muscle, cardiac muscle, endocrine, and exocrine glands. The autonomic nervous system is typically further divided anatomically and functionally into the sympathetic and parasympathetic subdivisions. [2, 4, 5]

In lower invertebrates of the animal kingdom, like Coelenterates or Cnidarians, the nervous system consist of specialized nerve cells of ectoderm called nerve net that consists of sensory and muscle cells diffusely distributed. [6, 7] The most highly evolved groups, like flatworms, show the first real CNS because their sensory cells are grouped into special anatomical collection forming a nerve ring or ganglia organized in the bilaterally symmetrical longitudinal body axis as nerve cords. Their ganglia can assume a segment-like structure as a result of the more or less regular array of cross-connections innervating the whole body. [8] In the head region, there are specialized structures, such as primitive "eye or ocelli". These structures are also be found in annelids, in which in the anterior end there is a distinct brain and segmented body plan, with ganglia organized into a ladder-like chain in each segment. [9] The dorsal brain is connected to the ventral chain of segmental ganglia via circumesophageal connectives. Each segmental ganglion, which typically is said to consist of about 1000 neurons, is organized in a bilaterally symmetrical way. Both halves are linked to one another by commissures and to neighboring ganglia by connectives. Peripheral nerves, typically three pairs, projects from each ganglion and innervate the segmental body wall. [10] All ganglia have a structure which is characteristic for higher invertebrates; neurons within cortex and the neuronal processes (dendrites and axons) lie in a neuropil in the core of the ganglion. In some annelids, distinctive giant neurons occur, and these play an important role in fast escape responses. [11] In some species the structure of optic ganglia is formed. [12, 13]

In arthropods, however, the body organization is different from that of annelids with articulated appendages and the fusion of originally unitary, metameric segments into the functional entities comprising the head, thorax, and abdomen. For such insects and crustaceans, their head region tends to form a complex brain consisting of extensively fused cerebral ganglia. These are often associated with the processing of information from specialized sensory organs, for example, a protocerebrum of insects, which receives visual sensory input from both compound eyes and from the simple ocelli, a deutocerebrum which receives sensory input from the antennae, and a tritocerebrum which receives input from the head surface. [14] These brain structures together contain about 90% of the neurons in the central nervous system, which in the larger crustaceans sum about one million nerve cells. In the higher arthropods, there are brain regions which consist of associative neuropil centres, cell body regions, and aggregates of neurosecretory cells. [15, 16] The requirement for accurate motor control of the articulated body appendages, especially the thoracic legs, has led to an increasing specialization of the ventral segmental ganglia. [17] There are the thoracic ganglia typically containing more interneurons, efferent projecting motor neurons and afferent sensory fibers than the abdominal ganglion. The latter, often associated with specialized structures, are located in the posterior end of the animal. In addition, there is a tendency toward fusion of the segmental ganglia into fewer (in some cases single) ganglia. [18] One such ganglion, the subesophageal, it is formed by several ganglia and controls the mouthparts – this is generally found enclosed in the head capsule. [19] The segmental specializations of the arthropod nervous system allow complex motor activity to be generated. This includes flying, running, jumping, manipulation, and sound production. [12, 18]

In molluscs, there are variations in the organization of the nervous system. In order to get sensations they have a collection of neurons in the ventral cord which are called ganglia. The basic organization of their CNS comprises about five pairs of ganglia which are arranged around the gut, normally near the head, and are linked to one another by connectives and commissures. It is possible to distinguish cerebral, buccal, pleural, pedal, and abdominal ganglia. [13, 20] The basic organizational plan can vary significantly among individual molluscan species, to the extent that the various ganglia can change their position and even fuse with one another. [12, 13, 20] In Gastropoda, Scaphopoda, Polyplacophora, Monoplacophora (slow-moving animals) and Cephalopoda (active predatory lifestyle), there is cephalisation. [7, 11, 21] There are numerous studies on the nervous system of the gastropod mollusc *Aphysia californica*, which is an animal model for the neurobiologists' study of behaviours, namely learning and memory. [22, 23] In bivalves, such as, clams, mussels, and scallops, there is bilateral symmetry and soft body. They have an interesting simple model of CNS, recognized as

very useful for studies ranging from neurobiochemistry to neurophysiology. [7, 24, 25] However, little seems to be known about the detailed anatomy of components in the bivalve nervous system. This Chapter reviews two major parts in bivalves. The first concerns the structure and function of nervous system. The second describes the neural control of reproduction.

General morphology and functions of nervous system in bivalves

Knowledge about the morphology and functioning of central nervous system in bivalves is still somewhat scarce and needing further study. The reasons for this limitation are varied. The histology, though studied to a certain extent, is different from that of vertebrates; most of the available forms are small, and the few experimental work has been performed using methods fruitful in vertebrates but, possibly, inadequate or insufficient for bivalves. A great feature of the bivalve nervous system is the small number of neuronal elements within ganglia and that contribute to the peripheral tissue. [20, 26] This makes possible a type of analysis that is difficult to achieve in vertebrates. Also, interesting direct correlations between the size of the ganglia and their function can be disclosed in bivalves.

The central nervous system

Anatomy of the ganglia

The basic plan of organization of all bivalves nervous system is bilaterally symmetric which each half body segment possessing a ganglion. In typical bivalves, they consist of three pairs of ganglia: cerebropleural (commonly called as cerebral), visceral and pedal; along with two pairs of long nerve cords. Both cerebral ganglia are interconnected to visceral and pedal ganglia by bilaterally running nerve cords. Each ganglion gives rise to nerve fibers that supply the organs and tissues in close proximity. [24, 26, 28] For instance, the cerebral ganglia innervate labial palps, anterior adductor muscle, anterior part of the mantle, and sensory organs, including statocysts (equilibrium organs) and osphradia (a chemo-mechanical sense organ). [29] The visceral ganglion innervates the gills, heart, posterior adductor muscle, posterior part of the mantle, siphons, and sensory organs in the mantle. [30] As in other bivalve species, the visceral ganglion of *Venus verrucosa* comes from the fusion of two original ganglia, thus showing bilateral symmetry; pairs of symmetrical nerves emerge from each pole and diverge. Lastly, the pedal ganglion, as the name indicates, innervates the foot. [28, 31, 32]

Cerebral ganglia

In most bivalves, the paired cerebral ganglia are well separated from each other (left and right) and they are usually triangular in shape, with the color varying from milky white to bright red. These ganglia are situated between the base of the labial palps and the first esophageal subdivision of the digestive tract, being shortly cross-connected by a commissure arching over the esophagus, as well as, longitudinal linked between pedal and visceral by connectives. In reality, they are formed by fusion of the cerebral and pleural ganglia around the anterior part, and that is why they are commonly referred as cerebropleural ganglia or cerebral ganglia in the literature [20, 32]; and herein we shall use the latter term henceforth for consistency. From each cerebral ganglion not only the principal two pair of nerves cords lead toward the posterior of the animal: one, cerebro-pedal connectives that extend posterior and ventrally to the pedal ganglia in the foot; another, cerebro-visceral connectives, running directly back from the cerebral ganglia to the visceral ganglion, which is located on the surface of the posterior adductor muscle. But, there are also the pallial nerves innervating the labial palp, anterior adductor muscle, gill [33], and part of mantle margin, as well as the statocysts and osphradia. In the absence of cephalic sense organs the cerebral ganglia are weakly developed and small. [33] In snails, the central ganglia are more concentrated and the visceral loop is so short that all of the principal ganglia are in the anterior nerve ring above the esophagus. [20, 21, 34]

Pedal ganglia

In general bivalves, the pedal ganglia is positioned below the esophagus and is anterior to the base of the foot. They have the same type of coloration but are larger than the cerebral ganglia and more rounded in appearance. The pedal commissures are rare; in most forms the right and left ganglia have met together in the middle line. Each ganglia extend the following nerves: 1) the pedal nerve, which innervates the foot, originates from the ventral posterior surface; 2) in genus *Mytilus*, the ventral byssus retractor nerve, innervating the byssus organ

and muscle and arising from the posterior ventral side of the ganglion; 3) the dorsal byssus retractor nerve, which also innervates the upper byssus muscles arise from the posterior dorsal of the ganglion. In *Crassostrea virginica*, there are as well no pedal ganglia in line with the lack of a foot for moving. [24, 32, 35]

Visceral ganglia

In typical bivalves the visceral ganglion is the largest ganglia, being derived from the fusion of two original ganglia. Visceral ganglia either appear as "rounded triangles" or else having multiple lobules, with milky white to bright red in colour at the ventral end of the visceral mass, on the anteroventral border next to adductor muscle. The visceral ganglia are much larger than the cerebral and nerves emanating from it innervate the mantle, gills, intestine, anus, skin, posterior part of the genital apparatus, kidney, the main digestive gland and posterior adductor muscle. [32-36] In addition to their usual autonomic functions, the visceral ganglia also receive sensory inputs from the sensory tentacles of the mantle. The tentacles are photoreceptive, mechanoreceptive, and even chemoreceptive organs. [24, 32] It is of interest to note that the distribution of the nerves which originate from the visceral ganglia is not always identical for each ganglion. Processes could be seen to extend from nerve cell bodies. Fibres could be seen in the cerebro-visceral connective and in the origin of the branchial nerve. [36] The large white visceral ganglion can be revealed by opening the exhalant chamber and cloaca and looking between the pyloric process and the posterior adductor muscle. [30, 37, 38]

Histology of the ganglia

Irrespective of the ganglia types, they typically consist of three layers, an outermost perineurium, the outer cortex and the inner medulla, which can be called neuropil. [20, 39] Accordingly, the typical structural organization of the ganglia , bivalves like those of most invertebrates, consist of a multilayered rind of neuronal cell bodies which send their processes to a central core, are sheathed by a connective tissue perineurium and contain two types of cells: nerve cells (neurons) and glial cells.[13, 20]

Perineurium

Ultrastructural analysis of the *V. verrucosa* ganglia shows — from the ganglion periphery the perineurium — a limiting envelope formed by a sheath of connective tissue that consists of collagen fibers and fibroblasts; they are arranged in a loose three-dimensional network, alternating with sheaths of dense microfilamentous material with the appearance of a basal lamina surrounding the ganglia. [36] As for its function, the perineurium is likely to provide not only a protective envelope, but also a permeability barrier, which may be particularly important in bivalve ganglia which probably lack a glial blood-brain barrier. [13, 20] But the perineurium in vertebrates is different from that in invertebrates, because it is a concentric layer of bundled nerves that it is a protective layer of connective tissue located around nerves in the body and the internal organs. Indeed, it is composed of concentric layers of connective tissue that form a protective sheath around bundles of nerve fibers. This structure is a transparent tube-shaped layer that is easily pulled away from the bundled nerves. Perineurium nerve coverings are a part of the peripheral nervous system (PNS), which is responsible for transmitting messages from the central nervous system (CNS) in the brain to the effectors, like arms, legs, and internal organs. [2]

The cortical part of ganglia (cortex)

The cortex, a multilayered area of neuron and satellite glial cells in *V. verrucosa* [36], is to be the complex network centre of neuronal cell bodies and glial cells. The cortex is not only involved in the control of many internal, homeostatic regulatory processes, but also in the production of complex behaviours. Many of nerve cell bodies located in the cortex were radially oriented and closely associated with the connective tissue sheath. Many of the neurons send their axons into the neuropil ganglion (inner) zone.[40]

Medulla or neuropil region

As previous mention, in the most invertebrate ganglia such as arthropods and annelids, the cell bodies of neurons occur in a thin rind on the periphery of the ganglion, and the core that contains axons and dendrites is called the neuropil, a ganglionic core containing the axonal processes of the cortical neurons in *V. verrucosa*. [36] These nerve

cell bodies appeared to be extensively innervated, as indicated by the specific staining of endings on their surface their process of the nerve cell body tapered as it extended from the body. The neuropil region has a fibre organization of axons in the nerve tracts that form clustered areas of complex synapses, i.e., glomeruli. [20, 37]

Ganglionic structure follows a common pattern in virtually all invertebrates, with an outer rind of neuronal somata surrounding an inner core of axons and dendrites. The somata are clustered in groups. The axonal processes of motor neurons leave the ganglion through the lateral nerves to innervate their targets in the periphery (often muscles). Most motor neurons have just one axon leaving the ganglion, but a few have axons in several nerves that innervate different targets. In this way a single motor neuron can exert coordinated control over sets of muscles that need to act together. Individual muscles are generally innervated by just one or a few excitatory motor neurons. [13, 20]

Neurons and glial cells (ganglionic cells)

There is no doubt that in all bivalves the number of central neurons is smaller if compared to more complex animals. Notwithstanding, each neuron has a specific and often complicated task to perform which involves receiving and making many synaptic connections.[41, 42] In certain instances, differences between the pair of neurons in each half of the central nervous system are slight, so that one can replace the other to a considerable extent. But in many other cases the loss of one fibre must involve considerable loss of function, which may be mitigated to a certain extent by the overlapping fields of different neurons. The nervous system also contains cells that surround, nourish, and support the neurons and their process, and these are called glial cells. [40]

Nerve cells or neurons

As in most invertebrates, unipolar neurons predominate, even though a few bipolar and even multipolar nerve cells have been described. [13, 20, 43] Neuronal cell bodies have overall ultrastructural features similar to those of most vertebrate and invertebrate neuron. They contain a pale round or oval nucleus with one or more prominent nucleoli. The cytoplasm is rich in granular and agranular endoplasmic reticulum, free ribosomes, mitochondria and glycogen deposits. Some mitochondria have a paracrystalline structure, similar to that found in the neurons of *Spisula solidissima* [32, 44], which may be related to the accumulation of proteins and lipid; as it is known to occur in a variety of vertebrate and invertebrate cells. Microtubules and microfilaments are bare. Golgi complexes are numerous and developed, being formed by long cured cisternae filled with finely granular electron-opaque material and by vesicular profiles of variable size and electron density. In most cell bodies, dense core vesicles are an important component and can be found in large amounts dispersed in the cytoplasm. They display a great variability of size, shape and electron-opacity and represent the only distinctive feature of the neuron, which are comparable in other ultrastructural respects. [37, 45]

Most neuronal bodies are in the cortex and close to the perineurium sheath of the ganglia. There are also the beginnings of the nerves fibres that are made of axons (i.e., neurites in unipolar neurons) and eventual dendrites. [46] Pigments can also be found within neuron, namely as granules designated by cytosomes or lipochondria, exactly alike described in gastropods. [20, 37, 47] The cytoplasmic membranes of neuronal cell bodies, which are in extensive reciprocal contact, do not show particular specializations, except for the presence of subsurface cisternae in peripheral neurons of *S. solidissima*. [44]

The neuronal cell process originates from a large, cone-shaped extension of the soma which gradually taper. The cytoplasm contains microtubules, neurofilaments, mitochondria and vesicles displaying the same ultrastructural heterogeneity as those in the cell bodies. The ganglionic core is formed by a complex network of processes of different diameters. Nerve processes containing cytoskeletal elements are intermingled with others filled with vesicles. Tracts are formed by wider axons of passage, while non-glomerular neuropil contains finer processes which arborise and establish synaptic contacts. [20] Different types of neurons can be identified from their branched process pattern and in terms of function, and so they can be grouped into three basic categories: a) neurons with specialized endings that respond to energy from the environment are called sensory neurons; b) neurons that have axons terminating on muscle fibers are called motor neurons c) all other neurons, that are interneurons. [1, 21, 48]

The majority of synaptic contacts occur in the neuropil between nerve processes, even if rare, axomatic synapses have also been recognized within the cortex. The presynaptic sides can be identified both the presence of neurotransmitter vesicles and of

electron dense areas collate to the membrane. In these synaptic areas, organelles such as mitochondria and cytoskeleton elements are sparse. Post synaptic sites are simpler, being the most significant feature the unevenness of the membrane. The synaptic space (cleft) typically does not vary in width (H" 20 nm) across the synapse. Despite this key features, more than one type of synaptic characteristics may occur. For instance, in the genus *Mytilus* there are synapses with vesicles that only have a lucid content while other have vesicles having either dense or clear cores. In addition to the vesicle discharges at synapses, it is accepted that neuromediators are released at non-synaptic sites; a process that is not exclusive of bivalves. More details on the above can be read elsewhere. [46, 49]

Glial cells

As in vertebrates, the glial cells of invertebrates have a vast array of structural and functional specializations. [50] They can be feature of the higher invertebrate groups like, the Arthropods, Annelids and Molluscs. Their location is around the neurons, especially at the nervous tissue interface. Glial cells have an oval nucleus with chromatin clumped in the periphery. Generally, two types of electron-dense, membrane-bound inclusions can be discerned: cytosome-like bodies and oval granules called gliosomes (450-650 nm in length and 250-350 nm in width). These later are a distinctive feature of glial cells in several bivalve species (and also gastropods). Their role in nervous activity appears to be necessary when the neurons become aggregated into ganglia. [13, 50, 52] In the *Mytilus edulis*, glial cells have an oval or indented nucleus with chromatin clumped in the periphery. Their cytoplasm is usually scanty but nevertheless contains microfilaments, mitochondria, cisternae of rough endoplasmic reticulum, free ribosomes, and small Golgi complexes.[53] Neuronal cell bodies in the cortex of the pedal ganglion are subdivided in clusters by septa formed by glial cell bodies and their processes, among which there is a system of intercellular channels, mainly evident in the subperineurial zone. In this region, even in well-fixed tissues, there are clusters of empty vesicular profiles of variable size, which seem to bud off from glial processes: the nature of dark glial cells characterized by a dense cytoplasm, which are present in the deepest regions of the cortex and in the neuropil. Glial cells appear less frequently in the ganglion central fiber core, being completely absent from wide neuropil regions. [20, 51]

The peripheral nervous system

The peripheral nervous system of bivalve is made up of sensory structures regulated through the lateral nerves. The organs are usually tentacles and most are typically mechanoreceptors and chemoreceptors. The sensory organs of bivalves are not well developed, and are largely a function of the posterior mantle margins. In scallops have complex eyes with a lens and retina, but most other bivalves have much simple eye or ocelli. In Septibranchs, the inhalant siphon is surrounded by vibration-sensitive tentacles for detecting prey.[7, 54]

Primary ciliary receptors

In bivalves, three types of ciliated sensory receptors were described. [55, 57] The most common consists of 35-40 nonmotile cilia on a cluster of four to six sensory neurons, apparently mechanoreceptors associated with a pair of glandular cells. The second type, a monociliary receptor, has a long, stiff kinocilium surrounded at the base by a corolla of nine short, club-shaped microvilli. The third type consists of 17-20 nonmotile cilia in a circle on a single sensory neuron that distally envelops a gland cell. These structures work as mechanoreceptors and can be seen in the tentacles of the scallop *Placopecten magellanicus* [58], mantle edge of *Donax serra* and *Donax sordidus* and on the siphon of *Macoma balthica*. [56]

Ocelli (eye spots)

Bivalves have two types of eyes: paired cerebral eyes, as well illustrated in the veliger (the planktonic larval stage) and adults of *M. edulis*, and pallial eyes. [32] The latter eyes are found on the siphons of *Cardium edule* and on the middle mantle fold of the *Pecten maximus*. [59] This organ is the light receptor, containing pigmented cells. In *M. edulis*, cerebral eyes appear as dark spots located at the bases of the first ctenidial filaments of the left and right inner demibranchs. Each ocellus is an open cup, and the retina is composed of sensory and pigment cells. Eyes in *P. magellanicus* are on the middle of the mantle skirt. [32] The photoreceptoral organelles are directed toward the incoming light. The sensory cell has a bulbous nuclear region, a slender cell process, and, apically, rhabdomeres, and, compared to the eye of genus *Pecten*, there are very few receptor cells. [59] In *Pecten maximus*, more than 60 eyes are located in the

sensory fold of the mantle. [58] Each consists of a cornea, a large cellular lens, a distal and proximal retina, a reflecting argentea, and a layer of pigment cell around the eye. The lens cells contain few organelles and rest on a thick basal lamina. Beneath the lamina there are nerve fibres of the distal retinal cells that bear few microvilli among numerous cilia at their distal surface. The axon leaves the distal retinal cell from the side, passes up to the basal membrane, and joins other distal nerve fibers to form the optic nerve. There are glial supporting cells between the distal and the proximal retina, the cells of which face in the opposite direction from the distal cells. [58, 59]

Statocyst

In bivalves, paired statocysts are located in both dorsolateral sides of the pedal ganglia, and there are nerve connecting them to the cerebral ganglia. In the genus *Pecten*, each statocyst consists of a sac of hair cells and supporting cells. Inside the sac is a statolith composed of crystals, and a static nerve extends from the sac and eventually connects to the cerebral ganglion. [60] Hair cells have kinocilia, microvilli at their distal ends, and one or more striated roots that pass deeply into the cell cytoplasm. They function to allow animal to maintain orientation. [32, 61]

Osphradium

The osphradium can detect incoming water as a chemo- or mechanoreceptor around the ctenidial axis, exhalent, and suprabranchial section of mantle cavity. In a number of bivalve species, osphradia have sensory processes, sensory cells, supporting cells, and innervation of the ridge by nerves from ventral ganglion. [29] The osphradium is an ancient sensory structure in Mollusca, and it is better developed in Gastropoda, where it is a strategically located chemo-mechanical organ in the pallial cavity. [32, 62]

Abdominal sense organs

Abdominal sense organs are situated on the ventral surface of the posterior adductor muscles in bivalves. [32] The sensory epithelium is tall and consists of two predominant cell types, electron-dense supporting cells with microvilli only, pigment granules and oval distal nuclei, and sensory cells with round proximal nuclei and electron-lucent cytoplasm. The narrow sensory processes always are bunched and reach the surface bearing long stiff cilia. Surrounding the cilium is nine 'stereomicrovilli' forming a basal plate in connection with the basal body. In the prosobranch *Nucula sulcata* there is the so-called Stempell's organ, a tube-like sense organ, situated immediately dorsal to the anterior adductor muscle. Collar receptors in the sensory portion of the organ indicated a mechanoreceptive function. [32]

The cellular components of an invertebrate nervous system include: sensory neurons, which convert physical variables (e.g., light level or muscle force) into electrical signals; motor neurons, which make synapses with muscles or other effector organs (e.g., light-producing organs, glands); interneurons, which transmit information between other neurons; and glial cells, which are electrically excitable, that influence the ionic environment surrounding neurons and the transmission of signals between them. [13] The transport of signalling of neurotransmitters is considered to be a major function of ganglia in most bivalves division of the ganglia. The central nervous system of bivalves have neurons that contain the biogenic amines dopamine (DA), norephinephrine (NE) and serotonin (5-HT), each type might inhibit the synthesis of the other transmitters.

Neuroactive substances

There are various techniques to study in nervous tissue of bivalves, and one of the important technique is immunocytochemistry, which for instance characterized the neurons containing neuroactive substances in *M. edulis*.[46]

Serotonin or 5-hydroxytrytamine (5-HT)

5-HT is found in the central nervous system of vertebrates and invertebrates. [63] It is thought as the key neurotransmitter that control reproductive process of many invertebrates, such as the crustacean *Macrobrachium rosenbergii*. [64] In *M. edulis*, serotonin immunoreactive neurons were seen in light microscopic immmunocytochemical studies. Most often, those neurons are unipolar (8.5-25 µm) and very numerous both in the pedal and the cerebral ganglia. [65] Moreover, a great number of labelled nerve processes were shown in the ganglionic cores, in the connectives and in the nerves. In the bivalves *Anodonta cygnea* and *Mactra stultorum*, auto radiographic studies indicated that there is a selective uptake of 3H5-HT by ganglionic nerve processes

containing dense core vesicles. The neuropil of the pedal ganglia has small dopamine-containing neurons closely associated with it. Situated ventrally in the pedal ganglia is a large group of 5HT-containing neurons. Both dopamine and 5-HT are present in the cells at the junction of the visceral and right parietal ganglia, and that dopamine and 5-HT varicosities are present in the neuropil of the pedal ganglia in molluscs. [63]

Neuropeptides

Neurons immunoreactive for gamma-aminobutyric acid (GABA) have been verified in all the ganglia using an antibody directed against the amino acid itself. [66] GABA immunoreactive neurons are represented more in the pedal and cerebral ganglia than in the visceral ganglia, but are less numerous than neurons displaying 5-HT-positivity. For the majority, GABA-positive neurons are small, unipolar (10 µm in diameter), the exceptions being represented by a few small bipolar and multipolar cells present almost exclusively in the pedal ganglia. [48] In these latter there are also two pairs of bilaterally symmetric, large (30 µm in diameter) multipolar neurons with long processes projecting widely throughout the neuropil. Immunoreactive processes form networks in the ganglionic cores and run in all the connectives and nerves; even so, GABAergic fibers are very rare in the foot. [48] Whether peptide releases occur at synaptic contacts remains to be fully elucidated, as synaptic terminals positive to neuropeptides have not yet been recognized. In addition to the substances above-mentioned, there is physiological and pharmacological evidence for the presence of other peptides, both in the central and peripheral nervous system, such as the case of FMRFamide (Phe-Met-Arg-Phe-NH2). [43]

Acetylcholine (Ach)

Acetylcholine has long been recognized as a neurotransmitter. In most bivalves Ach acts as an inhibitory neurotransmitter whereas in some it may have an excitatory role. Ach actions can be even inconsistent within a species. Ach has, therefore, a wide variety of effects, e.g., on the heart where it is a cardioinhibitory neurotransmitter. [67]

Dopamine

Dopamine is widely distributed in the invertebrate nervous system and has a diverse effect of reproduction in bivalves. [68] Dopamine was shown to inhibit spawning activity in serotonin-treated *Dreissena polymorpha* mussels, indicating that spawning activity is stimulated by serotonin but negatively controlled by dopamine (i.e. dopamine is linked to gametogenesis rather than spawning and fertilization) [69]. In the gonads of *Mizuhopecten yessoensis*, dopamine acts both as a neurotransmitter and neurohormone to rise the levels of cAMP, that seem to play a regulatory role in the reproduction. [70] This does not mean that dopamin have actions restricted to reproduction, exemplified by its role in the contro of ciliary beating as elegantely demonstrated in *C. virginica*. [42]

Mechanisms of neuronal transmission

Knowing that nerve impulses were mediated by chemical neurotransmitters, it became possible to isolate the inhibitory and excitatory effects of nerve stimulation and to identify the probable neurotransmitter substances.

The action potential

Just as a quick reminder, a basic function of most neurons is ability to produce nerve impulses or action potentials along the cell membrane. Potential differences cross the membrane known as the membrane potential. In the resting potential membrane, it is approximately -65 mV. When the membrane potential is raised enough to reach the threshold result in voltage-gated, sodium channels open up and allowing Na+ to flow into the cell and depolarizing the membrane. This is an action potential (AP), the rapid depolarization is soon opposed by the closing of Na+ channels (stopping its influx from the exterior) and opening of K+ channels (allowing the efflux of K+, during both the repolarisation and hyperpolarization phases for restoring the resting potential). Finally, both Na+ and K+ channels close and the membrane potential return to resting stage and along the membrane is passively extended and excited adjacent areas to do the same step. The presynaptic terminal contains synaptic vesicles-packets containing a chemical neurotransmitter. The type of neurotransmitter varies depending on the neuron. [1-5]

Neurotransmitter activity

We know that there are different neuropeptides and that small-molecule transmitters exist in the neuron bivalves, including acetylcholine, monoamines, and amino acids. [71] For the events underpinning impulse conduction, the synapse plays a critical role in integrating activities of the nervous system. This synapse is one in which transmission is chemically mediated, i.e., a substance liberated from the nerve ending of one cell brings about excitation in the plasma membrane of the next. In many cases acetylcholine fulfils this function just as it does in the classical myoneural junction. In other instances norepinephrine plays a similar role, although in these cases some structural differences in the synapse appear. Indeed, specialized low-resistance connections exist, coupling the pre and postsynaptic neurons and resulting in extremely rapid transmission. Finally, in all cases in which electrical transmission has been seen a particular structural type of intercellular junction has also been present. [1, 20]

Neural modulation of the physiology of the reproduction in bivalves

Many substances have been candidates as neurotransmitters in bivalves. Acetylcholine, 5-hydroxytryptamine, dopamine, and FMRF amide, they might be physiologically significant in a few species. Acetylcholine and 5-hydroxytryptamine are almost certainly neurotransmitter substances in the gonad whether or not any other neuroactive endocrine substances are release at sites remote from the gonad. [1, 65] Bivalves possess large identifiable nerve cells in their ganglia, and some of these have been shown to be reproductive-regulatory. [72]

For example, in green lipped mussel, *Perna canaliculus*, neurons in the visceral ganglia of both male and female were characterized by immunohistochemical techniques, and found that there are immunoreactivity of anti-5HT and anti-DA in large type and anti-APGWamide in small type of neurons.[38] In the gastropod *Haliotis asinina*, which has a predictable spawning cycle, there are various neuropeptides secreted from anterior ganglia that play a regulatory role in reproduction, like APGWamide, myomodulin, and FMRamide. [73]

Morphological and physiological aspects of gonads and breeding cycles

Sexual differentiation

Gonochorism is the condition of most bivalves, with no external morphological differences between the sexes. [7, 74, 75] However, the presence of some hermaphrodites in wild populations was reported, e.g. in the form of oocytes within the normal testicular tissue (ovotestis), namely in individuals of *Scrobicularia plana*.[76, 77] Some species are naturally predominantly hermaphrodites, with distinct male and female portions of the gonad, like seen in scallop *Pecten maximus*; the mature gonad is divided into two areas: dorsal testis with white colour and ventral ovary with orange-red colour. [7, 78] In *Anadara broughtoni* (48.3-52.5 mm in size), gonads are present at sexual maturity and the sexes were reported as being separated. In *Anadara senilis* from Nigerian coast, studies on the sexuality concluded that it is a protandrous hermaphrodite (monoecious), with animals developing as males first and then changing to be females. [79]

Gametogenesis

Gametogenesis involves the production of gametes in the gonad that occupy a major portion of the visceral mass as in bivalves. Spermatogenesis and oogenesis is related to a period of reproductive cycle that is influenced by external environmental factors. Spermatogenesis occurrence located along the inner periphery of acinus. Spermatogonia are the first cells to become primary spermatocytes by mitotic divisions, later these cells undergo into meiosis to become secondary spermatocytes and spermatids, respectively, then following the differentiation of mature spermatids into spermatozoa without further cell divisions. [28, 32] As to oogenesis, the primary oogonia have potential to do repeated mitosis and in the process differentiate to secondary oogonia, which ingress in the meiotic process until stopping at the prophase stage of meiosis I — the completion of meiosis occurs at fertilisation. During oogenesis, the oocytes greatly increase in size by a process named vitellogenesis, which basically consists in the assemblage of lipids and some glycogen in the ooplasm. [7, 78]

Spawning

In most bivalves, there are various stimuli suggested as being importance in control the breeding cycle, like water temperature, pH range, tide, latitude, and food abundance. [75-80] Whilst extreme temperatures may inhibit spawning, these seem to be less limiting in warmer climates than in temperate waters. It is a widely suggested that in each species may occur only over a critical spawning period and also depending on the physiological condition of the animals and / or their geographical distribution. [81, 82] Generally, gametes are discharged into the mantle cavity and then into the environment by valve movements, relaxation of adductor muscles, enlargement of ostia, and increased ciliary action of the ctenidia [32-79] and are fertilized externally. Internal fertilization in some bivalves females collect sperm in the mantle cavity or gill chamber and then the developing larvae are brooded. The zygote continues develop in various larval forms (trochophore and veliger) up to reaching the juvenile stage. [32] Differences exist even in species of the same genera. For example, the major period of spawning of *Anadara granosa* in southern Europe is from July to October with a peak in August, and larvae can be found for over a two month period. [83] This is different from *A. senilis,* as it appeared that the major spawning period is in October, and some spawning of *A. gmnosa* probably takes place throughout the year. [79] But there are evidences of a peak period in between June and September. *In A. broughtoni* from Japan has spawning time in beginning of August to the end of September. [79]

Evidence for neurosecretory (neuroendocrine) substances involved in reproduction

Bivalve reproduction consists of many critical steps, beginning in nerve centres and ending in the gonads. The steps include sexual development, gametogenesis, fertilization and spawning. On the whole, sexual differentiation processes of bivalves are still in doubt but some aspects are gaining a better understanding. Serotonin, dopamine and sex steroids are some agents that are involved in the sexual differentiation process. [84]

Monoamine oxidase (MAO) regulated by serotonin level is the main elimination pathway for monoamines such as dopamine, serotonin, octopamine and noradrenaline. The MAO activity could be induced by a variety of secondary amines in the environment and could likely modulate serotonin levels in nerve tissues and perhaps sex differentiation. For example, MAO activity in the nerve ganglia and gonad was shown to be induced with a concomitant decrease in serotonin and dopamine in mussels exposed for 90 day, 10 km downstream from a primary-treated municipal effluent plume. [85] Indolamines (serotonin and tryptomine) and catecholamines (i.e., dopamine and noradrenaline) are particular neurotransmitters involved in the integrated actions of neuronal populations that implicate at the sexual differentiation in bivalves.[86-87] The level of dopamine increases after injections of E2 in the sea scallop, but it dropped during active spawning period.[88] Moreover, dopamine was shown to inhibit spawning activity in serotonin-treated *D. polymorpha* mussels. [69] There has been a quest to locate the involved neurons. For instance, an immunohistochemical study was made in the green-lipped mussel, *Perna canaliculus*, using anti-sera raised against neuropeptides and neurotransmitters known to control reproduction and spawning. The authors concluded that there are neurons positive for serotonin (5-HT), dopamine (DA), APGWamide, and egg-laying hormone (ELH) within the visceral ganglia, despite not being able to prove the physiological functions in the control of the reproduction of the studied species. [38]

Many of the hormones in invertebrates are neurohormones, so they are produced by nerve cells. [89] As with conventional neurons, neurosecretory cells are able to receive signals from other neurons. However, unlike ordinary neurons that have cell-to-cell communication over short distances at synapses, neurosecretory cells ultimately release their product into an extracellular space that may be at some distance from the target cells. [89] In an organism with a circulatory system, the neurohormones are typically sent by the vascular route to their target. In contrast, in lower invertebrates that lack an organized circulatory system, the neurohormones apparently simply diffuse from the release site to the target. In molluscs, the neurosecretory cells and nerve cells in ganglia are described as endocrine cells producing neurohormones (dopamine, noradrenaline and serotonin). [89] In *V. verrucosa*, 5-HT was studied by immunohistochemistry, and it was found in serotonergic neurons that were located at a region of the cortex of the visceral ganglion, in serotonergic fibers at the root of branchial nerve, and along the walls of the ovarian follicles and also running between the seminiferous acini. [36] In *Lamelliden scorrianus,* two types of neurosecretory cells were observed on the dorsal surface of cerebropleural

ganglia, which accumulate the neurosecretory material at low temperature. [90]

By all the above, it is logically possible to hypothesize that there is a large potential for xenobiotic endocrine disruption effects on the nervous system controlled reproduction.

Effects of endocrine-disrupting chemicals on bivalves

Endocrine-disrupting chemicals (EDCs) are substances that can interfere with the endocrine system of animals, being this simplistic definition subject to refinements.[91] EDCs are known for a wide range of chemical compounds, including, natural estrogen and synthetic hormones (ethynylestradiol), industrial chemicals (such as alkylphenols, bisphenol A, ethoxylates and tributyltin) and pesticides (*eg*, chlormephos and atrazine). [92-94] Evidence of the effects of these compounds has been presented in the majority of studies with fish, crustaceans, annelids and molluscs. [95, 97] Certain alarming concerns have been increased in human health of EDCs, such as decline in sperm quality, increase in the frequency of development abnormalities of the male reproductive tract, precocious puberty, and altered neuronal development. [98, 99]

Aquatic organisms are being subjected to contact with these substances because they are discharged into the water, and thus appear in rivers, estuaries and sea. [100] This lead to numerous studies on wildlife and consequently the interest on endocrine disruption of invertebrates is obtaining more attention. Nowadays there are facts pointing that bivalves seem to be affected by EDCs, as revealed by the appearance of oocytes in the testes (ovotestis-intersex) of the peppery furrow shell, *S. plana*, from the Avon Estuary, United Kingdom, where there was a likely source of estrogenic chemical from agriculture, and also in the Guadiana Estuary, in Portugal, where the presence of EDCs was thought to mainly derived from urban, industrial and agricultural discharges.[76, 77] In the freshwater mussel, *Elliptio complanata*, waterborne exposure to estrogenic compounds present in municipal effluents (and also direct exposures by injection), were able to alter the metabolism of serotonin and dopamine (both players in the sexual differentiation), likely via E2 receptor-mediate pathway and serotonin receptors.[85] All these examples do show the current pertinence to address EDCs impacts over the nervous system of bivalves, and looking for the impacts of the gonadal maturation events.

References

1. Kay I. Introduction to Animal Physiology. Oxford: BIOS Scientific Publishers Ltd.; 1998.
2. Widmaier EP, Raff H, Strang KT. Human Physiology: The Mechanisms of Body Function. 9 ed. New York: The McGraw-Hill Companies; 2003.
3. Eisenstein EM. Review: Selecting a model system for neurobiological studies of learning and memory. Behav Brain Res. 1997; 82(2): 121-132.
4. Brodal P. The Central Nervous System. 4th ed. New York: Oxford University Press; 1992.
5. Rastogi SC. Essentials of Animal Physiology. 4th ed. New Delhi: New age international Ltd.; 2007.
6. Galliot B, Quiquand M, Ghila L, De Rosa R, Miljkovic-Licina M, Chera S. Origins of neurogenesis, a cnidarian view. Dev Biol. 2009; 332(1): 2-24.
7. Brusca RC, Brusca GJ. Invertebrates. 2nd ed. Massachusetts: Sinauer Associates, Inc.; 2003.
8. Kotikova EA, Raikova OI, Reuter M, Gustafsson MKS. The nervous and muscular systems in the free-living flatworm *Castrella truncata* (Rhabdocoela): an immunocytochemical and phalloidin fluorescence study. Tiss Cell. 2002; 34(5): 365-374.
9. Purschke G, Hessling R. Analysis of the central nervous system and sense organs in *Potamodrilus fluviatilis* (Annelida: Potamodrilidae). Zoolog Anz. 2002; 241: 19–35.
10. Orrha L, Muller MCM. Morphology of the nervous system of Polychaeta (Annelida). Hydrobiologia. 2005; 535(1): 79–111.
11. Moore J. An Introduction to the Invertebrates. 2nd ed. Cambridge: Cambridge University Press; 2006.
12. Reichert H. Introduction to Neurobilogy. 1st ed. Germany: University Press; 1992.
13. Matheson T. Encyclopedia of life sciences: Invertebrate nervous systems. Cambridge: Macmillan Publishers Ltd.; 2002.
14. Loesela R, Nässelb DR, Strausfelda NJ. Common design in a unique midline neuropil in the brains of arthropods. Arthropod Struct Dev. 2002; 31(1): 77-91.

15. Shiga S, Numata H. The Role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae.* Naturwissenschaften. 2000; 87(3): 125-128.

16. Padmaja M, Deecaraman M, Jaganathbose MT. Study of neurosecretory cells in sand lobster *Thenus orientalis* of royapuram coast-chennai. World J Fish & Marine Sci. 2010; 2(2): 82-85.

17. Harzsch S, Glötzner J. An immunohistochemical study of structure and development of the nervous system in the brine shrimp *Artemia salina* Linnaeus, 1758 (Branchiopoda, Anostraca) with remarks on the evolution of the arthropod brain. Arthropod Struct Dev. 2002; 30(4): 251-270.

18. Kirsch R, Richter S. The nervous system of *Leptodora kindtii* (Branchiopoda, Cladocera) surveyed with confocal scanning microscopy (CLSM), including general remarks on the branchiopod neuromorphological ground pattern. Arthropod Struct Dev. 2007; 36(2): 143-156.

19. Whitington PM, Mayer G. The origins of the arthropod nervous system: Insights from the Onychophora. Arthropod Struct Dev. 2011; 40(3): 193-209.

20. Bullock TH. Mollusca: Pelecypoda and Scaphopoda. In: Bullock TH, Horridge GA, editors. Structure and function of the nervous systems of invertebrates. London: W. H. Freeman & Co; 1965. p. 1387-1431.

21. Chase R. Behavior and its neural control in gastropod Molluscs. Oxford: University Press; 2002.

22. Glanzman DL. Habituation in *Aplysia*: The Cheshire cat of neurobiology. Neurobiol Learn Mem. 2009; 92(2):147-154.

23. Croll RP. Catecholamine-containing cells in the central nervous system and periphery of *Aplysia californica.* J Comp Neurol. 2001; 441: 91-105.

24. Barnes RD. Invertebrate Zoology. 2nd ed. Toronto: W. B. Saunders Company; 1968.

25. Pani AK, Croll RP. Pharmacological analysis of monoamine synthesis and catabolism in the scallop, *Placopecten magellanicus.* Gen Pharmacol.1998; 31(1): 67–73.

26. Khanna DR, Yadav PR. Biology of Mollusca. New Delhi: Discovery Publishing House; 2004.

27. Pennak RW. Fresh-water invertebrates of the United States: Protozoa to Mollusca. 3rd ed. New York: John Wiley and Sons; 1989.

28. Gosling E. Bivalve Molluscs: Biology, Ecology and Culture. Oxford: Blackwell Publishing; 2004.

29. Beninger PG, Donval A, Le Pemmec M. The osphradium in *Placopocten magellanicus* and *Pecten maximus* (Bivalvia, Pectinidae): histology, ultrastructure, and implications for spawning synchronisation. Mar Biol. 1995; 123(1) : 121-129.

30. Kraus DW, Doeller JE, Smith PR. A physiological comparison of bivalve mollusc cerebro-visceral connectives with and without neurohemoglobin. I. ultrastructural and electrophysiological characteristics. Biol Bull. 1988; 174(1): 54-66.

31. Morton B. Feeding and digestive in Bivalvia. In: Saleuddin ASM, Wilbur KM, editors. The Mollusca. New York: Academic Press; 1983. p. 65-147.

32. Harrison WF, Kohn JA. Microscopic anatomy of invertebrates. New York: Wiley-Liss, Inc.; 1997.

33. Ruppert EE, Fox RS, Barnes RB. Invertebrate Zoology: A functional evolutionary approach. 7th ed. Belmont CA: Brooks Cole Thomson; 2004.

34. Kamble NA, Londhe SR. Copper sulphate induced neuronal histological changes in the freshwater snail *Bellamya bengalensis* (L). Bioscan. 2011; 6(2): 351-354.

35. Martin K, Huggins T, King C, Carroll MA, Catapane EJ. The neurotoxic effects of manganese on the dopaminergic innervation of the gill of the bivalve mollusc, *Crassostrea virginica.* Comp Biochem Physiol C: Toxicol Pharmacol. 2008; 148(2): 152-159.

36. Siniscalchi A, Cavallini S, Sonetti D, Sbrenna G, Capuano S, Barbin L, et al. Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. Mar Biol. 2004; 144(6): 1205-1212.

37. Odiete WO. Fine-structure of the neurons in the mid-dorsal lobes of the visceral ganglion of the Lamellibranch mollusc *Scrobicularia plana* (da Costa). J Mollus Stud. 1978; 44: 305-321.

38. Mahmud S, Mladenov PV, Sheard P, Chakraborty SC. Characterization of neurons in the visceral ganglia of the green-lipped mussel (*Perna canaliculus*) using antibodies raised against neuropeptides and neurotransmitters. Bang J Anim Sci. 2008; 37 (1): 78 - 85.

39. Blankenship JE, Houck B. Nervous system (invertebrate). New York: McGraw-Hill Companies; 2008.

40. Laming PR, Kimelberg H, Robinsonc S, Salmd A, Hawrylakd N, Müller C , et al. Review: Neuronal–glial interactions and behaviour. Neurosci Bio behav Rev. 2000; 24(3): 295–340.

41. McLaughlin BJ, Howes EA. Structural connections between dense core vesicles in the central nervous system of *Anodonta cygnea* L. (Mollusca, Eulamellibranchia). Z Zellforsch. 1973; 144(1): 75-88.

42. Carroll MA , Catapane EJ. The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica.* Comp Biochem Physiol A Mol Integr Phsiol. 2007; 148(2): 445-450.

43. Henry M, Benlinmame N, Belhsen O, Jule Y, Mathieu M. Immunohistochemical localization of FMRFamide-containing neurons and nerve fibers in the ganglia and the gonad wall of the scallop, *Pecten maximus* (L). Neuropeptides. 1995; 28(2): 79-84.

44. Prior DJ, Lipton BH. An ultrastructural study of peripheral neurons and associated non-neural structures in the bivalve mollusc, *Spisula solidissima.* Tissue Cell. 1977; 9(2): 223-240.

45. Rosenbluth J. The visceral ganglion of *Aplysia californica.* Z Zellforsch. 1963; 60(2): 213-236.

46. Stefano GB. Neurobiology of *Mytilus edulis*. Manchester : Manchester University Press;1990.

47. Torska IV, Bilokrinitskyi VS, Burchinska LF, Genis YD. Properties of neurons of the central nervous system of the freshwater gastropod mollusc, *Planobis corneus.* Neurosci Behave Physiol. 1968; 14(4): 456-471.

48. Peter JS, Young D. Nerve cells and animal behaviour. 2nd ed. Cambridge: Cambridge University Press; 2003

49. Elekes K, Hiripi L, Nemcsók J. Ultrastructural effects of 6-hydroxy-dopamine and 5, 6-dihydroxytryptamine on the central nervous system of fresh-water mussel, *Anodonta cygnea* L. Acta Biol Hung. 1977; 28(3): 259-272.

50. Pentreath VW. Functions of invertebrate glia. In: Ali MA, editor. Nervous systems in invertebrates. New York: Plentum Press; 1987. p. 61-103.

51. Coles JA, Abbott JN. Signalling from neurones to glial cells in invertebrates. TINS. 1996; 19(8): 358-362.

52. Sonetti D, Ottaviani E, Bianchi F, Rodriguez M, Stefano M, Scharres B, et al. Microglia in invertebrate ganglia. Proc Natl Acad Sci U S A. 1994; 91(19):180-184.

53. Paemen LR, Porchet-Hennere E, Masson M, Leung MK, Hughes TK, Stefano GB. Glial localization of interleukin-1á in invertebrate ganglia. Cell Mol Neurobiol. 1992; 12(5): 463-472.

54. Miller SA, Harley JP. Zoology. 5th ed. Boston:The McGraw-Hill Companies; 2001

55. Owen G, McCrae JM. Further studies on the latero-frontal tracts of bivalves. Pro R Soc Lond [Biol]. 1976; 194: 527-544.

56. Pekkarinen M. Histology of the siphons of *Macoma balthica* (Bivalvia: Tellinidae). Ann Zool Fenn. 1986; 23:77-95.

57. Vitonis JEVV, Zaniratto CP, Machado FM, Passos FD. Comparative studies on the histology and ultrastructure of the siphons of two species of Tellinidae (Mollusca: Bivalvia) from Brazil. Zoologia. 2012; 29(3): 219-226.

58. Hardy D. Scallop Farming. Oxford: Fishing News Books; 1991.

59. Barber VC, Eileen EM, Land MF. The fine structure of the eye of the mollusc *Pecten maximus.* Z Zellforsch. 1967; 76: 295-312.

60. Cragg SM, Nott JA. The ultrastructure of the statocysts in the pediveliger larvae of *Pecten maximus* (L.) (Bivalvia). J Exp Mar Biol Ecol. 1977; 27(1): 23-36.

61. Frenkiel L, Moueza M. Ultrastructure des statocysts chez *Scrobicularia plana* et *Tellina tenuis* mollusques lamellibranches Tellinacea. J Mollus Stud. 1982. 482: 148-158.

62. Alexander CG. The osphradium of *Conus flavidus.* Mar Biol. 1970; 6:236-240.

63. Ferretti ME, Sonetti D, Pareschi MC, Buzzi M, Colamussi ML, Biondi C. Effect of serotonin and neuropeptides on adenylate cyclase of the central nervous system and peripheral organs of the freshwater snail *Planorbarius corneus.* Neurochem Int. 1996; 28(4): 417-424.

64. Tinikul Y, Mercier AJ, Sobhon P. Distribution of dopamine and octopamine in the central nervous system and ovary during the ovarian maturation cycle of the giant freshwater prawn, *Macrobrachium rosenbergii.* Tissue Cell. 2009; 41(6): 430-442.

65. Beiras R, Widdows J. Effect of the neurotransmitters dopamine, serotonin and norepinephrine on the ciliary activity of mussel

(*Mytilus edulis*) larvae .Mar Biol. 1995; 122(4): 597-603.

66. Hartenstein V. Review: The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. J Endocrinol. 2006; 190(3): 555-570.

67. Florkin M, Scheer BT. Chemical Zoology. London: Academic Press Inc.; 1972.

68. Tierney AJ, Kim T, Abrams R. Dopamine in crayfish and other crustaceans: Distribution in the central nervous system and physiological functions. Microsc Res Tech. 2003. 60(3): 325-335.

69. Fong PP, Noordhuis R, Ram JL. Dopamine reduces intensity of serotonin-induced spawning in the zebra mussel *Dreissena polymorpha* (Pallas). J Exp Zool. 1993; 266 (1): 79-83.

70. Khotimchenko YS, Deridovich II. The effect of dopamine and galoperidol on cyclic Amp in the gonad of the bivalve mollusc *Mizuhopecten yessoensis* and the sea urchin *Strongylocentrotus intermedius.* Comp Biochem Physiol C Comp Pharmacol. 1989; 92(1): 23-26.

71. Thorndyke MC, Goldsworthy GJ. Neurohormones in invertebrates. Cambridge: Cambridge University Press; 1988.

72. Saleuddin ASM, Wilbur KM. The Mollusca: Physiology Part 1. New York: Academic Press; 1983.

73. York PS, Cummins SF, Degnan SM, Woodcroft BJ, Degnan BM. Marked changes in neuropeptide expression accompany broadcast spawnings in the gastropods *Haliotis asinina*.Front Zool. 2012; 9(9): 1-16.

74. Joaquim S, Matias D, Lopes B, Arnold WS, Gaspar MB. The reproductive cycle of white clam *Spisula solida* (L.) (Mollusca: Bivalvia): Implications for aquaculture and wild stock management. Aquaculture. 2008; 281(1-4): 43-48.

75. Cardoso JFMF, Witte JIJ, van der Veer HW. Growth and reproduction of the bivalve *Spisula subtruncata* (da Costa) in Dutch coastal waters. J Sea Res. 2007; 57(4): 316-324.

76. Chesman BS, Langston WJ. Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? Biol Lett. 2006; 2(3): 420-422.

77. Gomes T, Gonzalez-Rey M, Bebianno MJ. Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal).Ecotox. 2009; 18(8): 104-109.

78. Helm MM, Bourne N, Lovatelli A. Hatchery culture of bivalves. A practical manual. FAO Fisheries Technical Paper. No. 471. Rome: Food and Agriculture Organization of the United Nations (FAO); 2004.

79. Broom MJ. The biology and culture of marine bivalve molluscs of the genus *Anadara*. Manila: The international center for living aquatic resources management; 1985.

80. Ruiz Y, Suarez P, Alonso A, Longo E, Villaverde A, Juan FS. Environmental quality of mussel farms in the Vigo estuary: Pollution by PAHs, origin and effects on reproduction. Environ Pollut. 2011; 159(1): 250-265.

81. Bayne BL. Marine mussels: Their ecology and physiology. 1976, London: Cambridge University Press

82. Sola JC. Reproduction, population dynamics, growth and production of *Scrobicularia plana* da Costa (Pelecypoda) in the Bidasoa estuary, Spain. Aquat Ecol. 1997; 30(4): 283-296.

83. Pathansali D, Soon MK. Some Aspects of Cockle (*Anadara granosa* L.) Culture in Malaya. Proc Indo-Pacific Fish Coun. 1958; 8(2): 26-31.

84. Croll RP, Nason J, Van Minnen J. Characterization of central neurons in bivalves using antibodies raised against neuropeptides involved in the control of egg-laying in gastropods. Inv Reprod Dev. 1993; 24(3): 161-168.

85. Gagne´ F, Blaise C. Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel *Elliptio complanata.* Comp Biochem Physiol C. 2003; 136(2): 117–125.

86. Tsai PS. Gonadotropin-releasing hormone in invertebrates: Structure, function, and evolution. Gen Comp Endocrinol. 2006; 148(1): 48-53.

87. Khotimchenko YS, Deridovich II. The effect of dopamine and galoperidol on cyclic AMP in the gonad of the bivalve mollusc *Mizuhopecten yessoensis* and the sea urchin *Strongylocentrotus intermedius.* Comp Biochem Physiol C Comp Pharmacol. 1989; 92(1): 23-26.

88. Osada M, Normura T. Estrogen effect on the seasonal levels of catecholamines in the scallop *Patinopecten yessooensis.* Comp Biochem Physiol. 1989; 93(2): 349-353.

89. Ketata I, Denier X, Hamza-Chaffai A, Minier C. Endocrine-related reproductive effects in molluscs. Comp Biochem Physiol C Toxicol Pharmacol. 2008; 147(3): 261-270.

90. Jadhav ML, Lomte SV. Neurosecretory Activity of the freshwater Bivalve, *Lamellidens corriaus* (Prasad) (Mollusca: Lamellibranchiata). Proc Indian natn Sci Acad A. 1981; 47(4): 496-500.

91. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS , Soto AM , et al. Review Endocrine-disrupting chemicals: An endocrine society scientific statement. Endocr Rev. 2009; 30(4): 293-342.

92. Overgaard A, Holst K, Mandrup KR, Boberg J, Christiansen S, Jacobsen PR, et al. The effect of perinatal exposure to ethinyloestradiol or a mixture of endocrine disrupting pesticides on kisspeptin neurons in the rat hypothalamus. NeuroToxicol. 2013; 37: 154-162.

93. Ceh K, Majdic G. Pesticides as Endocrine Disruptors.Slov Vet Res. 2010; 47(4): 163-166.

94. Cubero-Leon E, Ciocan CM, Hill EM, Osada M, Kishida M, Itoh N, et al. Estrogens disrupt serotonin receptor and cyclooxygenase mRNA expression in the gonads of mussels (*Mytilus edulis*). Aquat Toxicol. 2010; 98(2): 178-187.

95. Zhou J, Xiao-Shan Z, Zhong-Hua C. Endocrine disruptors: an overview and discussion on issues surrounding their impact on marine animals. J Mar Anim Ecol. 2009; 2(2): 7-17.

96. Hutchinson TH. Reproductive and developmental effects of endocrine disrupters in invertebrates: in vitro and in vivo approaches.Toxicol Lett. 2002; 131(1-2): 75-81.

97. Silva P, Rocha MJ, Cruzeiro C, Malhão F, Reis B, Urbatzka R, et al. Testing the effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens as found in the Douro River (Portugal) on the maturation of fish gonads—A stereological study using the zebrafish (*Danio rerio*) as model. Aquat Toxicol. 2012; 15(124–125): 1-10.

98. Norris DO, Carr JA. Endocrine disruption: Biological basis for health effects in wildlife and humans. New York: Oxford University Press; 2006.

99. World Health Organization (WHO). The world health report 2002: Reducing risks, promoting healthy life. Geneva (Switzerland); 2002.

100. Ribeiro C, Pardal MA, Martinho F, Margalho R, Tiritan ME, Rocha E, et al. Distribution of endocrine disruptors in the Mondego river estuary, Portugal. Environ Monit Assess. 2009; 149(1-4): 183-193.

CHAPTER2

THE PEPPERY FURROW SHELL (*SCROBICULARIA PLANA* (DA COSTA, 1778)) - AN OVERVIEW

The Peppery Furrow Shell (*Scrobicularia plana* (da Costa, 1778)) — An overview

[Formatted as a manuscript to be submitted for publication in an international journal. The version in this Thesis may change depending on further improvements from review by peers]

Sukanlaya Tantiwisawaruji [a,b,c]

[a]King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand.
[b]Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Porto, Portugal.
[c]Histomorphology, Physiopathology and Applied Toxicology Group, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto (U.Porto), Porto, Portugal.

Running title: The peppery furrow shell

Key words: ecology, biology, bivalve, *S. plana*

Correspondence to:

Sukanlaya Tantiwisawaruji
King Mongkut's University of Technology Thonburi (KMUTT)
Prachautid Road, Bangmod, Tungkru,
Bangkok 10140
Thailand
E-mail: sukanlaya_tan@hotmail.com

Abstract

Scrobicularia plana is one of the most important marine bivalve that habitats widely in intertidal soft sediment along the Atlantic coast from Europe to Africa. There have been various researches on this species such as distribution, growth, reproduction. *S. plana* is primarily gonochoristic, and the gonadal reproductive sequence can be classified in four stages: indifferent (resting stage); development (pre-active stage); mature (active stage); and spawning. External fertilization occurs within an hour after mature gametes of both sexes are released in the water. While in the early development stages, the larvae live as a part of the plankton and, after one month, it becomes pediveliger and then a juvenile. The post-larva stage thus chooses a suitable surface to settle on and grow into adults. The pattern of breeding cycle can present difference according to the latitude of habitat, which is attributed mostly to thermal differences and food availability. Studies of the nervous and sensory systems of *S. plana* are still rare. Beside commercial value (protein source), from fishery and aquaculture in various countries, there is a growing interest in studying and using that bivalves as a bioindicator, *i.e.*, to monitor aquatic environments, because of several characteristic, such as widespread location (namely in areas prone to pollution), abundance, easy to collect, considerable adaptability, and pollution tolerance.

2.1 Introduction

This Chapter gives a brief summary of the general biology of the bivalve *Scrobicularia plana*, which common name is peppery furrow shell (in Portugal "lambujinha"). The species is commonly found along the Northeast Atlantic coast (Bocher *et al*. 2007), up from the Norwegian Sea down to the Senegalese coast, occurring also in the Mediterranean Sea. It lives in shallow intertidal soft-sediment habitats, either in sandy or in (preferably) muddy seacoasts. This bivalve is a filter feeder, with long siphons, burying itself into the sediment in a vertical position and with the siphons raised above the substrate level (Boldina-Cosqueric *et al*. 2010; Pizzolla 2002) and, when buried, it leaves star-shaped marks on the sediment surface (Figure 1). The animal feeds on phytoplankton and benthic diatom, behaving slightly like a suspension feeder. The species' patchy pattern of geographical distribution along the wide range of latitude (and of environmental conditions) indicates a very good adaptability and high physiological tolerance (Guerreiro 1998).

Figure 1. Star-shaped marks (black arrows) on the sediment surface and habitat aspect of *Scrobicularia plana* in the Ria Formosa, Portugal. Image credit: Sukanlaya Tantiwisawaruji.

2.2 Taxonomy

Scrobicularia plana (from the Latin word *scrobiculus,* a diminutive of *scrobis*, a ditch) belongs to the class Bivalvia (Figure 2), whose other members, like mussels, oysters,

scallops and clams have two calcareous valves enclosing a thin mantle surrounding the soft body. A muscular foot allows locomotion. *S. plana* is mentioned as belonging to the Family Semilidae (ITIS 2013; WORMS 2013) or to Scrobiculariidae (MarLIN 2013); it is characterized by its round, flattened and thin shell with both valves being symmetrical along the hinge line at the dorsal ridge.

Kingdom: Animalia
Phylum: Mollusca
Class: Bivalvia
Order: Veneroida
Superfamily: Tellinoidea
Family: Semilidae or Scrobiculariidae
Genus: *Scrobicularia*
Species: *Scrobicularia plana*

Figure 2. The complete taxonomy of *Scrobicularia plana* (IT IS 2013).

2.3 Distribution and ecology

2.3.1 Food

Like most bivalves, *S. plana* is a deposit-feeder inhabiting aquatic sediments (Pizzola 2002; Sola 1997). Benthic microorganisms serve as the main food, as well as, *S. plana* is a prey species for a variety of species, including humans (Gosling 2004; Sola 1997). In the planktonic stage, the species is an important food source in the aquatic ecosystem (Hughes 1969; Morton 1983). As *S. plana* grows, its vulnerability decreases as it become sessile and its shell thickens. Adults are preyed upon by specific predators including starfish, crab, dog-whelk and birds (Wanink and Zwarts 2001).

2.3.2 Temperature

Temperature is a very important factor for bivalves. Variations in temperature bring changes in timing, composition and duration of nutritional algal blooms which affect growth, reproduction and survival of *S. plana* (Santos *et al*. 2011). These bivalves live in water temperatures ranging from 6 to 15.5°C in North Wales (Hughes 1971), or from 10 to 27°C in the Mediterranean Sea (Casagranda and Boudouresqe 2005). Experiments testing the effect of environmental temperature changes indicated that this abiotic factor is directly proportional to the animal's oxygen consumption, but inversely proportional to biomass and body growth (Lino 2010).

Salinity

Salinity plays a critical role in the estuarine habitat, for example by increasing the precipitation and aggregation of solids, which increases the water turbidity (Akberali and Davenport 1981; Sindermann 2006). Salinity varies with depth and shape of the estuary channel due to wind and tidal movement and evaporation from the water surface (Castro and Huber 2008). *S. plana* can be found in water salinities ranging from 11 ‰ (Green 1957) to 34.5 ‰ (Freeman and Rigler 1957).

2.3.3 Substrates

Sediment conditions are key in the distribution and physiology of benthic organisms. The substrate of most estuaries is sand or soft mud (Gosling 2004; Sindermann 2006). Mud is the combination of silt and clay, which is rich in organic compounds. Mud substrates have poor water movement within and frequently become anoxic at a shallow depth of substrate (Boldina-Cosqueric 2010; Castro and Huber 2008). *S. plana* can inhabit sandy substrates, but prefers the muddy ones. This could be verified in the Tagus River (Portugal) estuary, where the specimens densities are higher in the muddy than in the sandy sediments (Conde *et al.* 2011). In mud substrates, the animal can bury itself ≈ 20 cm from the substrate surface in summer, but in winter they can descend to twice this depth (Santos *et al.* 2005; Mouneyrac *et al*. 2008).

2.4 General morphology

Information on this topic exists in works made across many decades (ITIS 2013; Hughes 1969; Hodgson and Trueman 1981; Morton 1983; Pizzolla 2002; Lino 2010). In summary, *S. plana* has a thin, flattened, rounded, and typically bilaterally symmetrical shell (Figure. 3). Externally, the valves appear white, grey, yellow or straw in color and have thin growth lines arranged concentrically until the umbo (the oldest shell part). The internal surface varies less in color and is usually whitish. On the dorsal region, near the umbo, the internal shell consists of hinge and teeth (two teeth in the right valve and one in left valve), which are supported by ligaments. Incurrent and excurrent siphons locate at the posterior end of the shell.

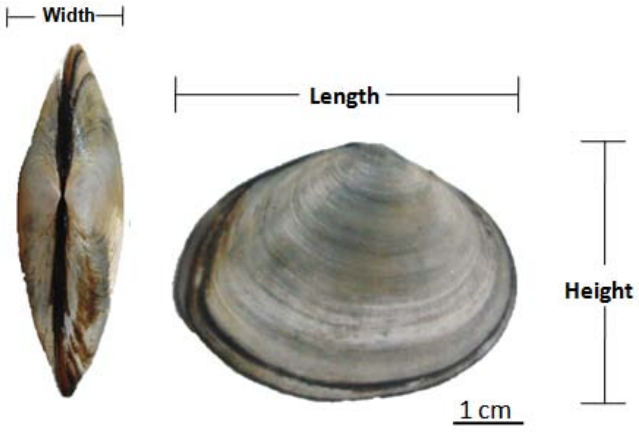

Figure 3. External views of the shell of *Scrobicularia plana*.
Image credit: Sukanlaya Tantiwisawaruji.

The internal organs are in the visceral mass, covered by mantle on both sides. Both sheets of the mantle are very thin and flat, with an epithelium rich in cilia that maintains constant the water current inside the mantle cavity, where the gills are situated. On the anterior area of the visceral mass, there is a pair of labial palps that bring food particles to the mouth. The foot emerges from the visceral ventral region. Within the latter and foot lie the digestive, excretory, circulatory, reproductive, and nervous systems (Figure 4). As the last two systems are the most relevant within the context of this Thesis, they are concisely described below.

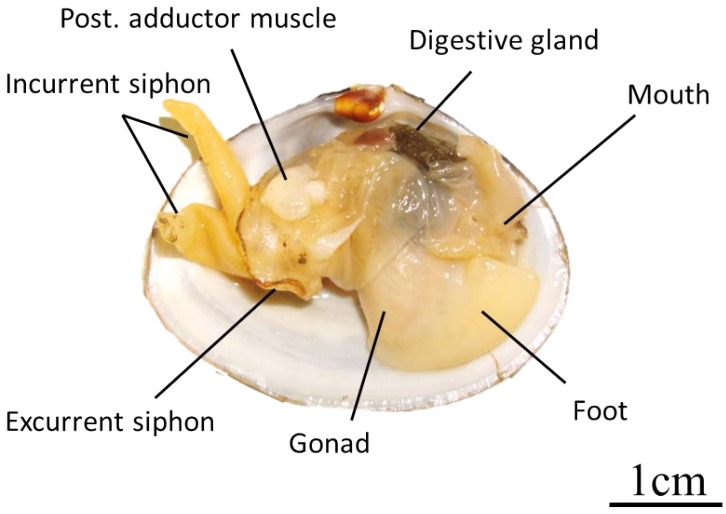

Figure 4. Internal structure of *Scrobicularia plana* without the right valve.
Image credit: Sukanlaya Tantiwisawaruji.

2.4.1 Reproduction

S. plana can reach sexual maturity at a mean length of ≈ 21.8 mm and at the age of 16 months (Sola 1997), despite other studies reported ages above two years (Paes-da-Franca 1956; Guerreiro 1998). This may depend on the population considering that Santos *et al*. (2011) reported that sexual development occurs in specimens that have minimal lengths from 14.8 (Portugal) to 25.0 mm (Norway).

S. plana is predominantly gonochoristic (Hughes 1971; Ruiz *et al*. 1995; Pizzolla 2002) but some cases of intersex (in males that also exhibit oocytes, in various extents) were documented at the southwest coast of the United Kingdom (Chesman and Langston 2006; Ciocan *et al.* 2012) and at the Guadiana estuary, in Portugal (Gomes *et al*. 2009).

Gonadal maturation happens after one year, during summer, with the gonads being then after retained in winter (Santos *et al*. 2011). In bivalves, gametogenesis can be classified via grading scales, such as using the stages defined by Sola (1997): **Stage 0**: There are few primary germ cells within small acini, localized near the wall of the digestive tubules. The sex cannot be identified at this stage; **Stage 1**: Sex cannot be identified but the walls of the acini are thicker; **Stage 2**: Sexual differentiation has occurred. The primary and secondary spermatocytes and a few spermatids are present in the acini of males. In females, the oogonia are arranged at the periphery of the acini; **Stage 3**: Acini are more grouped and bigger; **Stage 4**: Ripe sperm occupies most of the area of acini and ripe ova appear in the centre of the lumen. Some oocytes are attached to the acini wall by a thin stalk. When spawning starts, the free sperm and a number of ripe ova are released from the acini. Some are empty and appear isolated, with the outer wall broken down. Other evaluation schemes exist, such as that of Mouneyrac *et al*. (2008), in which the several stages of gonad in the reproductive cycle pass through: indifferent; development; mature (ripe); spawning; and spent. Summarizing, and for practical purposes, the gonadal development of *S. plana* can be classified in four developmental stages as in Table 1 (Sola 1997; Raleigh and Keegan 2006).

Table 1. The stages of gonadal development of *Scrobicularia plana.*

Stages	Male	Female
1: Resting	There are few germ cells.	There are some residual large oocytes and larger lumens.
2: Pre-active	Germ cells are relatively rare and usually consist of a small peripheral layer of spermatogonia.	There are no residual oocytes at all and only a limited number of oogonia and small oocytes occur peripherally.
3: Active	Central lumens inside large acini are small or absent.	There are few nutritive particles and follicular cells.
4: Spawning	Sperm are often visible lying loose within these spaces, in radial row in the center of the lumens, and are ready to be released.	The distal walls of many acini are interrupted and detached mature oocytes often occur within acini. Nutritive particles are found in some acini.

Post-spawning fertilization occurs externally, within an hour after mature gamete release (Hughes 1971; Sola 1997). Embryos develop in the larvae trochophore and then in the veliger stage (a plankton-eating predator). During the early development stages, the larvae live as a part of the plankton, and this may happen for a long time in this species (Raleigh and Keegan 2006). In particular, at 18ºC, it was estimated that veligers took one month to become pediveligers, which still needed some weeks before completing metamorphosis to become juveniles with perfectly functioning siphons (Frenkiel and Mouëza 1979). The post-larva stage chooses a suitable surface to settle on and grow into adults. The *S. plana* breeding cycle can present different patterns according to the latitude of its habitat; this is attributed mostly to thermal differences and food availability (Sola 1997; Wanink and Zwarts 2001; Santos *et al*. 2011). For example, in French estuaries, the spawning period of *S. plana* can be observed from May to July, then a long pause occurs until the end of the year, and gametogenesis starts again from January, in an indifferent stage, completing the cycle again in May. The same can be observed in Ireland (Raleigh and Keegan 2006). At lower latitudes (e.g., Spain), the spawning period starts earlier (March), the reproductive pause lasting only from October to December (Rodríguez-Rúa 2003).

2.4.2 Nervous and sensory systems

There are scarce studies on the nervous and sensory systems of *S. plana*. The study of Odeite (1978b) investigated the fine structure of neurons in the mid-dorsal lobes of the visceral ganglia. It was found that neurons contain numerous glycogen granules and organelles such as mitochondria, endoplasmic reticulum, and "Golgi bodies". Furthermore, there are large masses of orange or reddish multi-globular bodies in the extracellular space surrounding neural and glial cells and in the connective tissue associated to the ganglia.

The mantle and siphon possess chemoreceptors that respond to external and internal water flows. Their stimulation may elicit siphonal withdrawal and valve closure as a general stress avoidance response. *S. plana* is an osmoconforming bivalve, in which the valve closure is mediated by the detection of osmotic pressure change (Freeman and Rigler 1957). Ciliated tufts on the mantle and siphons behave as sensory organs that are involved in mechano- or chemoreception. Previous study suggests that the cruciform muscle and the papilla sense organ are chemoreceptors responsible for blood pressure regulation in the siphons. Moreover, it was proved that the papilla is a chemoreceptor and works together with the cruciform muscle (Odiete 1978a; Harrison and Kohn 1997).

2.5 The relevance of peppery furrow shell for human consumption and in toxicology

2.5.1 Economic importance

There are many varieties of bivalves and a large number are commercially valuable from fisheries and aquaculture production. They are a cheap source of protein and minerals in tropical and warm temperate areas of over 13 million tons in the recent year (FAO 2014; Idayachandiran *et al*. 2014). On the Atlantic coast of Western Europe, Mediterranean, and Western coast of Africa *S. plana* is one of the key species of the intertidal community, being harvested and exploited on an intensive commercial scale in various countries (Casagranda and Boudouresque 2005).

2.5.2 Bioindicators species

There has been a long continued interest in the impacts of contaminants on aquatic systems, and numerous studies have been conducted focusing on aquatic animals as bioindicators (Andrew *et al.* 2008; Porte *et al*. 2006; Solé *et al.* 2009). *S. plana* is widely known as a valuable species for studies biomonitoring metallic contamination in estuarine

sediments (Bryan and Hummerstone 1978; Cheggour *et al*. 2000; Coelho *et al*. 2006), tributyltin (TBT) bioavailability as deposit-feeding bivalve in sediments (Ruiz *et al*. 1995; Norris and Carr 2006), estrogens as endocrine disruption compounds (Langston *et al*. 2007). These investigations recommend *S. plana* as a suitable species for helping to understand and assess the biological impact of aquatic contaminants according to the typical criteria for ideal bioindicator organisms as following (Goldberg 1986; Mouneyrac *et al*. 2008):

a) They are sedentary and abundant in the Europe and Africa;

b) Their life span may be more than 7 years, which is sufficient to allow the sampling of more than one-year-class;

c) They are good bioaccumulators of metals and do signal changes in the environment;

d) They are easy to sample and strong enough to survive in the laboratory;

e) Their size allows sufficient tissue collection for the most diverse types of analyses.

As earlier mentioned, in estuarine English and Portuguese habitats some *S. plana* populations exhibited intersex (Chesman and Langston 2006; Gomes *et al*. 2009). This makes them potentially useful for measuring genetic changes related to intersex induction. Such condition is actually inducible with experimental exposure to endocrine disruptors (Ciocan *et al*. 2012).

Acknowledgments

The author expresses her gratitude for the scholarship that given by the Thai Government Science and Technology, and for the commentaries of Professor Eduardo Rocha, Professor Maria João Rocha and Professor Uthaiwan Kovitvadhi, that helped in improving this text.

3. References

Andrew, M. N., Dunstan, R. H., O'Connor, W. A., Van Zwieten, L., Nixon B. & MacFarlane, G. R. (2008) Effects of 4-nonylphenol and 17α-ethynylestradiol exposure in the Sydney rock oyster, *Saccostrea glomerata*: Vitellogenin induction and gonadal development. *Aquatic Toxicology* 88, 39-47.

Akberali, H. B. & Davenport, J. (1981) The responses of the bivalve *Scrobicularia plana* (da Costa) to gradual salinity changes. *Journal of Experimental Marine Biology and Ecology* 53, 251-259.

Bocher, P., Piersma, T., Dekinga, A., Kraan, C., Yates, M., Guyot, T., Folmer, E. & Radenac, G. (2007) Site- and species-specific distribution patterns of molluscs at five intertidal soft-sediment areas in northwest Europe during a single winter. *Marine Biology* 151, 577-594.

Boldina-Cosqueric, I., Amiard, J.-C., Amiard-Triquet, C., Dedourge-Geffard, O., Métais, I., Mouneyrac, C., Moutel, B. & Berthet, B. (2010) Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. *Ecotoxicology and Environmental Safety* 73, 1733-1741.

Bryan, G. W. & Hummerstone, L. G. (1978) Heavy-metals in burrowing bivalve *Scrobicularia plana* from contaminated and uncontaminated estuaries. *Journal of the Marine Biological Association of the United Kingdom* 58, 401-419.

Casagranda, C. & Boudouresqe, C. F. (2005) Abundance, Population Structure and Production of *Scrobicularia plana* and *Abra tenuis* (Bivalvia: Scrobiculariдae) in a Mediterranean Brackish Lagoon, Lake Ichkeul, Tunisia. *International Review of Hydrobiology* 90, 376–391.

Castro, P. & Huber, M. E. (2008) *Marine Biology*. McGraw-Hill, New york.

Cheggour, M., Langston, W. J., Chafik, A., Texier, H., Kaimoussi, A., Bakkas, S. & Boumezzough, A. (2000) Metals in the bivalve molluscs *Scrobicularia plana* (da Costa) and *Cerastoderma edule* (L.) and associated surface sediment from Oum er Rbia estuary (Moroccan Atlantic coast). *Toxicological and Environmental Chemistry* 77, 49-73.

Chesman, B. S. & Langston, W. J. (2006) Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? *Biology Letters* 2, 420-422.

Ciocan, C. M., Cubero-Leon, E., Peck, M. R., Langston, W. J., Pope, N., Minier, C. & Rotchell, J. M. (2012) Intersex in *Scrobicularia plana*: transcriptomic analysis reveals novel genes involved in endocrine disruption. *Environmental Science and Technology* 46, 936-942.

Coelho, J. P., Rosa, M., Pereira, E., Duarte, A. & Pardal, M. A. (2006) Pattern and annual rates of *Scrobicularia plana* mercury bioaccumulation in a human induced mercury gradient (Ria de Aveiro, Portugal). *Estuarine Coastal and Shelf Science* 69, 629-635.

Conde, A., Novais, J. M. & Dominguez, J. (2011) A field experiment on the reproductive success of the invasive clam *Mya arenaria* (Bivalvia) in the Tagus estuary: coexistence with the native clam *Scrobicularia plana*. *Scientia Marina* 75, 301-308.

FAO (2014) *World review of fisheries and a aquaculture*. Rome, p. 233.

Freeman, R. & Rigler, F. H. (1957) The responses of *Scrobicularia plana* (da Costa) to osmotic pressure changes. *Journal of the Marine Biological Association of the United Kingdom* 36, 553-567.

Frenkiel, L. & Mouëza, M. (1979) Développement larvaire de deux Tellinacea, *Scrobicularia plana* (Semelidae) et *Donax vittatus* (Donacidae). *Marine Biology* 55, 187-195.

Goldberg, E. D. (1986) The mussel watch concept. *Environmental Monitoring and Assessment* 7, 91-103.

Gomes, T., Gonzalez-Rey, M. & Bebianno, M. J. (2009) Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). *Ecotoxicology* 18, 1104-1109.

Gosling, E. (2004) *Bivalve Molluscs: Biology, Ecology and Culture*. Fishing News Books. Great Britain, p. 443.

Green, J. (1957) The Growth of *Scrobicularia plana* (da Costa) in the Gwendraeth estuary. *Journal of the Marine Biological Association of the United Kingdom* 36, 41-47.

Green, J. (1967) Activities of the siphons of *Scrobicularia plana* (da Costa). *Journal of Molluscan Studies* 37, 339-341.

Guerreiro, J. (1998) Growth and production of the bivalve *Scrobicularia plana* in two southern european estuaries. *Vie et Milieu* 48, 121-131.

Harrison, W. F. & Kohn, J. A. (1997) *Microscopic anatomy of invertebrates*. Wiley-Liss,Inc. New York.

Helm, M. M. (2004) Hatchery culture of bivalves: a practical manual. *FAO Fisheries Technical Paper*. Rome, Food and Agriculture Organization of the United Nations (FAO), p. 471.

Hodgson, A. N. & Trueman, E. R. (1981) The siphons of *Scrobicularia plana* (Bivalvia, Tellinacea) - Observations on movement and extension. *Journal of Zoology* 194, 445-459.

Hughes, R. N. (1969) A study of feeding in *Scrobicularia plana. Journal of the Marine Biological Association of the United Kingdom* 49, 805-823.

Hughes, R. N. (1971) Reproduction of *Scrobicularia plana*, da Costa (Pelecypoda: Semelidae) in North Wales. *Veliger* 14, 77-81.

Idayachandiran, G., Muthukumar, A., Kumaresan, S. & Balasubramanian, T. (2014) Nutritional value of marine bivalve, *Donax cuneatus* (Linnaeus, 1758) from Cuddalore coastal waters, southeast coast of India. *Inventi Impact: Life Style* 2014, 15-19.

ITIS (2013) Integrated Taxonomic Information System. Available online at http://www.itis.gov. [Accessed on 7 September 2013.]

Langston, W. J., Burt, G. R. & Chesman, B. S. (2007) Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. *Marine Ecology Progress Series* 333, 173-184.

Lino, F. C. (2010) *Effect of temperature in oxygen consumption and body mass condition during a starvation period in the peppery furrow shell - Scrobicularia plana (da Costa)*. Engineer Zoology. Master dissertation.

MArLIN (2013) The Marine Life Information Network. Available online at http://www.marlin.ac.uk/. [Accessed on 7 September 2013.]

Morton, B. (1983) The Mollusca : *Feeding and digestive in bivalvia*. Academic Press. New York.

Mouneyrac, C., Linot, S., Amiard, J. C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C. & Pellerin, J. (2008) Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. *General and Comparative Endocrinology* 157, 133-141.

Norris, D. O. & Carr, J. A. (2006) *Endocrine Disruption.* Oxford University Press. Oxford.

Odiete, W. O. (1978a) Cruciform muscle and its associated sense organ in *Scrobicularia plana* (da Costa). *Journal of Molluscan Studies* 44, 180-189.

Odiete, W. O. (1978b) Fine-structure of the neurons in the mid-dorsal lobes of the visceral ganglion of the Lamellibranch mollusc *Scrobicularia plana* (da Costa). *Journal of Molluscan Studies* 44, 305-321.

Odiete, W. O. (1979) Central nervous control of the adductor behavior of Lamellibranch mollusks. *Malacologia* 18, 499-506.

Paes-da Franca, M. L. (1956) Variação sazonal das gónadas em *Scrobicularia plana* (da Costa). *Arquivos do Museu Bocage* 27, 107-130.

Pizzolla, P. (2002) *Scrobicularia plana*. Peppery furrow shell. Marine Life Information Network: Biology and Sensitivity Key Information Sub-programme. Plymouth: Marine Biological Association of the United Kingdom. Available online at http://www.marlin.ac.uk/speciesinformation.php?speciesID=4316>. [Accessed on 7 September 2013.]

Raleigh, J. & Keegan, B. F. (2006) The gametogenic cycle of *Scrobicularia plana* (Mollusca: Bivalvia) in Mweeloon Bay (Galway, west coast of Ireland). *Journal of the Marine Biological Association of the United Kingdom* 86, 1157-1162.

Rodríguez-Rúa, A., Prado, M. A., Romero, Z. & Bruzón, M. (2003) The gametogenic cycle of *Scrobicularia plana* (da Costa, 1778) (Mollusc: Bivalve) in Guadalquivir estuary (Cádiz, SW Spain). *Aquaculture* 217, 157-166.

Ruiz, J. M., Bryan, G. W., Wigham, G. D. & Gibbs, P. E. (1995) Effects of tributyltin (TBT) exposure on the reproduction and embryonic development of the bivalve *Scrobicularia plana*. *Marine Environmental Research* 40, 363-379.

Santos, C. D., Granadeirop, J. & Palmeirim, J. M. (2005) Feeding ecology of Dunlin *Calidris alpina* in a southern european estuary. *Ardeola* 52, 235-252.

Santos, S., Cardoso, J. F. M. F., Carvalho, C., Luttikhuizen, P. C. & van der Veer, H.W. (2011) Seasonal variability in somatic and reproductive investment of the bivalve *Scrobicularia plana* (da Costa, 1778) along a latitudinal gradient. *Estuarine, Coastal and Shelf Science* 92, 19-26.

Sindermann, C. J. (2006) *Coastal Pollution: Effect on living resources and humans*. Taylor & Francis Group, Oxford.

Sola, J. C. (1997) Reproduction, population dynamics, growth and production of *Scrobicularia plana* da Costa (Pelecypoda) in the Bidasoa estuary, Spain. *Netherland Journal of Aquatic Ecology* 30, 283-296.

Solé, M., Kopecka-Pilarczyk, J. & Blasco, J. (2009) Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. *Environment International* 35, 523-531.

Wanink, J. H. & Zwarts, L. (2001) Rate-maximizing optimality models predict when oystercatches exploit a cohort of the bivalve *Scrobicularia plana* over a 7-year time span. *Journal of Animal Ecology* 70, 150-158.

WORMS (2013) World Register of Marine Species. [cited 7/09/2013]. Available online at http://www.marinespecies.org/aphia.php?p=taxdetails&id=141424. [Accessed on 7 September 2013.]

CHAPTER3

QUALITATIVE AND QUANTITATIVE INSIGHTS INTO THE 3D-MICROANATOMY OF THE NERVOUS GANGLIA OF THE PEPPERY FURROW SHELL *SCROBICULARIA PLANA* (BIVALVIA, TELLINOIDEA, SEMELIDAE)

Qualitative and quantitative insights into the 3D-microanatomy of the nervous ganglia of *Scrobicularia plana* (Bivalvia, Tellinoidea, Semelidae)

[Manuscript accepted for publication in the journal Molluscan Research. The version in this Thesis may be not equal to that in the final published article, after editorial changes.]

Sukanlaya Tantiwisawaruji[a,b,c], Uthaiwan Kovitvadhi[d],
Miguel Ângelo Pardal[e], Maria João Rocha[a,b] and Eduardo Rocha[a,b]

[a]Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), 4050-313, Porto, Portugal.
[b]CIIMAR/CIMAR - Interdisciplinary Centre of Marine and Environmental Research, University of Porto (U.Porto), 4050-123, Porto, Portugal.
[c]King Mongkut's University of Technology Thonburi (KMUTT), Bangkok 10140, Thailand.
[d]Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
[e]Centre for Functional Ecology(CFE), University of Coimbra, 3001-401 Coimbra, Portugal.

Running title: 3D-reconstruction of *S. plana* nervous ganglia

Key words: 3D-reconstruction, bivalve, histology, morphometry, nervous system

Correspondence to:

Eduardo Rocha
Laboratory of Histology and Embryology
Department of Microscopy
Institute of Biomedical Sciences Abel Salazar (ICBAS)
University of Porto (U.Porto)
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto
Portugal
Phone: 220 428 245
E-mail: erocha@icbas.up.pt

Abstract

The nervous system of bivalves is bilaterally symmetrical and consists of interconnected cerebral, pedal, and visceral ganglia, which may be partially to totally fused. We studied the microanatomy of the ganglia of *Scrobicularia plana* using three-dimensional (3D) reconstruction. We also address the hypothesis that intersex differences in the neural structure may exist. Each type of ganglion had a peculiar 3D-shape, and the cerebral ganglia shape was slightly asymmetrical. The visceral, pedal and cerebral ganglia are respectively smaller in volume, but only the pedal ganglion volume was positively correlated with the animal's length, height and width; suggesting functional implications. As to total surface area, correlations were found for the cerebral and visceral ganglia, but it is the visceral that consistently showed strong positive correlations with each biometric parameter. The medulla may often penetrate the cortex and touch the capsule in areas that (contrary to what could be suspected) are not connected with emerging nerves. Despite the differences in volume and surface area among ganglia, the volume ratio of cortex vs medulla is fairly stable (≈ 1.5), suggesting a functional optimum. Finally, we conclude that the ganglia of males and females do not show significant quantitative differences.

Introduction

The microanatomy of the nervous system of bivalves is somewhat simple in design, being overall bilaterally symmetrical and generally consisting of three pairs of ganglia (cerebral, pedal, and visceral), which are either partially, mostly or totally fused (Morse and Zardus1997; Gosling 2004). Despite the general aspects that are common among species, the detailed anatomy varies according to species. Even the types of ganglia that are either well separated or fused, and to what degree, varies among species (Morse and Zardus 1997). This kind of anatomical variability occurs across other invertebrates and its phylogenetic implications have long been investigated in the most diverse organisms (Landacre 1920); and continues to the present (e.g., Martynov *et al*. 2011). Studies of the adult central nervous system (CNS) and its ontogeny (e.g., Ellis and Kempf 2011) have provided fundamental phylogenetic and taxonomic information. The focus of morphological studies of the CNS of invertebrates, particularly in Mollusca, has not been so much on details of the nerves but rather on the nervous ganglia components, at times including its 3D-anatomy (Martynov *et al*. 2011). Considering the large abundance of species, and the vast ecologic and economic importance of bivalves (Smaal 1991; FAO 2004; Gosling 2004), there is a paucity of studies on the microanatomy of the CNS, even for the most common and/or economically important species. Moreover, sex-related differences in CNS anatomy are virtually unstudied in bivalves, although it is known that males and females can differ in morphofunctional aspects of their CNS, such as the significantly different response to serotonin, which has an important role in reproduction (Siniscalchi *et al*. 2004).

The peppery furrow shell (*Scrobicularia plana* (da Costa, 1778)) is an intertidal bivalve, usually found buried in mud or muddy sand. It can be found from offshore to estuarine coastlines, and mostly between Northwest Europe and South Africa (Santos *et al*. 2011). This species is economically relevant in many of those regions (Guerreiro 1998) and it is being used as a bioindicator species in biomarker-based approaches that, among other targets, includes the evaluation of neurotoxicity by biochemistry (Solé *et al* 2009; Boldina-Cosqueric *et al.* 2010). Nevertheless, there are no published detailed descriptions of the microanatomy of the nervous system. The only previous study of neuronal aspects in *S. plana* is that of Odiete (1978), which examined the fine-structure of neurons in mid-dorsal regions of the visceral ganglion.

Our aim herein was to conduct the first three-dimensional (3D) reconstruction of the *S. plana* ganglia, while estimating their total volumes and surface areas in the 3D models, and the relative volumes of the cortex and medulla. We also investigated whether sexual differences existed in ganglion size and internal composition. One rationale for the hypothesis relies on the key roles of the neurosecretory neurons in governing gonadal maturation, particularly in females.

Materials and methods

We used adult *S. plana* obtained from the estuary of the Mondego River, Portugal. Samples were taken in April, when animals have mature gonads. After field collection, the animals were kept for 24 hours in glass aquaria, with well aerated seawater (salinity 30‰), and at a constant temperature of 15°C. For this study, 6 males and 6 females were sampled and used for the 3D-dimensional reconstructions. The shell length (cm), width (cm) and height (cm) were measured with a Vernier caliper. Before dissection, the animals were anesthetized in a seawater solution of magnesium chloride (6%). The bodies were removed carefully from their shells and then immediately fixed in 10% buffered formalin, at room temperature. After fixation for 24 hours, the samples were washed in 70% ethanol, and further processed through 90% and 100% ethanol, and xylene, using an automatic tissue processor (Leica TP1020, Germany). The samples were then embedded in high quality paraffin (Paraplast Plus, Sigma-Aldrich), using a modular tissue embedding center (Leica EG 1140H, Germany). Each animal was entirely and serially sectioned in the sagittal plane. A fully motorized rotary microtome (Leica RM2155, Germany) was used to produce 12 μm thick sections, which were stained with hematoxylin and eosin, cleared in xylene, and mounted with DPX (Sigma-Aldrich). All the sections containing the ganglia were selected for software assisted 3D-dimensional reconstruction.

In every selected slide each ganglion was photographed under a light microscope (Olympus BX50, Japan), equipped with a digital camera (Olympus Camedia C-5050, Japan). Each photograph provided a high resolution image (JPEG, 2560x1920 pixels), and was taken with the 10 X objective lens for capturing each entire ganglion profile.
The three-dimensional reconstructions were made digitally from the original stacks of images, using the BioVis3D software (Ver. 3.0, BioVis3D, Uruguay). Final reconstructs presented in Results were exported as TIFF files. Estimates of surface areas, volumes and linear dimensions were computed by the software, after calibrating for magnification.

The statistical analysis was conducted with the software STATISTICA 12 (Statsoft). Two-way ANOVA was made (considering the sex and type of ganglia as fixed variables) for every quantitative parameter. Data normality was confirmed by the Shapiro-Wilks test. Homogeneity of variances was tested with the Levene test. The Newman–Keuls test post-hoc was used after a significant ANOVA, considering the interaction (sex vs ganglia type) and the sex and ganglia type separately, according to the ANOVA primary output. We also

conducted parametric correlation analyses, between the body size parameters of the animals and the total volumes and surface areas of each ganglion. The level of significance adopted was the usual standard of 0.05.

Results

Body morphometry and general microanatomy of the nervous system.

Data in Table 1 illustrates that the shell size of the males and females used were approximately the same size, both as to the mean values and overall interindividual variability. Consequently, no statistically significant differences were found regarding length, height or width. All the three parameters were highly linearly correlated, with coefficients of correlation (r) showing very strong positive associations: length vs height ($r = 0.95$; $p < 0.001$); length vs width ($r = 0.94$; $p < 0.001$); height vs width ($r = 0.928$; $p < 0.001$). The histological analysis involved the three types of neural ganglia (cerebral, pedal and visceral), interconnected by connectives (Figure 1).

Table 1. Body morphometry (cm) of the *S. plana* used in the study.

	Length	Height	Width
Males	2.8 (0.18)	2.1 (0.17)	0.9 (0.19)
Females	2.6 (0.20)	2.0 (0.18)	0.8 (0.25)
All together	2.6 (0.19)	2.0 (0.25)	0.8 (0.24)

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation). There are no significant differences.

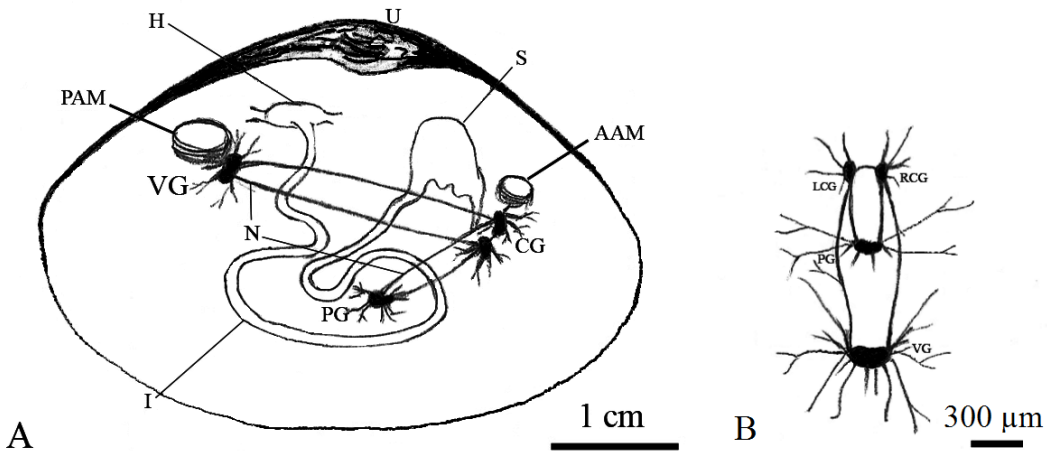

Figure 1. A diagrammatic representation of the nervous system of *Scrobicularia plana*. **A**) Sagittal view showing the position of ganglia and connectives with the left valve removed. **B**) Dorsal view of neural elements. AAM - anterior adductor muscle; CG - cerebral ganglia; H - heart; I - intestine; LCG - left cerebral ganglion; N - nerves; PG - pedal ganglion; PAM - posterior adductor muscle; RCG - right cerebral ganglion, S - stomach, U - umbo, VG - visceral ganglion.

3D anatomy of the cerebral ganglia

The cerebral ganglia were located just laterally to the mouth, between the latter and the posterodorsal end of the anterior muscle. The 3D reconstructions revealed that the cerebral ganglia were pear-shaped, somewhat elliptical or even roundish (Figure 2). Intriguingly, we found that in 90% of the analyzed animals the right ganglion could be considered pear-shaped, whereas in 10% of the cases the 3D shape was of the elliptical type. To the contrary, the left cerebral ganglion was consistently roundish. Also, in all the specimens that had the right pear-shaped ganglia, the "tip" of the "pear" pointed to the median position, as depicted in both images in Figure 2.

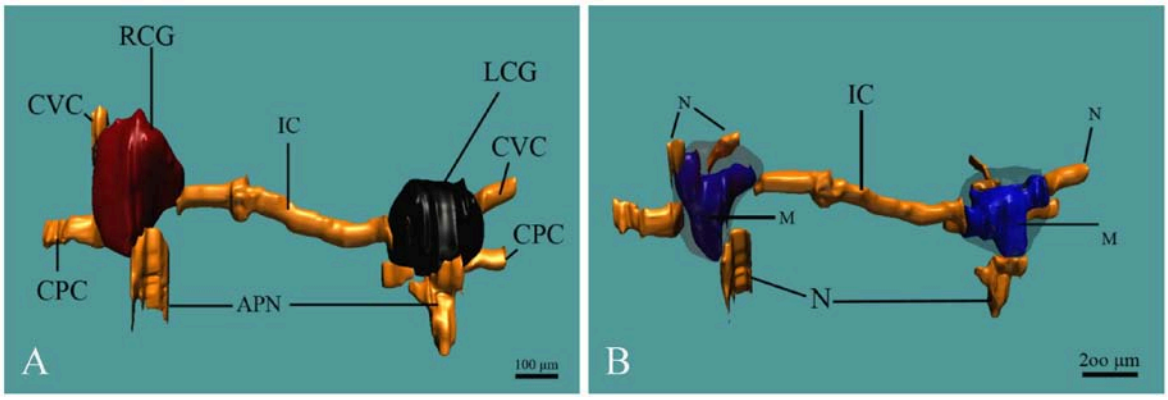

Figure 2. **A**) Frontal view of a 3D-reconstruction of the left (LCG) and the right cerebral ganglion (RCG) of *Scrobicularia plana*, showing the cerebral commissure (IC), together with the emergence of several nerves, which are shown truncated near the ganglia. **B**) A slightly rotated frontal view of the 3D-reconstruction of the pair of cerebral ganglia shown in A. In this semi-transparent mode the shape of medulla (M) (in blue) can be seen inside the ganglia. APN - anterior pallial nerves; CPC - cerebropedal connectives; CVC - cerebrovisceral connectives; IC - interganglionar commissure; N - nerves.

In addition to the overall 3D outline, the reconstruction of the medulla revealed the precise anatomical spatial positioning between the cortex and medulla. We noticed the medulla generally followed the overall global shape of the ganglia (Figure 2B), but not exactly in all cases, so that the cortex did not have the same thickness along each ganglion. Also, the medulla bulged at times towards the neuronal capsule, where it connected with the emerging nerves (Figure 2B). Accordingly, to assist in understanding the shape variations of the medulla, we have shown the five to six nerves emerging from each ganglion in the reconstruction and confirm that all major bulges coincided with the point of emergence of the nerves.

3D anatomy of the pedal ganglia

The two pedal ganglia were fused into one, being located in the median plane of the body between the gonad and the foot. The reconstruction shows a cylindrical to ovoid-barrel shape, usually with a slightly undulating contour along the long axis (Figure 3A). This long axis was always transverse with respect to the anterior-posterior axis of the animal. The tips of this ganglion were typically flat at the right side and pointed at the left (Figure 3A). The reconstructed medulla resembled a tube within a larger tube (Figure 3B). As seen in the cerebral ganglia, thin and flattened or thinner and roundish projections run towards the cortex. Such medullar projections ultimately anastomose with nerves (Figure 3C). Usually there are six nerves emerging from the ganglion but in a few specimens there were seven or even eight. Generally, three to four nerves emerge from both the anterior and the posterior surfaces of each ganglion. Sometimes medullar projections pierced into the cortex, reaching the capsule but not actually perforating it (Figures 3C, 3D). Thus, in such regions, immediately below the capsule there was medullar tissue instead of (the expected) cortex. Despite such kind of subcapsular medullar regions did not directly anastomose with an emerging nerve, they could be continuous with nearby medullar protrusions — that ultimately connected with a nerve.

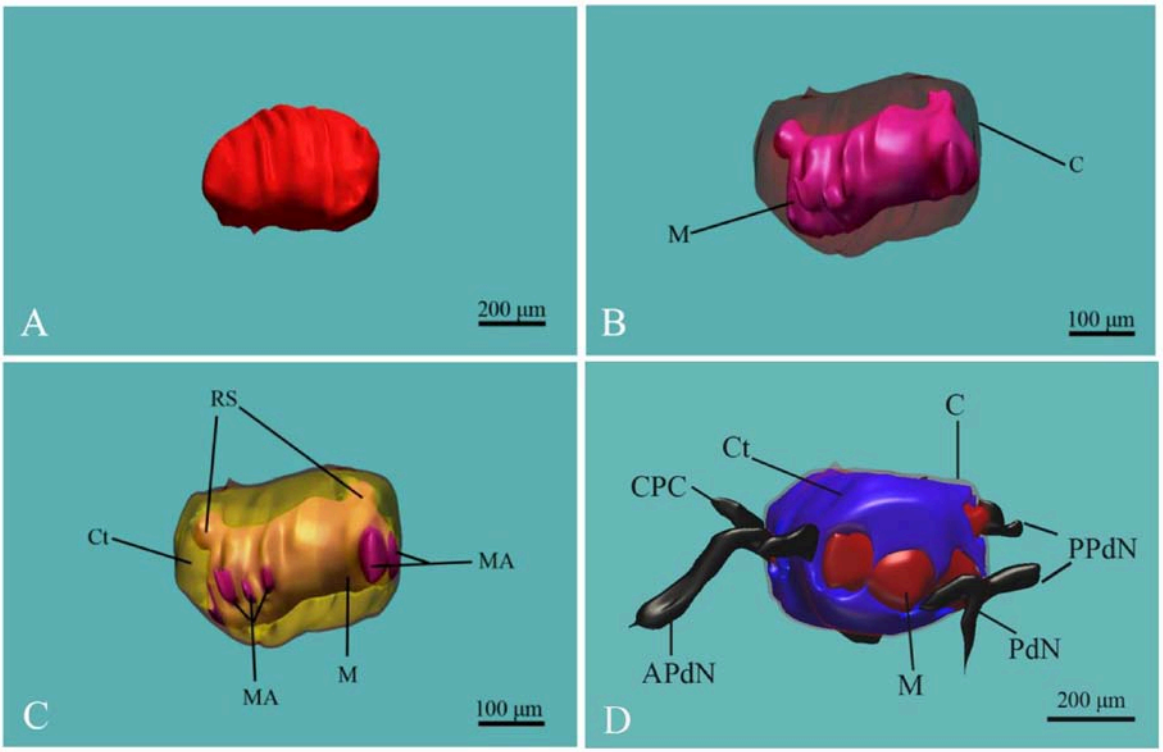

Figure 3. **A**) Ventral view of a 3D-reconstruction of the pedal ganglia of *Scrobicularia plana*. This specimen has an ovoid-barrel shape, with the more pointed tip located at the left. **B**) Anterior view of the 3D-reconstruction of the pedal ganglion shown in A. In this semi-transparent mode the

details of the medulla (M) (appearing in purple) can be observed, which has a fairly cylindrical shape. The capsule (C) is shown as a reference for the innermost medulla location. **C**) Anterior view of the 3D-reconstruction of the pedal ganglia shown in B, also in semi-transparent mode, but in which the purple colour is now restricted to the medullar areas (MA) connected with nerves. On the superior part of the medulla two medullar roundish sprouts (RS) can be seen that were not connected with any emerging nerve. Ct - cortex; M - medulla. **D**) Left view of a 3D-reconstruction of a pedal ganglion. In this case the medulla (M) (reddish) clearly pierced the all full thickness of the cortex (Ct) (in deep blue), thus reaching the ganglion capsule (C) (light grey). ApdN - anterior pedal nerve; CPC - cerebropedal connectives; PdN - pedal nerve; PPdN - posterior pedal nerves.

3D anatomy of the visceral ganglia

The visceral ganglia were located very close to the posterior adductor muscle. These ganglia were also totally fused into one, having an irregular lobular shape (Figure 4A) or a "deformed rectangle" (Figure 4B). There was no discernible left-right symmetry or shape consistency, except that the major axis of the fused ganglion was always perpendicular to the anterior-posterior axis of the animal. In contrast with the other ganglia, the medulla here did not follow so closely the overall shape of the ganglia, being particularly irregular in 3D. As seen in the other ganglia, there were medullar projections that pierced the capsule and merged with the nerves. Occasionally, the medulla touched the capsule, but did not actually cross it (Figure 4C). Usually, six to seven nerves are associated to a visceral ganglion, most often emerging from ventrolateral positions (Figure 4D).

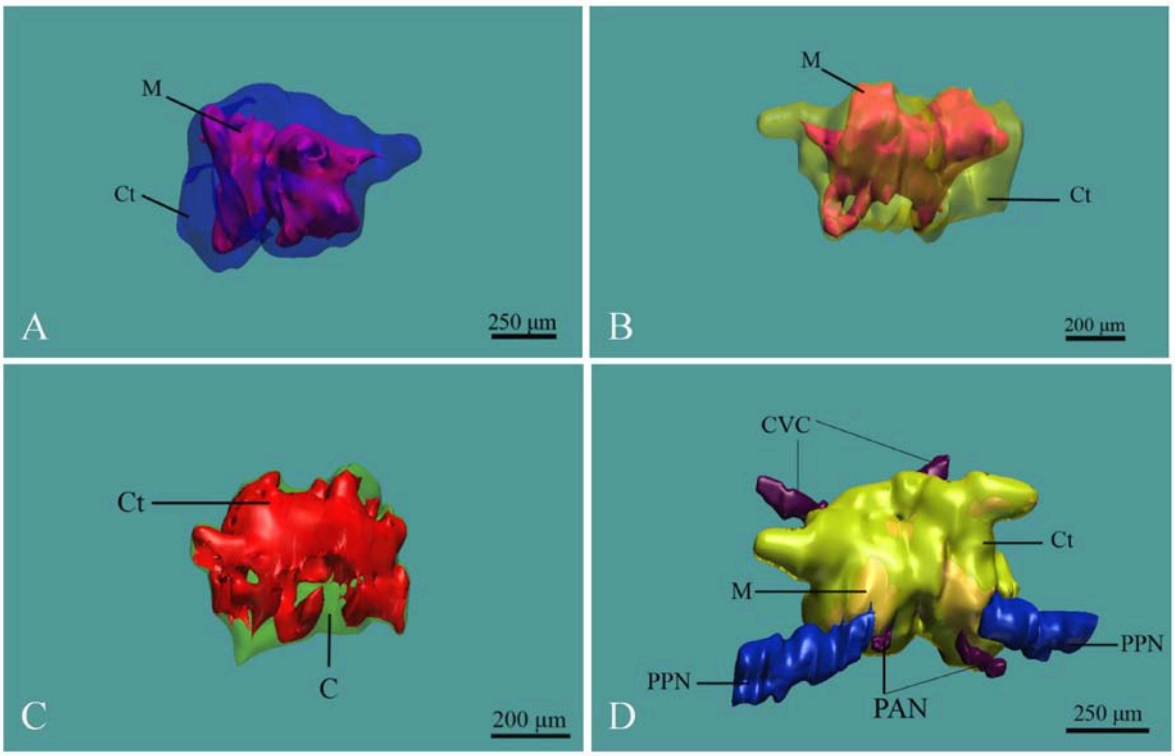

Figure 4. **A**) Anterior view of a 3D-reconstruction of a visceral ganglion of *Scrobicularia plana* showing the somewhat irregular shape. The semi-transparent mode reveals that the medulla (M) (purple) is somewhat irregular, and does not exactly follow the shape and contour of the cortex (Ct) (blue). **B**) Anterior view of a 3D-reconstruction of a visceral ganglion. The overall shape is somewhat irregular, but now resembling a deformed, more or less rectangular box. The semi-transparent mode reveals that the medulla (M) (reddish-orange) is quite irregular, not following the exact same shape and contour of the cortex (Ct) (green). **C**) Anterior view of a 3D-reconstruction of the visceral ganglion shown in B. In this semi-transparent view it can be seen on the top of the ganglion that the medulla (M) (reddish-orange) pierced the cortex (Ct), and on those locations the medulla directly touched the very thin external capsule (C) (lightest green, not shown at those places where the medulla touches the capsule). **D**) Posterior view of a 3D-reconstruction of a visceral ganglion. In addition to the irregular shape, the ventrolateral emerging nerves (N) can be seen, two more posteriorly (in blue) and three more anteriorly (in dark purple). The cortex (Ct) stands out in green, while the darker yellowish green corresponds to portions of the medulla (M). CVC - cerebrovisceral connectives, PAN - posterior adductor nerves; PPN - posterior pallial nerves.

Morphometry of the cerebral, pedal and visceral ganglia

The quantitative data derived from the reconstructions are given in Tables 2-5. As to the total volumes (Table 2) there are no intersex differences. Overall, the visceral ganglion is significantly larger than the other two ganglia, with the cerebrals the smallest. The mean

volume of the pedal ganglion differs significantly from both from the visceral and each cerebral ganglion. These display an almost perfect quantitative left-right symmetry as to the total volume (and also in the relative volumes of cortex and medulla; Tables 3-4). Contrasting with the other ganglia, the pedals have clear significantly linear correlations between the total volume and each of the body size parameters: length vs volume ($r = 0.65$; $p < 0.05$); height vs volume ($r = 0.66$; $p < 0.05$); width vs volume ($r = 0.74$; $p < 0.01$). The visceral ganglia volumes have lower moderate positive correlations, which are only statistically significant (despite marginally) with the height ($r = 0.65$; $p = 0.04$), and not significant regarding the length ($r = 0.58$; $p > 0.05$) and width ($r = 0.59$; $p > 0.05$). The cerebral volumes have residual positive correlations with each of the biometric parameters.

Table 2. Total volumes (μm^3) of the cerebral, pedal and visceral ganglia of *S. plana*.

	Cerebral		Pedal	Visceral
	Right	Left		
Males	$31.7x10^6$ (0.51)	$30.2x10^6$ (0.17)	$63.5x10^6$ (0.14)	$154.5x10^6$ (0.51)
Females	$39.1x10^6$ (0.41)	$36.7x10^6$ (0.53)	$62.0x10^6$ (0.18)	$139.4x10^6$ (0.28)
All together	$35.4x10^6$ (0.44)^A	$33.8x10^6$ (0.43)^A	$62.7x10^6$ (0.16)^B	$146.9x10^6$ (0.45)^C

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation).Within a row, mean values with different superscript letters differ statistically ($p < 0.05$). There are no significant differences between sexes (ANOVA sex effect: $p > 0.05$).

As to the relative volumes (Table 3-4), the cerebral and pedal ganglia display a very similar structure, with the cortex comprising ≈ 60% of the ganglion volume and the medulla ≈ 40%. Despite no significant differences in relative volumes, including by sex, ganglion type, or the interaction between them, it seems that the volume ratio of cortex vs medulla shows some small differences: ≈ 1.6 (cerebral), ≈ 1.5 (pedal), ≈ 1.3 (visceral).

Table 3. Relative volumes (%) of the cerebral, pedal and visceral ganglia cortex of *S. plana*.

	Cerebral Cortex		Pedal Cortex	Visceral Cortex
	Right	Left		
Males	60 (0.12)	61 (0.03)	62 (0.09)	54 (0.07)
Females	64 (0.04)	64 (0.14)	59 (0.09)	60 (0.14)
All together	62 (0.09)	62 (0.11)	60 (0.09)	57 (0.12)

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation).There are no significant differences.

Table 4. Relative volumes (%) of the cerebral, pedal and visceral ganglia medulla of *S. plana*.

	Cerebral Medulla		Pedal Medulla	Visceral Medulla
	Right	Left		
Males	40 (0.18)	39 (0.05)	38 (0.15)	46 (0.08)
Females	36 (0.07)	36 (0.25)	41 (0.14)	40 (0.21)
All together	38 (0.15)	38 (0.18)	40 (0.14)	43 (0.16)

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation). There are no significant differences.

As to the total surface area (Table 5), and in agreement with the volumetric trends, in both sexes there is a significant tendency for a smaller surface area in the cerebral ganglia, with the pedal intermediate and the visceral ganglion clearly having the largest surface area. The cerebral ganglia have matching averages for the surface area. As seen for both the total and relative volumes, there are no intersex differences, but the effect of ganglion type in the ANOVA is significant.

Table 5. Total surface area (μm^2) of the cerebral, pedal and visceral ganglia of *S. plana*.

	Cerebral		Pedal	Visceral
	Right	Left		
Males	$1.3x10^6(0.29)$	$1.2x10^6(0.12)$	$1.8x10^6(0.23)$	$4.1x10^6(0.40)$
Females	$1.3x10^6(0.32)$	$1.2x10^6(0.48)$	$1.7x10^6(0.20)$	$3.5x10^6(0.28)$
All together	$1.3x10^6(0.29)^A$	$1.2x10^6(0.34)^A$	$1.7x10^6(0.21)^B$	$3.8x10^6(0.35)^C$

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation). Within a row, mean values with different superscript letters differ statistically ($p < 0.05$).There are no significant differences between sexes (ANOVA sex effect: $p > 0.05$).

Table 6. Surface-volume ratio ($\mu m^2/\mu m^3$) of the cerebral, pedal and visceral ganglia of *S. plana*.

	Cerebral		Pedal	Visceral
	Right	Left		
Males	$3.4x10^{-2}$ (0.25)	$3.9x10^{-2}$ (0.08)	$2.8x10^{-2}$ (0.21)	$2.8x10^{-2}$ (0.12)
Females	$3.4x10^{-2}$ (0.48)	$3.0x10^{-2}$ (0.19)	$2.9x10^{-2}$ (0.17)	$2.4x10^{-2}$ (0.27)
All together	$3.4x10^{-2}$ (0.36)	$3.3x10^{-2}$ (0.20)	$2.8x10^{-2}$ (0.18)	$2.6x10^{-2}$ (0.21)

A total of 6 males and 6 females were used. Data given in mean (coefficient of variation). There are no significant differences.

However, the correlation analyses of the total surface area in relation to the total volumes (Table 6) showed that the visceral ganglion was consistently strongly and significantly positively correlated with each of the biometric parameters: length vs surface area ($r = 0.84$; $p < 0.01$); height vs surface area ($r = 0.82$; $p < 0.01$); width vs surface area ($r = 0.83$; $p < 0.01$). Both cerebral ganglia provide some moderate (mainly in the right side) to strong (in the left side) correlations, as follows: 1) right cerebral – length vs surface area ($r = 0.65$;

$p < 0.05$); height vs surface area ($r = 0.73$; $p < 0.05$); width vs surface area right cerebral ($r = 0.68$; $p < 0.05$); 2) left cerebral – length vs surface area ($r = 0.79$; $p < 0.01$); height vs surface area ($r = 0.84$; $p < 0.01$); width vs surface area ($r = 0.87$; $p < 0.01$).

Discussion

The value of computer assisted three-dimensional reconstructions for understanding either the macro or the microanatomy of cells, organs and even entire tiny organisms, based on serial histological sections, is well illustrated in the literature (e.g., Neusser *et al*. 2006, 2011; Da Costa *et al*. 2007; Ge *et al*. 2012; Geiselbrecht and Melzer 2013; Neves *et al*. 2013). The importance and wide range of potential applications of this strategy for anatomical research has long been recognised (Salisbury 1994), including the potential of deriving (besides qualitative aspects) unbiased/valuable morphometric data (namely object counting and volumetric measurements) from the reconstructs; sometimes even with advantages over other established approaches, like stereology. The program we used in this study for the 3D reconstructions from histological serial sections allowed us, with proper calibration, to additionally take both two dimensional (likes surface area) or three dimensional (namely volume) measurements, as well as detailed 3D features.

Typically, the 3D anatomy of the ganglia of bivalves has been described from gross observations from dissections and microscopic observation. That approach can provide realistic information, but is prone to caveats, such as: it is difficult or even impossible to properly appreciate details when the ganglia are small: the possibility of inducing damage or deformations during collection: the inability to assess the inner 3D anatomy. The final result (typically a drawing) may depend much on the artistic skills of the observer. Careful, detailed comparisons are sometimes necessary, for example, the evaluation or re-evaluation of the taxonomy of a species (e.g., Romera *et al*. 2013) or to properly look at the nervous system during larval development (e.g., Ellis and Kempf 2011). Drawings of the whole ganglia are rarely accompanied with photomicrographs, at least with respect to bivalves. Real images of ganglia are thus rare and normally from larger bivalves e.g., the visceral ganglia in the freshwater mussel *Hyriopsis bialatus* (Simpson, 1900) (Meechonkit *et al*. 2012).

From our experience with *Scrobicularia plana* it is difficult not only to fully isolate all the ganglia from one animal to appreciate its standard 3D anatomy and also variability, but

also to conciliate such invasive strategy (involving delicate dissecting) with 3D measurements of the isolates. As far as we know our study is the first to conduct a 3D-reconstruction of the ganglia on adult specimens of a bivalve. So far, we have found only one study that presents software supported 3D-reconstructs of the ganglia of the larvae of the brooding bivalve *Lasaea adansonii* (Gmelin, 1791) (Altnöder and Haszprunar 2008), but this did not provide insights into the medulla morphology or quantitative data. There are no previous studies comparing the ganglia in male vs female bivalves, despite recent evidence regarding their different function, such as the higher immunoreactivity for serotonin in the visceral ganglia of the female freshwater mussel *H. bialatus* (Meechonkit *et al*. 2012).

Despite the lack of significant quantitative differences between reproductively mature individuals of different sex in *S. plana* they are presumably under the influence of a different constellation of key reproduction modulators, such as sex steroids, neuropeptides and neurotransmitters (Croll and Wang 2007; Mahmud *et al*. 2008). However, as we provide only very general 3D size-related parameters, our data does not mean that there are no cytological or histochemical differences connected with the seasonal reproductive cycle. We aim to obtain in the future other parameters (e.g., neuron and glial cell counts) that may determine differences between sexes.

Differences between the various types of ganglia are obvious, including total volume, with the cerebral ganglia being the smallest and the visceral the largest. Interestingly, the sum of the volumes of each cerebral ganglion is close to the total volume of the fused pedal ganglion. Some bivalves have separate pedal ganglia, even if closely connected by a commissure; for example *Mytilus edulis* Linnaeus, 1758 (Stefano *et al*. 1990). Curiously, the different shapes of the cerebral and pedal ganglia resulted in the surface area of the former being not much different to the latter. That is, the somewhat pear-shaped appearance of the cerebrals present a summed larger (more than double) surface per unit of volume when compared with the somewhat more cylindrical pedal ganglion. It is not clear whether or not such morphological differences have functional implications, or if they represent some ancient form of “early folding” as seen in the long history of brain evolution (Roth and Dicke 2013). To our knowledge, these volume-surface interrelations have not been reported in other bivalves.

Despite differences in volume or surface area, it is interesting to note that the relative volumes of the cortex vs medulla are fairly constant across the different ganglia. Whatever the shape of each ganglion and the diversity in size, the outer cortex and the inner medulla maintained a fairly stable volume ratio of≈ 1.5 (with a small variance between 1.6 in cerebral and 1.3 in the visceral). This suggests an optimal and strictly regulated structural balance for the ganglia of this species. To our knowledge, only qualitative descriptions of the medulla and cortex are available in the literature, and if the rare qualitative observations that compared different ganglia in a species point to a equal cellular composition for each ganglia type — as very recently reported for the freshwater mussels *Villosa nebulosa* (Conrad, 1834), *Fusconaia cerina* (Conrad, 1838), and *Strophitus connasaugaensis* (Lea, 1858) (McElwain and Bullard 2014) — we seem to be the first to reveal this quantitative balance between the cortex and medulla bivalve ganglia. It would be interesting to study these aspects in other bivalves, as anatomical features such as cell densities and cortex volumes are relevant to understanding the evolution of the central nervous system (Roth and Dicke 2013).

The left and right cerebral ganglia were a structural mirror in basic volumes and surface areas. But, interestingly, their shape was not exactly the same (confirmed in all the analyzed animals), as if the same amount of nervous tissue is modeled in a slightly different way, with the right cerebral ganglion fairly pear-shaped whereas the left was always roundish. Whether these observations result from an ancient adaptative morphofunctional asymmetry in the cerebral ganglia of bivalves, or of this species and/or its relatives in particular deserves further study.

Finally, we wish to point out the linear correlations found in relation to the pedal volume and surface area vs the animals' biometric parameters. Within the size range of the specimens we studied, the bigger the specimen the bigger the pedal ganglia, but the size of the two other types of ganglia do not necessarily increase. The pedal correlations were consistently found in relation to all the biometric parameters. It is known that the pedal ganglion serves primarily to control the musculature of the foot (Bullock and Horridge 1965), and we noticed in dissections that specimen size appeared to be correlated with foot size, although this was not formally measured. Therefore, we hypothesize that the correlations found may be: 1) connected with the need of bigger animals to control the respectively larger foot, namely because of the coordination/power of its movements; 2)

and/or related with the fact that adults maturing their gonads (within the foot) may need bigger ganglia to cope with functional needs, namely to grant regulatory molecules and abundant nerve fibers coming from the pedal ganglia to the gonad wall (Henry *et al*. 1995; Tanabe *et al*. 2006).

Acknowledgments

We thank Fernanda Malhão and Célia Lopes for teaching technical details to the first author, and for overseeing her processing of the animals for histology. We also thank Catarina Cruzeiro for helping in bivalve handling in the field and in-house. We express our gratitude to reviewers and editorial advice that enabled us to improve this work. The first author was supported by a Thai Government Science and Technology Scholarship. This work was partially supported by the European Regional Development Fund (ERDF) funds through the Competitiveness and Trade Expansion Program (COMPETE) and by National Funds as provided by the Fundação para a Ciência e a Tecnologia (FCT), via the research projects PEst-C/MAR/LA0015/2013 and UID/Multi/04423/2013.

References

Altnöder, A. & Haszprunar, G. (2008) Larval morphology of the brooding clam *Lasaea adansonii* (Gmelin, 1791) (Bivalvia, Heterodonta, Galeommatoidea). *The Journal of Morphology* 269, 762-774.

Boldina-Cosqueric, I., Amiard, J.-C., Amiard-Triquet, C., Dedourge-Geffard, O., Métais, I., Mouneyrac, C., Moutel, B. & Berthet, B. (2010) Biochemical, physiological and behavioural markers in the endobenthic bivalve *Scrobicularia plana* as tools for the assessment of estuarine sediment quality. *Ecotoxicology and Environmental Safety* 73, 1733-1741.

Bullock, T. H. & Horridge, G. A. (1965) *Mollusca: Pelecypoda and Scaphopoda: Structure and function in the nervous systems of invertebrates*. Two volumes. W. H. Freeman & Co., London.

Croll, P. R. & Wang, C. (2007) Possible roles of sex steroids in the control of reproduction in bivalve molluscs. *Aquaculture* 272, 76-86.

Da Costa, S., Cunha C. M., Simone, L. R. L. & Schrödl, M. (2007) Computer-based 3-dimensional reconstruction of major organ systems of a new aeolid nudibranch subspecies, *Flabellinaengeli lucianae*, from Brazil (Gastropoda: Opisthobranchia). *Journal of Molluscan Studies* 73, 339-353.

Ellis, I. & Kempf, S. C. (2011) Characterization of the central nervous system and various peripheral innervations during larval development of the oyster *Crassostrea virginica*. *Invertebrate Biology* 130, 236-250.

Food and Agriculture Organization of the United Nations (2004) *The State of Food Insecurity in the World*, Rome, Italy.

Ge, S.-Q., Wipfler, B., Pohl, H., Hua, Y., Ślipiński, A., Yang, X.-K. & Beutel, R. G. (2012) The first complete 3D reconstruction of a Spanish fly primary larva (*Lytta vesicatoria*, Meloidae, Coleoptera). *PLOS One 7*, 1-17.

Geiselbrecht, H. & Melzer, R. R. (2013) Nervous systems in 3D: a comparison of Caridean, Anomuran, and Brachyuran zoea-I (Decapoda). *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution* 320, 511-524.

Gosling, E. (2004) *Bivalve Molluscs: Biology, Ecology and Culture*. Fishing News Books, Blackwell Publishing, Oxford.

Guerreiro, J. (1998) Growth and production of the bivalve *Scrobicularia plana* in two southern European estuaries. *Vie et Milieu* 48, 121-131.

Henry, M., Benlinmame, N., Belhsen, O. K., Jule, Y. & Mathieu, M. (1995) Immunohistochemical localization of FMRFamide-containing neurons and nerve fibers in the ganglia and the gonad wall of the scallop, *Pecten maximus* (L). *Neuropeptides* 28, 79-84.

Landacre, F. L. (1920) The origin of cerebral ganglia. *The Ohio Journal of Science* 20, 299-310.

Mahmud, S., Mladenov, P. V., Sheard, P. & Chakraborty, S. C. (2008) Characterization of neurons in the visceral ganglia of the green-lipped mussel (*Perna canaliculus*) using antibodies raised against neuropeptides and neurotransmitters. *Bangladesh Journal of Animal Science* 37, 78 - 85.

Martynov, A., Brenzinger, B., Hooker, Y. & Schrodl, M. (2011) 3D-Anatomy of a new tropical Peruvian nudibranch gastropod species, *Corambe mancorensis*, and novel hypotheses on dorid gill ontogeny and evolution. *Journal of Molluscan Studies* 77, 129-141.

McElwain, A. & Bullard, S. A. (2014) Histological atlas of freshwater mussels (Bivalvia, Unionidae): *Villosa nebulosa* (Ambleminae: Lampsilini), *Fusconaia cerina* (Ambleminae: Pleurobemini) and *Strophitus connasaugaensis* (Unioninae: Anodontini). *Malacologia* 57, 99-239.

Meechonkit, P., Asuvapongpatana, S., Jumromn, W., Kovitvadhi, U. & Weerachatyanukul, W. (2012) Sexual differences in serotonin distribution and induction of synchronous larval release by serotonin in the freshwater mussel *Hyriopsis bialatus*. *Journal of Molluscan Studies* 78, 297-303.

Morse, M. P. & Zardus, J. D. (1997) Bivalvia. In: Harrison, F. W. & Kohn, A. J. (Eds.), *Microscopic anatomy of invertebrates. Mollusca II Volume 6A,* Wiley-Liss New York, pp. 1-118.

Neusser, T. P., Fukuda, H., Jörger, K. M., Kano, Y. & Schrödl, M. (2011) Sacoglossa or Acochlidia? 3D reconstruction, molecular phylogeny and evolution of Aitengidae (Gastropod: Heterobranchia). *Journal of Molluscan Studies* 77, 332-350.

Neusser, T. P., Hess, M., Haszprunar, G. & Schrödl, M. (2006) Computer-based three-dimensional reconstruction of the anatomy of *Microhedyle remanei* (Marcus, 1953), an interstitial acochlidian gastropod from Bermuda. *Journal of Morphology* 267, 231-247.

Neves, R. C., Bailly, X., Leasi, F., Reichert, H., Sørensen, M. V. & Kristensen, R. M. (2013) A complete three-dimensional reconstruction of the myoanatomy of Loricifera:

comparative morphology of an adult and a Higgins larva stage. *Frontiers in Zoology* 10, 1-21.

Odiete, W. O. (1978) The fine structure of the neurons in the mid-dorsal lobes of the visceral ganglion of the Lamellibranch mollusk *Scrobicularia plana* (da Costa). *Journal of Molluscan Studies* 44, 305-321.

Romera, B. L. V., Simone, L. R. L. & Cunha, C. M. (2013) Redescription and anatomy of *Diplodonta portesiana* (d'Orbigny, 1846) (Bivalvia, Ungulinidae) from Brazil. *Zookeys* 275, 1-15.

Roth, G. & Dicke, U. (2013) Evolution of Nervous Systems and Brains. In: Galizia, C.G. & Lledo, P.-M. (Eds.), *Neurosciences - From Molecule to Behavior*, Springer-Verlag Berlin Heidelberg, pp. 19-45.

Salisbury, J. R. (1994) Three-dimensional reconstruction in microscopical morphology. *Histology and Histopathology* 9, 773-780.

Santos, S., Cardoso, J. F. M. F., Carvalho, C., Luttikhuizen, P. C. & van der Veer, H. W. (2011) Seasonal variability in somatic and reproductive investment of the bivalve *Scrobicularia plana* (da Costa, 1778) along a latitudinal gradient. *Estuarine, Coastal and Shelf Science* 92, 19-26.

Siniscalchi, A., Cavallini, S., Sonetti, D., Sbrenna, G., Capuano, S., Barbin, L., Turolla, E. & Rossi, R. (2004) Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology* 144, 1205-1212.

Smaal, A. C. (1991) The ecology and cultivation of mussels: new advances. *Aquaculture* 94, 245-261.

Solé, M., Kopecka-Pilarczyk, J. & Blasco, J. (2009) Pollution biomarkers in two estuarine invertebrates, *Nereis diversicolor* and *Scrobicularia plana*, from a Marsh ecosystem in SW Spain. *Environment International* 35, 523-531.

Stefano, G. B., Cadet, P., Sinisterra, J., Charles, R., Barnett, J., Kuruvilla, S. & Aiello, E. (1990) Functional neural anatomy of *Mytilus edulis*: Monoaminergic and opioid localization. In: Stefano, G. B. (Ed.), *Neurobiology of Mytilus edulis*. Manchester University Press, Manchester, pp. 38-56.

Tanabe, T., Osada, M., Kyozuka, K., Inaba, K. & Kijima, A. (2006) A novel oocyte maturation arresting factor in the central nervous system of scallops inhibits serotonin-induced oocyte maturation and spawning of bivalve mollusks. *General and Comparative Endocrinology* 147, 352-61.

CHAPTER4

OVERVIEW OF THE NEUROCYTOLOGY OF GANGLIA AND IDENTIFICATION OF PUTATIVE SEROTONIN-AND DOPAMINE-SECRETING NEURONS IN THE BIVALVE PEPPERY FURROW SHELL (*SCROBICULARIA PLANA*)

Overview of the neurocytology of ganglia and identification of putative serotonin- and dopamine-secreting neurons in the bivalve peppery furrow shell (*Scrobicularia plana*)

[Formatted as a manuscript to be submitted for publication in an international journal. The version in this Thesis may change after the revision to be made by all prospective authors.]

Sukanlaya Tantiwisawaruji[a,b,c], Fernanda Malhão[a,b], Célia Lopes[a,b], Ana Silva[b], Uthaiwan Kovitvadhi[d], Miguel A. Pardal[e], Maria J. Rocha[a,b] and Eduardo Rocha[a,b]

[a]Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Porto, Portugal.
[b]Histomorphology, Physiopathology and Applied Toxicology Group (PATH), Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto (U.Porto), Porto, Portugal.
[c]King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand.
[d]Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand.
[e]Centre for Functional Ecology (CFE), University of Coimbra, Coimbra, Portugal.

Running title: Neurocytological aspects of nervous ganglia of *S. plana*

Key words: bivalve, glial cells, histology, ultrastructure, monoamines, nervous system, neurons

Correspondence to:

Eduardo Rocha
Department of Microscopy
Laboratory of Histology and Embryology
Institute of Biomedical Sciences Abel Salazar (ICBAS)
University of Porto (U.Porto)
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto
Portugal
E-mail: erocha@icbas.up.pt

Abstract

The cytology of the three ganglia types of *Scrobicularia plana* was never studied in fine detail, except in some regions of the visceral ganglia. However, to support fundamental and applied studies using this species nervous ganglia, it appears constructive to us that a baseline structural descriptive study is made at light and electron microscopy. To start filling the knowledge gaps we made a general histological (including special stainings) and ultrastructural study in *S. plana*. Adults of both sexes and immature specimens were collected from the Ria Formosa lagoon, Portugal. For light microscopy observations, the animals were measured, anesthetized, dissected and then fixed in 10 % buffered formalin. Then after they were routinely processed for paraffin embedding and sectioned for diverse purposes along the sagittal plane, using a fully motorized microtome. A systematic survey was made for a first identification of neurons that contain serotonin and dopamine, as a first step to gather to knowledge of the presence and role of neuroendocrine neurons in *S. plana*. For transmission electron microscopy (TEM) dissected ganglia were fixed in 2.5 % glutaraldehyde, post-fixed in 1 % osmium tetroxide, and processed for epoxy embedding. Sections were produced with a diamond knife using an ultramicrotome. As we expected, the general histology of the ganglia matched what is known for bivalves in general, with an outer cortex and an inner medulla. In the cortex there are two basic categories of neurons: large and small. Almost all are unipolar neurons; only a few bipolar were identified. We found no evidences of myelin like material. In all ganglia there is a decreasing size of the neurons as they ate located closer to the medulla. Glial cells appear around neurons, and also amidst the axons that route into the medulla. They typically have smaller sizes and greater nucleus-cytoplasm ratio when compared with neurons. For description, we considered three basic cytological types: fusiform, roundish and triangular. Facing their shapes and diverse ultrastructure we can hypothesize either they are different sub-types of glia, or at least they correspond to diverse functional status — a matter of debate in bivalves. Facing our immediate objectives our focus was on light microscopy, but as to the ultrastructure we noted differences in relation to earlier findings, mainly in type of inclusions and vacuoles. This discrepancies seem also worth exploring in future, correlating with ganglia type and biotic and abiotic factors. At last, serotonergic and dopaminergic cell bodies and neurites were identified in all ganglia. Our data suggested an identical expression pattern in adults of both sexes and in the immature animals (with undefined sex). However, the extent/intensity of serotonin positivity in visceral ganglia support that mature animals have stronger expression than undifferentiated ones,

which may be connected the role of that ammine in helping bivalve gonad maturation and spawning. Overall, our study offers a range of baseline data that are useful for further studies in *S. plana*.

Introduction

The nervous system of invertebrates is composed of two main categories of cells, neurons and glial cells (Bullock and Horridge 1965; Coles and Abbott 1996; Harrison 1997; Oikonomou and Shaham 2011; Hidalgo *et al*. 2011), in line with what is seen in vertebrates (Brodal 1992; Galbiati *et al*. 2003; Laming *et al*. 2000). Also as in the latter group, there are neurosecretory cells in invertebrates, such as described in arthropods (Bharathi 2014; Padmaja *et al*. 2010; Perić-Mataruga 2011) and in molluscs (Meechonkit *et al*. 2010; Sleem 2003; Wijdenes *et al*. 1980). Regarding cell peculiarities, there are cytological characteristics that the neurons have when they are secretory, particularly lipid droplets and granules filled with secretion, which products — e.g., dopamine, noradrenaline and serotonin — can act as neurohormones (Carroll and Catapane 2007; Gagné and Blaise 2003). Being produced in somata, the neurohormones are packed in granules that are ultimately moved into the axonal endings, like the synaptic vesicles (Bullock and Horridge 1965; Roubos 1975; Thorndyke and Goldsworthy 1988). Neurosecretory cells receive inputs from other neurons, but, unlike the regular neuronal cells (i.e., non-secretory), which display cell-to-cell communications over very short distances, at the synapses, neurosecretory neurons release their products into an extracellular space, which may be at some distance from their target. In an organism with a closed circulatory system, the neurohormones are typically sent through the vascular route up to their site of action. Yet, in lower invertebrates, which lack an organized circulatory system, the neurohormones seem to simply diffuse from the release site up to the target (Ketata *et al*. 2008).

In molluscs there are many evidences of neurosecretory activity — for example, in the central ganglia of the sea hare, *Aplysia oculifera*, and the sea snail, *Neptunea arthritica*, three and four types of neurosecretory cells, respectively, were characterized (Sleem 2003; Yahata and Takahashi 1972). Also in bivalves correlations have been found between, on the one end, the neurosecretory activity and, on the other end, the neuronal lipid stores and the breeding cycle — for instance, in the ganglia of the mussel, *Crenomytilus grayanus*, whereas numerous neuronal lipid droplets were present in the spawning period, very low amounts of those inclusions were found in the pre-spawning period; i.e., the lipid contents were inversely proportional to the extent of neurosecretion (Reunova *et al*. 1997). In the scallop, *Nodipecten subnodosus*, there is an increased concentration of monoamines (including norepinephrine, dopamine and 5-HT) in the gonad, gill, and mantle tissues during the maturing stage, with a drop after spawning, suggesting that the animals used varied neurotransmitters during the

reproductive cycle (López-Sánchez *et al*. 2009). In bivalves, as in other invertebrates, the monoamines, dopamine and serotonin are considered neurohormones, because they are known to act in several tissues/organs, either as a neurotransmitter or as a neuromodulator of diverse processes (Bullock and Horridge 1965; Gagné *et al*. 2007).

Histological and ultrastructural studies about the neural cells (inc. neurosecretory) are very limited in the bivalve peppery furrow shell, *Scrobicularia plana*. Considering that this species is of economic importance and also that it is used as pollution bioindicator — that is prone to neuronal and reproductive disruption — a better knowledge of its nervous system is not only of a fundamental and comparative interest, but it also can be used for practical purposes. For instance, histopathological approaches for appreciating the impacts of pollutants do need a good baseline characterization of the normal histological and cytological features. In view of this background, we aimed: 1) to review the histology and cytology of the neural cells in *S. plana*, registering its main aspects and eventually looking after any still undescribed feature; and 2) to identify the neurosecretory neurons — viz. those putatively producing serotonin and dopamine — across the cerebral, pedal and visceral ganglia, and considering the gonadic sex.

Materials and methods

Animals

Adult mature and immature *S. plana* were collected at ebb tide from the intertidal zone of the Ria Formosa Lagoon, south of Portugal. After capture, the animals were transferred to in house facilities in the same day, and maintained in glass aquaria (10 L) filled with aerated seawater (salinity 30 ‰), at a temperature of 15 ^{o}C. In the next day, the animals were anesthetized by immersion in a solution of $MgCl_2$ (6 %) until total relaxation of the valves. Length, width, and height were measured using a Vernier caliper. The medium animals sized animals (3.4 ± 0.2 mm in length) were selected for light microscopy (including immunohistochemistry), and the bigger ones (3.9 ± 0.02 cm in length) were used for transmission electron microscopy (TEM).

Light microscopy — Fixation to paraffin embedding

Each animal was removed carefully from the shell and fixed *in toto* for 1-2 h, using 10 % neutral buffered formalin, at room temperature. Subsequently, most of the specimens were sliced with a sharp razor blade and 3 cross-sectioned slabs were taken around the body zones where the nervous ganglia are known to be positioned. The smallest animals were fixed only

in toto. In mature specimens the procedure allowed an immediate confirmation of the sex, due to the distinctive gross aspect of the gonad. After slicing, all the pieces were kept in the fixative for 24 h. The fixed organic fragments were washed in 70 % ethanol, dehydrated in increasing concentrations of ethanol (up to 100 %), cleared in xylene, and then infiltrated with paraffin (Histosec, Merck) in an automatic tissue processor (TP1020, Leica, Germany). Embedding of the pieces in plastic cassettes was made in a paraffin station (EG 1140H, Leica, Germany).

Light microscopy — Routine and special staining methods

Animals were cut on a fully motorized rotary microtome (RM2155, Leica, Germany), either in non-serial (5 µm thin) or in serial sections (30 µm thick), in cross or in sagittal planes. The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol (absolute up to 70 %), followed by water, and finally either subjected to routine hematoxylin and eosin (H&E) stain, Kluver-Barrera method (with cresyl violet counterstaining – a classic nucleic acid stain for highlighting the soma), Weil's stain (usually used for detecting myelin), and, finally, to Bielschowsky's silver stain (for highlighting neuronal fibres). For observation of slides and recording of images we used a light microscope (BX50, Olympus, Denmark), equipped with a digital camera (Camedia C-5050, Olympus, Japan).

Transmission electron microscopy (TEM)

For TEM, ganglia were isolated from adult (biggest) animals and cut under a stereomicroscope (LSM 510 Meta, Zeiss Inc., Germany) into tiny pieces (approximately 1 mm^3). The cells were fixed in 2.5 % glutaraldehyde, in 0.2 M sodium cacodylate-hydrochloric acid buffer, pH= 7.2, for 2 h, at 4ºC, and then washed twice in the same buffer, 10 min each. Post-fixation was made in 1 % osmium tetroxide, also in 0.2 M sodium cacodylate-hydrochloric acid buffer at pH = 7.2, for 2 h, at 4ºC. The fixed pieces were then dehydrated in increasing series of ethanol (from 50 % to absolute ethanol p.a.), immersed, 2 times, in propylene oxide for 15 min, and then in mixtures of propylene oxide and epoxy resin (3:1; 1:1; 1:3, in this order, each for 1 hour). Embedding was made in the same resin. After 2 days of polymerization in the oven, at 60ºC, the hardened blocks were trimmed and cut with a diamond knife (Diatome, Switzerland), in an ultramicrotome (Reichert Supernova, Leica, Germany). One µm thick semithin sections were stained with a mixture 1:1 of 1 % methylene blue and 1 % azure II. Ninety nm thick ultrathin sections were placed into 200 mesh hexagonal copper grids, and contrasted with uranyl acetate and lead citrate. Sections of

several ganglia were observed and representative images taken with an electron microscope JEOL 100CXII, operated at 60 kV.

Immunohistochemistry against serotonin and dopamine

Paraffin blocks of 5 males, 5 females, and 5 undifferentiated animals were sectioned in the above referred motorized microtome, at 3 μm thick, either in cross or in sagittal planes. The resulting sections were placed on 3-aminopropyltriethoxysilane coated glass slides and kept at -80 ºC. The sections having the left and right cerebral, pedal, and visceral ganglia were brought up to room temperature, deparaffinized in xylene, and then rehydrated as described above. For immunohistochemistry an antigen retrieval method using citrate buffer (0.01 M, pH = 6) was made using a pressure cooker. After this, the, sections were cooled up to room temperature and then washed in distilled water. Afterwards, endogenous peroxidase was quenched by treatment with 0.3 % H_2O_2 in methanol (v/v), for 10 min at room temperature, to reduce non-specific binding. After washing in tap water and then PBST (phosphate buffered saline with 0.05 % of Tween 20), pH = 7.5, for 3 x 2 min, the sections were incubated in a 10 % goat non-immune serum blocking solution (Histostain kit, Invitrogen, USA) for 1 h. After draining the sections, they were incubated in a moist chamber overnight, at 4 ºC, either with the primary antibody against dopamine (rabbit polyclonal anti-dopamine antibody, Enzo, USA) or against serotonin (rabbit polyclonal anti-serotonin antibody, Sigma-Aldrich, USA), used diluted in PBS, pH = 7.5, at 1:3000 and 1:10000, respectively. Afterwards, the sections were washed, 3 x 2 min with PBST, and then incubated with a biotinylated secondary antibody (Histostain Kit, Invitrogen, USA), for 25 min, and rinsed, 3 x 2 min with PBS. This step was followed by the application of the enzyme conjugation streptavidin-peroxidase reagent (Histostain kit, Invitrogen, USA), for 25 min. After subsequent rinsing, the sections were incubated with 3, 3'-diaminobenzidine (DAB) (Novolink Max DAB, Leica, UK), for 5 min, and counterstained with Mayer's hematoxylin (Merck, Germany), for 2 min. Negative controls, in which the primary antibody was omitted were always included. After dehydration and clearing, sections were coverslipped with DPX mountant (Sigma-Aldrich, USA). Study and image recording were made as for H&E staining.

Semi-quantitative grading of serotonin- and dopamine-secreting neurons

The extent of immunostaining intensity was semi-quantitatively rated by overall looking at all ganglia type of males, females and undifferentiated specimens. Grading followed the criteria exposed in Table 1; these were established after a pilot blind overview to evaluate the tagging

extent and intensity. Results were given as, median, minimum and maximum, and differences were statistically analyzed by the non-parametric Kruskal–Wallis test. Computations used the software STATISTICA 12.0 (StatSoft, USA). The significance level was set at 0.05.

Table 1 – Semi-quantitative grading scale used for serotonin and dopamine immunostaining.

Grade	Criteria
1	Less than 20% of sectioned neurons are labelled. Staining intensity is typically very faint.
1.5	20 to 40% of sectioned neurons are labelled. Staining intensity is typically faint.
2	40 to 60% of sectioned neurons are labelled. Staining intensity is typically moderate.
2.5	60% to 80% of sectioned neurons are labelled. Staining intensity is typically strong.
3	More than 80% of sectioned neurons were labelled. Staining intensity is typically the strongest.

Results

Light and transmission electron microscopy aspects of the neurocytology of S. plana

Irrespective of details about the size and shape of the ganglia, all display the same fundamental histology, with the outer cortex being exuberant in cellularity of both neurons and glial cells, in contrast with the medulla that is rich in neurites and in projections of glial cells (Fig. 1). The bigger neurons clearly stand out from the other cells, being primarily located in at the periphery (cortex) of the ganglia, emerging immediately below the capsular connective sheath. Glial cells are typically smaller than neurons and with very scanty cytoplasm, often not even distinct at light microscopy level. Images illustrative of the staining procedures used in the observations at light microscopy are presented in Figure 2. The soma richness in RNA is well patent, both with the routine H&E (Fig. 2A) and particularly with cresyl violet in Kluver-Barrera's stain (Fig. 2B). The neurites run from the cortex into the ganglia centrum (the neuropil or medulla). The absence of positive staining around the neurites with the Weil's technique attested that the neuronal projections are unmyelinated axons (Fig. 2C.). With the Bielschowsky's stain it was confirmed that almost all neurons are unipolar (Fig. 2D-F); despite rare bipolar neurons exist. Despite thorough scrutiny in the silver stained sections, multipolar neurons were not detected. Scattered in cortex are pigmented cells, distinct by the yellowish cytoplasmic colour and by the fact that the nucleus tends to be located at the margin of the cell body (Fig. 2G-H); those cells also exist in the medulla, but being seen much more rarely when compared with the cortex.

The electron microscopy visualization (Fig. 3) confirmed in three ganglia the presence of the three cell types seen at light microscopy: glial cells, neurons, and pigmented cells. Glial cells exist in the medulla and cortex, displaying three morpho-phenotypes: fusiform, roundish, and triangular, with shape of the nucleus with the overall morphology of the cell body (Fig. 3A-B). As perceivable in light microscopy, two morphotypes of nerve cells can be considered, based on their size, and that we name, for practical purposes, as small and large neurons. The large ones typically present a roundish euchromatic nucleus, bearing a prominent nucleolus, and the cytoplasm have sparse dispersed rough endoplasmic reticulum cisternae and plenty of mitochondria (Fig. 3B). The small neurons, often laying side by side with the larger ones, but predominating in inner half of the cortex, have also a roundish and centrally located nucleus; that is not so euchromatic and being less prominent than in large neurons. In line with size, smaller neurons have also a comparatively reduced amount of cytoplasm, without dense granules (at least they were not detected herein), and overall with a lesser load of organelles. Irrespective of the neuron size, the mitochondria content declined from the soma to the neurites.

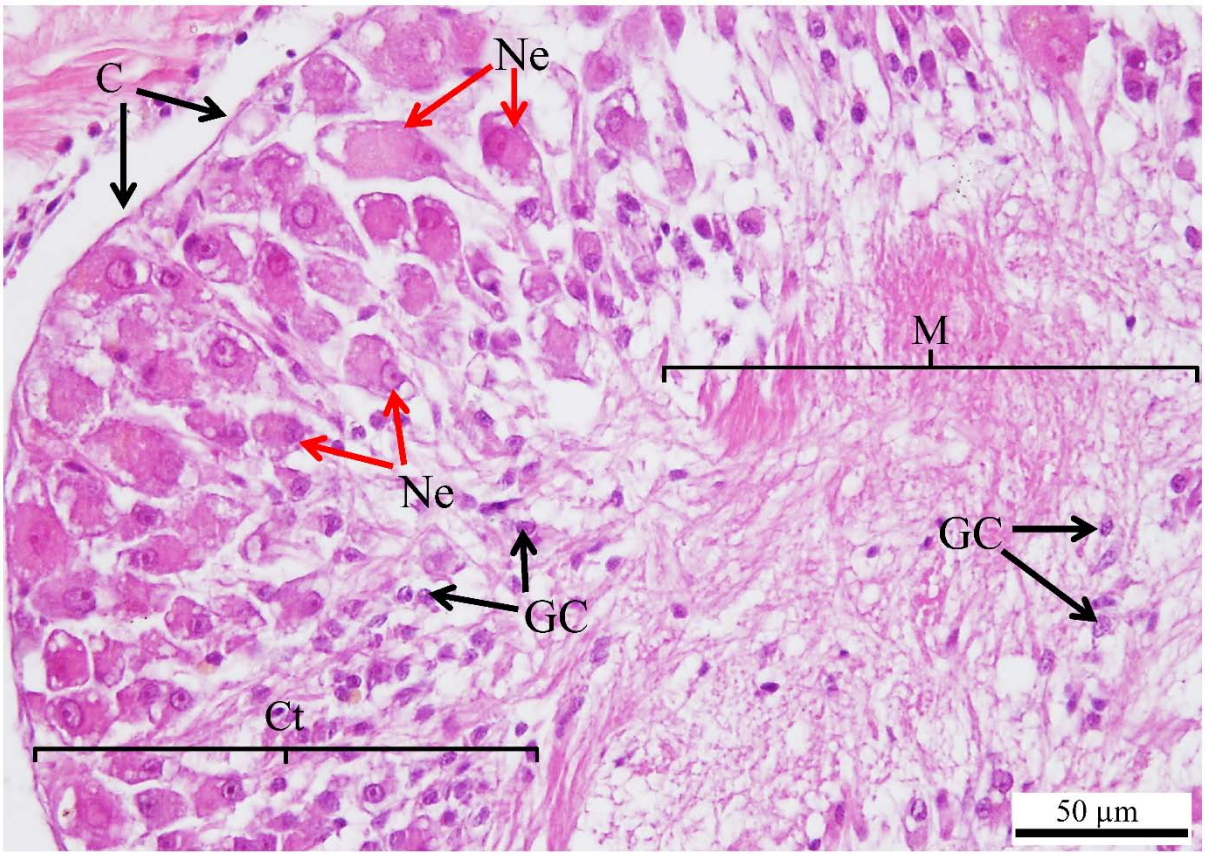

Figure 1. Photomicrograph of the cerebral ganglion of *Scrobicularia plana*, showing the cortex rich in neural cells — with the largest appearing in the outer cortex — contrasting with the inner neuropil rich medulla. H&E stain. C – capsule; Ct – cortex; GC – glial cells; M – medulla; Ne – neurons.

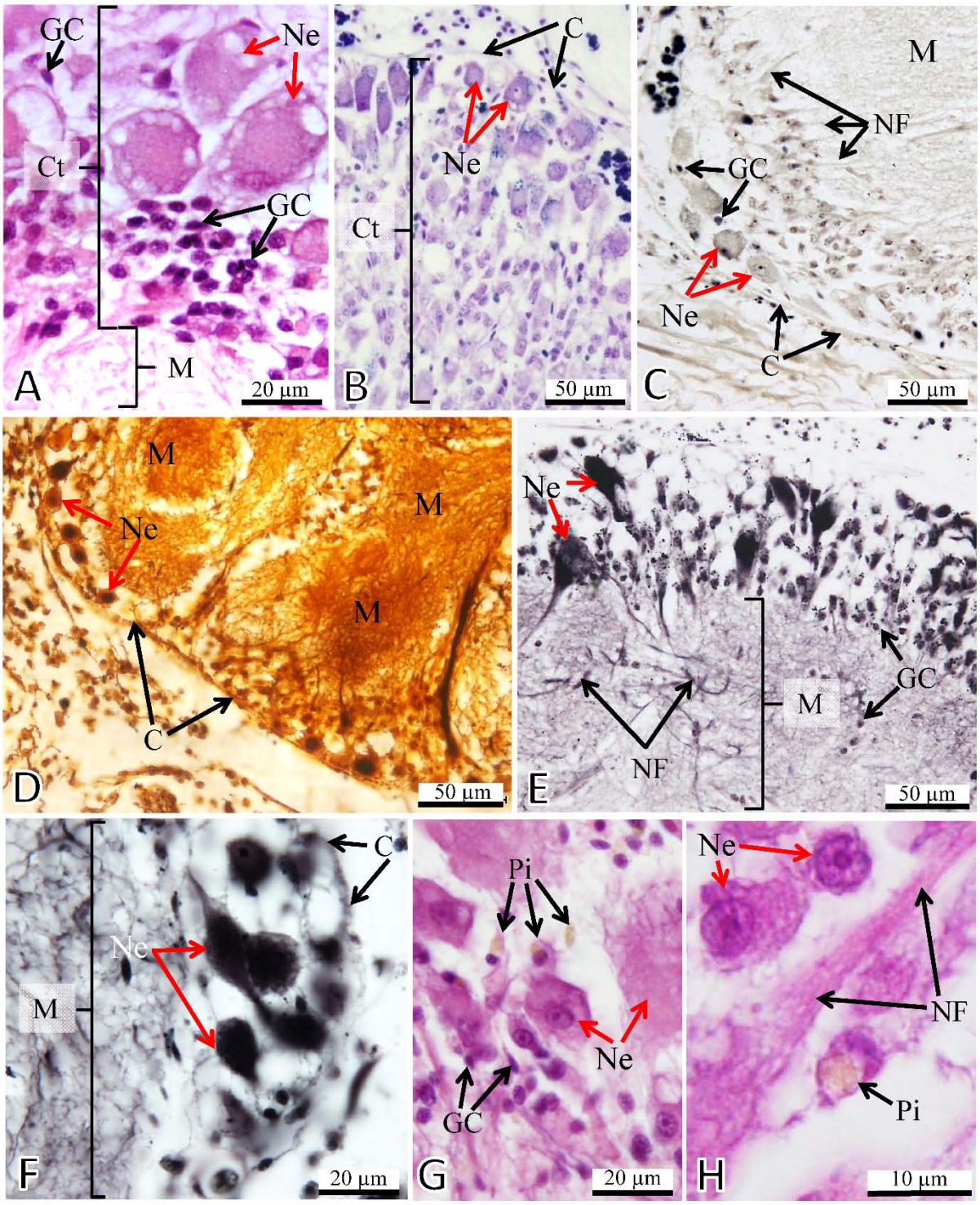

Figure 2. Histological details of the nervous ganglia cortex and medulla of *Scrobicularia. plana*. **A)** H&E stained section showing neurons and glial cells at the cortex of a pedal ganglion. **B)** Kluver-Barrera stained section showing the violet stained neurons, with greater ones clustered in the outer cortex. **C)** Weil stained section showing neurons, also with larger ones crowded at a subcapsular location. The nerve fibers (neurites) run from the cortex into the medulla. **D)** Bielschowsky's silver staining, without gold toning, highlighting the intricate medullar network of nerve fibers and glial cell processes. **E)** Bielschowsky's silver staining, toned with gold, unveiling the presence of thick nerve fibers. **F)** Higher magnification from a Bielschowsky's silver staining section, toned with gold. **G)** H&E stained section of a visceral ganglion, showing pigmented cells in cortex, nearby neurons and

glial cells. H**)** Detail of H&E stained cerebral ganglion, with a magnification that details one pigmented cell, nearby neuronal soma and a thick nerve fiber (axon). C – capsule; Ct – cortex; – GC – glial cells; M – medulla; NF – nerve fibers; Ne – neurons; Pi – pigmented cells.

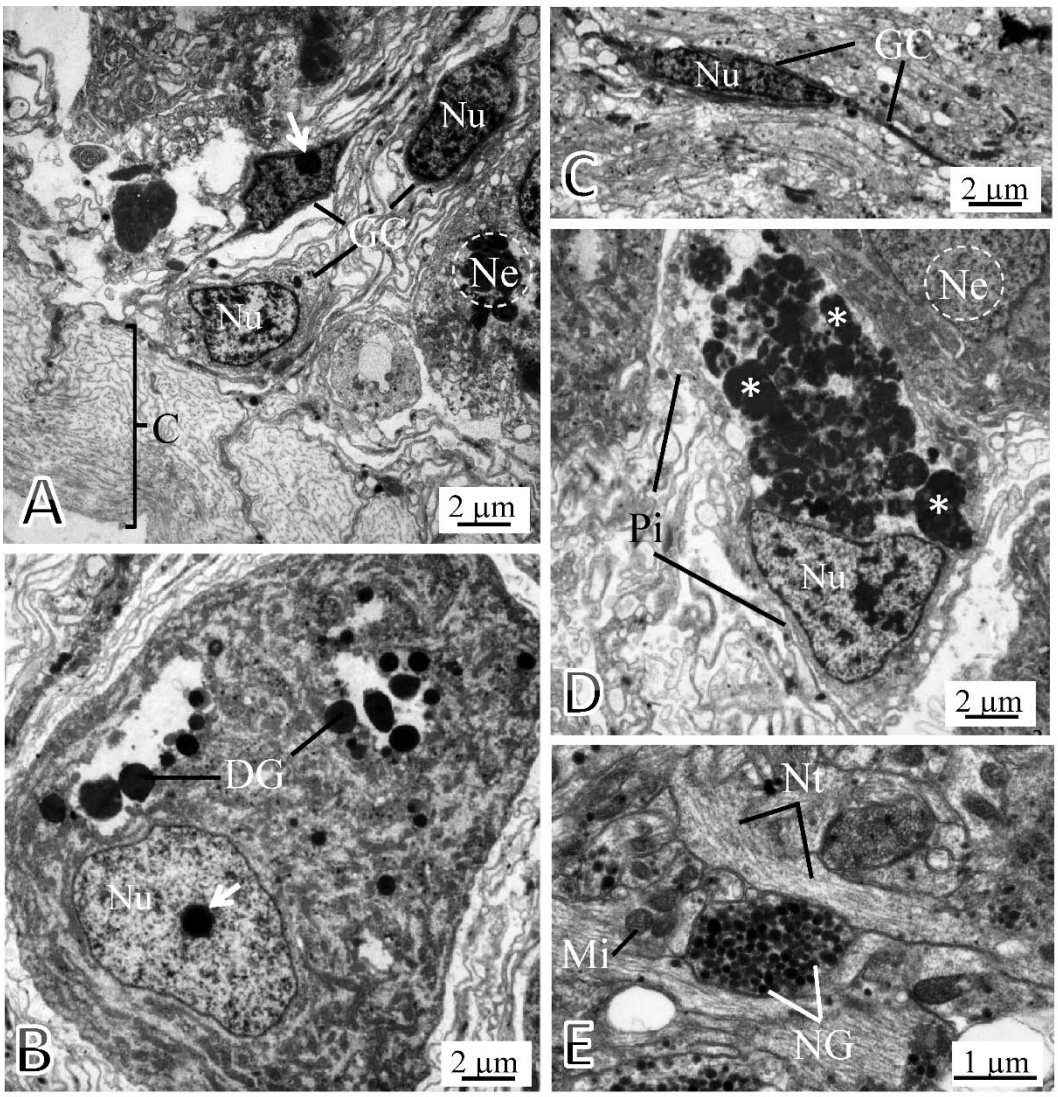

Figure 3. Transmission electron micrographs of varied neural cells of *Scrobicularia plana*. **A)** Image from an outer ganglionic region, below the connective tissue capsule. Two elongated (electron dense) and one (electron lucent) triangular/roundish glial cells are seen close to a larger nearby neuron. **B)** Large neuron with a euchromatic nucleus, bearing a central nucleolus, and with the cytoplasm packed with organelles; these include dense granules. **C)** Detail of a medullar fusiform type of glial cell, from which apical slim projections emerge. **D)** Pigmented cell nearby a neuron, exposing the typical peripheral nucleus. **E)** Region with neurofilament-rich neurites and also exhibiting one that shows a varicosity filled with homogenously dense vesicles. C – Capsule; DG – dense granules; GC – glial cells; Mi – mitochondria; Ne – neurons; NG – neurotransmitter granules; Pi – pigmented cells; White arrows – Nucleolus.

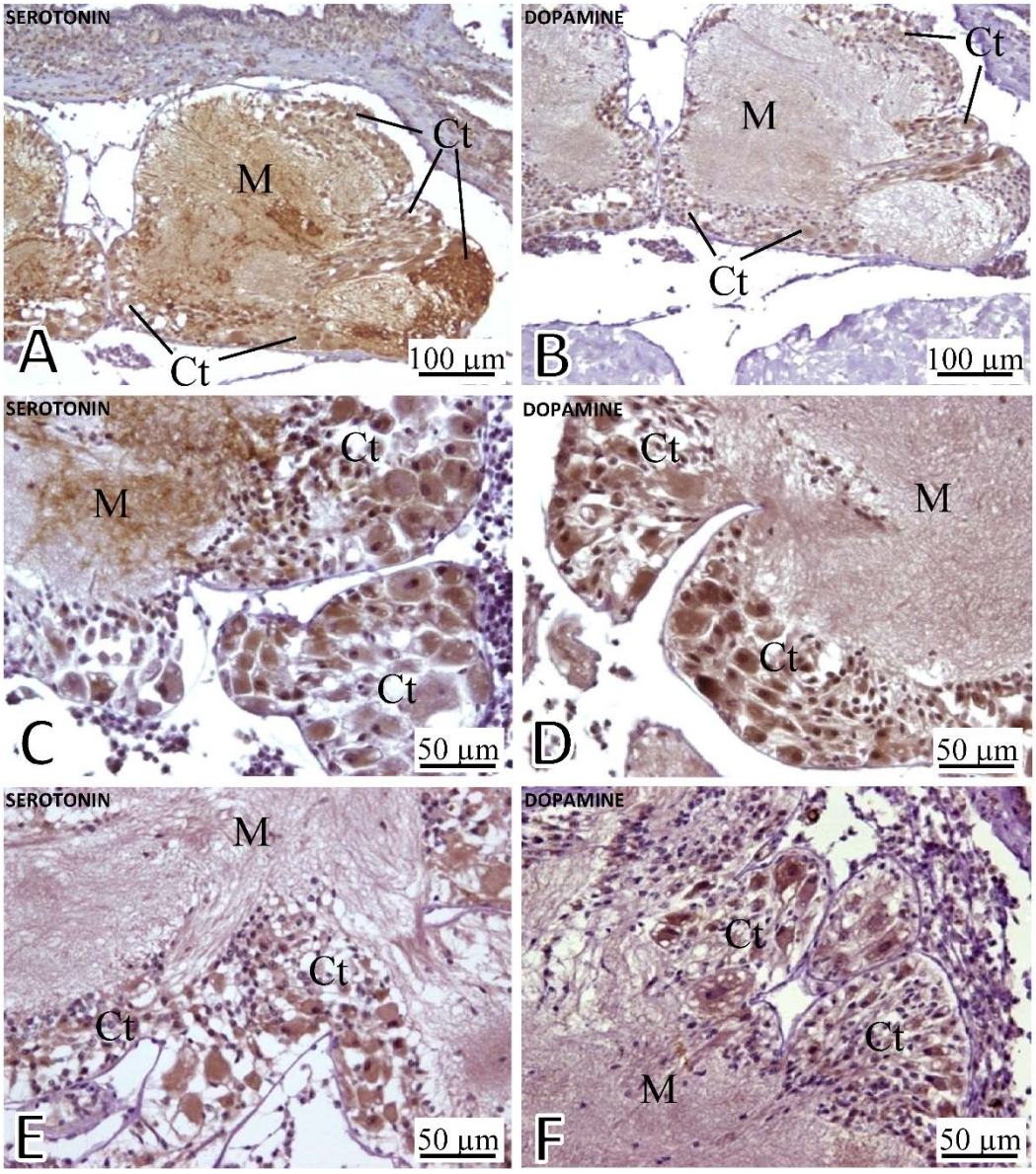

Figure 4. Serotonin and dopamine immunostaining (as brown colour essentially in the neuronal soma) in the visceral ganglia of *Scrobicularia plana*. **A)** Serotonin and **B)** Dopamine in a male – Grade 3 (according with the criteria explained in Table 1). **C)** Serotonin and **D)** Dopamine in a female – Grade 3. **E)** Serotonin and **F)** Dopamine in an undifferentiated animal – Grade 2.5. Ct – cortex; M – medulla.

Identification and semi-quantitative grading of serotonin- and dopamine-secreting neurons

All ganglia exhibit serotonin and dopamine positive immunoreactivity of neurons, which seem relatively stronger in the larger neurons (Fig. 4). The negative (no antibody) controls sections do not evidence immunomarking (not shown). Also, the small glial cells with scanty cytoplasm do not stain at all or, at most, stained very faintly (and so interpreted as no specific background staining). The positive tagging is essentially located in the soma, but neurites, including in the medulla, show also some positivity, despite much weaker. There was a

degree of heterogeneity as to the signal strength within each ganglion, i.e., not all neurons stain equally. Qualitatively, no global patterns of immunostaining are seen as to the effect of sex status or of ganglia type.

Grading data for the immunomarking is given on Table 2. The median grades were either 2.5 or 3, despite values varied from 1.5 to 3. No animal was rated with the lowest possible score of 1. The most common scenario found was quite stable, with 60% to 80%, or more, of the sectioned neurons being labelled, and the staining intensity being strong to very intense. Only one significant difference was found, and in the visceral ganglion, with the females having a higher median score for serotonin when compared to males and undifferentiated specimens.

Table 2. Immunologic intensity of positive neuron in cerebral, pedal and visceral ganglia.

Ganglia and sex condition	**Serotonin**		**Dopamine**	
Cerebral	Median	(Min-Max)	Median	(Min-Max)
Males	3.0	1.5-3.0	3.0	3.0-3.0
Females	2.5	2.5-3.0	3.0	2.5-3.0
Undifferentiated animals	3.0	2.5-3.0	3.0	2.5-3.0
Pedal	Median	(Min-Max)	Median	(Min-Max)
Males	3.0	2.0-3.0	3.0	2.5-3.0
Females	3.0	2.5-3.0	2.5	2.0-3.0
Undifferentiated animals	3.0	3.0-3.0	3.0	2.5-3.0
Visceral	Median	(Min-Max)	Median	(Min-Max)
Males	3.0[a]	3.0-3.0	2.5	2.0-3.0
Females	3.0[a]	2.5-3.0	3.0	2.5-3.0
Undifferentiated animals	2.5[b]	2.5-3.0	2.5	2.0-2.5

* Median values bearing different superscript letter differ significantly ($P < 0.05$).

Discussion

General light and electron microscopy aspects

The general histological organization of the ganglia was the expected one, in accordance with that already studied in a 3D perspective (in the Chapter 3 of this Thesis). Our observations thus served the purpose to note any specificity as to the structure of the neural cells and/or of their distribution within the ganglia. As far as we know, in *S. plana* only Odiete (1978) studied the neurocytology of the visceral ganglia, reporting aspects of their fine structure. However, that study neither revealed the general histology nor provided data about eventual phenotypes of neurons (particularly regarding the size) and glial cells (namely as to size or particular shapes). In current study, we found that the neurons contrast much in size — for the sake of simplicity we just classified them as large and small — and that most of large neurons are located at the periphery of all three ganglia. Irrespective of the size and position of the neurons in the cortex, somata typically have a single process directed towards the medulla. Occasionally we spotted two processes emerging from the soma of large neurons. This finding is in accordance with the literature, as bipolar neurons are consistently found to be the far less dominant neuron types in ganglia of most invertebrates (Orrha and Muller 2005; Croll 2001). The nomenclature about neuronal projections is often used in the literature in a non-systematic and unanimous form. According with the review recommendations of Richter *et al.* (2010) for invertebrates, "all cell processes of neurons" should "collectively be referred to neurites", and, importantly in our context, "The single main process emerging from the soma of unipolar neurons and connecting them to dendrites and axons is then called primary neurite". These aspects are worth citing here because it has been recognized for long that in invertebrates it is difficult to distinguish axons form dendrites, both in structural and functional points of view (Bullock and Horridge 1965). Here and elsewhere we use the term neurite and axon as neurites can be separated into primary neurites, axons (here mostly from unipolar neurons), and dendrites (Richter *et al.* 2010).

The special staining procedures and the electron microscopy observations did not evidenced the presence of myelin-like material around neurites. However, Odiete (1978) illustrated in this species a sort of concentrically lamellar figures that were deemed as myelinated axons (but this only in small sized ones), stating, however, that most axons entering the medulla were typical unmyelinated. Our electron microscopy approach was not an extensive study, and, eventually, we may have missed such kind of structures. This is a matter worth further

study, namely in light of the divergent data and continuous research about the presence of early-to-recent forms of myelination throughout its complex evolution process (Zalc 2006; Castelfranco and Hartline 2015). Apropos, it must be stressed that axonal sheaths of true myelin (i.e., as in vertebrates) seem not to exist in invertebrates, including for instance the multi-lamellate glial sheets described in bivalves, crustaceans and annelids (Hartline 2008; Roots 2008). As to neurons cytoplasm, especially of large ones, there are vacuoles/vesicles, which appear as light roundish spaces at light and as membrane bounded bodies of varying electron-density when seen at electron microscopy. Some of these structures had large dense granules inside, much greater than the electron-dense or electron-lucent small vesicles that appeared at varicosities or pre-synaptic regions of the axons. These vesicles are thought to serve multiple purposes, from accumulation of proteins and lipids to storage of neurohormones and neurotransmitters, and they are known to appear in various invertebrates (Golding and Pow 1988; Siniscalchi *et al*. 2004; Meechonkit *et al*. 2010). The structure of neuronal vesicles is quite diverse in bivalves, and attempts were made to advance some systematization. For example, for *Mytilus edulis* the neuronal vesicles were described as small granular, large granular, large opaque, and pleomorphic vesicles, with variations (Vitellaro-Zuccarello and Biasi 1990). The diversity of structure is known for long to match a variety of neurotransmitters, in bivalves and other molluscs (Endean 1972), and studies have been made to know the vesicles/neural zones that have each neurochemical (e.g., Karhunen *et al.* 1993; Karhunen *et al.* 2004; Meechonkit *et al.* 2010). We did not find the so-called "yellow globules" or "cytosomes" that Odiete (1978) described in some neurons of the mid-dorsal lobes of the visceral ganglia of *S. plana*; such globules were then characterized by displaying an electron-dense cortex and electron-lucent centrum. Most likely, such cytosomes occur in particular regions of the ganglia, and our electron study was quite general, based on random samples of the cortex that are not enough to discriminate between particular regions. As to other features of *S. plana* neuronal somata, histology and ultrastructure revealed neurons with one euchromatic round-to-oval nucleus with one salient nucleolus, and cytoplasm with the usual key organelles, standing out mitochondria; overall in line with the other bivalve species (Bullock and Horridge 1965; Stefano, 1990; Morse and Zardus 1997; Odiete 1978).

As to glial cells, they were identified herein by their morphology and positioning, appearing in the cortex and medulla. In bivalves, the glial cells histology/cytology has deserved much less attention than neurons, but their comparatively smaller cell size and scanty cytoplasm has

been described, particularly in ganglia of *M. edulis* (Vitellaro-Zuccarello and Biasi 1990; Paemen *et al*. 1992). In *S. plana*, Odiete (1978) focused the attention on the diversity of glial granules. Our operational definition of the glial cells into fusiform, roundish, and triangular, is one first attempt to at least systematize their description, and it may not have functional implications. It is possible that these forms can be mere stages of the same cell type or it may correspond to different roles. Only more in depth studies can answer this question, but in our posterior studies we will maintain this operational definition, namely when making differential cell counting. The difficulty in characterizing the invertebrate glia based on clear consistent criteria is a well-recognized caveat, and there is no unique set of "morphological glial markers" (Hartline 2011). In the particular case of bivalves, it was recognized that the ultrastructure (not to mention function) of the glia within the ganglia is far from understood, and that tandem questions exist, such as if there is one or more glial subtypes and if environmental factors influence the often seen structural variations (Vitellaro-Zuccarello and Biasi 1990). Despite all this, there are some consistent features appearing in different bivalves, such as the existence in the ganglia of glial cells that have an electron-dense cytoplasm and thin projections, for instance in *M. edulis* Vitellaro-Zuccarello and Biasi 1990 and *Spisula solidissima* (Prior and Lipton 1977), as we saw in *S. plana*. Whatever the variability and poorly characterized morphofunctional subtypes, it is well-recognized that glial cells are prominent in invertebrates, including molluscs, where they contact and cover neurons, and are absolutely critical for as increasing number of known functions, resembling those much better known in mammalian glial cells (Coles 2009).

In all types of ganglia, we consistently found neuronal cells that showed a brown-yellowish colour at light microscopy. We labelled them as pigmented cells. They appear mostly in the cortex, but also in the medulla. In the vast majority of occasions these cells were of small size — compatible with that of many glial cells but also with that of small neurons — and showed an ovoid to ellipsoidal contour. For operational reasons and facing a degree of uncertainty about its true nature, we opted for presenting these cells as a separate neural cell entity. One of the reasons is the fact that pigments can be found in bivalves within both neurons and glial cells, namely as organelles. A diversity of pigmented cytoplasmic structures may appear in bivalves and other molluscs, such as the yellow globules or cytosomes of neurons and the multiglobular bodies/ fused yellow globules of glial cells, as described in the mid dorsal lobes of *S. plana* (Odiete, 1978). Membrane bound bodies with yellowish pigments in neural cells are not exclusive of bivalves. For example cytosomes (also named lipochondria) have been

studied in gastropods (*e.g.*, Lay and Rogers 1956; Zs-Nagy 1971; Sugaya and Onozuka 1978; Robles *et al*. 1986). At light microscopy, the morphology of the pigmented cells we spotted were consistently more compatible with glial cells, and no large neurons displayed such yellowish inclusions/vacuoles. Despite this, further studies are needed to understand the differences in the pigmented vacuoles/globules we observed herein and those reported by Odiete (1978). Such divergences can be the reflex of biotic and abiotic factors, namely when knowing that pigment (lipochrome) serves various uses, such as anoxic endogenous oxidation that helps surviving low oxygen tension (Zs-Nagy 1971, 1974). In view of other functions, as for lipofuscin and/or lipochrome pigments in neurons of the gastropod *Aplysia californica* (Henkart 1975; Schwartz *et al*. 1979), more studies are warrant in *S. plana*.

The serotonin- and dopamine-secreting neurons

Although serotonin and dopamine have been reported within the ganglia of some other bivalves (Gagné *et al*. 2007; López-Sánchez *et al*. 2009; Meechonkit *et al*. 2010), our work is the first to report their immunoreactivity in mature (males and females) *vs*. undifferentiated *S. plana*. We observed cells with consistent positivity for serotonin and dopamine in all ganglion types. Qualitatively there were no perceivable interganglionic differences, but knowing that neuro-signalling is critical for the success of adult bivalve reproduction, we used semi-quantification of the staining as a pilot approach to search for clues of intersex and age-related differences. After implementing our pre-defined grading scores, both serotonin- and dopamine secreting neurons were always present and showing what seems a continuous evident expression of the two peptides. However, one significant difference was found in visceral ganglia, where serotonin expression for undifferentiated animals (as measured by the extent and strength of immunostaining) was lower than in mature males and females. Such differences were not seen regarding dopamine. Assuming that the significant difference is not a mere occasional event, it suggests that adult visceral neurons of *S. plana* produce (use, release) greater quantities of serotonin, eventually connected with gonad maturation events. This interesting possibility is worth exploring in the future, using more refined techniques, including chemical determinations of neurotransmitters. Indeed, interactions of serotonin and dopamine in nervous tissue are recognized to be important for reproduction and other functions in bivalves, including muscle contraction in spawning and respiration (Beiras and Widdows 1995; López-Sánchez *et al*. 2009; Meechonkit *et al*. 2012). Our results make particular sense considering that serotonin is known to induce spawning in bivalves (Gibbons and Castagna, 1984); which agrees with lower levels in immature *S. plana*. Nevertheless this

explanation is partial, as it does not fit in the no difference between adult and immature specimens as to dopamine. Indeed, the latter monoamine is also evidently connected with gonadal maturation and spawning events (Osada *et al.* 1987; Klouche *et al*. 2015). Studies on distribution of monoamine production neurons in bivalves reported diverse patterns. For example, in *M. edulis*, histofluorescent localization in the cerebral ganglia showed that they enclose dopamine and serotonin positive cells, that the pedal contained mainly serotonin, and that the visceral possesses smaller amounts of dopamine; no data about the sex of the animals used was given (Stefano and Aiello 1975). In contrast, the more intense staining of serotonin was detected in the nerve fibres and termini of the visceral ganglia of *Hyriopsis bialata* (Meechonkit *et al.* 2010). In the latter study, the large neurons were the ones that staining stronger both with paraldehyde fuchsin and against serotonin, which is in accordance with our observation in neurons of *S. plana*. We can anticipate that interspecies differences can be due either to biotic (*e.g.*, Burrell and Stefano 1981) and/or abiotic factors (*e.g.*, Hiripi *et al.* 1982), but we lack more analyses to be able to perceive global patterns. In view of the absence of studies, research on neuroamines in *S. plana* is warrant because they should play roles as neurotransmitters and neurohormones, expectable in accordance with those seen in bivalves so biodiverse as *Mya arenaria*, *Misuhopecten yessoensis*, *Hyriopsis bialatus*, and other (Carroll and Catapane 2007; Khotimchenko and Deridovich 1989; Meechonkit *et al*. 2012). We found here that immunostaining for serotonin and dopamine was not restricted to the soma but that it was also present in neurites. Thus, it can be concluded that *S. plana* has a network of dopaminergic and serotonergic neurites distributed throughout the ganglionic medulla. Serotonergic neurites were also noticed in visceral ganglia and gonad of the bivalve *Venus verrucosa* (Siniscalchi *et al*. 2004). Despite the few studies, we know that serotonergic and dopaminergic networks can be formed soon in the bivalve embryonic development, as early as 24 h post-fertilization, as established in *S. solidissima* (Kreyling *et al.* 2001). Such knowledge on monoaminergic systems and the current new data for *S. plana*, offer large potential for basic and applied purposes, both in aquaculture (Khotimchenko and Deridovich 1991; FAO 2004) and toxicology (Klouche *et al.* 2015).

Acknowledgments

The first author was supported by a Thai Government Science and Technology Scholarship. This work was partially supported by the European Regional Development Fund (ERDF) funds through the Competitiveness and Trade Expansion Program (COMPETE) and by National Funds as provided by the Fundação para a Ciência e a Tecnologia (FCT), via the research projects PEst-C/MAR/LA0015/2013 and UID/Multi/04423/2013. The studies at electron microscopy level additionally benefited from the project EUCVOA (NORTE-07-0162-FEDER-000116), co-financed by the North Portugal Regional Operational Program (ON.2 O Novo Norte), under the National Strategic Framework (NSRF), via the ERDF.

References

Beiras, R. & Widdows, J. (1995) Effect of the neurotransmitters dopamine, serotonin and norepinephrine on the ciliary activity of mussel (*Mytilus edulis*) larvae. *Marine Biology* 122, 597-603.

Bharathi, A., Sarojini, N. & Padmaja, M. (2014) Comparative study of neurosecretory cells in female *Penaeus indicus* after unilateral eyestalk ablation. *International Journal of Scientific and Research Publications* 4, 1-4.

Brodal, P. (1992) *The Central Nervous System*. New York: Oxford University Press.

Bullock, T. H. & Horridge, G. A. (1965) *Mollusca: Pelecypoda and Scaphopoda: Structure and function in the nervous systems of invertebrates*. Two volumes. W. H.

Burrell, D. E. & Stefano, G. B. (1983) Analysis of monoamine accumulation in the neuronal tissues of *Mytilus edulis* (Bivalvia)—IV. Variations due to age. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 74**,** 59-63.

Carroll, M. A. & Catapane, E. J. (2007) The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology* 148, 445-450.

Castelfranco, A. M., & Hartline, D. K. (2015) The evolution of vertebrate and invertebrate myelin: a theoretical computational study. *Journal of Computational Neuroscience* 38, 521-538.

Coles, J. A. (2009) Glial cells: invertebrate. In: Squire, L. R. (ed.) *Encyclopedia of Neuroscience*. Elsevier: Amsterdam, pp. 749-759.

Coles, J. A. & Abbott, J. N. (1996) Signalling from neurones to glial cells in invertebrates. *TINS* 19, 358-362.

Croll, R., Boudko, D. & Hadfield, M. (2001) Histochemical survey of transmitters in the central ganglia of the gastropod mollusc *Phestilla sibogae*. *Cell and Tissue Research* 305, 417 - 432.

Endean, R. (1972) Aspects of Molluscan Pharmacology. In Florkin, M. & Sheer, B. T. (Eds.), *Chemical Zoology* Vol. 8 pp. 421-466. Academic Press, New York & London.

Food and Agriculture Organization of the United Nations (2004) *The State of Food Insecurity in the World*, Rome, Italy.

Galbiati, M., Martini, L. & Melcangi, R. C. (2003) Role of glial cells, growth factors and steroid hormones in the control of LHRH-secreting neurons. *Domestic Animal Endocrinology* 25, 101-108.

Gagné, F. & Blaise, C. (2003) Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel *Elliptio complanata*. *Comparative Biochemistry and Physiology Part C* 136, 117–125.

Gagné, F., Blaise, C., Pellerin, J. & André, C. (2007) Neuroendocrine disruption in *Mya arenaria* clams during gametogenesis at sites under pollution stress. *Marine Environmental Research* 64, 87-107.

Gibbons, M. C. & Castagna, M. (1984) Serotonin as an inducer of spawning in six bivalve species. *Aquaculture* 40**,** 189-191.

Golding, D. W. & Pow, D. V. (1988) The new neurobiology – ultrastructural aspects of peptide release as revealed by studies of invertebrate nervous systems. In: Thorndyke, M.C. &and Goldsworthy, G.J. (Eds.) *Neurohormones in Invertebrates*. Cambridge University Press. pp. 7-18.

Harrison, W. F. & Kohn, J. A. (1997) *Microscopic anatomy of invertebrates*. Wiley-Liss,Inc., New York.

Hartline, D. K. (2008) What is myelin? *Neuron Glia Biology* 4, 153-163.

Hartline, D. K. (2011) The evolutionary origins of glia. *Glia* 59, 1215-1236.

Henkart, M. (1975) Light-induced changes in the structure of pigmented granules in *Aplysia* neurons. *Science* 188, 155-157.

Hidalgo, A., Kato, K., Sutcliffe, B., McIlroy, G., Bishop, S. & Alahmed, S. (2011) Trophic neuron-glia interactions and cell number adjustments in the fruit fly. *Glia* 59, 1296-1303.

Hiripi, L., D. E. Burrell, M. Brown, P. Assanah, Stanec, A. & Stefano, G. B. (1982) Analysis of monoamine accumulations in the neuronal tissues of *Mytilus edulis* and *Anodonta cygnea* (Bivalvia)—III. Temperature and seasonal influences. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 71, 209-213.

Karhunen, T., Matti, S., Airaksinen, Tuomisto, L. & Panula, P. (1993) Neurotransmitters in the nervous system of *Macoma balthica* (Bivalvia). *The Journal of Comparative Neurology* 334, 477-88.

Ketata, I., Denier, X., Hamza-Chaffai, A. & Minier, C. (2008) Endocrine-related reproductive effects in molluscs. *Comparative Biochemistry Physiology Part C: Toxicology and Pharmacology* 147, 261-270.

Khotimchenko,Y. S. & Deridovich I. I. (1989) The effect of dopamine and galoperidol on cyclic AMP in the gonad of the bivalve mollusc *Mizuhopecten yessoensis* and the sea urchin *Strongylocentrotus intermeius*. *Comparative Biochemistry and Physiology-Part C: Toxicology and Pharmacology* 92, 23-26

Khotimchenko, Y. S. & Deridovich, I. I. (1991) Monoaminergic and cholinergic mechanisms of reproduction control in marine bivalve molluscs and echinoderms: a review *Comparative Biochemistry and Physiology- Part C: Toxicology and Pharmacology* 100, 311-31

Klouche, M. S., De Deurwaerdère, P., Dellu-Hagedorn, F., Lakhdar-Ghazal, N. & Benomar, S. (2015) Monoamine content during the reproductive cycle of *Perna perna* depends on site of origin on the Atlantic Coast of Morocco. *Scientific Reports* 5, 13715.

Kreiling, J. A., Jessen-Eller, Miller, K., J., Seegal. R. F. & Reinisch, C. L. (2001) Early development of the serotonergic and dopaminergic nervous system in *Spisula solidissima* (surf clam) larvae. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 130, 341-351.

Lacy, D. & Rogers, G. E. (1956) Recent observations by light and electron microscopy on the cytoplasmic inclusions of the neurons of *Patella vulgate. Journal of the Royal Microscopical Society* 75, 172-175.

Laming, P.R., Kimelberg, H., Robinsonc, S., Salmd, A., Hawrylakd, N., Mullere, C. & Rootsf, K.B.N. (2000) Review: Neuronal–glial interactions and behaviour. *Neuroscience and Biobehavioral Reviews* 24, 295–340.

López-Sánchez, J. A., Maeda-Martínez, A. N., Croll, R. P. & Acosta-Salmón, H. (2009) Monoamine fluctuations during the reproductive cycle of the Pacific lion's paw scallop *Nodipecten subnodosus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 154, 425-428.

Meechonkit, P., Kovitvadhi, U., Chatchavalvanich, K., Sretarugsa, P. & Weerachatyanukul, W. (2010) Localization of serotonin in neuronal ganglia of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus*. *Journal of Molluscan Studies* 76, 267-274.

Meechonkit, P., Asuvapongpatana, S., Jumromn, W., Kovitvadhi, U. & Weerachatyanukul, W. (2012) Sexual differences in serotonin distribution and induction of synchronous larval release by serotonin in the freshwater mussel *Hyriopsis bialatus. Journal of Molluscan Studied* 78, 297-303.

Morse, M.P. & Zardus, J.D. (1997) Bivalvia. *In*: Harrison, F.W. & Kohn, A.J. (Eds). *Microscopic Anatomy of Invertebrates: Mollusca II.* New York, Wiley-Liss Inc., vol. 6A, p. 7-118.

Odiete, W.O. (1978) Fine-structure of the neurons in the mid-dorsal lobes of the visceral ganglion of the Lamellibranch mollusc *Scrobicularia plana* (da Costa). *Journal Molluscan Studies* 44, 305-321.

Oikonomou, G. & Shaham, S. (2011) The Glia of *Caenorhabditis elegans*. *Glia* 59, 1253-1263.

Orrha, L. & Muller, M.C.M. (2005) Morphology of the nervous system of Polychaeta (Annelida). *Hydrobiologia* 535, 79-111.

Osada, M., Matsutani, T. & Nomura, T. (1987) Implication of catecholamines during spawning in marine bivalve molluscs. *International Journal of Invertebrate Reproduction and Development* 12, 241-251

Padmaja, M., Deecaraman, M. & Jaganathbose, M. T. (2010) Study of neurosecretory cells in sand lobster *Thenus orientalis* of Royapuram Coast-Chennai. *World Journal of Fish and Marine Sciences* 2, 82-85.

Paemen, L., E. Porchet-Hennere, M. Masson, M. Leung, T. Hughes, Jr. & Stefano, G. (1992) Glial localization of interleukin-1α in invertebrate ganglia. *Cellular and Molecular Neurobiology* 12, 463-472.

Perić-Mataruga, V., Mrdaković, M., Vlahović, M., Ilijin, L., JakovićTomanić, M. & Mircić, D. (2011) Biogenic amines in protocerebral A2 neurosecretory neurons of *Lymantriadispar* L. (Lepidoptera : Lymantriidae) - Response to trophic stress *Archives of Biological Science Belgrade* 63, 571-577.

Prior, D. J. & Lipton, B. H. (1977) An ultrastructural study of peripheral neurons and associated non-neural structures in the bivalve mollusc, *Spisula solidissima*. *Tissue Cell* 9, 223-240.

Reunova, O., Kalinina, G. & Motavkin, P. (1997) Neurosecretory activity and dynamics of the lipid content of CNS neurons in Gray's mussel, a bivalve mollusk. *Neuroscience and Behavioral Physiology* 27, 524-532.

Richter, S., R. Loesel, G. Purschke, A. Schmidt-Rhaesa, G. Scholtz, T. Stach, L. Vogt, A. Wanninger, G. Brenneis, C. Doring, S. Faller, M. Fritsch, P. Grobe, C. Heuer, S. Kaul, O. Moller, C. Muller, V. Rieger, B. Rothe, Stegner, M. & Harzsch, S. (2010) Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary. *Frontiers in Zoology* **7**, 1-29.

Robles L. J., Breneman J. W., Anderson E. O., Nottoli V. A. & Kegler, L. L. (1986). Immunocytochemical localization of a rhodopsin-like protein in the lipochondria in photosensitive neurons of *Aplysia californica*. *Cell and Tissue Research* 244, 115

Roubos, E. W. (1975) Regulation of neurosecretory activity in the freshwater pulmonate, *Lymnaea stagnalis* (L.) with particular reference to the role of the eyes. *Cell and Tissue Research* 160, 291-314.

Roots, B. I. (2008) The phylogeny of invertebrates and the evolution of myelin. *Neuron Glia Biology* 4, 101-109.

Schwartz, J. H., Shkolnik, L. J. & Goldberg, D. J. (1979) Specific association of neurotransmitter with somatic lysosomes in an identified serotonergic neuron of *Aplysia californica*. *Proceedings of the National Academy of Sciences of the United States of America* 76, 5967-5971.

Siniscalchi, A., Cavallini, S., Sonetti, D., Sbrenna, G., Capuano, S., Barbin, L., Turolla, E. & Rossi, R. (2004) Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology* 144, 1205-1212.

Sleem, S. H. (2003) Neurosecretory cells in some principal ganglia of the sea hare, *Aplysia ocuhfera.Egyptian Journal of Aquatic Biology and Fisheries* 7, 21-43.

Sugaya, E. & Onozuka, M. (1978) Intracellular calcium: its movement during pentylenetetrazole-induced bursting activity. *Science* 200, 797-799.

Stefano G. B. (1990) *Neurobiology of Mytilus edulis*. Manchester: Manchester University Press.

Stefano, G. B. & Aiello, E. (1975) Histofluorescent localization of serotonin and dopamine in the nervous system and gill of *Mytilus edulis* (Bivalvia). *Biological Bulletin of the Marine Biological Laboratory* 148, 141-156.

Thorndyke, M. C. & Goldsworthy, G. J. (1988) *Neurohormones in invertebrates* Cambridge: Cambridge University Press.

Vitellaro-Zuccarello, L., De Biasi, S. & Amadeo, A. (1990) Immunocytochemical demonstration of neurotransmitters in the nerve plexuses of the foot and the anterior byssus retractor muscle of the mussel, *Mytilus galloprovincialis. Cell and Tissue Research* 261, 467-476.

Wijdenes, J., Minnen, J. & Boer, H. H. (1980) A comparative study on neurosecretion demonstrated by the alcian blue-alcian yellow technique in three terrestrial pulmonates (Stylommatophora). *Cell and Tissue Research* 210, 47-56.

Yahata, T. & Takahashi, H. (1972) Demonstration of possible neurosecretory cells in the nervous system of neptune Whelk, *Neptunea arthritica. Bulletin of the Faculty of Fisheries, Hokkaido University* 23, 135-143.

Zalc, B. (2006) The acquisition of myelin: a success story. In: Chadwick, D.J. & Goode, J. (Eds), *Purinergic Signalling in Neuron-Glia Interactions*, No. 276 Wiley, Chichester. pp. 15-25.

Zs-Nagy, I. (1971) The lipochrome pigment of molluscan neurons as a possible electron acceptor. *Comparative Biochemistry and Phsyiology Part A: Comparative Physiology* 40, 595-602.

Zs-Nagy, I. (1974) Some quantitative aspects of oxygen consumption and anaerobic metabolism of molluscan tissues — A review. *Comparative Biochemistry and Physiology Part A: Comparative Physiology* 49, 399-405.

CHAPTER 5

A STEREOLOGICAL STUDY OF THE VOLUMES AND CELLULARITY OF THE NERVOUS GANGLIA OF MALES, FEMALES AND UNDIFFERENTIATED ADULTS OF THE PEPPERY FURROW SHELL (*SCROBICULARIA PLANA*)

A stereological study of the volumes and cellularity of the nervous ganglia of males, females and undifferentiated adults of the peppery furrow shell (*Scrobicularia plana*)

[Formatted as a manuscript to be submitted for publication in an international journal. The version in this Thesis may change after the revision to be made by all prospective authors.]

Sukanlaya Tantiwisawaruji[a,b,c], Ana Silva[b], Uthaiwan Kovitvadhi[d], Miguel A. Pardal[e], Maria J. Rocha[a,b] and Eduardo Rocha[a,b]

[a]Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Porto, Portugal; [b]Histomorphology, Physiopathology and Applied Toxicology Group, CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, U.Porto, Porto, Portugal; [c]King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand; [d]Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand; [e]Centre for Functional Ecology (CFE), University of Coimbra, Coimbra, Portugal.

Running title: Stereology of the neural ganglia of *S. plana* considering sex and gonadal stage.

Key words: bivalves, cell numbers, glia cells, nervous ganglia, neurons, sex differences

Correspondence to:

Eduardo Rocha
Laboratory of Histology and Embryology
Institute of Biomedical Sciences Abel Salazar (ICBAS)
University of Porto (U.Porto)
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto
Portugal
E-mail: erocha@icbas.up.pt

Abstract

Neurotransmitters do play key roles as to the maturation of gonads in bivalves. Also, sex steroids were suggested to have also critical functions in the reproductive control. Yet, it remains virtually unknown what kind of interplay may exist with those two kinds of controls, and if there are underlying differences in the nervous system structure between sexes. To help investigating this issue, a quantitative stereological study was made on the nervous ganglia of adult peppery furrow shell, *Scrobicularia plana*; a bioindicator species with relevancy for local economies. Mature males, mature females, and undifferentiated animals were collected at Ria Formosa Lagoon (Portugal), fixed with 10% buffered formalin, and processed for paraffin embedding, for studies at light microscopy. The animals were serially cut into 35 μm thick sections that were routinely stained with hematoxylin-eosin. Sections having the cerebral, pedal, and visceral ganglia were studied. The parameters of interest were the absolute volumes of the ganglia and the total and relative volumes of their cortex and medulla, and, finally, the total number of cells (neurons, glial, and pigmented) in the whole ganglia and each compartment. The volumes and the cell numbers were estimated, respectively, by the Cavalieri's principle, and by the optical fractionator method. Data were analyzed by ANOVA and post-hoc tests. Apart from the fact that females have a higher glia-to-neuron numerical ratio, we do not find other major differences between maturing males and females. These have a greater ganglionic volume when compared with undifferentiated adults; with males showing intermediate values. These facts point that size of the ganglia is related somehow with the sex and gonad maturation. The numerical data suggest that cell size differences may be at the basis of the differences, because there are no significant differences in the total cellularity among the gender studied. The three types of ganglia differ in total volumes and volume ratio of cortex versus medulla. The significantly greater volumes of the pedal ganglia (in relation to the cerebral ones) and of the visceral ganglia (in relation to all other) imply more voluminous cortexes and medullae, but more neuronal and non-neuronal cells only in the visceral. We disclose for the first time that a small bivalve as *S. plana* can have a mean total number of neural cells that may reach over 68000 in the visceral ganglia. The new fundamental data herein hopefully can help sustaining better interpretations as to the bivalve neurophysiology, and how it relates with unsolved issues in malacology, such as those related to nociceptive behavior and its implications in animal welfare.

Introduction

Neuroscience research has shown that female and male brains can be different in various ways (Leenaars *et al*. 2013; Leong and Packard 2014; Cosgrove *et al*. 2007), and that gonadal steroidal hormones not only are involved in the regulation of reproduction, but they can also induce sexually dimorphic brain development and organization (Arnold 2003). All these facts make the brain – and by proxy the whole general central nervous system – a key target to study the basic questions that prevail as to intersexual differences. For example, it is still in doubt if the brains (cells) work differently for specific skills between sexes (Burgaleta *et al*. 2012). Moreover, neuronal survival and degeneration are related with sex-steroids such as estrogens, progesterone, and testosterone (Garcia-Segura and Balthazart 2009; Gillies and Mcarthur 2010). These steroid hormones are thought to link behaviors either with an internal mechanism, like ovulation, or with an external factor, such as a nutritional condition (Gillies and Mcarthur 2010). In some animals the sexual dimorphism is often definitely observed. There are studies covering a range of animals, for instance birds, lamb, and rat, in which specific zones of the brains (individual behavior) of different sexes were investigated, namely by comparing the organ's volume and the neuron number (Arnold 2003; Sahin *et al*. 2001).

For invertebrates, the relation between the sex of individuals and differences in its nervous system structure and function is much less clear. However, some findings show that differences may exist; for instance, fruit flies show signs of the sex differences on decision-making behavior in their mating – these modulations may occur by expression of neurons and networks in the fly's brain (Dickson 2008). Similar findings as to olfactory preferences were found, in relation to behavior for movement and reproduction of nematodes, in which sexual dimorphism is related to specific groups of neurons within a core nervous system shared by both sexes. This specific sensory behavior seems to take place from functional modulation of common neural circuits controlled by sex chromosomes (Lee and Portman 2007).

There are already a few examples of nervous system dimorphism in bivalves too. For instance, in the Pacific lion's paw scallop, the detection of specific monoamines – including dopamine (DA), serotonin (5HT), and norepinephrine (NA) – was higher in male or females depending on the periods of the reproductive cycle (López-Sánchez *et al*. 2009). These monoamines were found in gonad, digestive gland, gill, and mantle, where it may modulate mechanisms involved in motor behaviors. Most of the 5HT was registered in the male gonad,

at nearly all the maturation stages, except at the spent stage, whereas norepinephrine was abundant in the female gonad (López-Sánchez *et al*. 2009). In the New Zealand mussel's visceral ganglia, a few selectively targeted monoamines and neuropeptides were identified by immunohistochemistry, with peculiar expressions found either in small or in large neurons of both males and females (Mahmud *et al*. 2008). Although the latter study did not search for differences between sexes, it pointed the presence of substances labeled as "responsible for different aspects of reproduction and spawning", and stressed the need to know the effects of seasons/gonad stages; dialogues between the nervous and gonad systems may logically occur. It should be noted that, as seen in other animal taxa, bivalve sex-steroid hormones are mostly produced in the gonads (Pazos and Mathieu 1999; Croll and Wang 2007; Yan *et al*. 2011).

In addition to the neural mechanisms underlying the sexual differentiation/maturation, we know that many toxicants can disrupt the nervous system (including of bivalves), and this is a reason why much better descriptions of that system's normal morphology and physiology in bivalves are in need. These will surely contribute to better diagnose, appreciate and predict the neurotoxic impacts that have been described in these organisms (e.g., Matozzo *et al*. 2005; Martin *et al*. 2008). Other reasons are more fundamental in nature, such as understanding the evolution of the nervous system intersexual differentiation, and the potential use of bivalves as experimental organisms, even in biomedicine, to get new mechanistic insights (Nelson *et al*. 2010). In line with the all the above considerations, we hypothesize herein that because of the key involvement of the nervous ganglia in the gonad differentiation and maturation in bivalves, the microscopic morphology of such ganglia can vary between sexes or maturation status. Also, because each ganglia type seems to have specific functions, we further theorize that the location/function of the ganglia shapes both its size and cellularity. To start tackling these hypotheses we did a stereological study on the nervous ganglia of gonad maturing and of exhausted peppery furrow shell, in similarly sized adult animals, looking into differences between gender and ganglia types. We elected their global and compartment volumes and the cellularity, using up-to-date gold-standard (design-based, unbiased, and efficient) stereology (Mayhew and Lucocq 2015). The species is of considerable ecological and economical value (Worrall *et al*. 1983), and has been used as a bioindicator organism for various pollutants (Chesman and Langston 2006; Gomes *et al*. 2009; Petridis *et al*. 2009; Ahmad *et al*. 2011).

Materials and methods

Animals and histological procedures

Wild adult peppery furrow shell (*Scrobicularia plana*) were collected at Ria Formosa Lagoon, Portugal. The animals were transferred to in house facilities in the day of capture, and maintained in glass aquaria (10 L), with aerated seawater (salinity 30 ‰) and at a temperature of 15°C. In the day after, arbitrarily sampled animals were anesthetized by immersion in a seawater solution of magnesium chloride (6%), kept at room temperature (≈ 20ºC). Their length, width, height, and fresh and total mass were measured before processing.

Each sampled animal (later identified, through histology, as 6 males, 6 females, and 6 animals with undifferentiated/spent gonad) were removed carefully from the shell and fixed *in toto* for 24 h, using 10% buffered formalin, at room temperature. After fixation, the samples were washed in 70% ethanol, dehydrated with increasing concentrations of alcohol (70% to 100%), cleared in xylene, and infiltrated with paraffin. Dehydration to infiltration was carried out using an automatic tissue processor (Leica TP1020, Germany). Paraffin embedding used a station (Leica EG 1140H, Germany).

Each animal was cut into serial sections (35 µm in thickness), on a motorized rotary microtome (Leica RM2155, Germany), and kept onto 3-aminopropyltriethoxysilane coated slides before hematoxylin-eosin (H&E) staining, xylene clearing, and DPX mounting. Sections having neural ganglia were used for stereology (other were occasionally used for sexing the animal). The left cerebral, right cerebral, pedal, and visceral ganglia were the targets. General qualitative observations were made with a light microscopy (BX50, Olympus).

Stereological analyses

The Cavalieri's principle was used for estimating the volume (V) of each ganglion (and separately of its cortex and medulla), based on the formula: $V = t \cdot \sum A$, where t is the mean distance between analyzed section planes, and A the sectional area of the target of interest (Gundersen and Jensen 1987). The volume of the ganglia was determined semi-automatically, using the stereological workstation CAST-Grid (version 1.5, Olympus Denmark), running with a light microscope (BX50, Olympus), equipped with a microcator (Heidenhain MT-12), a motorized stage with 1 µm X-Y movement accuracy (Prior), and a CCD video camera (Sony) displaying live image in a 17'' CRT monitor (Sony). Analyses were done under the x10

objective lens. For each ganglion, the areas of the cortex and medulla were registered in every section across them, so to later apply the above cited formula. The t for a ganglion was confirmed by measuring the section thickness with the microcator (see below). The total volumes were used to estimate the relative volumes (V_V) of cortex and medulla in the ganglion: V_V (compartment, ganglion) = V (medulla or cortex) ÷ V (ganglion).

The number of nervous ganglia cell was estimated via the optical disector-fractionator combination (Gunderson 1986) the total number (N), making use of the general formula:

$$N = Q \cdot (1 / ssf) \cdot (1 / asf) \cdot (1/ hsf),$$

where Q refers to the total number of cells actually counted in all the optical disectors; hsf is the height sampling fraction, captures the ratio of the section thickness that was screened; asf is the area sampling fraction, i.e., the ratio between the area of the counting frame and the area covered by each x,y movement; ssf is the section sampling fraction, i.e., the fraction of total sections sampled. Herein, half of total sections of each ganglia were sampled and a minimum of 100 neurons and 100 glia cells were counted per ganglia. The procedure was also enforced semi-automatically, with the above stereological workstation. Counting was done under the x100 (NA = 1.35) oil immersion lens, in systematically sampled fields. To check and account for any eventual non-uniform deformation, t was measured in every field, and as we did not notice such deformation the averaged t was used for hsf = h/t (Dorph-Petersen *et al*. 2001). Here, the average t was 33 µm and disector h was 25 µm. We set a minimal top guard zone of 3 µm as there is no heterogeneous distribution of cells across the z-axis (von Bartheld 2002).

As to cellularity, data is given in various forms, including splitting the N in numbers per cellular contingents defined by morphology, viz. large and small neurons, also fusiform, roundish, and triangularly shaped glial cells, and, finally, pigmented neural cells. Despite the concept of large and small may seem dubious, under the microscope it is easy to ensure; each counted cell is tagged as a "large" (oval/round, often with larger cytoplasmic granules, Ø of ≈ 18 x 25 µm) or as a "small" neuron (oval/round, thick heterochromatin rim, Ø ≈ 7 x 15 µm).

Statistical analysis

The statistical analyses were performed using the software STATISTICA (version 12.0 StatSoft Inc.). Data sets were checked for normality and homogeneity of variances — using the Shapiro–Wilk's W-test and Levene's test, respectively — prior to implement a two-way analysis of variance (two-way ANOVA). After a significant ANOVA, multiple comparisons

were made simultaneously using the Tukey's and Newman-Keuls' tests; in case only one test would indicate significance, the result would be considered as marginally significant. In some cases, logarithmic and square root transformations were carried out for normalizing and/or homogenization of variances of the raw data. When transformation was unsuccessful, a non-parametric Kruskal–Wallis ANOVA was used, followed by Mann-Whitney U tests for pairs, with a sequential Bonferroni correction. The significance level was set at the usual 5%. Data in Tables are given either as mean (CV – coefficient of variation = standard deviation / mean) and data in graphs are given as mean connected to the respective 95% confidence interval.

Results

Qualitative histological observations

The general structure of three nervous ganglia types is presented in Figure 1, being easily distinguishable the outer basophilic and highly cellular cortex, which contrasts with the very eosinophilic inner medulla, essentially composed of neural cell processes.

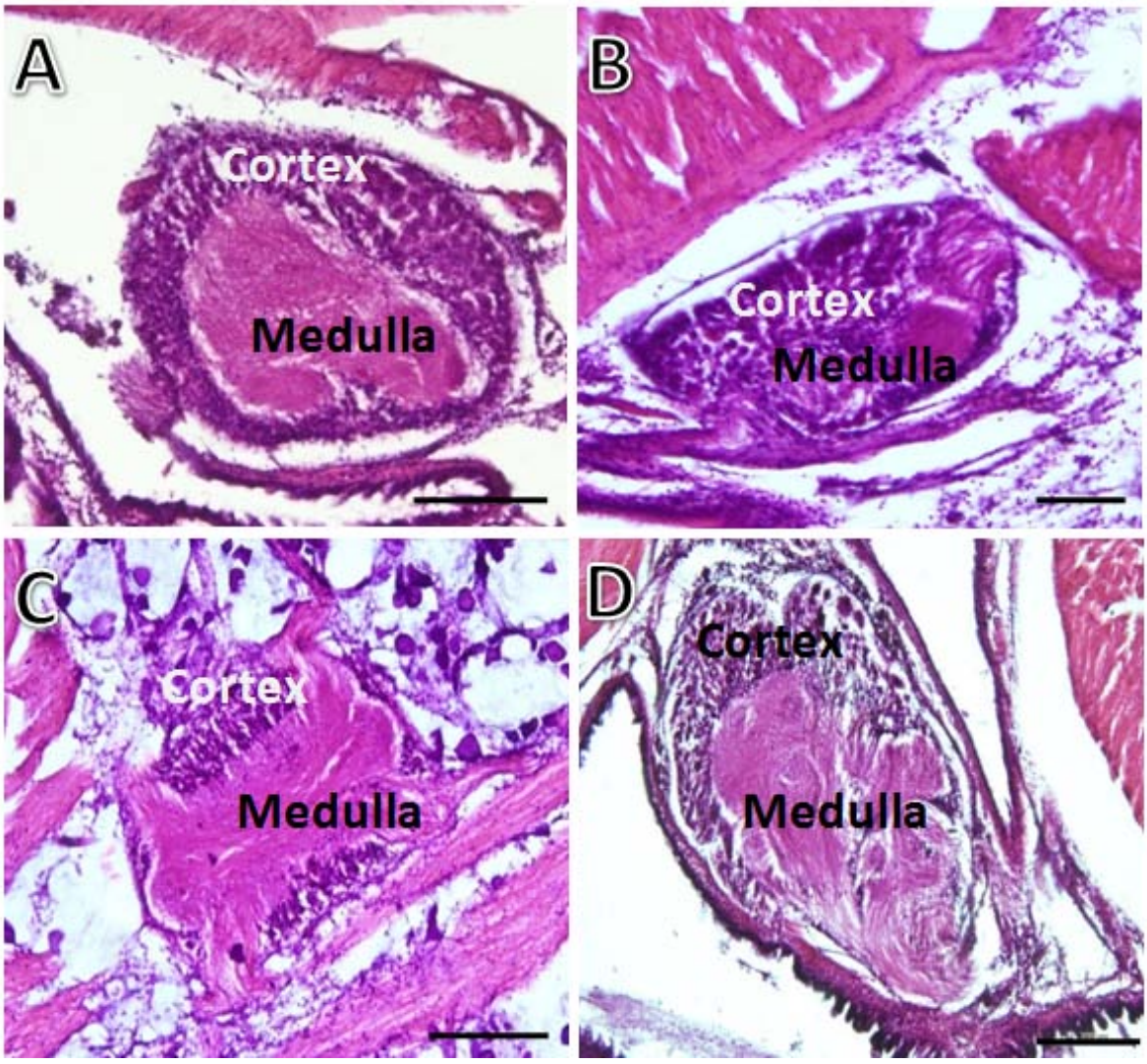

Figure 1. Transversely sectioned nervous ganglia of *S. plana*, picked randomly for illustration aims. **A**) Left cerebral ganglion. **B**) Right cerebral ganglion. **C**) Pedal ganglion. **D**) Visceral ganglion. The outer cellular cortex and the contrasting inner medulla are labeled. H&E staining. Scale bar = 200 μm.

The neurons are recognizable for their bigger size, having a single roundish nucleus, typically with one nucleolus (Fig. 2A). The larger neurons usually are at the outermost cortex and the smaller ones are aggregated in between the larger neurons, and predominate in the inner cortex. Typical neurons and pigmented cells appear in the medulla as well, scarcely but

systematically. As for glial cells, they are scattered across the ganglia, amidst neurons and medullar neurites (Fig. 2B). They show three phenotypes: fusiform, roundish, and triangular.

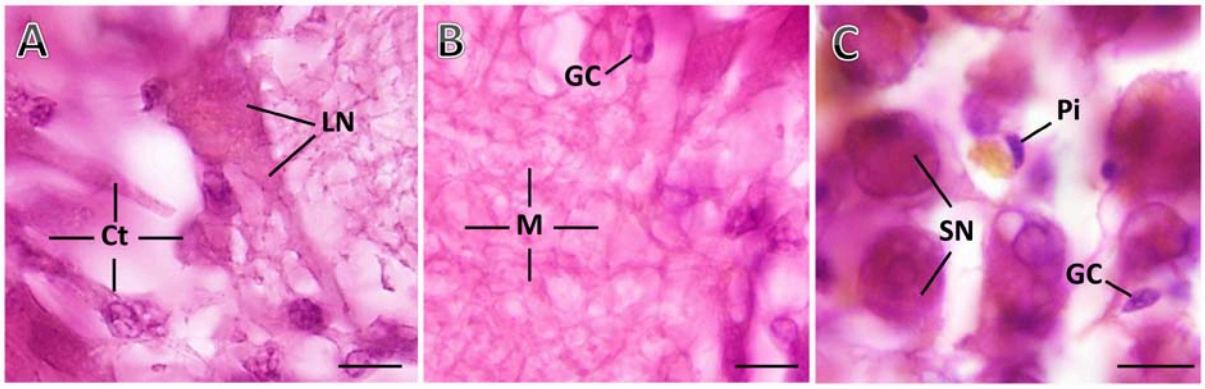

Figure 2. Photomicrographs taken from histological thick sections of a nervous ganglia of *S. plana*. **A**) Cortex (Ct), with a standing out large neuron (LN). **B**) Medulla (M), with neuronal and glial eosinophilic projections, and somata of glial cells (GC). **C**) Detail of cortex, where smaller neurons (SN), an elongated glial cell (GC) and a pigment cell (Pi) are seen. H&E Staining. Scale bar: 10 µm.

For determining the sex/gonadal status of each animal, we looked at the foot zones near the pedal ganglion. The serial sections allowed us to definitely pin-point for each case which kind of gametes, if any, were differentiating, rating the animals as males, females or as undifferentiated (Fig. 3); in the latter, the gonadal acini do not exhibit active gametogenesis.

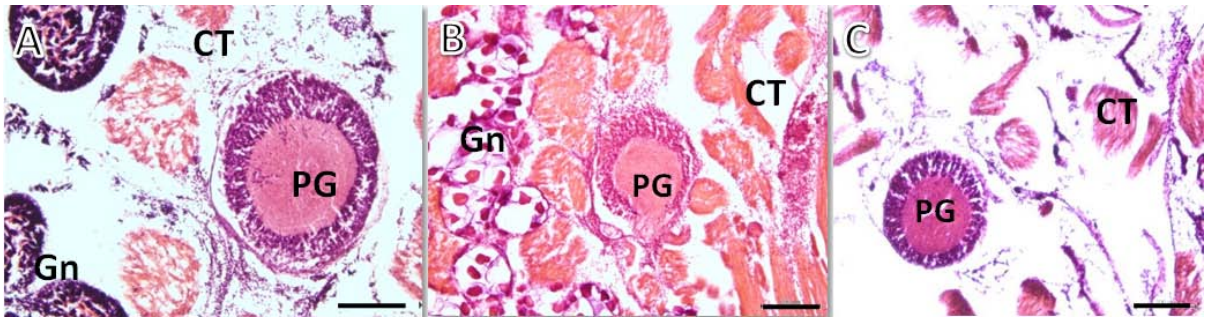

Figure 3. Photomicrographs taken from histological thick sections of a nervous ganglia of *S. plana*. **A**) Male: spermatogenesis is evident within the gonadal acini (Gn); at upper and lower left corners of the image. **B**) Female: gonadal acini (Gn) are filled with roundish maturing oocytes. **C**) Image from one undifferentiated animal, with atrophic acini devoid of maturing gametes, occasionally appearing within the connective tissue (CT). PG – Pedal ganglion. H&E staining. Scale bar: 200 µm.

The qualitative observations did not allow to discern cellularity differences between animals of different sexes (defined as per their gonad maturation/differentiation degree), or across the three nervous ganglia categories — left cerebral ganglion (LCG); right cerebral ganglion (RCG); pedal ganglion (PG); visceral ganglion (VG) —, and so we could conclude only

about very obvious features, like the fact that the cortex is more cellular than the medulla and that the visceral ganglion is the biggest among all; despite we clearly could not infer about how much bigger it was in relation to the other types.

Quantitative data - Body morphometry

The body morphometric parameters of the animals are in Table 1, for all genders. No statistically significant differences exist. The mass was the most variable parameters.

Table 1. Body morphometry of *S. plana* used in the study.

Gender	Length (cm)	Height (cm)	Width (cm)	Fresh mass (g)	Total mass (g)
Males	3.1 (0.18)	2.5 (0.13)	0.9 (0.12)	1.28 (0.24)	3.03 (0.29)
Females	3.3 (0.06)	2.7 (0.09)	1.1 (0.12)	1.67 (0.22)	4.55 (0.26)
Undifferentiated	3.2 (0.13)	2.4 (0.17)	1.0 (0.21)	1.67 (0.53)	4.57 (0.58)

Six animals per gender were used. Data given as mean (coefficient of variation).

Quantitative data – Total and relatives volumes

The volumes (V) of the three ganglia types are given in Table 2, split by gender, and in Figure 4A with all genders grouped. The two-way ANOVA highlights a significant effect for the "ganglion type", where the visceral ganglion V is significantly bigger (3 to 4-fold) than the other ganglia (Fig. 4A). Additionally, there is an overall statistical significance as to the parameter "gender", with females having a greater global/summed ganglionic V than that of undifferentiated specimens (Fig. 4B); with males do not differing from the other genders.

Table 2. Total volumes (μm^3) of the cerebral, pedal and visceral ganglia of *S. plana*.

Genders	Cerebral		Pedal	Visceral
	Left	Right		
Males	$38.3x10^6$ (0.19)	$35.6x10^6$ (0.23)	$59.0x10^6$ (0.29)	$164.2x10^6$ (0.26)
Females	$49.8x10^6$ (0.23)	$48.3x10^6$ (0.29)	$55.8x10^6$ (0.12)	$191.7x10^6$ (0.32)
Undifferentiated	$36.8x10^6$ (0.34)	$31.9 x10^6$ (0.42)	$51.6x10^6$ (0.48)	$133.0x10^6$ (0.32)

Six animals per gender were used. Data given as mean (coefficient of variation).

The volumetric patterns for the ganglia analyzed as a whole also occur when we look at the two key structural ganglionic compartments separately. Indeed, both the cortex and the medulla follow the exact same trends, as it can be seen in Figure 5, with the visceral ganglion compartment standing out from all the other and with females differing from undifferentiated.

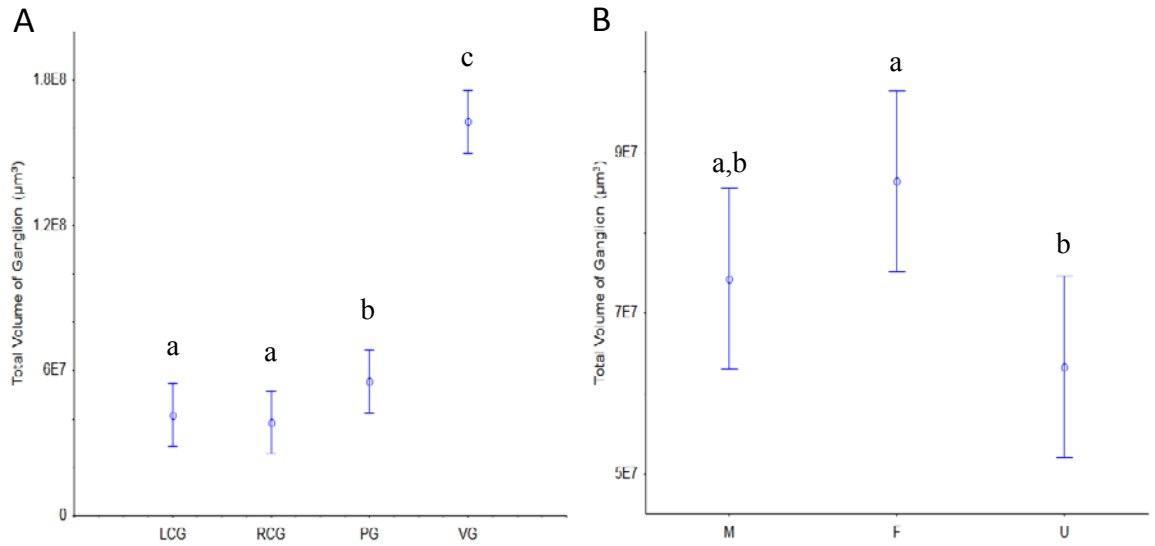

Figure 4. Volumes of the nervous ganglia of *S. plana*. **A**) Per ganglion. **B**) All ganglia per gender. Different letters mean significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion; M: males; F: females; U: undifferentiated.

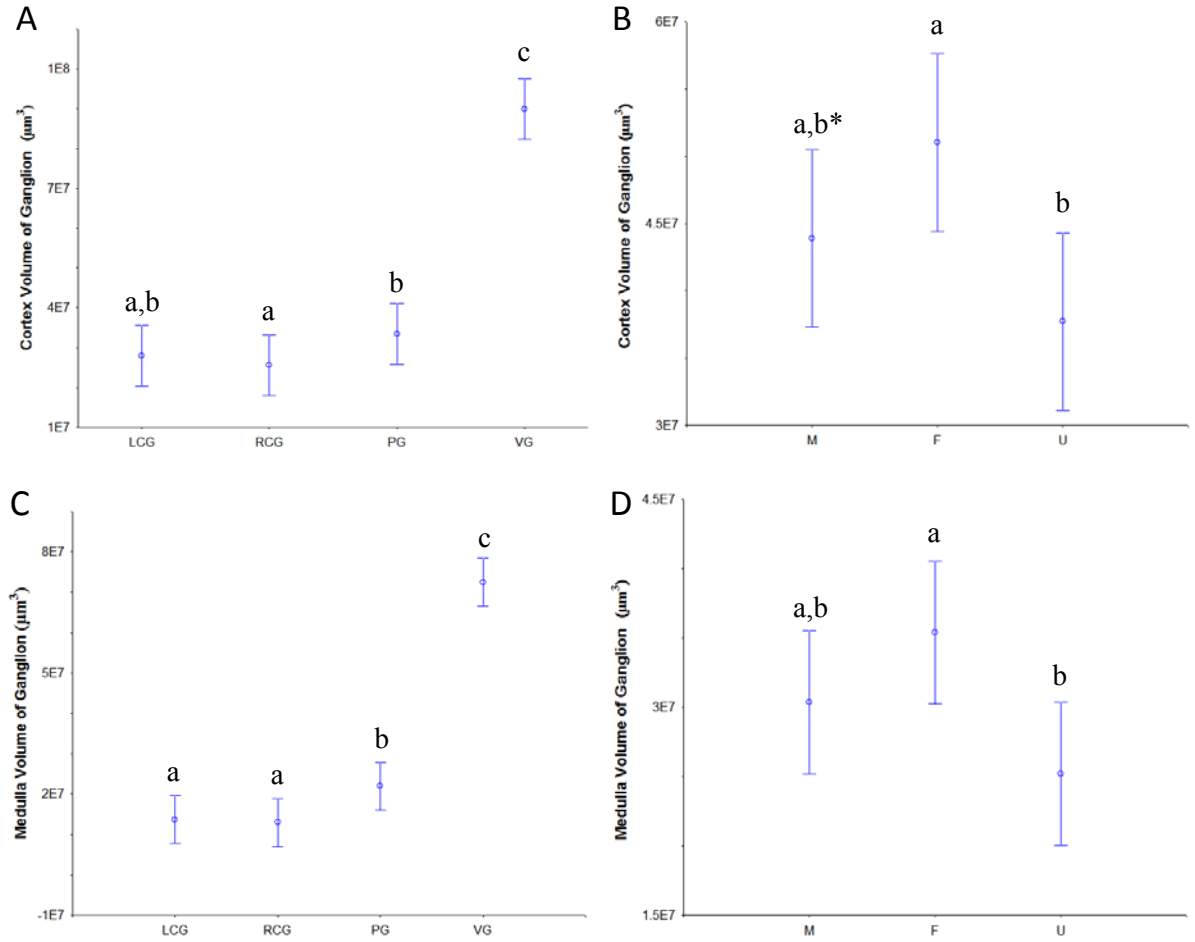

Figure 5. Volumes of the nervous ganglia cortex and medulla of *S. plana*. **A**) Cortex per ganglion. **B**) All cortexes per gender. **C**) Medulla per ganglion. **D**) All medullae per gender. Different letters mean significant differences. Data as mean and 95% confidence interval. LCG, RCG, PG, VG, M, F, and U as in Fig. 4. *Based on our criteria, there is a marginally significant difference between M and F (Newman-Keuls' test, p = 0.038).

The V_V (cortex, ganglion) and V_V (medulla, ganglion) per gender are displayed on Tables 3 and 4. In all ganglia types the cortex occupies over 50% of the total volume. There are no differences at to the gender effect, but the analysis reveals a significant effect for the ganglia type ($p<0.05$), with the V_V (cortex, ganglion) decreasing anterior-posteriorly, from the cerebral ganglia (that do not differ bilaterally) towards the visceral ganglion; in contrast, the V_V (medulla, ganglion) follows a significant reverse pattern, raising towards the visceral. The patterns and the detailed statistical differences between ganglia are displayed in Figure 6.

Table 3. Relative volumes (%) of the cerebral, pedal and visceral ganglia cortex of *S. plana*.

Gender	Cerebral Cortex		Pedal Cortex	Visceral Cortex
	Left	Right		
Males	64 (0.02)	63 (0.06)	59 (0.03)	57 (0.12)
Females	68 (0.03)	67 (0.04)	61 (0.09)	54 (0.03)
Undifferentiated	69 (0.04)	68 (0.05)	62 (0.04)	57 (0.09)

Six animals per gender were used. Data given as mean (coefficient of variation).

Table 4. Relative volumes (%) of the cerebral, pedal and visceral ganglia medulla of *S. plana*.

Genders	Cerebral Medulla		Pedal Medulla	Visceral Medulla
	Left	Right		
Males	36 (0.04)	36 (0.05)	41 (0.05)	43 (0.17)
Females	32 (0.06)	33 (0.08)	39 (0.13)	46 (0.04)
Undifferentiated	31 (0.09)	32 (0.10)	38 (0.06)	43 (0.12)

Six animals per gender were used. Data given as mean (coefficient of variation).

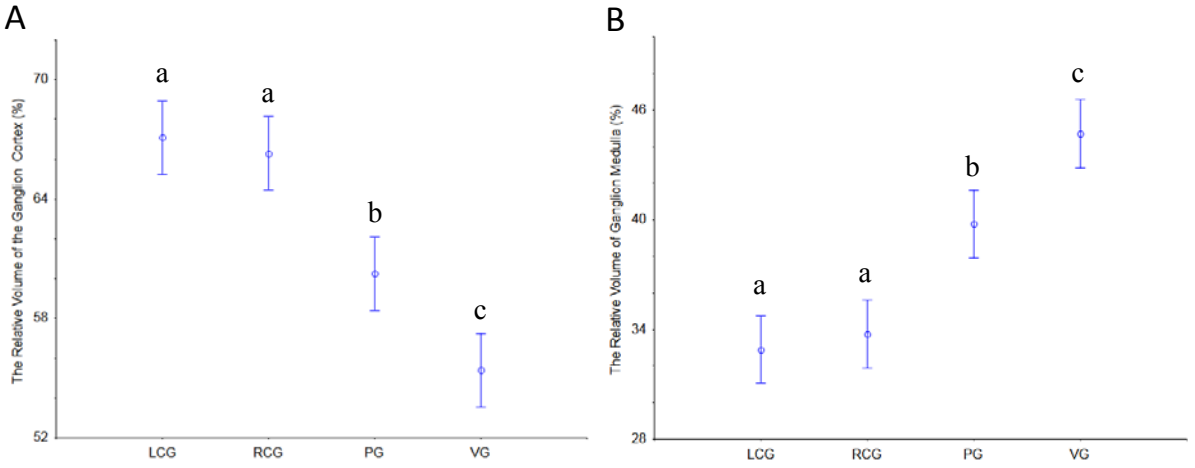

Figure 6. Relative volumes (%) of the cerebral, pedal and visceral ganglia cortex of *S. plana*, with all genders combined. **A**) V_V (cortex, ganglion). **B**) V_V (medulla, ganglion). Different letters means significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

Quantitative data – Number of neural cells

Estimates of the (total) N of neurons (including their partition in the general groups of large and small), of glial cells (sub-divided into fusiform, roundish, and triangular shaped), and, finally, of pigmented cells data are shown in Tables 5-7 and Figures 7-8; the latter offer data sets for which statistically significant differences exist. In the tabular data the ganglia type and gender are taken into consideration, being genders grouped in Figures 7-8. Table 5 refers to the whole ganglia, whereas Tables 6 and 7 refer to cortex and medulla, respectively.

There are no significant differences between genders as to cellularity, despite there is a consistent pattern towards a higher N of pigmented cells in the undifferentiated animals. In parallel to a high variability (expressed as high CV); which precludes a significant difference. As to the N considering the ganglia type, the ANOVA unveils a significant effect ($p<0.001$), with the visceral ganglion having statistically significant more neurons and glial cells, but not of pigmented cells. The N of these neural cells types are significantly greater both when we look at the whole ganglia (Fig. 7) and looking at each of its structural compartments (Fig. 8).

Tables 5-7 illustrate that the subtypes of neurons and glial cells follow similar patterns as when considering all types. One exception is that the neurons N in medulla of the visceral ganglion is higher, as in for the whole ganglion; but this is so at the cost of the small neurons ($p<0.05$), as the bigger ones do not statistically change. The data in Tables 5-7 translate into numbers the fact that there are more cells in the cortex, but further demonstrates that the statistics is true for any type of neural cell, and not only for neurons. Despite being a minority in the medulla, the N of neurons still reach from hundreds to ≈ two thousands; as those cells are more erratic in the medulla, the variability associated to the N is higher than in the cortex – as it is seen by the relatively higher CV (Table 7) and wider confidence intervals (Fig. 8C).

Based on the N, we further estimated the so called “glia-to-neuron” ratio (Table 8 and Figs. 9 and 10). Looking at Table 8, for the whole ganglia, there is a trend for slightly more glial cells than neurons, but if we look at the cortex the glia-to-neuron ratio is more balanced, at least in the cerebral ganglia. Indeed, the ratio in cortex is ≈ 1 (LCG ≈ 0.9; RCG ≈ 0.9), and rises in PG ≈ 1.3 and VG ≈ 1.2. In the whole ganglia, thus including medullar neurons and glial cells, the ratio expectably rises in all three ganglia to ≈ 1.5 (LCG ≈ 1.2; RCG ≈ 1.3; PG ≈ 1.7; VG ≈ 1.8). The ratio in the medulla is by nature very high, and as neurons are erratic the CVs are higher. The ANOVA revealed no significant interaction between the factors type

of ganglia and gender, but each of the factors significantly impacted in the ratio (Figs. 9 and 10). In summary, we can say that the ratio is greater in the PG and particularly in the VG, that the females do have a larger ratio than males, and that the undifferentiated appear in between.

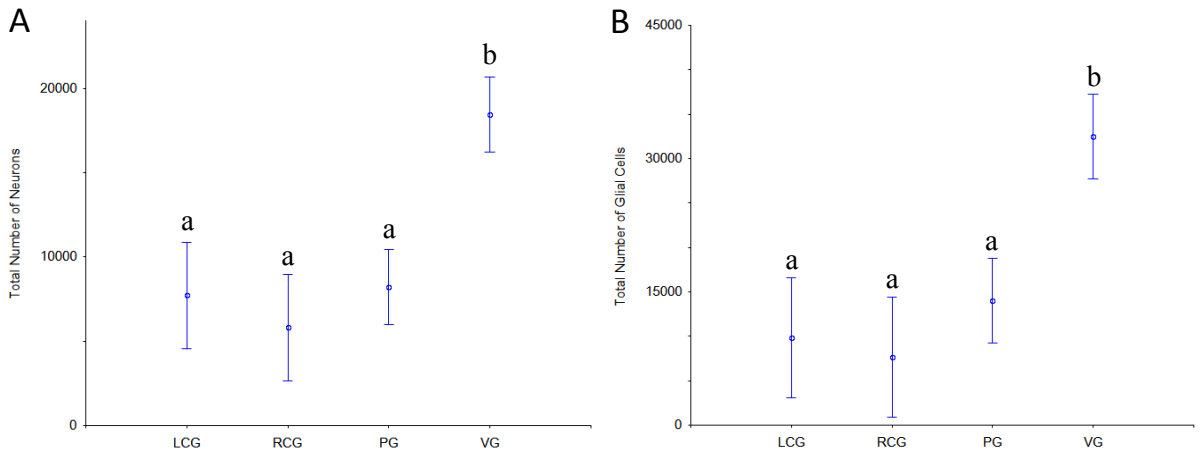

Figure 7. Number of neurons and glial cells in the nervous ganglia of *S. plana*; data from all genders combined. **A**) Neurons. **B**) Glial cells. Different letters means significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

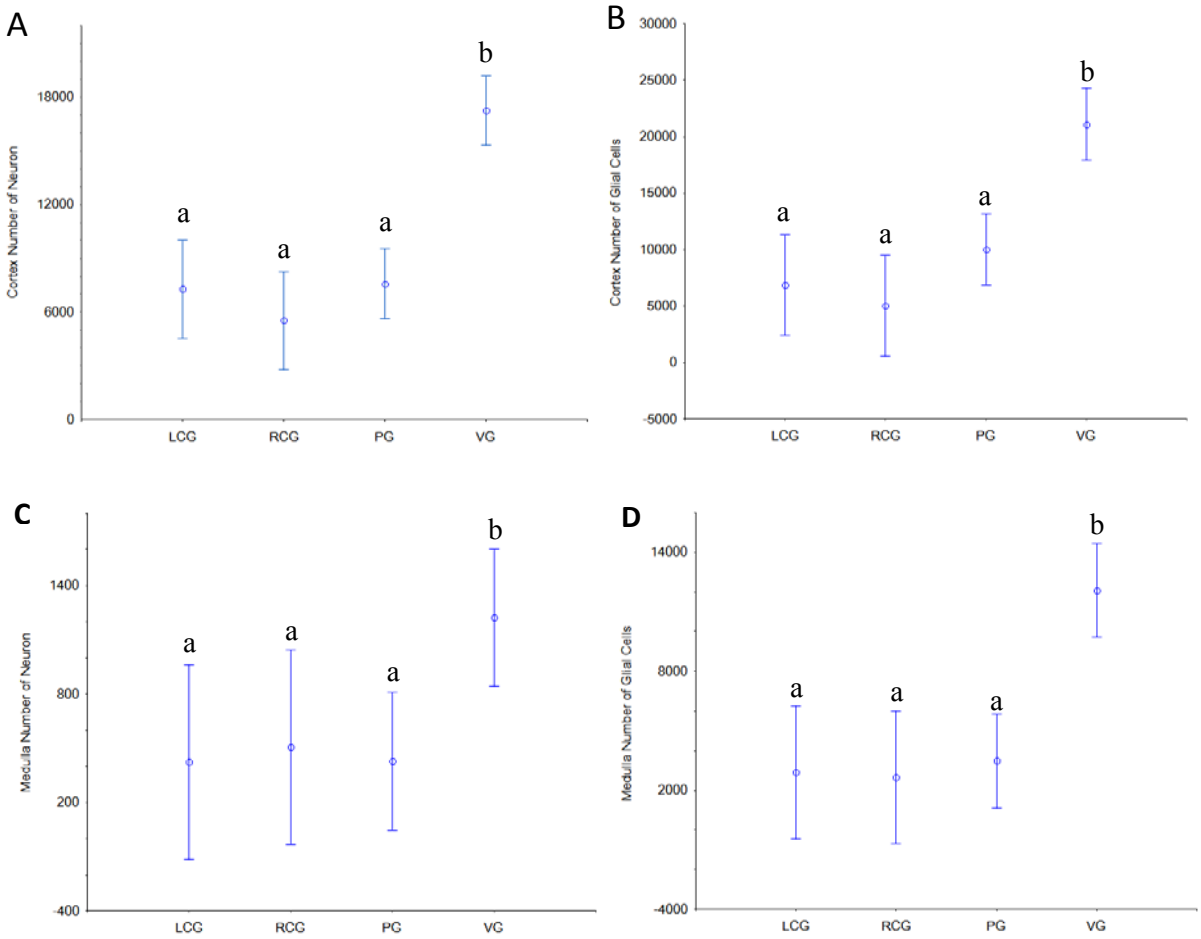

Figure 8. Number of neurons and glial cells in the cortex and medulla of nervous ganglia of *S. plana*; data from all genders combined. **A**) Neurons in the cortex. **B**) Glial cells in the cortex. **C**) Neurons in the medulla. **D**) Glial cells in the medulla. Different letters means significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 5. Total mean number (N) of neural cells, by ganglia type and gender in *S. plana*.

Ganglia	Gender	Neurons			Glial cells				Pigmented cells
		Small	Large	Total	Fusiform	Roundish	Triangular	Total	
LCG	Males	2,636 (0.3)	4,056 (0.3)	**6,692 (0.3)**	1,613 (0.1)	2,461 (0.4)	1,672 (0.3)	**5,746 (0.2)**	**997 (0.4)**
	Females	2,886 (0.3)	4,977 (0.4)	**7,863 (0.2)**	2,047 (0.6)	3,904 (0.2)	3,584 (0.2)	**9,535 (0.1)**	**1,595 (0.8)**
	Undifferentiated	3,524 (0.4)	5,061 (0.2)	**8,585 (0.3)**	3,088 (0.4)	5,960 (0.6)	4,915 (0.5)	**13,963 (0.5)**	**2,785 (1.3)**
RCG	Males	2,430 (0.1)	3,052 (0.3)	**5,482 (0.1)**	1,379 (0.3)	2,416 (0.2)	1,926 (0.4)	**5,721 (0.2)**	**817 (0.9)**
	Females	2,268 (0.1)	3,555 (0.1)	**5,823 (0.1)**	2,866 (0.3)	4,248 (0.6)	2,365 (0.3)	**9,479 (0.3)**	**1,458 (0.4)**
	Undifferentiated	3,149 (0.6)	3,647 (0.5)	**6,796 (0.6)**	2,151 (1.0)	2,697 (0.9)	3,099 (0.2)	**7,947 (0.6)**	**2,785 (1.3)**
PG	Males	4,692 (0.4)	3,897 (0.2)	**8,589 (0.2)**	2,871 (0.4)	5,503 (0.4)	3,182 (0.4)	**11,556 (0.3)**	**1,336 (0.5)**
	Females	3,805 (0.2)	3,388 (0.3)	**7,193 (0.2)**	1,682 (0.3)	3,182 (0.5)	7,195 (0.2)	**14,709 (0.3)**	**1,688 (0.5)**
	Undifferentiated	4,745 (0.5)	3,534 (0.5)	**8,279 (0.5)**	2,894 (0.8)	7,438 (0.7)	3,914 (0.3)	**14,246 (0.5)**	**2,906 (0.8)**
VG	Males	7,408 (0.2)	8,578 (0.5)	**15,986 (0.3)**	6,200 (0.7)	11,455 (0.4)	8,670 (0.4)	**26,325 (0.4)**	**3,241 (0.8)**
	Females	8,964 (0.1)	9,339 (0.4)	**18,303 (0.3)**	7,833 (0.3)	14,407 (0.2)	10,207 (0.3)	**32,447 (0.2)**	**3,554 (0.6)**
	Undifferentiated	12,437 (0.6)	8,736 (0.4)	**21,173 (0.5)**	7,531 (0.7)	22,138 (0.8)	11,070 (0.4)	**40,739 (0.7)**	**6,251 (1.3)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 6. Total mean number (N) of neural cells in the cortex, by ganglia type and gender in *S. plana*.

Ganglia	Gender	Neurons			Glial cells				Pigmented cells
		Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
LCG	Males	2310 (0.3)	4001 (0.3)	**6311 (0.3)**	1218 (0.1)	1848 (0.4)	1335 (0.4)	**4401 (0.2)**	**785 (0.4)**
	Females	2745 (0.3)	4798 (0.4)	**7543 (0.2)**	1354 (0.6)	2806 (0.3)	2390 (0.2)	**6550 (0.1)**	**1373 (0.8)**
	Undifferentiated	3125 (0.4)	4887 (0.2)	**8012 (0.3)**	2173 (0.4)	4075 (0.6)	3345 (0.5)	**9593 (0.4)**	**3274 (0.7)**
RCG	Males	2171 (0.3)	2975 (0.3)	**5146 (0.2)**	1017 (0.3)	1513 (0.3)	1303 (0.4)	**3833 (0.2)**	**498 (0.7)**
	Females	2059 (0.1)	3273 (0.1)	**5332 (0.1)**	2112 (0.4)	2871 (0.6)	1571 (0.2)	**6554 (0.4)**	**1027 (0.4)**
	Undifferentiated	2801 (0.6)	3296 (0.4)	**6097 (0.5)**	1313 (1.1)	1477 (1.1)	2025 (0.4)	**4815 (0.8)**	**2269 (1.3)**
PG	Males	4382 (0.4)	3798 (0.3)	**8180 (0.2)**	2085 (0.4)	4358 (0.5)	2196 (0.4)	**8639 (0.4)**	**978 (0.3)**
	Females	3628 (0.3)	3206 (0.3)	**6834 (0.2)**	2467 (0.6)	5496 (0.2)	3188 (0.4)	**11151 (0.3)**	**1407 (0.6)**
	Undifferentiated	4346 (0.5)	3409 (0.5)	**7755 (0.5)**	2207 (0.8)	5230 (0.8)	2865 (0.4)	**10302 (0.6)**	**2224 (0.9)**
VG	Males	6903 (0.2)	8326 (0.5)	**15229 (0.3)**	4362 (0.7)	7279 (0.4)	5091 (0.4)	**16732 (0.5)**	**2178 (0.7)**
	Females	8371 (0.1)	9062 (0.4)	**17433 (0.2)**	5045 (0.3)	9595 (0.2)	6355 (0.3)	**20995 (0.2)**	**2719 (0.5)**
	Undifferentiated	10809 (0.5)	8322 (0.4)	**19131 (0.5)**	5398 (0.7)	12424 (0.6)	7752 (0.4)	**25574 (0.6)**	**5000 (1.2)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 7. Total mean number (N) of neural cells in the medulla, by ganglia type and gender in *S. plana*.

Ganglia	Gender	Neurons			Glial cells				Pigmented cells
		Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
LCG	Males	325 (0.6)	55 (1.0)	**380 (0.4)**	394 (0.1)	613 (0.5)	337 (0.6)	**1344 (0.2)**	**212 (0.9)**
	Females	141 (1.7)	180 (0.9)	**321 (1.3)**	693 (0.6)	1098 (0.1)	1194 (0.5)	**2985 (0.3)**	**222 (0.7)**
	Undifferentiated	398 (0.5)	174 (1.2)	**572 (0.6)**	916 (0.4)	1885 (0.6)	1569 (0.6)	**4370 (0.5)**	**623 (0.8)**
RCG	Males	259 (1.5)	77 (0.9)	**336 (1.3)**	362 (0.5)	903 (0.4)	623 (0.9)	**1888 (0.4)**	**319 (1.3)**
	Females	209 (0.9)	282 (0.5)	**491 (0.5)**	754 (0.2)	1377 (0.6)	794 (0.5)	**2925 (0.2)**	**431 (0.4)**
	Undifferentiated	347 (1.0)	351 (1.5)	**698 (1.2)**	837 (0.8)	1221 (0.6)	1075 (0.3)	**3133 (0.4)**	**516 (1.3)**
PG	Males	310 (1.2)	99 (1.3)	**409 (1.2)**	786 (0.6)	1145 (0.6)	986 (0.6)	**2917 (0.4)**	**358 (1.4)**
	Females	178 (1.1)	182 (1.3)	**360 (1.1)**	715 (0.4)	1700 (0.7)	1143 (0.3)	**3558 (0.3)**	**280 (0.3)**
	Undifferentiated	399 (0.7)	126 (1.3)	**515 (0.8)**	687 (0.9)	2208 (0.8)	1049 (0.3)	**3945 (0.6)**	**682 (1.2)**
VG	Males	505 (0.7)	252 (1.2)	**757 (0.8)**	1838 (0.7)	4176 (0.3)	3579 (0.4)	**9593 (0.4)**	**1063 (1.1)**
	Females	592 (0.8)	276 (0.8)	**868 (0.6)**	2789 (0.4)	4812 (0.5)	3851 (0.5)	**11452 (0.4)**	**835 (0.9)**
	Undifferentiated	1628 (1.0)	414 (1.0)	**2042 (0.9)**	2133 (0.7)	9714 (1.1)	3318 (0.5)	**15165 (0.8)**	**1251 (1.8)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 8. Glia-to-neuron (number) ratio in the cerebral, pedal and visceral ganglia medulla of *S. plana*.

Ganglia	Gender	Glia-to-neuron ratio		
		Whole ganglion	Cortex	Medulla
LCG	Males	0.9 (0.11)	0.7 (0.17)	3.7 (0.24)
	Females	1.3 (0.25)	0.9 (0.25)	26.7 (0.83)
	Undifferentiated	1.3 (0.40)	1.2 (0.23)	8.9 (0.56)
RCG	Males	1.1 (0.14)	0.7 (0.16)	6.9 (0.54)
	Females	1.2 (0.50)	1.2 (0.34)	16.9 (0.98)
	Undifferentiated	1.1 (0.40)	0.7 (0.30)	9.7 (1.13)
PG	Males	1.5 (0.17)	1.0 (0.23)	17.2 (0.91)
	Females	2.2 (0.37)	1.7 (0.21)	18.8 (1.05)
	Undifferentiated	1.7 (0.15)	1.3 (0.14)	6.8 (0.14)
VG	Males	1.6 (0.16)	1.1 (0.26)	13.7 (0.58)
	Females	1.8 (0.13)	1.2 (0.10)	22.6 (0.96)
	Undifferentiated	1.8 (0.22)	1.3 (0.10)	12.1 (1.00)

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

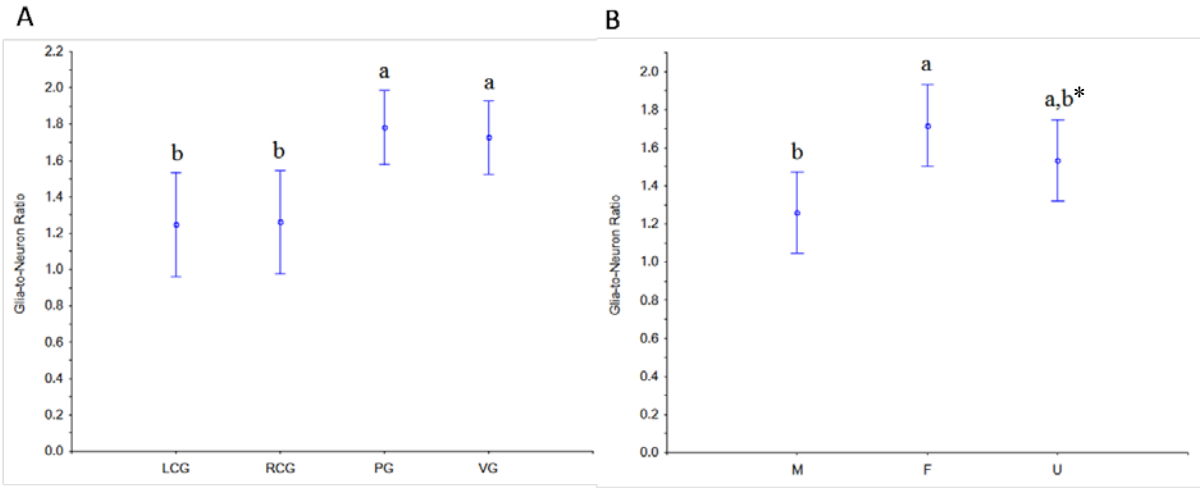

Figure 9. Glia-to-neuron (number) ratio in the nervous ganglia of *S. plana*, considering the whole ganglia. **A**) Data per ganglion type, irrespective of gender. **B**) Results from all ganglia, grouped per gender. Different letters mean significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion; M: males; F: females; U: undifferentiated. *Based on our criteria, there is a marginal statistical difference between M and U (Newman-Keuls' test, p = 0.031).

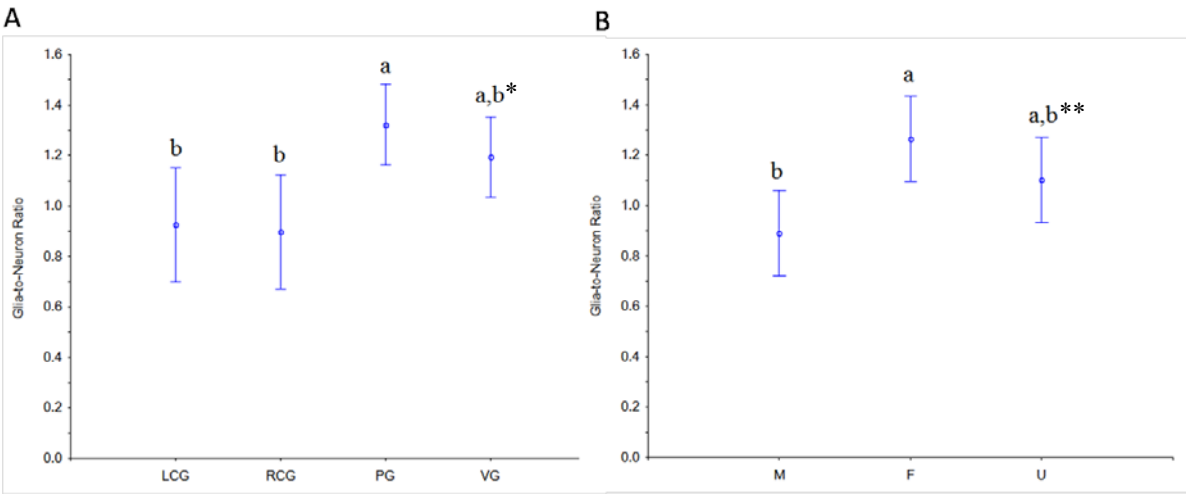

Figure 10. Glia-to-neuron (number) ratio in the cortex of the nervous ganglia of *S. plana*. **A**) Data per ganglion type, irrespective of gender. **B**) Results from all ganglia, grouped per gender. Different letters mean significant differences. Data as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion; M: males; F: females; U: undifferentiated. *Based on our criteria, there is a marginal statistical difference between VG and both LCG and RGC (Newman-Keuls' test, $p = 0.014$ and $p = 0.011$, respectively). **Based on our criteria, there is a marginal statistical difference between M and U (Newman-Keuls' test, $p = 0.026$).

Discussion

To our knowledge this is the first study that makes quantifications of bivalve cells and organs with design-based (or unbiased) stereological tools, which do not rely on the older methods' unrealistic and uncontrolled assumptions about the structure's geometric shape or spatial orientation and distribution of structures in 3D space. The advantages of the unbiased methods have been established, illustrated and refined over the last three decades (Gundersen 1986; Gundersen and Jensen 1987; Dorph-Petersen *et al*. 2001, Mayhew and Lucocq 2015). In bivalves we find only one article that used the optical fractionator, not for estimating any component of the animal, but rather for revealing the exact number of the infecting protozoan parasite *Perkinsus marinus* in the mantle of *Crassostrea virginica* (Remacha *et al*. 2008). The latter study and ours illustrate well how the same stereological techniques can tackle so varied questions, not to mention the latterly discussed key potential to be part of the new field of "morphomics", in line with other "omics" (Mayhew 2015; Mayhew and Lucocq 2015). The stereology tools we used to study the nervous ganglia of *S. plana* are well recognized in vertebrate neurocytology, and have been paramount to sustain advances (Schmitz and Hof 2005; Walløe *et al*. 2014). Our study is significant not only because it tests the technology and unveils new data for *S. plana*, suited to tackle hypotheses and sustain morphofunctional inferences, but also since it encourages further use of unbiased stereology in bivalve research.

The quantitative approach herein aimed to compare a 3D-relevant size of the nervous ganglia of *S. plana* (absolute and additionally relative volumes of the whole ganglia and of its cortex and medulla) and of the cellularity, measured as the number of their constituent cells. The hypothetical background was a plausible fundamental influence of the animal sex (when it can be disclosed) in the microanatomy of the bivalve nervous system. We studied animals that were either males or females, as explicitly identified by their maturing gametes, and also specimens that could not be sexed because their gonads were spent and atrophic. Facing the key physiological modelling actions of the nervous system on the gametogenesis of bivalves (Siniscalchi *et al*. 2004; Gagné *et al*. 2007; López-Sánchez *et al*. 2009), and eventual (but not well established) feedback loops, at least in theory we studied animals that should be as functionally dissimilar as *S. plana* adults of a different sex could be. We thus opted to analyze the three "gender types" as a way to promote the odds of capturing a difference, it if existed. Also, this strategy helped to increase power for studying differences between ganglia types,

within the two-way ANOVA, particularly in case of a no significant effect for gender – this was relevant here as we wished to back/extend earlier suggested inter-ganglionic differences.

As to gender differences, one that is statistically confirmed concerns the total volume of the ganglia, which is greater in females than in the undifferentiated, with the males do not differing from either of the other groups. The cortex and medulla evidence basically the same differences, with one additional marginal being found in cortex; with males having a smaller volume (Fig. 5B). The not significant difference between males and females concords with our previous data, that despite based on another technic (3D-reconstruction), offered estimates in the same order of magnitudes and close to those herein (Tantiwisawaruji *et al*. 2015). It would be speculative to point one particular reason for the difference between females versus undifferentiated, but it is a fact in perfect accordance with our hypothesis that the sex/gonadal status "shapes" the bivalve nervous system structure — either because of the activity of the latter in influencing gonads (e.g., Siniscalchi *et al*. 2004) or by effects of factors originated in the gonad (e.g., sex-steroids) and impacting on neural elements (e.g., Stefano *et al*. 2003). Irrespective of the functional implications, what makes females having greater volumes than the undifferentiated and tendentiously more than males? Are there more neuronal cells and/or bigger ones? Finally, are there any differences in the amount/size of neural processes? This study was not designed to answer all these questions, but later in this Discussion we will go back to those so interesting and puzzling differences, after debating the data on cell numbers.

Despite there is no interaction between gender and ganglia type, there is a statistically significant effect of the latter in the volumes of the ganglia and of their compartments. The two CG are similar in size, but volumes overall increase significantly towards the PG, which is greater than the cerebral and much smaller than the VG. The cortical and medullar parts do significantly follow the trends of the whole ganglia. Once more, the facts nicely agree with our prior work, in which we unveil the same pattern in males and females (Tantiwisawaruji *et al*. 2015). We can thus confidently suggest that the size differences between all ganglia types are independent of the gender and of the condition of being in a process of gonad maturation.

In addition to the absolute volumes, we look at the relative volumes (V_V) of the cortex and medulla, quantifying that overall the cortex is≈ 60% and the medulla 40%. Yet, if gender does not seem to matter for the cortex to medulla ratio, there is a statistically significant

effect of the type of ganglia in V_V, with the less voluminous CG showing the highest mean values for the cortex V_V, with PG being intermediate, and the VG having the smallest values; conversely, the V_V for the medulla followed a matching opposite pattern (Fig. 6). It is worth mentioning that we noted an approximate trend in our previous study, but we could not prove significance (Tantiwisawaruji *et al*. 2015). Such fine structural differences between the V_V of the ganglia likely are not a random event and should have a rational and a functional impact. One possible reason can be related with the number of neurites that emerging from the cortex go into the medulla, that in absolute terms is expected to be greater at least in VG, facing the higher total cellularity this ganglion has when compared with the others. A higher number and/or size of neuronal and glial projections would promote a relatively greater V_V (medulla, ganglion), when compared with other ganglia types. The lowest cellularity of the LCG and RCG, logically with less projections going into the medullar neuropil, would also explain the smallest V_V (medulla, ganglion). On the other hand, this sort of rationale does not explain the intermediate value of the PG, as in this case the total cellularity is not greater than that in either type of CG (see discussions on cellularity below). So, a mixture of morphofunctional factors must contribute to the differences in V_V. Among them, we can also think about still unstudied differences as to the neuron and glia cell volumes, also the degree of complexity in their interconnections, particularly in the cortex of the ganglia, which can have impacts on the volume ratio of cortex to medulla, between each ganglion type, in view of their functions.

To support the rational of our discussion we recall that each ganglia types has specific degrees of organization and function. For instance, the VG is viewed as the most differentiated central nervous system structure in bivalves (Bullock 1965; Harrison and Kohn 1997). Some evidences pointed that this ganglion is responsible for influencing the cardiac rhythm and motilities of the shell, mantle, siphons and gills (Stefano 1945; Bullock 1965; Carroll and Catapane 2007). The PG responds to stimulations of the foot, with local contractions, but requires the cerebral connection to allow digging (Bullock, 1965). The CG play roles in the anterior adductor control, in coordination of visceral and pedal actions, and it is dominant on behavioral rhythms (Bullock 1965); there can a dominance of the cerebral function (Wilkens, 2006). Along with the VG, the CG have roles in respiratory metabolism (Mane *et al*. 1990; Jadhav *et al*. 2012). An update view of the ganglia functions can be found in Gosling (2015).

As to cellularity, despite the differences between genders as to the total V of ganglia, and also the differences between the volumes of the different ganglia types irrespective of the gender, there were no major dissimilarities as to the N of neurons, glial and pigmented cells, when comparing the LCG and RCG with the PG; despite the latter being significantly bigger. By other hand, the VG consistently — i.e., in whole ganglia, cortex and medulla — showed a significantly higher N of neurons and glial cells; but not of pigments cells. The higher total N in the VG is most surely directly related with the fact that they directly/functionally control a vast area, as recently stressed by Gosling (2015), which must be based on more neural cells.

Above in the Discussion, a propos of the differences in the absolute volume of ganglia between females, viz. with the undifferentiated specimens — with females having greater volumes — we rose questions about what could structurally sustain the dissimilarities. As we did not found differences in the absolute numbers, this fact means that females must have a higher relative cellularity, or relative volume of cells per unit volume, typically represented in stereology as the N_V. By dividing the V of a ganglion (or one of its compartment) by the N of cells it contains we get an estimate of the N_V (cell, containing space). If we investigate this, in the neurons or glia in the cortex, we get a N_V in the undifferentiated (of$\approx 4.3 \times 10^5$ neurons /mm^3 and 5.5×10^5 glia cells/mm^3) that more than doubles the values of females (of $\approx 2.0\times10^5$ neurons/mm^3 and 2.5×10^5 glia cells/mm^3); with males situated in between both other gender. If we make this exercise with all neural cells, in all ganglia, we find that the undifferentiated animals have$\approx$ 2.3 more cells per unit of ganglionic volume ($\approx 11\times10^6$ cells/mm^3), when compared with either females ($\approx 4.9\times10^6$ cells/mm^3) or males ($\approx 4.7\times10^6$ cells/mm^3), that are globally quite similar. These inferences suggest that, overall, undifferentiated animals have a similar N of cells in their ganglia fitted into less volume, implying that both the neurons and glial cells are more "concentrated", and so likely smaller in size; or else the ganglia volume would not be smaller. Overall, our data strongly point that in *S. plana* there are gender/gonad stage related undisclosed differences in the mean volume of neurons and/or glial cells, and/or of their projections — a matter of countless studies in vertebrate neuroscience (Schmitz and Hof 2005) but totally "untouched" in bivalves. Thus, looking at cell sizes is worth studying in the future to better understand the cytology and physiology of the bivalve nervous system.

Still about cellularity, it is worth pointing that this is the first study in a bivalve that provides estimates of glia-to-neuron ratios, a fundamental aspect that has been hotly debated in

vertebrates and mostly for the human brain, with the once well-established 10:1 ratio being recently challenged with rigorous estimates pointing to a 1:1 ratio, supporting the conclusion that humans have an "isometrically scaled-up primate brain" (Azevedo *et al*. 2009; Hilgetag and Barbas 2009). Our global data for *S. plana* (i.e., all ganglia and gender combined) suggest a ≈ 1:1 glia-to-neuron ratio in the cortex, and when joining the medulla the ratio rises to ≈ 1:1.5. However, the exact ratio depends on the gender and on the ganglia type — either factor acting independently — with cerebral ganglia having significantly lower ratios, and females showing the highest ratio. In view of the neural supportive functions glial cells have across phylogeny, our data likely have functional effects. Speculations about the new facts would be farfetched, but it is very interesting to note that differences between sexes as to glia-to-neuron ratio were kept along evolution, up to humans, and that they may be also dependent on neural regions (Pelvig *et al*. 2008; Oliveira-Pinto *et al*. 2014). Our new findings add one more piece to the puzzle of the evolutionary origins of the glia and of their always acquired news roles (Hartline 2011), and offer "ancient roots" in line with the notion that, once there, brains gained non-neuronal cells in parallel with neuronal additions, resulting in fairly constant relative densities/ratios of non-neuronal cells (Herculano-Houzel 2011).

Regardless of the interesting agreement among our study and other recent ones as to that cell ratio, and although we used a "gold-standard "technically counting procedure, we must view our data with caution, namely because we are identifying neurons versus glial cells based only on their morphology as seen at light microscopy. Despite we made a preceding histological and ultrastructural study to get further morphological insights about *S. plana* neural cells (see Chapter 4 of this Thesis), we cannot discard the possibility that a marginal number of very small neurons could be identified/counted as glial cells; being the opposite situation much more unlikely to occur, in our opinion. The first design-based stereological study (technically harder to do at the time) that estimated neurons and glial cells in humans dealt with this caveat too (Pakkenberg and Gundersen 1988). Only more recently there are antibodies that allow an unambiguous distinction between those cells types in humans and in commonly used rodents (Herculano-Houzel and Lent 2005; Lyck *et al*. 2008). Despite a few continuous attempts to produce and/or test antibodies for disclosing neuronal subpopulations in bivalves (Croll *et al*. 1993; Mahmud *et al*. 2008; Meechonkit *et al*. 2010), the current lack of tested and accepted specific immunomarkers for neurons and glial cells in bivalves is one

more aspect that needs progress, to allow for the characterization of the types of glial cells and of their roles, providing ways for sound phenotypical anchoring. Whatever caveats we may have in identification, we stress that irrespective of our operational divisions of neurons and glial cells we got the same patterns in relation to those disclosed for the total neural cells, proposing an ongoing steadiness at least of morphological cell subtypes.

Conclusion

In summary, this is the first study that used unbiased stereology techniques to estimate the volume of the three types of nervous ganglia (including of their cortex and medulla parts) and the neuronal and non-neuronal cell number, in the whole ganglia and in every partition. We do not disclose many differences between adult maturing males and females, but these have an overall greater ganglionic volume when compared with other adults that could not be sexed because they had atrophic (exhausted) gonads – males exhibited intermediate values. So, this tells us that something connected to maturing relates somehow with the ganglia size. The numerical data suggest that cell size differences may be at the basis of the differences, because there are no significant differences in the total cellularity among the gender studied. Yet, females show a greater glia-to-neuron number ratio than males, and the undifferentiated are in between. This key ratio is significantly highest in the VG and lowest in both CG. We further show that the three types of ganglia have other fundamental differences, namely in the volume ratio of cortex versus medulla, and that the significantly greater volumes of the PG (in relation to the CG) and of the VG (in relation to all other) imply more voluminous cortex and medulla, but more neuronal and non-neuronal cells only in the VG. We disclose for the first time that a small bivalve as *S. plana* has a mean total number of neural cells that spans from over 12000 (in CG) to over 68000 (in VG). This is not only new data for malacology but makes think on how much intricate and integrative neural networks it offers to the animal, and how it relates with unsolved issues in mollusks physiology, such as related to nociceptive behavior, with currently at stake repercussions in animal welfare (Crook and Walters 2011).

Acknowledgments

The author Sukanlaya Tantiwisawaruji was supported by a Thai Government Science and Technology Scholarship. This research was partially supported by the Strategic Funding UID/Multi/04423/2013, through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the program PT2020. Further support was given by ICBAS, via its Ph.D. Program in Biomedical Sciences. We do thank Fernanda Malhão and Célia Lopes their valued histotechnical advices.

References

Ahmad, I., Coelho, J. P., Mohmood, I., Pacheco, M., Santos, M. A., Duarte, A.C. & Pereira, E. (2011) Immunosuppression in the infaunal bivalve *Scrobicularia plana* environmentally exposed to mercury and association with its accumulation. *Chemosphere* 82, 1541-1546.

Arnold, A. P. (2003) The gender of the voice within: the neural origin of sex differences in the brain. *Current Opinion in Neurobiology* 13, 759-764.

Azevedo, F. A., Carvalho, L. R., Grinberg, L. T., Farfel, J. M., Ferretti, R. E., Leite, R. E., Jacob Filho, W., Lent, R. & Herculano-Houzel, S. (2009) Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. The *Journal of Comparative Neurology* 513, 532-541.

Bullock, T.H. (1965) Mollusca: Pelecypoda and Scaphopoda. In: Bullock, T.H. & Horridge, G.A. (Eds.), *Structure and function of the nervous systems of invertebrates*. London: W. H. Freeman & Co; pp. 1387-1431.

Burgaleta, M., Head, K., Álvarez-Linera, J., Martínez, K., Escorial, S., Rchard, H. & Colom, R. (2012) Sex differences in brain volume are related to specific skills, not to general intelligence. *Intelligence* 40, 60-68.

Carroll, M. A. & Catapane, E. J. (2007) The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology - Part A: Molecular and Integrative Physiology* 148, 445-450.

Chesman, B. S. & Langston, W. J. (2006) Intersex in the clam *Scrobicularia plana*: a sign of endocrine disruption in estuaries? *Biology Letters* 2, 420-422.

Cosgrove, K. P., Mazure, C. M. & Staley, J. K. (2007) Evolving knowledge of sex differences in brain structure, function and chemistry. *Biological Psychiatry* 62, 847-855.

Croll, R. P., Nason, J. & Minnen, J. V. (1993) Characterization of central neurons in bivalves using antibodies raised against neuropeptides involved in gastropod egg-laying behavior. *Invertebrate Reproduction & Development* 24, 161-168.

Croll, P. R. & Wang, C. (2007) Review article: Possible roles of sex steroids in the control of reproduction in bivalve molluscs. *Aquaculture* 272, 76-86.

Crook, R. J. & Walters, E. T. (2011) Nociceptive behavior and physiology in molluscs: Animal welfare implications. *ILAR Journal 52*, 185-195.

Dickson, B. J. (2008) Review: Wired for Sex: The neurobiology of *Drosophila* mating decisions. *Science* 322, 904-909.

Dorph-Petersen, K. A., Nyengaard, J. R. & Gundersen, H. J. G. (2001) Tissue shrinkage and unbiased stereological estimation of particle number and size. *Journal of Microscopy* 204, 232-246.

Garcia-Segura, L. M., & Balthazart, J. (2009) Steroids and neuroprotection: new advances. *Frontiers in Neuroendocrinology* 30, 5-9.

Gagné, F. Blaise, C. Pellerin, J. & André C. (2007) Neuroendocrine disruption in clams during gametogenesis at sites under pollution stress. *Marine Environmental Research* 64, 87-107.

Gillies, G. E. & Mcarthur, S. (2010) Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. *Pharmacological reviews* 62, 155–198.

Gomes, T., Gonzalez-Rey, M. & Bebianno, M. J. (2009) Incidence of intersex in male clams *Scrobicularia plana* in the Guadiana Estuary (Portugal). *Ecotoxicology* 18, 1104-1109.

Gosling, E. (2015) *Marine Bivalve Molluscs*, 2nd ed. Jonh Wiley & Sons, Ltd. Oxford.

Gundersen, H. J. (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *Journal of Microscopy* 143, 3-45.

Gundersen, H. J. & Jensen, E. B. (1987) The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 147, 229-263.

Harrison, W. F. & Kohn, J. A. (1997) *Microscopic anatomy of invertebrates*. New York: Wiley-Liss, Inc.

Hartline, D. K. (2011) The evolutionary origins of glia. *Glia* 59, 1215-1236.

Herculano-Houzel, S. (2011) Not all brains are made the same: new views on brain scaling in evolution. *Brain, Behavior and Evolution* 78, 22-36.

Herculano-Houzel, S. & Lent, R. (2005) Isotropic fractionator: A simple, rapid method for the quantification of total cell and neuron numbers in the brain. *The Journal of Neuroscience* 25, 2518-2521.

Hilgetag, C. C. & Barbas, H. (2009) Are there ten times more glia than neurons in the brain? *Brain Structure and Function* 213, 365-366.

Jadhav, M. L., Gulave, A. & Bawane, V. (2012) Role of cerebral ganglia in regulation of oxygen consumption of freshwater bivalve mollusc, *Lamellidens marginalis* from Godavari river during summer season. *Bioscience Discovery* 3, 337-341.

Lee, K. & Portman, D. S. (2007) Neural sex modifies the function of a *C. elegans* sensory circuit. *Current Biology* 17, 1858-1863.

Leenaars, C. H., Girardia, C. E., Joosten, R. N., Lakoa, I. M., Ruimschotel, E., Hanegraafa, M. A., Dematteis, M., Feenstra, M. G. & Someren, E. J. (2013) Instrumental learning: An animal model for sleep dependent memory enhancement. *Journal of Neuroscience Methods* 217, 44-53.

Leong, K. C. & Packard, M. G. (2014) Exposure to predator odor influences the relative use of multiple memory systems: Role of basolateral amygdala. *Neurobiology of Learning and Memory* 109, 56-61.

López-Sánchez, J. A., Maeda-Martínez, A. N., Croll R. P. & Acosta-Salmón, H. (2009) Monoamine fluctuations during the reproductive cycle of the Pacific lion's paw scallop *Nodipecten subnodosus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 154, 425-428.

Lyck, L., Dalmau, I., Chemnitz, J., Finsen, B., & Schrøder, H. D. (2008) Immunohistochemical markers for quantitative studies of neurons and glia in human neocortex. *Journal of Histochemistry and Cytochemistry* 56, 201–221.

Mane, U. H., Rao, K. R., Muley, S. D. & Vedpathak, A. N. (1990) Probable role of nerve ganglia in respiration of the estuarine clam *Katelysia opima*. *Indian Journal of Comparative Animal Physiology* 8, 21- 27.

Mahmud, S., Mladenov, P. V., Sheard, P. & Chakraborty, S. C. (2008) Characterization of neurons in the visceral ganglia of the green-lipped mussel (*Perna canaliculus*) using antibodies raised against neuropeptides and neurotransmitters. *Bangladesh Journal of Animal Science* 37, 78 - 85.

Martin, K., Huggins, T., King, C., Carroll, M. A. & Catapane, E. J. (2008) The neurotoxic effects of manganese on the dopaminergic innervation of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 148, 152-159.

Matozzo, V., Gagné, F., Gabriella, M.M., Ricciardi, F. & Blaise, C. (2005) Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: A review. *Environment International* 34, 531-545.

Mayhew, T. M. & Lucocq, J. M. (2015) From gross anatomy to the nanomorphome: stereological tools provide a paradigm for advancing research in quantitative morphomics. *Journal of Anatomy* 226, 309-321.

Mayhew, T. M. (2015) Morphomics: An integral part of systems biology of the human placenta. *Placenta* 36, 329-340.

Meechonkit, P., Kovitvadhi, U., Chatchavalvanich, K., Sretarugsa, P. & Weerachatyanukul, W. (2010) Localization of serotonin in neuronal ganglia of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialata*. *Journal of Molluscan Studies* 76, 267-274.

Nelson, M., Huggins, T., Licorish, R., Carroll, M. A. & Catapane, E. J. (2010) Effects of p-Aminosalicylic acid on the neurotoxicity of manganese on the dopaminergic innervation of the cilia of the lateral cells of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 151, 264-270.

Oliveira-Pinto, A. V., Santos, R. M., Coutinho, R.A., Oliveira, L. M., Santos, G. B., Alho, A. T., Leite, R. E., Farfel, J. M., Suemoto, C. K., Grinberg, L. T., Pasqualucci, C. A., Jacob-Filho, W. & Lent, R. (2014) Sexual dimorphism in the human olfactory bulb: females have more neurons and glial cells than males. *PLoS One* 9, 1-9.

Pakkenberg, B. & Gundersen, H. J. G. (1988) Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. *Journal of Microscopy* 150, 1–20.

Pazos, A. J. & Mathieu, M. (1999) Effects of five natural gonadotropin-releasing hormones on cell suspensions of marine bivalve gonad: Stimulation of gonial DNA synthesis. *General and Comparative Endocrinology* 113, 112-120.

Pelvig, D. P., Pakkenberg, H., Stark, A. K. & Pakkenberg, B. (2008) Neocortical glial cell numbers in human brains. *Neurobiology of Aging* 29, 1754-1762.

Petridis, P., Jha, A. N. & Langston, W. J. (2009) Measurements of the genotoxic potential of (xeno-) oestrogens in the bivalve mollusc *Scrobicularia plana*, using the Comet assay. *Aquatic Toxicology* 94, 8-15.

Remacha-Trivino, A., Borsay-Horowitz, D., Dungan, C., Gual-Arnau, X., Go´mez-Leon, J., Villamil, L. & Gomez-Chiarri, M. (2008) Numerical quantification of *Perkinsus marinus* in the American oyster *Crassostrea virginica* (GMELIN, 1791) (Mollusca:Bivalvia) by modern stereology. *Journal of Parasitology* 94, 125-136.

Sahin, B., Aslan, H., Unal, B., Canan, S., Bilgic, S., Kaplan, S. & Tumkaya, L. (2001) Brain volumes of the lamb, rat and bird do not show hemispheric asymmetry: A stereological study. *Image Analysis & Stereology* 20, 9-13.

Schmitz, C. & Hof, P. R. (2005) Design-based stereology in neuroscience. *Neuroscience* 130, 813-831.

Siniscalchi, A., Cavallini, S., Sonetti, D., Sbrenna, G., Capuano, S., Barbin, L., Turolla, E. & Rossi, R. (2004) Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology* 144, 1205-1212.

Stefano, G. B. (1945) *Neurobiology of Mytilus edulis*. Great Britanin Biddles Ltd., Guildford and King's Lym.

Stefano, G. B., Zhu, W. Mantione, K. Jones, D., Salamon, E., Cho, J. J. & Cadet, P. (2003) 17-β estradiol down regulates ganglionic microglial cells via nitric oxide release: Presence of an estrogen receptor β transcript. *Neuroendocrinology Letters* 24, 130-136.

Tantiwisawaruji, S., Kovitvadhi, U., Pardal, M. A., Rocha, M. J. & Rocha, E. (2015) Qualitative and quantitative insights into the 3D-microanatomy of the nervous ganglia of *Scrobicularia plana* (Bivalvia, Tellinoidea, Semelidae). *Molluscan Research* (*article in press*).

von Bartheld, C. (2002) Counting particles in tissue sections: choices of methods and importance of calibration to minimize biases. *Histology and Histopathology* 17, 639-648.

Yan, H., Li, Q., Liu, W., Ke, Q., Yu, R. & Kong, L. (2011) Seasonal changes of oestradiol-17β and testosterone concentrations in the gonad of the razor clam *Sinonovacula constricta* (Lamarck, 1818). *Journal of Molluscan Studies* 77, 116-122.

Walløe, S., Pakkenberg B. & Fabricius, K. (2014) Stereological estimation of total cell numbers in the human cerebral and cerebellar cortex. *Frontiers in Human Neuroscience* 8, 1-9.

Wilkens, L. A. (2006) Neurobiology and behaviour of the scallop. Chapter 5. In: Shumway, S. E. & Parsons, G. J. (Eds.), *Scallops: Biology, Ecology and Aquaculture*. Elsevier, pp. 317-356.

Worrall, C. M., Widdows, J. & Lowe, D. M. (1983) Physiological ecology of three populations of the bivalve *Scrobicularia plana*. *Marine Ecology Progress Series* 12, 267-279.

CHAPTER6

IMPACTS OF AGE IN THE NERVOUS GANGLIA VOLUME AND CELLULARITY IN TWO ADULT SIZE-CLASSES OF THE BIVALVE PEPPERY FURROW SHELL (*SCROBICULARIA PLANA*)

Impacts of age in the nervous ganglia volume and cellularity in two adult size-classes of the bivalve peppery furrow shell (*Scrobicularia plana*)

[Formatted as a manuscript to be submitted for publication in an international journal. The version in this Thesis may change after the revision to be made by all prospective authors.]

SukanlayaTantiwisawaruji[a,b,c], Maria J. Rocha[a,b], Ana Silva[b], Uthaiwan Kovitvadhi[d], Maja Jordanova[e], Miguel A. Pardal[f]and Eduardo Rocha[a,b]

[a]Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Porto, Portugal.
[b]Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto (U.Porto), Porto, Portugal.
[c]King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand.
[d]Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand.
[e]Institute of Biology, Faculty of Natural Sciences and Mathematics, Ss. Cyril & MethodiusUniversity, Skopje, Macedonia.
[f]Centre for FunctionalEcology (CFE), Universityof Coimbra, Coimbra, Portugal.

Running title: Does size/age matters for neural ganglia of the peppery furrow shell?

Key words: age, bivalve, cell number, growth, glia, ganglia, neurons, stereology, volume

Correspondence to:

Eduardo Rocha
Department of Microscopy
Laboratory of Histology and Embryology
Institute of Biomedical Sciences Abel Salazar (ICBAS)
University of Porto (U.Porto)
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto
Portugal
E-mail:erocha@icbas.up.pt

Abstract

Scientists are now certain that the nervous ganglia of bivalves control many of the animal's life essential aspects, including growth and gonadal maturation and spawning-related events. Basic questions on the structure and function of the bivalve nervous system remain unanswered, such as eventual intrinsic influences and changes related with development and age and also gender. Within the scope of the structural characterization we are conducting on *Scrobicularia plana*, we start studying interganglionic and sex-related differences, and herein we propose to expand our investigation scope by looking at the size of the ganglia and neural cellularity with age. In view of the generally higher plasticity of the invertebrate nervous system, we hypothesise that if the adult animal continues to grow its ganglia could continue to develop too, increasing for example its cellularity, *i.e.*, getting more neural elements, particularly neurons and glial cells. Twenty four adult specimens of *S. plana*, with well-defined gonads, sampled in the estuary of the Mondego River estuary, were used; six animals per size-class and per gender. Considering that size is a proxy of age, the animals were split into two-size classes, that we named "Small" (age 2+ years) and "Big" (age 3+ years). Formalin-fixed and paraffin-embedded animals were cut into 35 μm thick sections that were stained in hematoxylin-eosin and used for stereological analyses. These estimated total volumes of ganglia, their cortex and medulla relative volumes, and total number of earlier characterised neural cells: neurons, glial cells and pigmented cells. In animals not differing in size/length and mass, we found interganglionic, sex-related and size-related significant effects upon the ganglionic volumes, relative volumes of cortex and medulla, and total numbers of both neurons, glial cells, and pigmented cells. Under multi-way analysis of variance, the effect of size (age) was consistently marked, and statistically significant, with the older specimens having approximately twice as bigger ganglia (irrespective of its type and of sex), that contained significantly more neural cells of all types. The increase in cellularity took place in each whole ganglion, and in both cortex and medulla. Data support our hypothesis that neurogenesis continues to occur in adult *S. plana*, irrespective of the animals' gender. New questions arise from our results, namely about the nature and sources of neural cell progenitors. In line with recent interest of researchers as to the utility of bivalves as models in neurosciences, we think that *S. plana* can also be a stimulating model for neurogenesis and age-related studies.

Introduction

Aging is a key event for living organisms, and typically entails continuous changes along life, from birth to maturity and then after by gradual declining towards senescence (Campisi and d'Adda di Fagagna 2007), which can ultimately lead to critical deterioration followed by death — considering the wide range of physiological mechanisms and outcomes, a plethora of deleterious changes typically occur in neural activities with the passage of time (Tardy 2003; Betts *et al*. 2005). Mainly because of the frequent health problems related with Human aging, mammals have been particularly studied in what concerns the profusion of nervous system changes with aging. Such studies include innumerous morphological evaluations, including the use of quantitative approaches (such as stereological techniques), mostly made in rats, mice, monkeys and humans (*e.g.*, Samorajski and Rolsten 1973; Monteiro *et al.* 1991; Henrique *et al*. 2001). Research on neuroscience of molluscs has been mainly made and increased based both on gastropods (Croll *et al.* 1993; Franchinf*et al.* 1985; Janse *et al.* 1996; Simmons and Young 1999; Torrska *et al.* 1968) and cephalopods (Amano *et al.* 2008; Di Cristo 2013; Takuwa-Kuroda *et al.* 2003). Yet, there are a few studies with bivalves, such as one with *Mytilus edulis* showing that aging is linked to a decline of dopamine-stimulated adenylate cyclase activity in the pedal ganglion (Stefano 1982), and alter the monoamine accumulation in the neuronal tissue (Burrell and Stefano 1983). So, aging makes a difference in what respects the nervous system of bivalves, and therefore these organisms can be much better understood in that respect, with potential gains for the overall understanding of aging. Indeed, it is recognized by research community that invertebrates have been very important to get new insights about the physiology of aging, working both with emerging and established models for the study of human aging (Yeoman and Faragher 2001; Yeoman *et al.* 2012)

Despite the research potential of molluscs, and bivalves in particular, for studying the nervous system aging and plasticity, there are practical problems too. Particularly, there are difficulties for estimating the bivalve's exact age when using incremental changes in growth lines or annual marks on the shell (Gosling 2004); even so, increases in shell size are well correlated with age. For instance, in the genus *Prototheca* animals reached a mean length of 3.7 cm at the age of approximately 3.5-4 years, in Columbia (Shaw 1986), and in the genus *Scrobicularia* it was in the range of 2.2 cm at the age of about 1.4 year, in Bidasoa estuary (Sola 1997). On the side of advantages, it should be stressed that bivalves display a wide range of lifespans, from 1 to awesome ± 400 years as reviewed by Abele *et al.* (2009), which

makes them potentially prone to tackle quite different questions. To this aspect we must add the fact that the bivalves nervous system has a perceived (although not yet quantified) low number of neural cells, making them very attractive organisms for studying neural networks (Kotsyuba and Kotsyuba 2002). Despite relevancy and opportunities, rather few studies were conducted in the bivalves' nervous system, and virtually none concerning its quantitative morphology.

Irrespective of details, it is well known that the basic types of bivalve ganglia (cerebral, pedal, and visceral) contain neurons that are critical for controlling the portfolio of fundamental responses needed for the individual, and ultimately for the species, to survive, *viz*. reproduction (Khotimchenko 1991), feeding (Margaret *et al.* 2007), movement (Hodgson and Trueman 1981), and cardiac functions (Kodirov 2011). It is also evident that the ganglia cortex (the ganglionic external region) includes the vast majority of neural cells (Stefano *et al.* 1990). Both the neurogenesis and neuroplasticity along life are still poorly studied topics in bivalves, and the few existing publications are devoted not so much to what happens in the adults but focus instead the very earlier maturing stages (Flyachinskaya 2000; Raineri 2009). In true, adult neurogenesis (including brain regeneration) has been neglected, contrarily to the great attention that has been paid to this issue in invertebrate and vertebrate animal models (Chen *et al*. 2013; Kizil *et al*. 2012). In view of caveats, Voronezhskaya *et al.* (2008) emphasized that in spite of "understanding of neuronal development in Trochozoa has progressed substantially in recent years, relatively little attention has been paid to the bivalve molluscs in this regard".

In a previous work we devoted our attention to look after the size and cellularity of the nervous ganglia of the peppery furrow shell (*Scrobicularia plana*), quantitatively detailing and comparing each ganglia type in maturing males and females, and in animals that did not have a differentiated/maturing gonad to allow sexing them. Herein, we continue those new research efforts, by advancing the hypothesis that not only the sex but also the age factor, in adults, may influence the microscopic anatomy of the nervous system of this species; particularly the cellularity of the ganglia, with eventual implications for the size (volume) of the ganglia. Our rationale relies of the fact that neural plasticity existsin the nervous systems of invertebrates and vertebrates, both along development and in adults (Moffet 1996; Kizil *et al*. 2012; Ashton 2013; Chen *et al*. 2013). The quest for knowing if adults loose (or if gain)

neural cells along life has been a greater focus of attention to neuroscientists, and even the once reputable views of neurogenesis in humans have been continuously overturned (Curtis *et al*. 2011). As most bivalves grow continuously, perhaps *S. plana* and others can generate new neural cells as juveniles and during adult life; at least before senescence. To start studying our questions, we did a stereological study on ganglia of two size-cohorts of adult maturing males and females, knowing that the body size in bivalves correlates well with longevity (Ridgway *et al.* 2011).

Materials and methods

Animals and histological procedures

Wild adult peppery furrow shell (*S. plana*) were collected in the Mondego River estuary, in Portugal, in April. After capture, the animals were transferred to in house facilities in the same day, and maintained in glass aquaria (10 litres), with aerated seawater (salinity 30 psu), and at 15^{o}C of water temperature. The animals' length (L), width (W), and height (H) were measured, and two size-classes were created and designated for the sake of simplicity and readability as: a) "Small", measuring 2.4 (1.3), 0.7 (0.4), and 1.8 (0.9) cm, respectively for L, W, and H; and b) "Big", 3.8 (1.7), 1.2 (0.8), and 2.9 (1.8) cm — data given as mean (variation coefficient) (CV). In the next day, sampled animals were anesthetized by immersion in a seawater solution of magnesium chloride (6%), and kept at room temperature (≈ 20ºC).

Each arbitrarily sampled animal used for this study — and later identified, by histology, as 6 males and 6 females, per size-class, summing a total of 24 specimens — were removed carefully from the shell and then were fixed *in toto* for 24 hours, using 10% buffered formalin, at room temperature. After fixation, the samples were washed in 70% ethanol, then dehydrated with increasing concentrations of that alcohol (70% to 100%), cleared in xylene, and infiltrated with paraffin. Dehydration to infiltration was carried out using an automatic tissue processor (Leica TP1020, Germany). Paraffin embedding used a station (Leica EG 1140H, Germany).

Each animal was cut into serial sections (35µm in mean thickness), on a motorized rotary microtome (Leica RM2155, Germany), and kept onto 3-aminopropyltriethoxysilanecoated slides before hematein-eosin staining, xylene clearing, and DPX mounting. Sections having neural ganglia were used for stereology (other were occasionally used for sexing the animal). The left cerebral (LCG), right cerebral (RCG), pedal (PG), and visceral ganglia (VG) were all targets of study. Their presence was confirmed at light microscopy, using an Olympus BX50.

Stereological analyses

The Cavalieri's principle was used for estimating the volume (V) of each ganglion (and separately of its cortex and medulla), based on the formula: $V = \cdot \sum A$, where t is the mean distance between analysed section planes, and A the sectional area of the target of interest

(Gundersen and Jensen 1978). The volume of the ganglia was determined semi-automatically, using the stereological workstation CAST-Grid (version 1.5, Olympus), running in a light microscope Olympus BX50, equipped with a microcator (Heidenhain MT-12), a motorized stage with 1 µm X-Y movement accuracy (Prior), and a CCD video camera (Sony) displaying live image in a 17'' monitor (Sony). Analyses were done under a x10 objective lens. For each ganglion in an animal, the areas of the cortex and medulla were registered in every section the ganglion appeared, so to later apply the above cited formula. The *t* for a ganglion was confirmed by measuring the section thickness with the microcator (see below). The final total volumes were used to estimate the volume densities (V_V) of cortex and medulla in the ganglion: V_V (medulla or cortex) = V (medulla or cortex) ÷ V (ganglion).

The total number (N) of cells within each nervous ganglia was estimated via the optical disector-fractionator combination (Gunderson 1986), making use of the general formula:

$$N = Q \cdot (1 \div ssf) \cdot (1 \div asf) \cdot (1 \div hsf),$$

where Q refers to the total number of cells actually counted in all the optical disectors; hsf is the height sampling fraction, captures the ratio of the section thickness that was screened; asf is the area sampling fraction, ie, the ratio between the area of the counting frame and the area covered by each X-Y movement; ssf is the section sampling fraction, ie, the fraction of total sections sampled. Herein, half of total sections of each ganglia were sampled and a minimum of 100 neurons and 100 glia cells were counted per ganglia. The procedure was also enforced semi-automatically, with the above stereological workstation. The counts were made under the x100 (NA=1.35) oil immersion lens, in systematically sampled fields. To check and account for any eventual non-uniform deformation, t was measured in every field, and, as we did not notice such deformation, the averaged t was used for hsf = h/t (Dorph-Petersen *et al*. 2001).

As to cellularity, data is given in various forms, including splitting the total numbers in number per type of cellular contingent, defined by morphology, *viz*. large and small neurons, fusiform, roundish, and triangularly shaped glial cells, and, finally, pigmented (neural) cells.

Statistical analysis

The statistical analyses were performed using the software STATISTICA (version 12.0. StatSoft Inc.). Data sets were checked for normality and homogeneity of variances prior to make a two-way analysis of variance (two-way ANOVA). After each significant ANOVA multiple comparisons were made using simultaneously the Tukey' and Newman-Keuls' test. In some cases, logarithmic and square root transformations were carried out for normalizing and/or homogenization of variances of the raw data. When transformation was unsuccessful, a non-parametric Kruskal–Wallis ANOVA was used, followed by Mann-Whitney U tests for pairs, with a sequential Bonferroni correction. The significance level was set at the usual 5%.

Results

Body morphometry

Table 1 presents the morphometric data of the animals according to size (operationally divided into small and big) and gender. The ANOVA shows that within a size class there are no statistically significant differences in any of the parameters (no gender effect), but that the effect of size class was significant for all parameters, with greater values in bigger animals.

Table 1. Body morphometry of *S. plana* used in the study, according with gender and size class.

Size	Gender	Length (cm)	Height (cm)	Width (cm)	Fresh mass (g)	Total mass (g)
Small	Males	2.4 (0.06)	1.8 (0.06)	0.7 (0.04)	0.57 (0.20)	1.13 (0.19)
	Females	2.4 (0.05)	1.8 (0.05)	0.6 (0.06)	0.51 (0.21)	1.02 (0.16)
Big	Males	3.8 (0.04)	2.9 (0.07)	1.1 (0.06)	2.12 (0.20)	5.25 (0.23)
	Females	3.8 (0.05)	3.0 (0.05)	1.2 (0.06)	2.03 (0.20)	5.48 (0.27)

Six animals per gender in each size class. Data given as mean (coefficient of variation). For all the parameters, the Big animals have significantly greater mean values (Tukey's test, $p < 0.001$, irrespective of the parameter).

Total volumes of ganglia

The absolute volumes (V) of the three ganglia types are given in Table 2 and Figures 1 and 2. From Table 2 we sense that the pattern point for greater values in bigger animals, which also tend to have a higher variability (their CVs are all higher against the corresponding ones in the smaller specimens). The V tends to increase anterior-posteriorly, *i.e.*, from the CB to the VG, which is evidently several times larger than the other. In agreement with to the descriptive trends, the MANOVA reveals highly significantly independent effects for the factors size ($p < 0.001$), ganglia type ($p < 0.001$), and for gender ($p = 0.044$, a significant but marginal effect).

Table 2.Total volumes (μm^3) of the cerebral, pedal and visceral ganglia, as per size-class and gender.

	Small		Big	
Ganglia	**Males**	**Females**	**Males**	**Females**
LCG	19.6×10^6 (0.24)	15.4×10^6 (0.16)	46.8×10^6 (0.32)	40.9×10^6 (0.39)
RCG	17.6×10^6 (0.19)	17.3×10^6 (0.10)	48.1×10^6 (0.28)	35.1×10^6 (0.53)
PG	31.5×10^6 (0.19)	32.7×10^6 (0.12)	63.4×10^6 (0.28)	59.4×10^6 (0.21)
VG	90.6×10^6 (0.15)	78.5×10^6 (0.16)	172.0×10^6 (0.29)	177.7×10^6 (0.23)

Six animals per gender in each size class. Data given as mean (coefficient of variation).

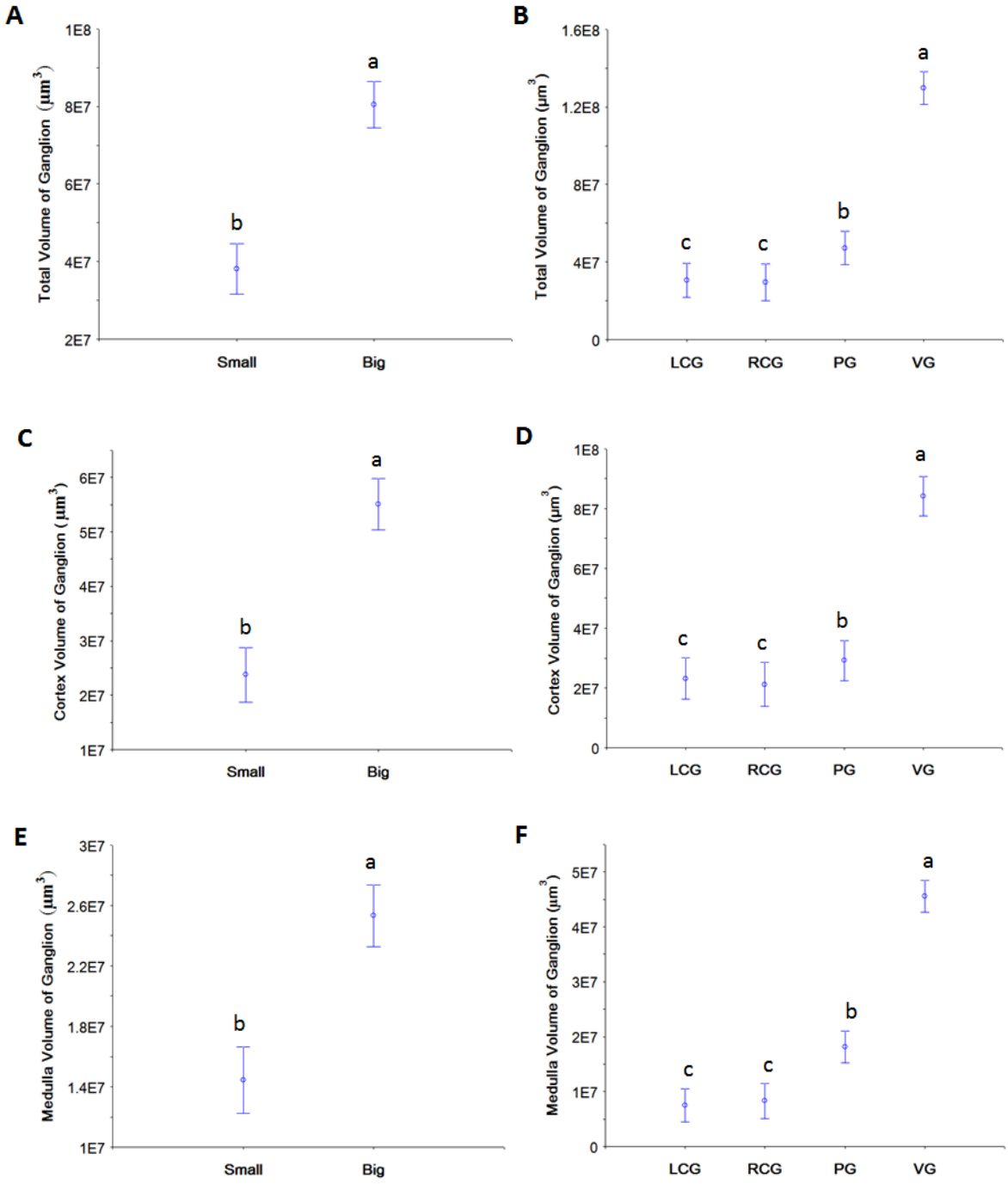

Figure 1. Volumes of the nervous ganglia and of their respective cortexes and medullae in *S. plana*. **A**) Whole ganglia, per the defined size classes Small and Big. **B**) Whole ganglia, per ganglia type. **C**) Cortex, per size classes. **D**) Whole ganglia, per ganglia type. **E**) Medulla, per size classes. **F**) Medulla, per ganglia type. Different letters mean significant differences. Data given as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

The Figure 1 illustrates the data grouped according to the independent effects of size and ganglia type. As to whole ganglia V, overall the bigger animals have definitely the highest mean, and, regardless of the animals' size or sex, while the VG is the most voluminous of all

ganglia, the PG is significantly greater than both LCG and RCG, and these do not differ (Fig. 1A, 1B). When splitting the whole V into cortex and medulla, both compartments follow similar significant trends than those for whole ganglia (Figs. 1C-E). Globally, *i.e.*, considering all animals, irrespective of size and ganglia type, males tend to have a significantly lesser V, both of the entire ganglia ($p = 0.044$) and the cortex ($p = 0.023$) (Fig. 2); but not for medulla.

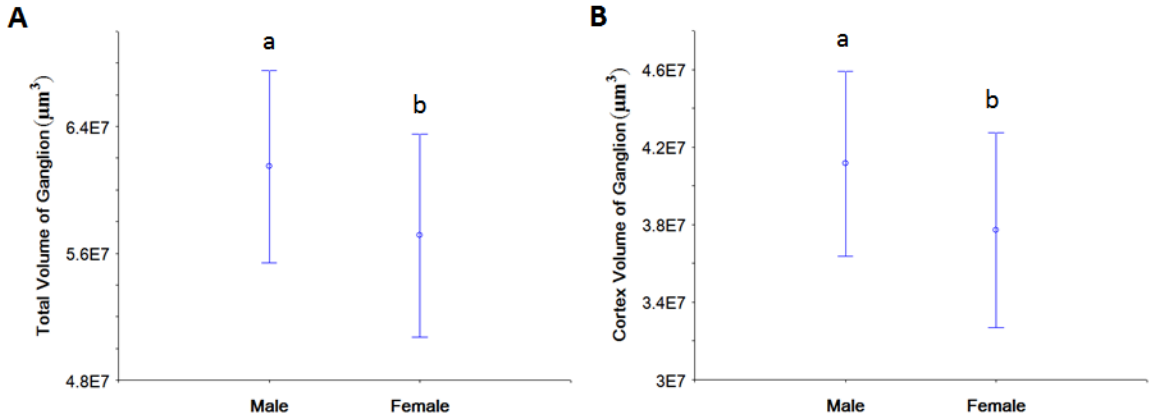

Figure 2. Volumes of the nervous ganglia and of their cortexes in *S. plana*. **A**) Whole ganglion per gender. **B**) Cortex per gender. Different letters mean significant differences. Data given as mean and 95% confidence interval.

Relative volumes of cortex and medulla

Tables 3 and 4 present data for the V_V (cortex, ganglion) and V_V (medulla, ganglion), respectively. The cortex always occupies more than half the ganglionic volume, and variability is overall low (many of the CVs are below 10%) to moderate (higher CVs seen in the bigger animals). The descriptive data is suggestive that V_V of the cortex tend to decrease from the CG towards VG; also, specimens sized as Big seem to have that consistently higher mean values.

Significant differences between size-class animals were observed — males and females being equal — with the V_V (cortex, ganglion) of bigger specimens being significantly greater ($p<0.001$) than that of smaller ones (Fig. 3A). In accord, the V_V (medulla, ganglion) is smaller ($p<0.001$) in bigger animals (Fig. 3C). When considering the data per ganglia type (Figs. 3B, 3C), there are no differences between genders but there are significant effects for ganglia type ($p<0.001$), with the V_V (cortex, ganglion) decreasing anterior-posteriorly, from the cerebral ganglia (that do not differ bilaterally) towards the visceral ganglion; the V_V (medulla, ganglion) follows a significant reverse pattern ($p<0.001$), increasing the % towards the visceral ganglia.

Table 3. Relative volumes (%) of the cerebral, pedal and visceral ganglia cortex of *S. plana*.

	Small		Big	
Ganglia	**Males**	**Females**	**Males**	**Females**
LCG	73 (0.04)	70 (0.08)	77 (0.05)	77 (0.07)
RCG	68 (0.05)	67 (0.03)	75 (0.05)	72 (0.08)
PG	59 (0.08)	56 (0.07)	63 (0.03)	65 (0.07)
VG	63 (0.09)	58 (0.07)	66 (0.06)	66 (0.11)

Six animals per gender in each size class. Data given as mean (coefficient of variation).

Table 4. Relative volumes (%) of the cerebral, pedal and visceral ganglia medulla of *S. plana*.

	Small		Big	
Ganglia	**Males**	**Females**	**Males**	**Females**
LCG	27 (0.11)	30 (0.18)	23 (0.18)	23 (0.23)
RCG	32 (0.11)	33 (0.06)	25 (0.16)	28 (0.21)
PG	41 (0.11)	44 (0.09)	37 (0.06)	35 (0.13)
VG	37 (0.16)	42 (0.09)	34 (0.12)	34 (0.22)

Six animals per gender in each size class. Data given as mean (coefficient of variation).

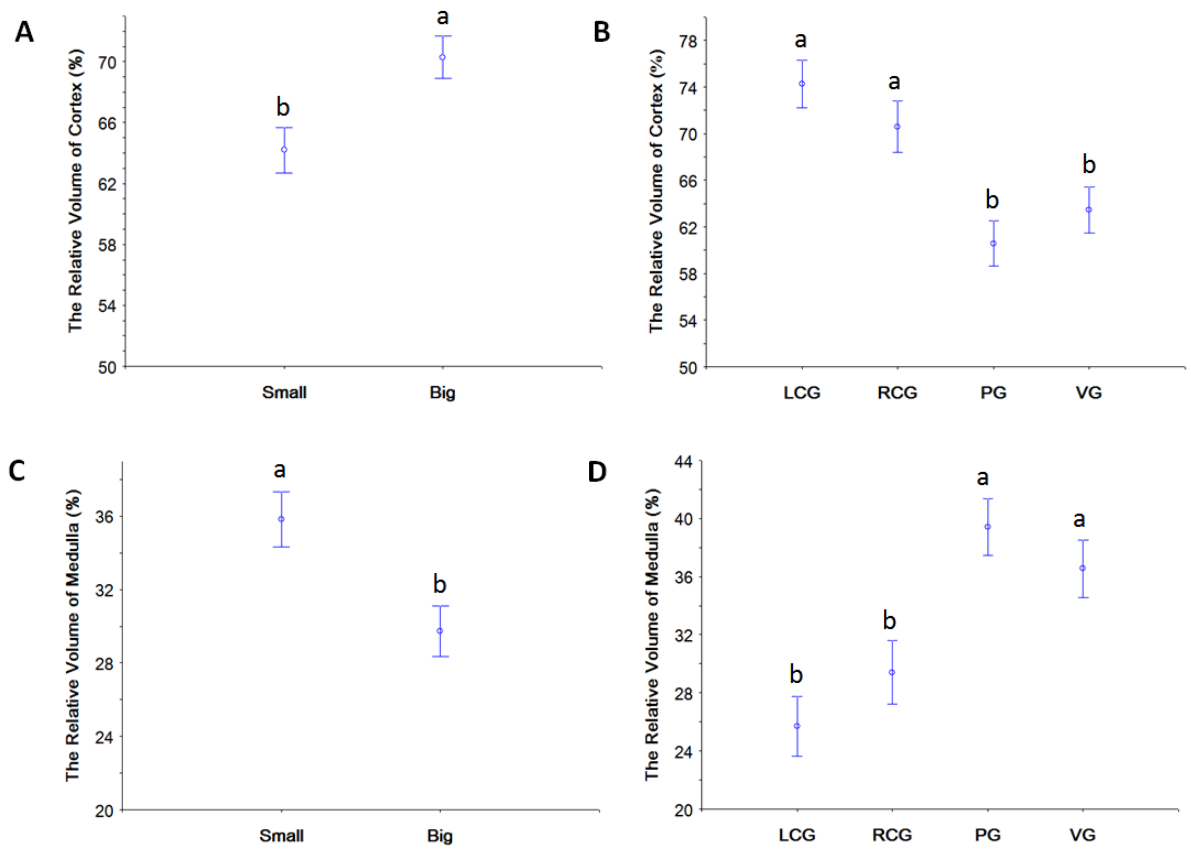

Figure 3. Relative volumes (%) of the cerebral, pedal and visceral ganglia cortex and medulla of *S. plana*: **A**) V_V (cortex, ganglion), accordingly to size class and **B**) accordingly to ganglia type. **C**) V_V (medulla, ganglion), accordingly to size class and **D**) accordingly to ganglia type. Different letters represent significant differences. Data given as mean and 95% confidence interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

Total number (N) of neurons, glial cells, and pigmented cells per ganglion

Three types of cells (neurons, glial cells and pigmented cell) are identified in the ganglia of *S. plana*. The most numerous are neurons and glial cells (combined they make ≈ 94 % of the neural cell population), with pigmented cells representing ≈ 6 %.

Tables 5 and 6 display the N of ganglionic cells, represented by the two types of neurons (small, large), glial cells (separated by phenotypes: fusiform, roundish and triangular) and the pigmented cells. For better readability, data sets are organized per body size-class, gender and type of ganglia (cerebral, pedal, and visceral). To better appreciate the significant differences, graphical outputs are additionally provided (Figs. 4, 5). The statistics revealed significant effects for size, gender and ganglion type, but with no significant interactions between factors; there was one exception, mean N of roundish glial cells (size *vs*. gender) that was not valorised.

As to the mean N of neurons per ganglion, the bigger specimens had almost the double of cells ($p<0.001$) (Fig. 4A). The neuronal mean N was almost three times greater in the visceral ganglia when compared with all the other ($p<0.001$) (Fig. 4B). Finally, the mean neuronal N altogether was significantly higher in males than in females ($p = 0.013$); however, it is worth noticing that inter-gender differences were not disclosed when analysing only larger neurons.

As to the mean N of glial cells, the patterns followed those of neurons (Figs. 4D-F). On average, bigger animals had more cells ($p<0.001$), which were more numerous in the visceral ganglia ($p<0.001$), and with males having more numbers of those cells than females ($p<0.001$).

As for the average N of pigmented cells (Figs. 5A, 5B), there were significant effects for size-class ($p<0.001$), and type of ganglia ($p<0.001$). The bigger animals showed ≈ 9 times more cells than the smaller ones, and the visceral ganglia presented about twice the average N when compared with all other. No significant differences existed between genders ($p = 0.07$).

Total number (N) of neurons, glial cells, and pigmented cells per ganglionic regions

In addition to the above analyses, we organized the data sets considering the location of the cells. Accordingly, the neural cells considered were separated as elements in the ganglia cortex (Tables 7, 8, and Figs.6, 7) and in the medulla (Tables 9, 10, and Fig. 8).

Patterns found in cortex (Figs. 6, 7) were generally in line with those in ganglia as a whole (*i.e.*, considering cortex and medulla all together), with ANOVA unveiling significant effects of factors body size ($p<0.001$), ganglia type ($p<0.001$) and gender ($p = 0.025$), for neural cells, effects of factors body size ($p<0.014$) and ganglia type ($p<0.001$), for glial cells, and at last the effects of body size ($p<0.001$) and ganglia type ($p=0.003$) for pigmented cells.

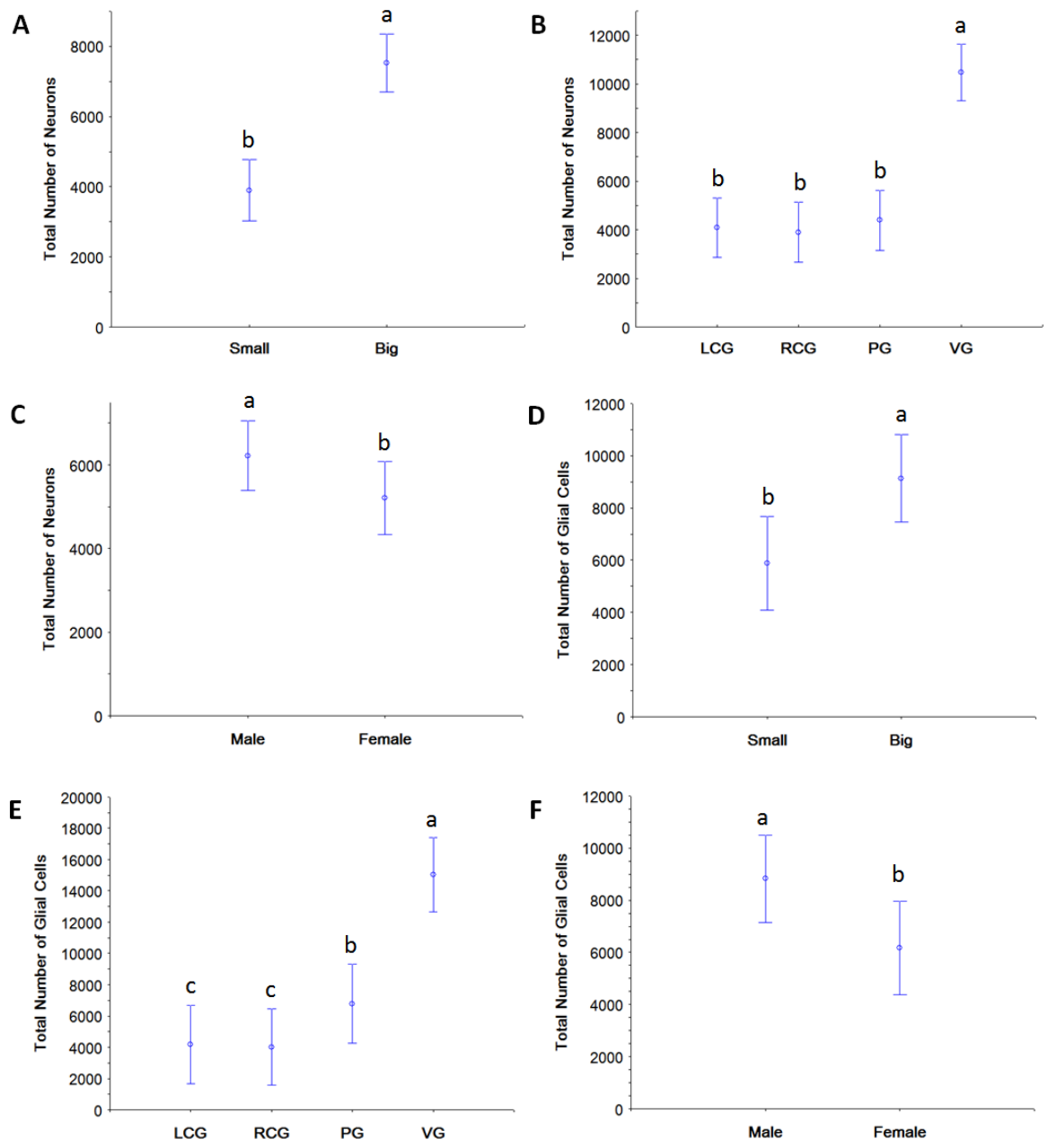

Figure 4.Total mean number of neurons and glial cells in the ganglia of *S. plana*.**A**) Neurons per size class, **B**) per ganglion, and **C**) per gender. **D**) Glial cells per size class, **E**) per ganglion, and **F**) per gender. Different letters signify significant differences. Data given as mean and 95% confidential interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

When considering only those cells that were located in medulla, variability of data sets is overall much higher compared with that in cortex; the coefficients of variance are much higher in Tables 9 and 10 when compared with those appearing is Tables 5-8. The statistically significant effects were restricted to glial and pigmented cells (Fig. 8). As to glial cells, patterns found in cortex match those in whole ganglia, with bigger animals having more cells ($p<0.001$), visceral ganglia having more cells ($p<0.001$), and males having more cells ($p=0.034$). As to the pigmented cells (Fig. 8D), the body size effect is illustrated by the greater values seen in bigger animals ($p<0.001$).

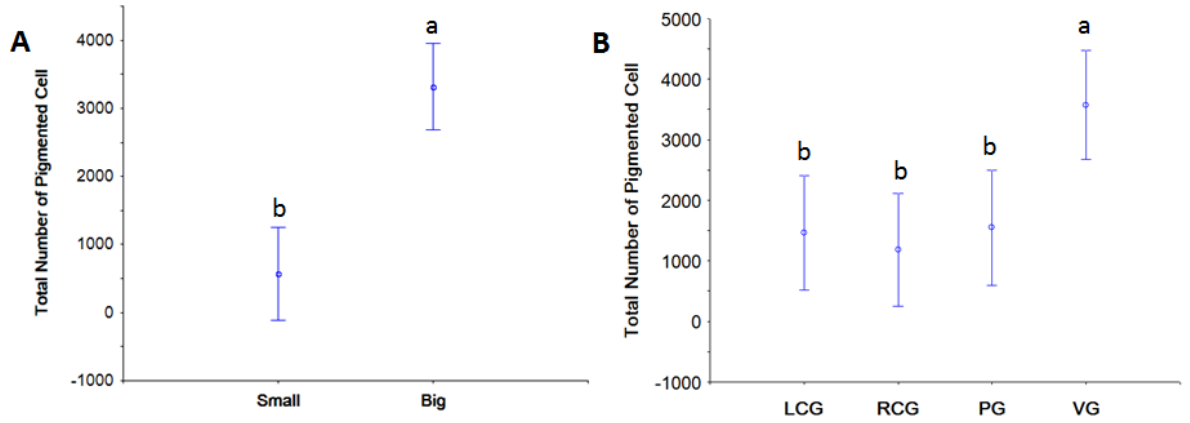

Figure 5.Total mean number of pigmented cells in the ganglia of *S. plana*. **A**) Cells per size class and **B**) per ganglion. Different letters mean significant differences. Data given as mean and 95% confidential interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

Table 5. Total mean number (N) of neural cells in body size class Small, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Small	LCG	**Males**	1381 (0.2)	1640 (0.2)	**3021 (0.2)**	551 (0.7)	1539 (0.4)	1722 (0.1)	**3812 (0.2)**	**482 (0.8)**
		Females	965 (0.4)	1356 (0.2)	**2321 (0.2)**	509 (0.5)	1022 (0.3)	1065 (0.4)	**2596 (0.3)**	**245 (0.9)**
		Mean	1173 (0.3)	1498 (0.2)	**2671 (0.2)**	530 (0.5)	1281 (0.4)	1393 (0.3)	**3204 (0.3)**	**364 (0.9)**
	RCG	**Males**	1046 (0.2)	1532 (0.3)	**2578 (0.2)**	554 (0.1)	1193 (0.3)	1234 (0.6)	**2981 (0.3)**	**303 (0.8)**
		Females	1062 (0.2)	1512 (0.1)	**2574 (0.1)**	325 (0.4)	1094 (0.6)	1276 (0.4)	**2155 (0.4)**	**312 (0.5)**
		Mean	1052 (0.2)	1524 (0.3)	**2576 (0.1)**	463 (0.3)	1153 (0.4)	1251 (0.5)	**2606 (0.4)**	**307 (0.7)**
	PG	**Males**	1769 (0.4)	2004 (0.4)	**3773 (0.3)**	749 (0.2)	3375 (0.2)	2477 (0.4)	**6601 (0.2)**	**497 (0.6)**
		Females	1606 (0.3)	1680 (0.1)	**3286 (0.2)**	727 (0.5)	3021 (0.3)	2056 (0.2)	**5804 (0.2)**	**621 (0.9)**
		Mean	1704 (0.3)	1874 (0.3)	**3578 (0.3)**	740 (0.3)	3233 (0.2)	2309 (0.3)	**6282 (0.2)**	**547 (0.7)**
	VG	**Males**	3155 (0.3)	3549 (0.3)	**6704 (0.3)**	1637 (0.3)	6136 (0.4)	4896 (0.5)	**12668 (0.3)**	**1096 (0.9)**
		Females	3021 (0.3)	3979 (0.3)	**6999 (0.3)**	1930 (0.4)	4074 (0.1)	3835 (0.4)	**9839 (0.2)**	**587 (0.6)**
		Mean	3094 (0.3)	3744 (0.3)	**6838 (0.3)**	1770 (0.3)	5199 (0.3)	4414 (0.5)	**11382 (0.3)**	**1053 (0.8)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 6. Total mean number (N) of neural cells in body size class Big, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Big	LCG	Males	2368 (0.3)	4071 (0.3)	**6439 (0.2)**	1395 (0.8)	2601 (0.1)	2300 (0.4)	**6297 (0.2)**	**3101 (0.7)**
		Females	1442(0.5)	3341 (0.4)	**4783(0.4)**	861 (1.0)	1050 (0.6)	2102 (0.7)	**4013 (0.4)**	**2039 (0.5)**
		Mean	1863 (0.4)	3673 (0.3)	**5536 (0.3)**	1104 (0.8)	1755 (0.5)	2192 (0.5)	**5051 (0.5)**	**2522 (0.6)**
	RCG	Males	2196 (0.3)	3715 (0.3)	**5911 (0.2)**	1606 (0.7)	2329 (0.3)	2461 (0.7)	**6396 (0.4)**	**2381 (0.5)**
		Females	2164 (0.4)	2543 (0.3)	**4707 (0.3)**	728 (0.6)	1113 (0.4)	2188 (0.5)	**4029 (0.4)**	**1738 (0.9)**
		Mean	2169 (0.4)	3129 (0.3)	**5279 (0.3)**	1167 (0.9)	1721 (0.6)	2325 (0.6)	**5213 (0.6)**	**2060 (0.5)**
	PG	Males	3451 (0.4)	2451 (0.4)	**5902 (0.4)**	1048(0.6)	4105 (0.3)	3421 (0.4)	**8574 (0.3)**	**2340 (0.4)**
		Females	2113 (0.2)	2494 (0.3)	**4607 (0.2)**	560 (0.7)	2506 (0.3)	3120 (0.5)	**6186 (0.3)**	**2735 (0.3)**
		Mean	2843 (0.4)	2471 (0.4)	**5314 (0.3)**	826 (0.7)	3379 (0.4)	3284 (0.4)	**7489 (0.3)**	**2519 (0.3)**
	VG	Males	6528 (0.3)	6781 (0.3)	**13309 (0.3)**	3501 (0.7)	7760 (0.5)	7846 (0.6)	**19107 (0.5)**	**5422 (0.4)**
		Females	5873 (0.3)	7358 (0.3)	**13231 (0.3)**	2743 (0.8)	5891 (0.4)	6505 (0.8)	**15138 (0.6)**	**5321 (1.1)**
		Mean	6200 (0.3)	7070 (0.3)	**13270 (0.3)**	3122 (0.7)	6825 (0.5)	7175 (0.7)	**17122 (0.5)**	**5371 (0.8)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 7. Total mean number (N) of cortical neural cells in body size class Small, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Small	LCG	Males	1329 (0.2)	1611 (0.2)	**2940 (0.4)**	400 (0.7)	985 (0.3)	1250 (0.2)	**2635 (0.2)**	**342 (0.6)**
		Females	862 (0.3)	1304 (0.3)	**2166 (0.2)**	361 (0.5)	828 (0.3)	770 (0.4)	**1959 (0.3)**	**194 (1.1)**
		Mean	1096 (0.3)	1457 (0.3)	**2553 (0.3)**	380 (0.6)	907 (0.3)	1010 (0.4)	**2297 (0.3)**	**268 (0.8)**
	RCG	Males	1018 (0.2)	1494 (0.3)	**2512 (0.2)**	423 (0.3)	920 (0.3)	894 (0.6)	**2237 (0.2)**	**257 (0.9)**
		Females	1047 (0.1)	1462 (0.1)	**2509 (0.1)**	188 (0.8)	798 (0.6)	897 (0.4)	**1883 (0.5)**	**218 (0.4)**
		Mean	1030 (0.2)	1481 (0.2)	**2511 (0.2)**	329 (0.5)	871 (0.4)	895 (0.5)	**2095 (0.3)**	**241 (0.7)**
	PG	Males	1673 (0.4)	1940 (0.3)	**3613 (0.3)**	575 (0.2)	2887 (0.3)	1836 (0.4)	**5298 (0.2)**	**383 (0.5)**
		Females	1492 (0.2)	1645 (0.1)	**3136 (0.2)**	587 (0.6)	2419 (0.3)	1571 (0.3)	**4577 (0.2)**	**424 (1.0)**
		Mean	1601 (0.3)	1822 (0.3)	**3423 (0.3)**	580 (0.4)	2700 (0.3)	1730 (0.3)	**5009 (0.2)**	**400 (0.7)**
	VG	Males	3052 (0.3)	3501 (0.3)	**6553 (0.3)**	1034 (0.3)	4684 (0.3)	2979 (0.5)	**9697 (0.3)**	**850 (0.9)**
		Females	2933(0.3)	3902 (0.3)	**6835 (0.3)**	1282 (0.5)	3282 (0.2)	2688 (0.4)	**7252 (0.2)**	**707 (0.5)**
		Mean	2998 (0.3)	3683 (0.3)	**6681 (0.3)**	1147 (0.4)	4047 (0.3)	2847 (0.4)	**8041 (0.4)**	**785 (0.8)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 8. Total mean number (N) of cortical neural cells in body size class Big, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Big	LCG	Males	2251 (0.3)	4000 (0.3)	**6251(0.2)**	1071 (0.6)	1986 (0.2)	1476 (0.3)	**4533 (0.4)**	**2807 (0.7)**
		Females	1087 (0.6)	3221 (0.6)	**4308 (0.5)**	606 (0.9)	892 (0.6)	1647 (0.6)	**3145 (0.6)**	**1596 (0.4)**
		Mean	1616 (0.5)	3575 (0.4)	**5191 (0.3)**	818 (0.8)	1389 (0.5)	1569 (0.5)	**3776 (0.5)**	**2147 (0.7)**
	RCG	Males	2117 (0.3)	3676 (0.2)	**5793 (0.2)**	1116 (0.7)	1842 (0.3)	1735 (0.6)	**4693 (0.4)**	**1870 (0.5)**
		Females	2104 (0.4)	2496 (0.3)	**4600 (0.3)**	533 (0.7)	779 (0.5)	1484 (0.5)	**2796 (0.4)**	**1348 (0.8)**
		Mean	2110 (0.4)	3086 (0.3)	**5196 (0.3)**	825 (0.8)	1311 (0.6)	1609 (0.6)	**3745 (0.4)**	**1609 (0.5)**
	PG	Males	3350 (0.4)	2396 (0.5)	**5746 (0.4)**	642 (0.6)	3076 (0.3)	2276 (0.4)	**5994 (0.2)**	**1848 (0.4)**
		Females	2079 (0.2)	2438 (0.3)	**4517 (0.2)**	379 (0.5)	1958 (0.3)	2399 (0.6)	**4736 (0.4)**	**2214 (0.2)**
		Mean	2772 (0.4)	2415 (0.4)	**5187 (0.3)**	522 (0.6)	2568 (0.4)	2332 (0.5)	**5422 (0.3)**	**2014 (0.3)**
	VG	Males	6143 (0.2)	6361 (0.3)	**12505 (0.3)**	2184 (0.5)	5601 (0.3)	4864 (0.6)	**12649 (0.4)**	**3861 (0.2)**
		Females	5521 (0.3)	7216 (0.3)	**12738 (0.3)**	1776 (0.8)	4868 (0.4)	2012 (0.7)	**8656 (0.5)**	**3984 (1.0)**
		Mean	5832 (0.3)	6789 (0.3)	**12621 (0.3)**	1980 (0.6)	5234 (0.3)	3438 (0.8)	**10652 (0.5)**	**3922 (0.7)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

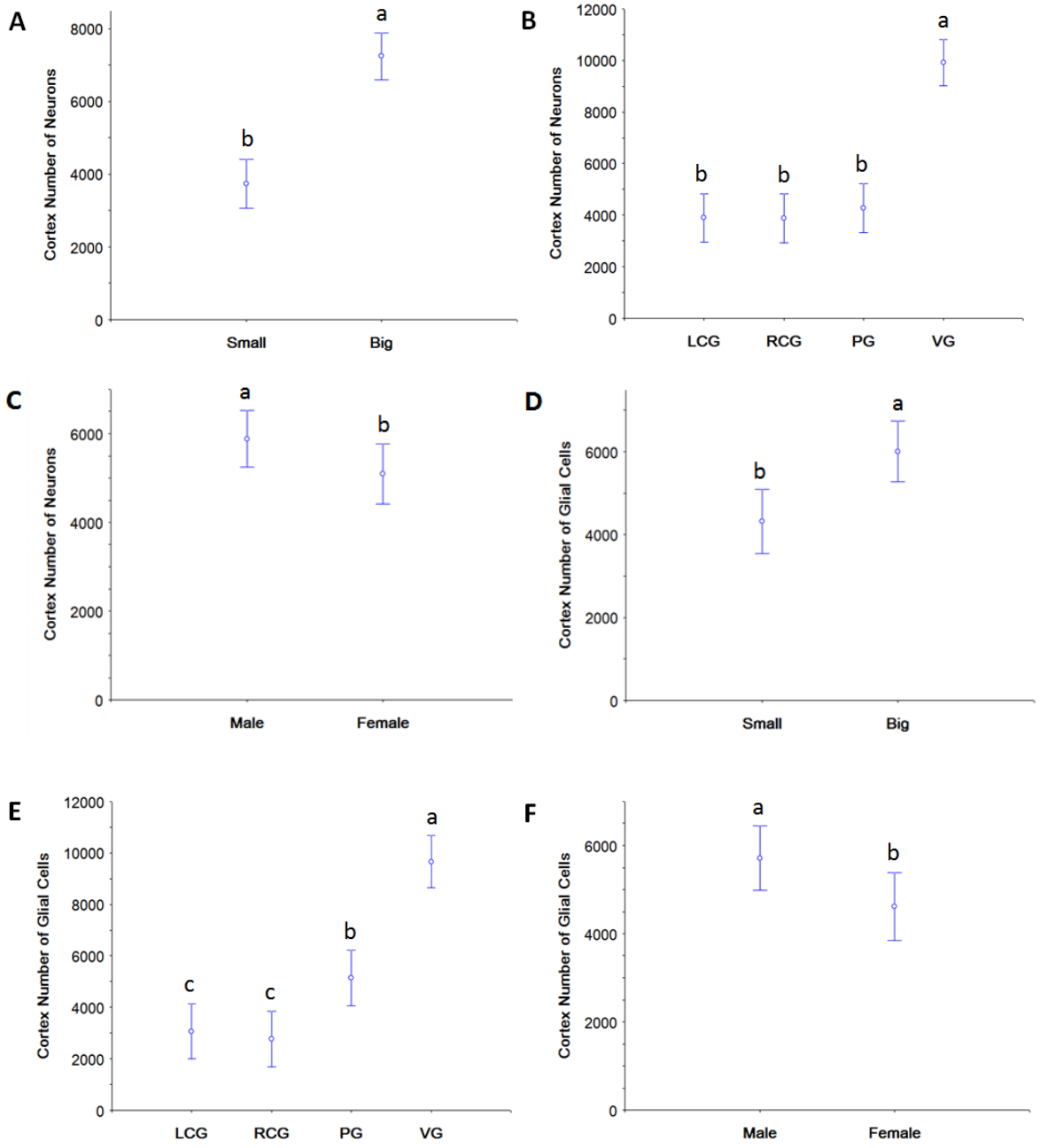

Figure 6. Total mean number of neurons and glial cells in the cortex of ganglia of *S. plana*. **A**) Neurons per size class, **B**) per ganglion, and **C**) per gender. **D**) Glial cells per size class, **E**) per ganglion, and **F**) per gender. Different letters mean significant differences. Data given as mean and 95% confidential interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

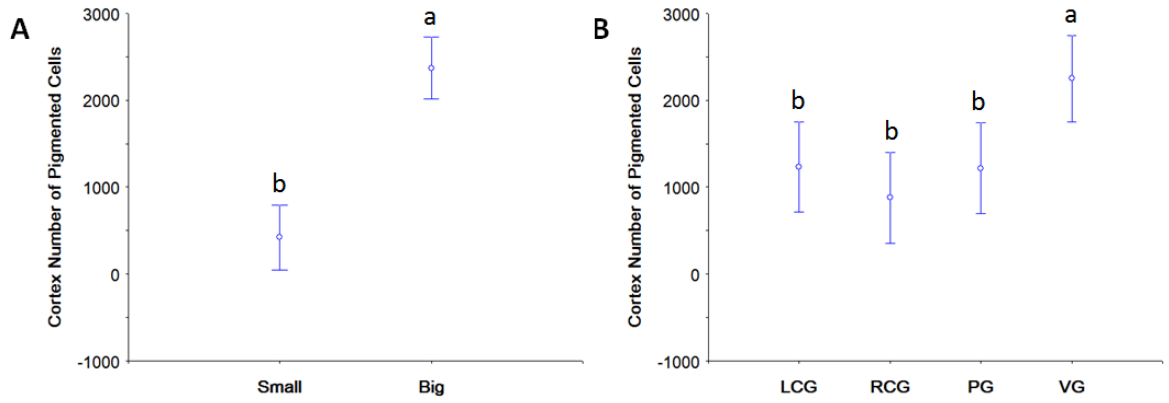

Figure 7. Total mean number of pigmented cells in the ganglia cortex of *S. plana*. **A**) Cells per size class and **B**) per ganglion. Dissimilar letters mean significant differences. Data given as mean and 95% confidential interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

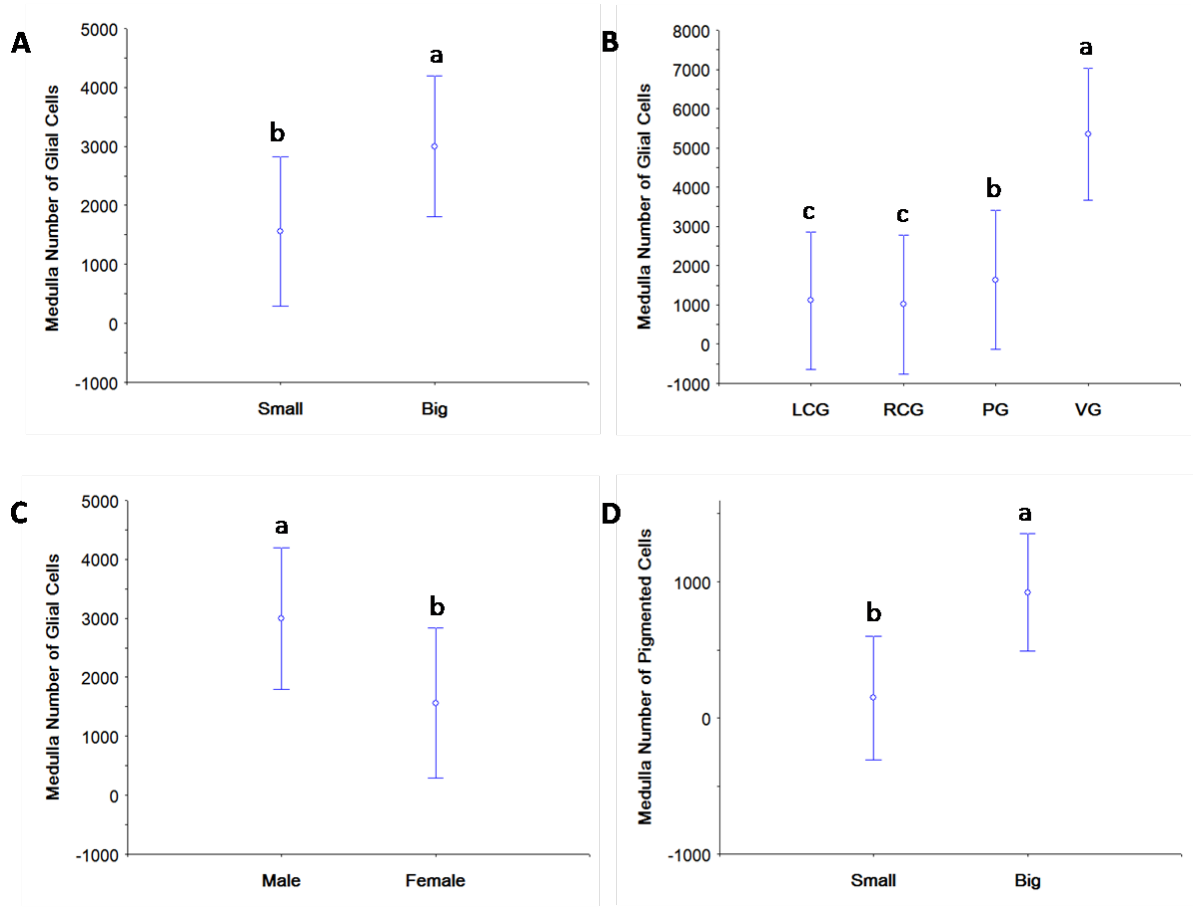

Figure 8. Total mean number of glial cells and pigmented cells in the medulla of the ganglia of *S. plana*. **A**) Glial cells per class size, **B**) per ganglia type, and **C**) per gender. **D**) Pigmented cells per size class. Different letters signify significant differences. Data given as mean and 95% confidential interval. LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglia; VG: visceral ganglia.

Table 9. Total mean number (N) of medullar neural cells in body size class Small, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Small	LCG	Males	52 (1.7)	29 (1.9)	**81 (1.8)**	151 (0.8)	554 (0.9)	472 (0.8)	**1177 (0.8)**	**141 (1.7)**
		Females	103 (1.2)	52 (1.7)	**155 (1.0)**	148 (0.8)	195 (0.8)	295 (0.9)	**638 (0.7)**	**52 (0.5)**
		Mean	77 (1.3)	41 (1.6)	**118 (1.0)**	150 (0.8)	374 (1.0)	383 (0.9)	**907 (0.9)**	**96 (1.7)**
	RCG	Males	28 (0.9)	38 (1.8)	**66 (1.3)**	131 (0.8)	273 (0.4)	340 (1.0)	**744 (0.6)**	**46 (1.3)**
		Females	14 (1.6)	50 (0.4)	**64 (0.6)**	137 (0.3)	296 (0.6)	379 (0.6)	**812 (0.4)**	**95 (0.7)**
		Mean	23 (1.3)	43 (1.2)	**66 (1.0)**	134 (0.6)	282 (0.5)	356 (0.8)	**772 (0.5)**	**66 (1.0)**
	PG	Males	96 (0.4)	63 (1.3)	**159 (0.7)**	175 (0.9)	488 (0.9)	641 (0.6)	**1304 (0.7)**	**114 (1.1)**
		Females	114 (0.8)	36 (0.8)	**150 (0.7)**	140 (0.6)	602 (0.4)	485 (0.2)	**1227 (0.2)**	**198 (0.8)**
		Mean	103 (0.6)	52 (1.3)	**155 (0.7)**	161 (0.8)	534 (0.7)	579 (0.5)	**1274 (0.5)**	**148 (0.9)**
	VG	Males	104 (0.7)	47 (0.9)	**151 (0.7)**	1452 (0.5)	1917 (0.5)	1452 (0.5)	**4821 (0.4)**	**246 (1.1)**
		Females	87 (0.8)	77 (0.8)	**164 (0.7)**	792 (0.6)	1147 (0.5)	792 (0.6)	**2731 (0.4)**	**294 (1.2)**
		Mean	96 (0.7)	61 (0.9)	**157 (0.6)**	1152 (0.6)	1567 (0.5)	1152 (0.6)	**3871 (0.5)**	**268 (1.1)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Table 10. Total mean number (N) of medullar neural cells in body size class Big, by ganglia type and gender of *S. plana*.

Size	Ganglia	Gender	Neurons			Glial cells				Pigmented cells
			Small	Large	Sum	Fusiform	Roundish	Triangular	Sum	
Big	LCG	Males	117 (1.2)	71 (1.8)	**188 (1.4)**	324 (1.3)	615 (0.7)	824 (0.7)	**1763 (0.5)**	**293 (0.8)**
		Females	66 (1.7)	121 (1.0)	**188 (1.0)**	255 (1.1)	158 (0.6)	455 (1.2)	**868 (1.0)**	**442 (0.8)**
		Mean	90 (1.3)	98 (1.2)	**188 (1.0)**	286 (1.1)	366 (1.0)	623 (0.9)	**1275 (0.7)**	**375 (0.8)**
	RCG	Males	79 (1.5)	39 (0.8)	**118 (1.1)**	489 (1.1)	488 (0.5)	727 (1.0)	**1703 (0.7)**	**511 (0.6)**
		Females	38 (0.8)	9 (2.4)	**47 (0.9)**	195 (1.0)	334 (0.9)	704 (0.8)	**1233 (0.6)**	**391 (1.2)**
		Mean	59 (1.7)	24 (3.8)	**83 (1.4)**	342 (1.2)	411 (0.6)	715 (0.9)	**1468 (0.8)**	**451 (0.7)**
	PG	Males	102 (1.2)	55 (1.5)	**157 (0.9)**	406 (0.8)	1030 (0.5)	1144 (0.5)	**2580 (0.4)**	**492 (0.5)**
		Females	34 (1.4)	56 (1.1)	**90 (0.9)**	181 (1.0)	548 (0.6)	720 (0.3)	**1450 (0.3)**	**521 (1.0)**
		Mean	71 (1.2)	56 (1.3)	**126 (0.8)**	304 (0.9)	811 (0.6)	952 (0.5)	**2066 (0.5)**	**505 (0.7)**
	VG	Males	385 (1.4)	420 (1.3)	**805 (1.3)**	1317 (1.1)	2159 (1.0)	2982 (0.8)	**6458 (0.9)**	**1561 (0.9)**
		Females	351 (0.6)	142 (1.3)	**493 (0.5)**	966 (0.8)	1023 (0.5)	4493 (0.9)	**6482 (0.7)**	**1337 (1.4)**
		Mean	368 (1.1)	281 (1.5)	**649 (1.2)**	1142 (1.0)	1591 (1.0)	3738 (0.9)	**6470 (0.8)**	**1449 (1.1)**

Six animals per gender were used. Data given as mean (coefficient of variation). LCG: left cerebral ganglion; RCG: right cerebral ganglion; PG: pedal ganglion; VG: visceral ganglion.

Discussion

This study was undertaken on adult *S. plana* of two size classes to investigate if and in what extent the age factor can influence microscopic anatomy of central ganglia of this species, and in particular the number of neural cells. Our fundamental interest and question are founded on the possibility that as the animal grows the number of cells could eventually increase with age. If true, this event could be explored in the future, both in fundamental and applied research. Because of the unknown impacts of the animals' sex in this (and other) bivalves neurocytology, we opted to study males and females, which could allow disclosing novel interactions between age and gender; in this way we extend prior pioneer work on intersex differences (Chapter 5).

Before discussing and concluding about our primary question, it is relevant to address the validity of using a bigger size of the animal as a proxy of age. If by one hand, as stressed in Introduction, body size in bivalves correlates well with age (Ridgway *et al.* 2011), by other hand there are already specific information about the relation of shell size and age in *S. plana*. Whereas our size cohort Small has an average length of 2.4 cm, for both sexes, the group Big averages a significantly higher 3.8 cm (in both sexes as well). In view of earlier works with *S. plana* specimens sampled in Mondego River estuary (Verdelhos *et al.* 2005) and in Ria de Aveiro coastal lagoon (Coelho *et al.* 2006), our Small group has animals regarded as 2+, *i.e.*, having from 2 to 3 years of age, and our Big group integrates 3+ year old animals, and so having from 3 to 4 years of age. However, this should be viewed as an approximate age because as the length of the animal's shell increases the age estimation seem to become more inaccurate (Green 1957). It is also relevant to mention that *S. Plana* can live at least as long as 18 years (Green 1957), but its longevity in southern Europe is reported to be of about 5 years (Verdelhos *et al*. 2005). Irrespective of the lifespan range, and some uncertainty, there is no doubt that we worked with adult animals that differed in ≈1 year in age, and that were not extremely old.

This study generated numerous data sets that were presented for allowing the readership to appreciate the global scenario and so having the chance to build its own critical thinking. Additional data can even be derived from the current data sets, such as the glia-to-neuron ratio. The present discussion will focus on the main message that can be explored further in future. Importantly for comparative purposes regarding total numbers of neuronal cells, while

older specimens were bigger and heavier, females and males of the same age did not differ in mass. As in our previous studies, the visceral ganglia are more voluminous, followed in size by the pedal, which was bigger than each of the cerebrals. Considering the effect of sex, and overall, males have a marginally greater ganglionic volume, matching with a bigger cortex and medulla. Interganglionic differences were also found when looking at the relative importance of the cortex and medulla, with the visceral and the pedal ganglia having significantly greater relative volumes of the cortex (and smaller in what concerns medulla) compared with the cerebral ones. This scenario is partially in agreement with our previous study (see Chapter 5), in the sense that earlier we found no differences in the relative volume compartments of visceral *vs*. pedal ganglia. These aspects are still very poorly studied, and for instance age can be on factor that influences both the relative and total volumes. Indeed, in our earlier study we used animals that have mean size values (and therefore age) that are in between those of size-cohorts used here. Our idea is supported by the fact that relative volumes found herein are significantly influenced by age, with big/older animals having greater cortical (and lesser medullar) relative volumes.

In our previous study looking for gender differences and impact of gonadal maturation status in adults (Chapter 6) we could not demonstrate many differences in between males and females. Herein, having somewhat more statistical power, we detect a significant gender effect, with males having a global higher mean number of neurons and glial cells (but not of pigmented cells), both when considering the entire ganglia and when considering each compartment. This sex effect likely justifies the slightly (but significant) greater mean ganglion volume in males.

Notwithstanding the influences of ganglia type and/or sex of the animal over the studied parameters, the size/age effect was consistently significant. It can be estimated that within an average period of ≈ 1 year (*i.e.*, 2+ *vs*. 3+ years of age), the volumes of every ganglia doubled, and the number of neuronal cells were at the core mechanism of those global size increments. Without significant interactions either with sex or with ganglia type, generation of new neural cells occurred, largely irrespective of the structural phenotype and both in cortex and medulla. So, age does matter as to what concerns the nervous ganglia of this bivalve, and it is quite likely that the same kind of findings exit in other species, with adult neurogenesis ongoing throughout life, at least for a long period (years). Our data implicates

that adult neurogenesis thus occur in *S. plana*. New puzzling questions instantly emerge, e.g., what sources of new neural cells are? Bivalve neurogenesis has been studied in a few species, but very rarely and typically focusing the nervous system formation during early ontogeny, namely in embryos and larvae, eventually up to the young adult (*e.g.*, Raineri 1995; Voronezhskaya *et al.* 2008; Ramsmayer 2014). In other invertebrates, including aquatic species, adult neurogenesis has been deserving attention, namely for identifying the sources of precursor cells, their proliferation and migratory patterns. On this matter, sequences of articles on decapods beautifully illustrate the search for insights on such new fundamental questions (Zhang *et al.* 2009, 2011; *Benton et al*. 2011, 2013). These and other studies have been strongly supporting that neurogenesis emerge from local or non-neuronal precursors derived from hematopoiesis (Beltz *et al.* 2011; Chaves da Silva *et al*. 2015). In view of the potential longevity of *S. plana* and considering our current data that show that adults keep incrementing in an impressive way the numbers of their "neural soldiers", in every ganglia and irrespective of gender, we propose using this and other baseline knowledge of the species to make it one more stimulating bivalve model for studying adult neurogenesis aging, and even resistance to senescence. Our vision is perfectly in line with that expressed by Abele *et al.* (2009), when stating that "Bivalves are newly discovered models of natural aging", adding to the great interest in the exceptionally long-lived bivalves (Philipp and Abele 2010).

Conclusion

In summary, this is the second study that used unbiased stereology techniques to estimate the volume of the three types of nervous ganglia (including of their cortexes and medullae) and the neuronal and non-neuronal cell numbers, in the whole ganglia and in each compartment. All this in a study comprising adult specimens of two size cohorts. Because animals were caught from the same locations at the estuary of the Mondego River, sizes are comparable and can be faced as proxies of the individuals' age; corresponding to individuals with 2+ and 3+ years old. In animals that did not differ in size/length and mass, we disclosed interganglionic, sex-related and size-related significant effects upon the volumes of the ganglia, relative volumes of cortex and medulla, and total numbers of neurons (large and small phenotypes), glial cells (fusiform, roundish and "triangular" phenotypes), and pigmented cells. Extending previous findings we were able to disclose significant differences between males and females; in view of the ganglia together, the females displayed a smaller

average total number of neurons and glial cells. The comparisons between the animals of the two size classes Small (younger) and Big (older) supported that neurogenesis continues to occur in adult *S. plana*, with increasing numbers of neurons, glial cells, and pigmented cells in every ganglia, and irrespective of the animals' sex. Further statistical exploration of the data is worth their functional significance are at question: which factors govern increased cellularity; does the increase continues during lifespan; what is the nature of the neural cell progenitors; are increases in numbers occurring in parallel with bursts and/or cycles of cell death *vs.* renewal — among other, are fascinating questions worth pursuing. Facing the species abundance, wide distribution and range of biotic/abiotic factors, and new wealth of neural data being generated, we now view *S. plana* as one promising model for neurogenesis and age-related researches; well beyond its current use in ecology/toxicology.

Acknowledgments

The author Sukanlaya Tantiwisawaruji was supported by a Thai Government Science and Technology Scholarship. This research was partially supported by the Strategic Funding UID/Multi/04423/2013, through national funds provided by FCT – Foundation for Science and Technology and by European Regional Development Fund (ERDF), in the framework of the program PT2020. Further support was given by ICBAS, via its Ph.D. Program in Biomedical Sciences. We do thank Fernanda Malhão and Célia Lopes for their wise histotechnical advices.

References

Abele, D., Brey, T. & Philipp, E. (2009) Bivalve models of aging and the determination of molluscan lifespans. *Experimental Gerontology* 44, 307-315.

Amano, M., Oka, Y., Nagai, Y., Amiya, N. & Yamamori, K. (2008) Immunohistochemical localization of a GnRH-like peptide in the brain of the cephalopod spear-squid, *Loligo bleekeri*. *General and Comparative Endocrinology* 156, 277-284.

Ashton, Q.A. (2013) *Issues in Neuroscience Research and Application*. Scholarly Editions, Atlanta, Georgia.

Beltz, B.S., Zhang, Y., Benton, J.L. & Sandeman, D.C. (2011) Adult neurogenesis in the decapod crustacean brain: a hematopoietic connection? *European Journal of Neuroscience* 34, 870-883.

Benton, J.L., Chaves da Silva, P.G., Sandeman, D.C. & Beltz, B.S. (2013) First-generation neuronal precursors in the crayfish brain are not self-renewing. *International Journal of Developmental Neuroscience* 31, 657-666.

Benton, J.L., Zhang,Y., Kirkhart, C.R., Sandeman, D.C. & Beltz, B.S. (2011) Primary neuronal precursors in adult crayfish brain: replenishment from a non-neuronal source. *BMC Neuroscience* 2, 12- 53.

Betts, L.R., Taylor, C.P., Sekuler, A. B. & Bennett, P. J. (2005) Aging reduces center-surround antagonism in visual motion processing. *Neuron* 45, 361-366.

Burrell, D.E. & Stefano, G.B. (1983) Analysis of monoamine accumulation in the neuronal tissues of *Mytilus edulis* (Bivalvia). IV. Variation due to age. *Comparative Biochemistry Physiology: Part C* 74, 59-63.

Jansen, R.F., Pieneman, A.W. & ter Maat, A. (1996) Spontaneous switching between ortho- and antidromic spiking as the normal mode of firing in the cerebral giant neurons of freely behaving *Lymnaea stagnalis*. *Journal of Neurophysiology* 76, 4206-4209.

Campisi, J. & d'Adda di Fagagna, F. (2007) *Cellular senescence: when bad things happen to good cells*. *Nature Reviews Molecular Cell Biology* 8**,** 729-740.

Chaves da Silva, P.G., Santos de Abreu, I., Cavalcante, L.A., Monteiro De Barros, C.& Allodi, S. (2015) *Role of hemocytes in invertebrate adult neurogenesis and brain repair*.

Chen, C.-H., Chen, Y.-C., Jiang, H.-C., Chen, C.-K. & Pan, C.-L. (2013) Neuronal aging: learning from *C. elegans*. *Journal of Molecular Signaling* 8, 14-14.

Coelho, J.P. Rosa, M. Pereira, E. Duarte, A. & Pardal , M.A. (2006) Pattern and annual rates of *Scrobicularia plana* mercury bioaccumulation in a human induced mercury gradient (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science* 69, 629-635.

Croll, R.P., Nason, J. & VanMinnen, J.A. (1993) Characterization of central neurons in bivalves using antibodies raised against neuropeptides involved in gastropod egg-laying behavior. *Invertebrate Reproduction and Development* 24, 161-168.

Curtis, M.A., Kam, M. & Faull, R.L. (2011) Neurogenesis in humans. *European Journal of Neuroscience* 33, 1170-1174.

Di Cristo, C. (2013) Nervous control of reproduction in *Octopus vulgaris*: a new model. *Invertebrate Neuroscience*13, 27-34.

Dorph-Petersen, K.A., Nyengaard, J.R. & Gundersen, H.J.G. (2001) Tissue shrinkage and unbiased stereological estimation of particle number and size. *Journal of Microscopy* 204, 232-246.

Flyachinskaya, L.P. (2000) Localization of serotonin and fmrfamide in the bivalve mollusc *Mytilus edulis* at early stages of its development. *Journal of Evolutionary Biochemistry and Physiology* 36, 66-70.

Franchinf, A., Ottavianp, E. & Caselgrandi, E. (1985) Biogenic amines in the snail brain of *Helicella virgata* (Gastropoda, Pulmonata). *Brain Research* 347, 132-134.

Gosling, E. (2004) *Bivalve Molluscs: Biology, Ecology and Culture*. Fishing News Books, Blackwell Publishing, Oxford.

Green, J. (1957) The Growth of *Scrobicularia plana* (da Costa) in the Gwendraeth estuary. *Journal of the Marine Biological Association of the United Kingdom*36, 41-47.

Gundersen, H.J. (1986) Stereology of arbitrary particles: A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *Journal of Microscopy* 143, 3-45.

Gundersen, H.J. & Jensen, E.B. (1987) The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 147, 229-263.

Henrique, R.M.F., Rocha, E., Silva, M.W. & Monteiro, R.A.F. (2001) Age-related changes in rat cerebellar basket cells: a quantitative study using unbiased stereological methods. *Journal of Anatomy* 198, 727-736.

Hodgson, A.N. & Trueman, E.R. (1981) The siphons of *Scrobicularia plana* (Bivalvia, Tellinacea) Observations on movement and extension. *Journal of Zoology* 194, 445-459.

Kodirov, S.A. (2011) The neuronal control of cardiac functions in Molluscs. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 160, 102-116.

Kizil, C., Kaslin, J., Kroehne, V. & Brand, M. (2012) Adult neurogenesis and brain regeneration in zebrafish. *Developmental Neurobiology* 72, 429-461.

Khotimchenko, Y.S. (1991) Biogenic monoamines in oocytes of echinoderms and bivalve molluscs. A formation of intracellular regulatory systems in oogenesis. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 100, 671-675.

Kotsyuba, E.P. & Kotsyuba, A.E. (2002) Ultrastructural characteristics of interneuronal connections of the central nervous system of bivalve molluscs. *Journal of Evolutionary Biochemistry and Physiology* 38, 330-335.

Margaret, A., Carroll, E. & Catapane, J. (2007) The nervous system control of lateral ciliary activity of the gill of the bivalve mollusc, *Crassostrea virginica*. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*.148, 445-450.

Moffett, S.B. (1996) *Nervous System Regeneration in the Invertebrates*. Springer, New York.

Monteiro, R.A.F. (1991) Age-related quantitative changes in the organelles of rat neocerebellar Purkinje cells. *Histology and Histopathology* 6, 9 -20.

Philipp, E.E. & Abele, D. (2010) Masters of longevity: lessons from long-lived bivalves--a mini-review.*Gerontology* 56, 55-65.

Raineri, M. (1995) Is a mollusc an evolved bent metatrochophore? A histochemical investigation of neurogenesis in *Mytilus* (Mollusca: Bivalvia). *Journal of the Marine Biological Association of the United Kingdom* 75, 571-592.

Raineri, M. (1995) Is a mollusc an evolved bent metatrochophore? A histochemical investigation of neurogenesis in *Mytilus* (Mollusca: Bivalvia). *Journal of the Marine Biological Association of the United Kingdom*75, 571-592.

Ramsmayer, P.D. (2014) Neurogenesis in *Nucula tumidula* and *Kurtiella bidentata* (Mollusca: Bivalvia) as revealed by immunocytochemistry and confocal laser scanning microscopy.Master thesisUniv.-Prof. DDr. Andreas Wanninger.

Ridgway, I., Richardson, C.A. & Austad, S.N. (2011) Maximum shell size, growth rate, and maturation age correlate with longevity in bivalve molluscs. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 66A, 183-190.

Samorajski, T. & Rolsten, C. (1975) Nerve fiber hypertrophy in posterior tibial nerves of mice in response to voluntary running activity during aging. *Journal of Comparative Neurology*159, 553-558.

Shaw, W.N. (1986) Species profiles: life histories and environmental requirements of coastal fishes and invertebrates (Pacific Southwest)-common littleneck clam. In: U.S. Fish & Wildlife Service Biological Report 82 (11.60): U.S. Army Corps of Engineer.

Sola, J.C. (1997) Reproduction, population dynamics, growth and production of *Scrobicularia plana* da Costa (Pelecypoda) in the Bidasoa estuary, Spain. *Aquatic Ecology* 30, 283-296.

Simmons, P.J. & Young, D. (1999) Nerve cells and animal behaviour, 2 ed: Cambridge University Press.

Stefano, G.B. (1982) Comparative aspects of opioid-dopamine interaction. *Cellular and Molecular Neurobiology* 2, 167-178.

Stefano, G. B., Cadet, P., Sinisterra, J., Charles, R., Barnett, J., Kuruvilla, S. & Aiello, E. (1990) Functional neural anatomy of *Mytilus edulis*: Monoaminergic and opioid localization. In: G. B. Stefano (Ed.), Neurobiology of *Mytilus edulis*, Manchester University Press, Manchester pp. 38-56.

Takuwa-Kuroda, K., Iwakoshi-Ukena, E., Kanda, A. & Minakata, H. (2003) Octopus, which owns the most advanced brain in invertebrates, has two members of vasopressin/oxytocin superfamily as in vertebrates. *Regulatory Peptides* 115, 139-149.

Tardy, M. (2003) Brain aging: insights into neuron-glia interactions. *Journal* of *Biology and Medicine*, *Salvador* 2, 114-122.

Torrska, I.V., Bilokrinitskyi, V.S, Burchinska, L.F. & Genis, Y.D. (1968) Properties of neurons of the central nervous system of the freshwater gastropod mollusc, *Planobis corneus*. *Neuroscience Translations* 2, 745-755.

Verdelhos,T., Neto, J.M., Marques, J.C. & Pardal, M.A. (2005) The effect of eutrophication abatement on the bivalve *Scrobicularia plana. Estuarine, Coastal and Shelf Science* 63, 261–268.

Voronezhskaya, E., Nezlin,L. Odintsova, N. Plummer, J. & Croll, R. (2008) Neuronal development in larval mussel *Mytilus trossulus* (Mollusca: Bivalvia). *Zoomorphology*. 127, 97-110.

Yeoman, M.S. & Faragher, R.G.A. (2001) Ageing and the nervous system: insights from studies on invertebrates. *Biogerontology* 2, 85-97.

Yeoman, M., Scutt, G. & Faragher, R. (2012) Insights into CNS ageing from animal models of senescence. *Nature Reviews Neuroscience* 13, 435-445.

Zhang, Y., Benton, J.L. & Beltz, B.S. (2011) 5-HT receptors mediate lineage-dependent effects of serotonin on adult neurogenesis *in Procambarus clarkii*. *Neural Development* 6, 2-2.

Zhang, Y., Allodi, S.D., Sandeman C. & Beltz, B.S. (2009) Adult neurogenesis in the crayfish brain: proliferation, migration, and possible origin of precursor cells *Developmental Neurobiology* 69**,** 415-436.

CHAPTER7

DO ESTROGENS INFLUENCE THE BIVALVE NERVOUS GANGLIA SIZE AND CELLULARITY? A STUDY ON THE PEDAL GANGLIA OF THE PEPPERY FURROW SHELL*SCROBICULARIA PLANA* ACUTELY EXPOSED TO ETHINYLESTRADIOL

Do estrogens influence the bivalve nervous ganglia size and cellularity? A study on the pedal ganglia of the peppery furrow shell *Scrobicularia plana* acutely exposed to ethinylestradiol

[Formatted as a manuscript to be submitted for publication in an international journal. The version in this Thesis may change after the revision to be made by all prospective authors.]

Sukanlaya Tantiwisawaruji[a,b,c*], Catarina Cruzeiro[a,b*], Ana Silva[b], Uthaiwan Kovitvadhi[d], Maria J. Rocha[a,b] and Eduardo Rocha[a,b]

*Joint first authors

[a] Laboratory of Histology and Embryology, Department of Microscopy, Institute of Biomedical Sciences Abel Salazar (ICBAS), University of Porto (U.Porto), Porto, Portugal.
[b] Histomorphology, Physiopathology and Applied Toxicology Group, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto (U.Porto), Porto, Portugal.
[c] King Mongkut's University of Technology Thonburi (KMUTT), Bangkok, Thailand.
[d] Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand.

.

Running title: Pedal ganglia volume and cellularity in adult *S. plana* acutely exposed to an estrogen

Keywords: EDCs, nervous ganglia, glial cells, neurons, bivalves, ethinylestradiol

Correspondence to:

Eduardo Rocha
Laboratory of Histology and Embryology
Institute of Biomedical Sciences Abel Salazar (ICBAS)
University of Porto (U.Porto)
Rua de Jorge Viterbo Ferreira n.º 228
4050-313 Porto
Portugal
E-mail: erocha@icbas.up.pt

Abstract

Differences between sexes and influences of sex-steroids in the structure and function of the nervous system in bivalves are still poorly studied. Yet, that system's activity and presence of estrogens (viz. of 17β-estradiol) influence vital aspects of those organisms, and particularly reproduction. Water/sediment pollution by xenoestrogens is common nowadays and impose risks for biota because of the physiological disruption they may cause. The synthetic estrogen 17α-ethinylestradiol (EE_2) is an active ingredient of many contraceptive pills. Due to its large use and resistance to degradation, EE_2 pollutes aquatic ecosystems, particularly those receiving urban inputs. Toxicological impacts of EE_2 (particularly on reproduction) are far better known for fish than for invertebrates, including bivalves. We hypothesize that estrogens may cause impacts on the structure/cellularity of these organisms' nervous system. Therefore, the estuarine bivalve *Scrobicularia plana* was collected from Mondego River (Portugal) to start testing our hypothesis that EE_2 can cause neural impacts. Animals were exposed for 5 days to EE_2 (0.05 or 5 μg/L), for comparison with a solvent control group (0.01% ethanol). Each treatment was done in triplicate, each comprising 12-15 adults. At the end, the animals were euthanized and routinely processed for histology. The three experimental groups did not differ in body morphometry. The pedal ganglion was selected as target, being totally serially cut into 35 μm thick sections that were stained with hematoxylin-eosin. Stereology was used to unbiasedly estimate the: 1) total number (N) of neurons, glial and pigmented cells, by the optical fractionator method; and 2) total volume (V) of the ganglia, by the Cavalieri's principle. Data were analyzed via ANOVA, which did not uncover statistically significant differences. Thus, the current data did not support that estrogenic stimuli acutely impact on the cellularity and volume of the nervous ganglia of *S. plana*. Anyway, facing the literature background and possible modes of action of steroidal estrogens in both vertebrates and invertebrates, including bivalves, more studies are justified, viz. using more technical approaches/targets, both with acute and chronic exposures, and, in parallel, exploring in vitro assays.

Introduction

Considering what is known about the immense range of physiological roles that sex-steroids play in vertebrates, we can consider that the same kind of knowledge in invertebrates is still at its infancy. As to mollusks, and bivalves in particular, sex-steroids have been continuously associated with the physiology of reproduction, but many general doubts do exist, not to mention that exact mechanisms remain so far from clear that even the sources of estrogens and androgens is debated (Ciocan *et al*. 2011; Liu *et al*. 2014). Despite the knowledge gaps, we know already that bivalves are sensitive to physiological influences of estrogens, being capable of showing "vertebrate-like" responses after exposure to natural or synthetic estrogens (via waterborne or by injections); including up-regulation of estrogen receptors (Ciocan *et al*. 2010; Li *et al.* 1998) or increased expression of egg yolk proteins (Ciocan *et al*. 2010; Osada *et al*. 2003). Nonetheless, the standard vertebrate estrogen responsive genes may also fail to be up-regulated after bivalve exposure to estrogens, evoking varied explanatory hypotheses for the differences (Puinean *et al*. 2006). Anyway, tests looking at differentially expressed genes in the mussel *Mytilus edulis* do support that bivalves can be negatively impacted by exogenous estrogen exposure (Ciocan *et al*. 2010). One key aspect of bivalve reproduction is that it is controlled by neurotransmitter and neurohormones (Siniscalchi *et al*. 2004), and that estrogens correlate with the reproductive cycle and modulate the process, e.g., by controlling the levels of serotonin and of its receptor (Cubero-Leon *et al*. 2010; Osada *et al*. 2003; Liu *et al*. 2014). Nonetheless, if the overall functional role and mechanistic aspects of estrogens in bivalves remains meagre (Ciocan *et al*. 2010), the fine modulation, the impacts and mode(s) of action of the endogenous/exogenous estrogens in the bivalve nervous system are nearly unknown. Fundamental and practical reasons call for more studies.

From over 25 years, there has been a concern about pollutants from anthropogenic sources that enter aquatic systems and that, by mimicking natural hormones, physiologically act as endocrine disrupting chemicals (EDCs), leading to mild-to-severe disruptive effects on both vertebrates and invertebrates, particularly on the reproductive organs and the closely related physiology (IEH 1999; Gauthier-Clerc *et al*. 2006; Porte *et al*. 2006; Segner *et al*. 2003), and beyond them (Canesi *et al*. 2007). Despite regulatory measures, waterborne EDCs are still prevalent in Europe and elsewhere (Rocha and Rocha 2015). Bivalves, like vertebrates, can be feminized by exposure to such EDCs (Langston *et al*. 2007).

Among the most potent estrogenic EDCs is the synthetic steroid 17α-ethinylestradiol (EE_2), which is widely used in oral contraceptives and presents a chemical structure derivate from that of the natural hormone 17β-estradiol (Soto and Sonnenschein 2005). Typically, after internalization, EE_2 strongly binds to intracellular estrogen receptors, that once dimerized and translocated to the cell nucleus cause activation or inhibition of specific functions — besides being high relative estrogenic potency, EE_2 is a most often found waterborne xenoestrogen (Aris *et al*. 2014; Rocha and Rocha 2015). Yet, the fact is that to this date estrogen receptors from mollusks have been shown to be insensitive to activation by steroidal estrogens, therefore we do not know yet what exact mechanisms mediate estrogen signaling and disruption (Keay *et al*. 2006; Matsumoto *et al*. 2007; Keay and Thornton 2009).

In view of the above context, because both estrogens and neuro-mediated signaling pathways do play roles in governing the bivalve reproduction, because estrogens have seasonal fluctuation patterns that may impact on the nervous system structure and/or function (Gauthier-Clerc *et al*. 2006; Liu *et al*. 2014), because nervous system plasticity exists in adults, particularly in invertebrates (Cayre *et al*. 2002; Beltz *et al*. 2015), and, at last, because estrogenic stimulus is linked to neurogenesis, including neuronal proliferation and survival, in various organisms (Fowler *et al*. 2005; Fowler *et al*. 2008; Li *et al*. 2011), we hypothesize that neural ganglia cellularity in bivalves can be impacted by exposure to EE_2. To start investigating this hypothesis we used as model organism the peppery furrow shell *Scrobicularia plana* and elected the pedal ganglia as target for counting the number of neuronal cells. In parallel, we estimated the ganglionic volume and of its major compartments (cortex and medulla). To our best knowledge, this is the first experimental study with bivalves using design-based unbiased stereological methods capable of producing technically sound estimations of numbers of neural cells.

Materials and methods

Collection and maintenance

Wild bivalves were collected manually at 0.1-1 m depth from a brackish site in the Mondego River Estuary. The bivalves were sampled during low tide in May 2013 (pre-spawning). Specimens were transferred into 10 L boxes with local sediment, and then transported to the laboratory. For in-house acclimatization, they were kept in glass aquaria, without sediment, with well-aerated artificial sea water (30 ‰ salinity, as in the field), at 15^{o}C. The animals were fed with chlorella during adaptation.

Chemicals

The EE_2 (≥ 98%) was purchased from Sigma-Aldrich (Germany). The stock solution (1 mg/L) was prepared in ethanol (99.9%), bought from Merck (Germany), and stored in an amber flask at -20ºC.

Experiment

Two nominal concentrations of EE_2 were used on this trial (environmental and 100-fold higher), the environmental one (0.05 µg/L) was based on concentrations found in Douro River estuary (≈ 0.05 µg/L) (Ribeiro *et al*. 2009; Rocha *et al*. 2011). Each EE_2 treatment concentration (T1 = 0.05 and T2 = 5 µg/L) had 3 replicates of 12-15 adult animals (practically with uniform size). The parallel control groups were subjected to ethanol only (0.01% (v/v)). The experiment was set under semi-static conditions, in glass aquaria, with a daily renewal of 100% of water volume (5 L), during 5 days. The animals were kept spatially well separated from each other. In every replicate, the water temperature was measured daily and the ammonia, nitrite and salinity were monitored at least twice a week.

Histological preparation

After the exposure period the animals were anesthetized using magnesium chloride (60 g/L; ± 98%) (Butt *et al*. 2008), and their length, width, height, and fresh and total mass were measured.

The soft body of each specimens was removed carefully and then prepared following a standard procedure for histology. Briefly, the fragments were fixed in 10% buffered formalin overnight, at room temperature. After 24 hours, the samples were dehydrated with increasing concentrations of ethanol (from 70% to 100%), cleared in xylene and infiltrated with paraffin

(Histosec, Merck); the dehydration and infiltration procedures were done using an automatic tissue processor (Leica TP1020, Germany). Final paraffin embedding was performed in an laboratory bench station (Leica EG 1140H, Germany).The paraffin blocks of randomly sampled animals from each treatment were serially sectioned (35-µm thick) on a motorized rotary microtome (Leica RM2155, Germany), and placed onto APES (3-amino propyltriethoxysilane) coated slides. The resulting slides were stained with hematoxylin and eosin and mounted with DPX media (Sigma-Aldrich, Germany).

Stereological analyses

Stained sections from the three treatments (solvent control: Sc, 0.05 µg/L: T1, and 5 µg/L: T2) were examined under a light microscope (Olympus BX50, Japan) equipped with a digital camera (Camedia C-5050, Olympus, Japan). The efficient Cavalieri's principle (Gundersen and Jensen 1987) estimated the volume (V) of the pedal ganglion (and separately of its cortex and medulla), based on the formula:

$$V = t \cdot \sum A,$$

where t is the mean distance between analyzed section planes, and A is the sectional area of the target of interest. The areas were determined via the stereological workstation (Olympus CAST-Grid, version 1.5, Denmark), running in a light microscope (Olympus BX50, Japan), equipped with a length gauge (microcator) (Heidenhain MT-12, Germany), a motorized stage with 1 µm X-Y movement accuracy (Prior, USA), and a CCD video camera (Sony, Japan) displaying live image in a 17'' monitor (Sony, Japan). Analyses were done in fields captured with the 10 × objective lens. The areas of the cortex and medulla were read semi-automatically in every section, by manually delineating the region of interest. The t was confirmed by measuring the section thickness with the microcator (see below). The total ganglionic volumes were further used to estimate the volume densities (V_V) of the cortex and medulla in the ganglion: V_V (medulla or cortex) = V (medulla or cortex) / V (ganglion).

The total number (N) of the diverse neural cells in the ganglia was estimated via the optical disector-fractionator combination (Gunderson 1986), using the general formula:

$$N = Q \cdot (1 / ssf) \cdot (1 / asf) \cdot (1 / hsf),$$

where Q refers to the total number of cells actually counted in all the optical disectors; hsf is the height sampling fraction, which captures the ratio of the section thickness that was screened; asf is the area sampling fraction, *i.e.*, the ratio between the area of the counting frame and the area covered by each x,y movement; ssf is the section sampling fraction, *i.e.*,

the fraction of total sections sampled. Herein, half of total sections of each pedal ganglia were systematically sampled, and a minimum of 100 neurons and 100 glial cells were counted per ganglia. The procedure used the cited stereological workstation. Counts were done in live images captured under the 100× (NA=1.35) oil immersion objective lens. A systematic random sampling procedure was made for selecting the fields. The area of the counting frame (with forbidden lines) was 9600 μm^2, and the XY-sampling step was set at 507 μm. To check and account for eventual non-uniform deformations, t was measured in every field; as we did not notice much variability the averaged t was used for hsf = h/t (Dorph-Petersen *et al.* 2001). Herein the average t was 33 μm and the disector h was 25 μm. We set a small top guard zone of 3 μm, viz. because we found no heterogeneous distribution of cells across the z-axis (von Bartheld 2002).

As to cellularity, the data are given herein as N of neural cell, and further presented as N of neurons N of glial cells and, finally, N of pigmented cells. Additionally, when recoding the N of neurons each cell was tagged either as a "large" neuron (oval/round, often with cytoplasmic granules, diameter of ≈ 18 x 25 μm) or and as "small" neuron (oval/round, thick heterochromatin rim, diameter ≈ 7 x 15 μm).

Statistical analyses

The analyses were performed using the software SigmaStat (version 3.5; Systat software Inc.), where all data were tested for normality and homogeneity of variances, using the Shapiro–Wilk's W-test and Levene's test, respectively; square root transformation was applied to obey both conditions. Data were analyzed by one-way ANOVA, considering the three treatments (Sc, T1 and T2), a priori choosing the post hoc Tukey's test in case of statistical significance of the ANOVA (i.e., $p < 0.05$). Data are given as mean ± standard deviation. Because the males and females did not show significant differences in what concerns the currently studied parameters, they were grouped in the presentation of the Results.

Results

During the experiment physicochemical water parameters, viz. ammonia and nitrites, ranged from 0.13 to 1.18 mg/L and 0.01 to 0.24 mg/L, respectively. The mortality was 10%, with no statistical differences between groups. The morphometry of the animals sampled for the stereological analysis did not differ among the experimental groups; excluding the animal size as a possible confounding factor that could affect the results. The grouped morphometric results are as follows: length (3.6 ± 0.4 cm); width (1.1 ± 0.2 cm); height (2.7 ± 0.3 cm); fresh weight (1.8 ± 0.7 g); total weight (3.9 ± 1.5 g).

Looking at the stereological data we found no significant differences in any of the studied targets in relation with the exposure conditions. The total mean V of the pedal ganglion and of its cortex and medulla were fairly constant across groups (Figure 1). The V_V (cortex, ganglion) and V_V (medulla, ganglion) was stable too, overall: 61 ± 5% and 39 ± 5%, respectively. Such stable volumes are in line with the N of all neural cells summed in the pedal ganglion, which were: Cs = 23895 ± 4056; T1 = 19430 ± 6119, T2 = 21962 ± 7599. Figure 2 offers a view of the total results for the N of glial cells, neurons, and pigmented neural cells, in decreasing order of abundance. Splitting the neurons in two size-related classes — the less numerous “larger” and the more abundant “smaller” nerve cells —no differences existed between across groups all assays; the N were: A) larger: Cs = 1235 ± 464; T1 = 1992 ± 1515; T2 = 1199 ± 665; B) smaller: Cs 4540 ± 1807; T1 = 4087 ± 1401; T2 = 5296 ± 2076.

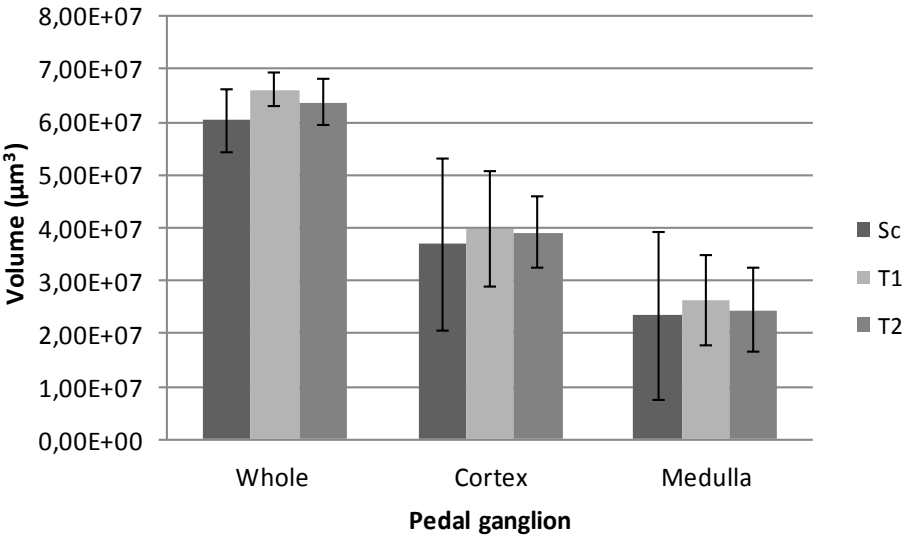

Figure 1. Total volume (μm^3) of the whole pedal ganglion, and of its cortex and medulla, in animals from the three experimental treatments. Sc: Solvent control (ethanol at 0.01%); T1: EE_2 at 0.05 µg/L; T2: EE_2 at 5 µg/L. Data given as: mean ± SD.

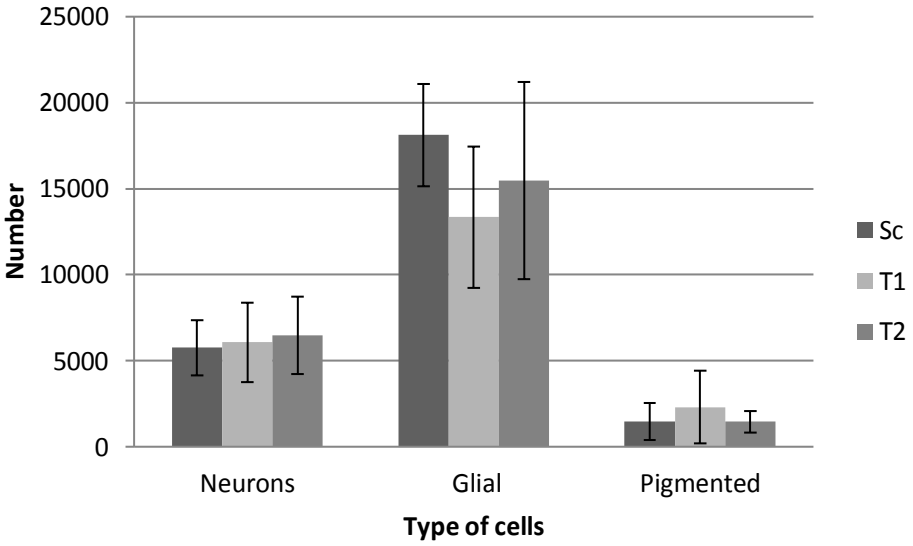

Figure 2. Total number of neurons, glial cells and pigmented cells in the pedal ganglion of animals from the three experimental treatments. Sc: Solvent control (ethanol at 0.01%); T1: EE_2 at 0.05 µg/L; T2: EE_2 at 5 µg/L. Data given as: mean ± SD.

Discussion

Herein, adult specimens of *S. plana* were exposed for a period of 5 days to nominal concentrations of 0.01% of ethanol (used as solvent) or to EE_2, either at 0.05 or at 5 µg/L. This first experiment targeted the volume of the pedal ganglia and its cellularity as structural proxies of neural influences, having as a baseline framework pillars, among other, the knowledge that the invertebrate nervous system is quite plastic, that steroidal estrogens seem to model diverse physiological processes on bivalves by still unclear ways, that at least some neurons control these organisms reproductive processes, and that their neuropeptides and neurohormones may functionally interplay with other elements (viz. steroids). In addition, but relevant in the context, estrogenic action was shown able to interfere with agents, e.g., mitogen-activated protein kinases, which are known to govern cell mitosis (Canesi *et al.* 2004, 2007).

Considering that neither concentration of the synthetic estrogen could trigger quantitative changes in the structural proxies of pedal ganglion, our first exploratory data sets do not endorse the hypothesis that neural ganglia cellularity in bivalves can be impacted by acute exposure to EE_2; at least within the range of the concentrations tested, that cover environmental scenarios. Additionally, even if some neurotrophic effects may have occurred, they did not impact on the gross volume of the ganglia, and both in the cellular cortex and in the neural fiber rich medulla. However, we must bear in mind that our findings do not imply that other sort of structural or functional changes may have occurred, or that longer exposure periods will not impact on the neuronal cell numbers. Indeed, in higher taxa we now know that estrogens, viz. 17β-estradiol, do have trophic effects on a variety of brain regions and in key neurocytological aspects, such as spine density and axonal outgrowth/retraction (de Lacalle 2006). The estrogenic effects attain not only neurons but also glial cells, and include functional and structural (morphometric) parameters and proliferation, both in adult and developing brains (Fowler *et al.* 2005; McCarthy 2008). We could argue that it could be far reaching to hypothesize that the same sort of impacts could attain the nervous system of lower taxa, and particularly bivalves. Yet, the notion that estrogen stimulation can impact on neuronal elements of bivalves is strongly backed up by quite direct evidences. Particularly, Stefano *et al.* (2003a) elegantly proved that there is estrogen signaling in the pedal ganglia of *M. edulis* and that 17β-estradiol model the activation of its ganglionic microglia. Additionally, Stefano *et al.* (2003b) demonstrated that 17β-estradiol and an ERβ receptor were both present in the pedal ganglia of the same species, and that those elements governed

local NO release. In the latter work, the authors concluded that "estrogen processes appear to have been developed much earlier in evolution than previously thought", and we do agree that more research is needed.

Herein we used pre-spawning animals of both sexes that were grouped in the data presentation, because they did not show any major significant differences in the selected parameters. This is in agreement with our previous studies, where maturing animals of both sexes did not differ in those parameters either. Despite we do not expect that bivalves have the same kind of well-described endocrine feedback mechanisms like those the hypothalamic–pituitary–gonadal axis of vertebrates, one aspect that we can postulate that may influence the animal's responses to experimental exposure to estrogens is the endogenous status in terms of amount of sex-steroids, and particularly of estradiol. Notably, at least in several bivalve species, including *S. plana* at the spawning period, we must bear in mind that animals of both sexes may not differ significantly as to organic levels of sexual steroids, and particularly of 17β-estradiol (Wang and Croll 2006; Ketata *et al.* 2007; Mouneyrac *et al.* 2008). With this in mind, if by one hand there are signs of differential responses between males and females when exposed to an estrogen (e.g., Wang and Croll 2006), evidences support that experimentally induced estrogen responses seem depend on the gonadal maturation status. For instance, adult *M. edulis* exposed to estrogens (for 10 days) displayed a significant rise in estrogen receptor mRNA, as far as the mussels were exposed at an early stage of gametogenesis (Ciocan *et al.* 2010). Contrarily, exposure of mature *M. edulis* to the estrogens did not affect the mRNA level (Ciocan *et al.* 2010). In view of the above, it is justified to extend our tests to less maturate and particularly to undifferentiated specimens. This idea is also justified by the fact that despite the above cited interestingly similarity between males and females on (at least certain) sex steroid levels in tissues, there are also reports of seasonal changes, and not necessarily equal in either sex (Ketata *et al.* 2007; Mouneyrac *et al.* 2008).

The value of publishing "negative results" has been often stressed (Dirnagl and Lauritzen 2010; Matosin *et al.* 2014). So, despite our "negative findings", in view of supportive literature and logical rational, we propose to test our hypothesis further, by adding new tools and targets for shorter/acute exposures, conducting longer exposure assays, and by exploring in vitro assays with isolated ganglia. Importantly, this work stands as the 1st that used

unbiased stereology tools in bivalve experiments, and consequently the new quantitative data constitutes a sound baseline reference for future studies.

Acknowledgments

The author Sukanlaya Tantiwisawaruji was supported by a Thai Government Science and Technology Scholarship. This research was partially supported by the Strategic Funding UID/Multi/04423/2013, through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the program PT2020. We also recognize the financial support of the ICBAS – U.Porto, through its Ph.D. Program in Biomedical Sciences. We thank Fernanda Malhão and Célia Lopes for their valued histotechnical advices.

References

Aris, A. Z., Shamsuddin, A. S. & Praveena, S. M. (2014) Review: Occurrence of 17α-ethynylestradiol (EE_2) in the environment and effect on exposed biota. *Environment International* 69, 104–119.

Beltz, B. S., Zhang, Y. & Benton, J. L. (2015) Serotonin modulates adult neurogenesis in an invertebrate model: Approaches to receptor localization and function. *Serotonin Receptor Technologies Neuromethods* 95, 205-222.

Butt, D., O'Connor, S.J., Kuchel, R., O'Connor, W. A. & Raftos, D. A. (2008) Effects of the muscle relaxant, magnesium chloride, on the Sydney rock oyster (*Saccostrea glomerata*). *Aquaculture* 275, 342-346.

Canesi, L., Ciacci, Betti, C. M., Lorusso, L., Marchi, C. B., Burattini, S., Falcieri, E. & Gallo, G. (2004) Rapid effects of 17 beta-estradiol on cell signaling and function of *Mytilus* hemocytes. *General and Comparative Endocrinology* 136, 58-71.

Canesi, L., Lorusso, L. C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G. & Marcomini, A. (2007) Immunomodulation of Mytilus hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: In vitro and in vivo studies. *Aquatic Toxicology* 81, 36-44.

Cayre, M., Malaterre, J., Scotto-Lomassese, S., Strambi, C. & Strambi, A. (2002) The common properties of neurogenesis in the adult brain: from invertebrates to vertebrates. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 132, 1-15.

Ciocan, C. M., Cubero-Leona, E., Puinean, A. M., Hill, E., Minier, M., Osada, C. M., Fenlon, K. & Rotchell, J. M. (2010) Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. *Environmental Pollution* 158, 2977–2984.

Coles, J. A. (2009) Glial Cells: Invertebrate. In Larry, R.S. (Ed.), *Encyclopedia of Neuroscience* Oxford: Academic Press. pp. 749-759.

Cubero-Leon, E., Ciocan, C. M., Hill, E. M., Osada, M., Kishida, M., Itoh, N., Kondo, R., Minier, C. & Rotchell, J. M. (2010) Estrogens disrupt serotonin receptor and cyclooxygenase mRNA expression in the gonads of mussels (*Mytilus edulis*). *Aquatic Toxicology* 98, 178-187.

de Lacalle, S. (2006) Estrogen effects on neuronal morphology. *Endocrine* 29, 185-190.

Dorph-Petersen, K. A., Nyengaard, J. R. & Gundersen, H. J. G. (2001) Tissue shrinkage and unbiased stereological estimation of particle number and size. *Journal of Microscopy* 204, 232-246.

Dirnagl, U. & Lauritzen, M. (2010) Fighting publication bias: introducing the Negative Results section. *Journal of Cerebral Blood Flow & Metabolism* 30, 1263–1264.

Fowler, C. D., Johnson, F. & Wang, Z. (2005) Estrogen regulation of cell proliferation and distribution of estrogen receptor-alpha in the brains of adult female prairie and meadow voles. *Journal of Comparative Neurology* 489, 166-179.

Fowler, C. D., Liu, Y. & Wang, Z. (2008) Estrogen and adult neurogenesis in the amygdala and hypothalamus. *Brain Research Reviews* 57, 342-351.

Gauthier-Clerc, S., Pellerin, J. & Amiard, J.C. (2006) Estradiol-17 beta and testosterone concentrations in male and female *Mya arenaria* (Mollusca, Bivalvia) during the reproductive cycle. *General and Comparative Endocrinology* 145, 133-139.

Gundersen, H.J. (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. *Journal of Microscopy* 143, 3-45.

Gundersen, H. J. & Jensen, E. B. (1987) The efficiency of systematic sampling in stereology and its prediction. *Journal of Microscopy* 147, 229-263.

IEH (1999) Assessment on the ecological significance of endocrine disruption: Effects on reproductive function and consequences for natural populations (Assessment A4), Leicester, UK, MRC Institute for Environment and Health.

Keay, J., Bridgham, J. T. & Thornton, J. W. (2006) The *Octopus vulgaris* estrogen receptor is a constitutive transcriptional activator: evolutionary and functional implications. *Endocrinology* 147, 3861-3869.

Keay, J. & Thornton, J. W. (2009) Hormone-activated estrogen receptors in annelid invertebrates: implications for evolution and endocrine disruption. *Endocrinology* 150, 1731-1738.

Ketata, I., Guermazi, F., Rebai, T. & Hamza-Chaffai, A. (2007) Variation of steroid concentrations during the reproductive cycle of the clam *Ruditapes decussatus*: a one year study in the gulf of Gabès area. *Comparative Biochemistry and Physiology A Molecular and Integrative Physiology* 147, 424-431.

Langston, W. J., Burt, G. R. & Chesman, B. S. (2007) Feminisation of male clams *Scrobicularia plana* from estuaries in Southwest UK and its induction by endocrine-disrupting chemicals. *Marine Ecology Progress Series* 333, 173-184.

Li, Q., Osada, M., Suzuki, T. & Mori, K. (1998) Changes in vitellin during oogenesis and effect of estradiol-17β on vitellogenesis in the Pacific oyster *Crassostrea gigas*. I*nvertebrate Reproduction & Development* 33, 87-93.

Li, J., Siegel, M., Yuan, M., Zeng, Z., Finnucan, L., Persky, R., Hurn, P. D. & McCullough, L.D. (2011) Estrogen enhances neurogenesis and behavioral recovery after stroke. *Journal of Cerebral Flow & Metabolism* 31, 413-425.

Liu, J., Zhang, Z., Zhang, L., Liu, X., Yang, D. & Ma, X. (2014) Variations of estradiol-17β and testosterone levels correlated with gametogenesis in the gonad of Zhikong scallop (*Chlamys farreri*) during annual reproductive cycle. *Canadian Journal of Zoology* 92, 195-204.

Matosin, N., Frank, E., Engel, M., Lum, J. S. & Newell, K. A. (2014) Negativity towards negative results: a discussion of the disconnect between scientific worth and scientific culture. *Diseases Models & Mechanisms* 7, 171–173.

Matsumoto, T., Nakamura, A. Mori, K. Akiyama, I., Hirose, H. & Takahashi, Y. (2007) Oyster estrogen receptor: cDNA cloning and immunolocalization. *General and Comparative Endocrinology* 151, 195-201.

Mouneyrac, C., Linot, S., Amiard, J.-C., Amiard-Triquet, C., Métais, I., Durou, C., Minier, C. & Pellerin, J. (2008) Biological indices, energy reserves, steroid hormones and sexual maturity in the infaunal bivalve *Scrobicularia plana* from three sites differing by their level of contamination. *General and Comparative Endocrinology* 157, 133–133.

McCarthy, M. M. (2008) Estradiol and the developing brain. *Physiological Reviews* 88, 91-124.

Osada, M., Takamura, T., Sato, H. & Mori, K. (2003) Vitellogenin synthesis in the ovary of scallop, *Patinopecten yessoensis*: Control by estradiol-17β and the central nervous system. *Journal of Experimental Zoology Part A: Comparative Experimental Biology* 299A, 172-179.

Puinean, A. M., Labadie, P., Hill, E. M., Osada, M., Kishida, M., Nakao, R. & Rotchell, J. M. (2006) Laboratory exposure to 17β-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. *Aquatic Toxicology* 79, 376-383.

Porte, C., Janer, G., Lorusso, L. C., Ortiz-Zarragoitia, M., Cajaraville, M. P., Fossi, M. C. & Canesi, L. (2006) Endocrine disruptors in marine organisms: Approaches and perspectives. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 143, 303-315.

Ribeiro, C., Pardal, M. A., Martinho, F., Margalho, R., Tiritan, M. E., Rocha, E. & Rocha, M. A. (2009) Distribution of endocrine disruptors in the Mondego river estuary, Portugal. *Environmental Monitoring and Assessment* 149, 183-193.

Rocha, M. J., Cruzeiro, C., Ferreira, C. & Rocha, E. (2011) Occurrence of endocrine disruptor compounds in the estuary of the Iberian Douro River and nearby Porto Coast (NW Portugal). *Toxicological & Environmental Chemistry* 94, 252-61.

Rocha, M. J. & Rocha, E. (2015) Estrogenic compounds in estuarine and coastal water environments of the Iberian Western Atlantic coast and selected locations worldwide — Relevancy, Trends and Challenges in View of the EU Water Framework Directive. In: Andreazza, A.C. & Scola, G. (Eds.), *Toxicology Studies - Cells, Drugs and Environment* InTech, Rijeka, Croatia, pp. 153-193.

Segner, H., Caroll, K., Fenske, M.C., Janssen, R., Maack, G., Pascoe, D., Schafers, Vandenbergh, C. G., Watts, F. M. & Wenzel, A. (2003) Identification of endocrine-disrupting effects in aquatic vertebrates and invertebrates: report from the European IDEA project. *Ecotoxicology and Environmental Safety* 54, 302-314.

Siniscalchi, A., Cavallini, S., Sonetti, D., Sbrenna, G., Capuano, S., Barbin, L., Turolla, E. & Rossi, R. (2004) Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology* 144, 1205-1212.

Soto, A. M. & Sonnenschein, C. (2005) Estrogens, xenoestrogens, and the development of Neoplasms. In: Naz, R.K. (Ed.), *Endocrine disruptors: Effects on Male and Female Reproductive System,* CRC Press, Florida, p. 444.

Stefano, G. B., Zhu, W. Mantione, K. Jones, D., Salamon, E., Cho, J. J. & Cadet, P. (2003a) 17-β estradiol down regulates ganglionic microglial cells via nitric oxide release: Presence of an estrogen receptor β transcript. *Neuroendocrinology Letters* 24, 130-136.

Stefano, G. B., Cadet, P., Mantione, K., Cho, J. J., Jones, D. & Zhu, W. (2003b) Estrogen signaling at the cell surface coupled to nitric oxide release in *Mytilus edulis* nervous system. *Endocrinology* 144, 1234-1240.

von Bartheld, C. S. (2002) Counting particles in tissue sections: choices of methods and importance of calibration to minimize biases. *Histology and Histopathology* 17, 639–648.

Wang, C. & Croll, R. P. (2006) Effects of sex steroids on spawning in the sea scallop, *Placopecten magellanicus*. *Aquaculture* 256, 423–432.

CHAPTER8

FINAL REMARKS

Final Remarks

This Thesis contributed to advance the knowledge of the structure of the nervous ganglia of *Scrobicularia plana*, thus contorting to better understanding of the bivalve neuroscience in general. This study was the first to implement 3D-reconstruction techniques and stereology (firstly applied to the bivalve nervous system) to generate new qualitative and quantitative data on this species, considering the diverse ganglia types, the gonadal maturation status, and the age as facts and factors that could influence the neurocytology of this and other species. Also, we made a first effort to examine if exposure to estrogens impact on ganglia structure.

Typically, 3D anatomy of the ganglia of bivalves has been described from gross observations from dissections and from general microscopic observations. These approaches can provide realistic information. Yet, there was no information about the 3D aspect of *S. plana* nervous ganglia (NG), and this fundamental aspect was studied and fully described in Chapter 3, in order to qualify and quantify that sort of structure. Total volumes, surfaces and NG relative proportions of cortex and medulla were measured for the first time. Data reveals that the size (total volume) and the internal composition (cortex and medulla) of NG no differ among sexes across ganglia. This study also proves that both males and females display specific differences among the different types of ganglia. These are seen firsthand in the total volumes, with the cerebral ganglia being these smaller than the visceral ones. Curiously, the different shapes of the cerebral and pedal ganglia resulted in the surface area of the former being not much different to the latter. That is, the somewhat pear-shaped appearance of the cerebrals present a summed larger (more than double) surface per unit of volume when compared with the somewhat more cylindrical pedal ganglion. It is not clear whether or not such morphological differences have functional implications, or if they signify some ancient form of “early folding” as seen in the long history of brain evolution. To our knowledge, these new volume-surface interrelations and facts seem still unreported in other bivalves.

Herein we provide broad 3D size-related parameters that do not disclose major differences between sexes. However, this fact does not mean that there are no cytological, histological and other differences connected with the sex and seasonal reproductive cycle. We thus aimed to get other parameters (*e.g.*, neural cell counts) that may illustrate differences between sexes.

Despite differences in volume or surface area, it is interesting to note that the relative volumes of the cortex *vs*. medulla are fairly constant across the different ganglia. Whatever the shape of each ganglion and the diversity in size, the outer cortex and the inner medulla maintained a fairly stable volume ratio of ≈ 1.5 (with a small variance between 1.6 in cerebral and 1.3 in the visceral). This suggests an optimal and strictly regulated structural balance for the ganglia of this species. It would be interesting to study these aspects in other bivalves, as anatomical features such as cell densities and cortex volumes are relevant to understand the evolution of the central nervous system (Roth and Dicke 2013). Finally, we wish to point out the linear correlations found in relation to the pedal volume and surface area *vs*. the animals' biometric parameters. Within the size range of the specimens we studied, the bigger the specimen the bigger the pedal ganglia, but the size of the two other types of ganglia do not necessarily increase. The pedal correlations were consistently found in relation to all the biometric parameters. Maybe this is connected somehow with the fact that the pedal ganglion serves primarily to control the musculature of the foot (Bullock and Horridge 1965).

Due to the findings reported above, it was considered vital to characterize the normal features of the neurocytology of *S. plana.* In this vein, further histological and ultrastructural studies were done in the nervous system of *S. plana* (Chapter 4). These studies revealed that the ganglionic cell population can be divided into three main categories (neurons, glial cells and pigmented cells). Nonetheless, two types of neurons were classified taking in account their size and cytological characteristics. So, one type of neurons showed bigger dimensions with a single main process emerging from the soma (unipolar neurons). Because in invertebrates it is often difficult to distinguish axons form dendrites, both in structural and functional points of view (Bullock and Horridge 1965), we also use the term neurite, which can be divided into primary neurites, axons (here mostly from unipolar neurons), and dendrites (Richter *et al.* 2010). Rarely, we saw two processes emerging from the soma of large neurons. This finding is in accordance with the literature, because bipolar neurons are consistently found to be the far less dominant neuron types in ganglia of most invertebrates (Croll *et al* 2001; Orrha and Muller 2005). Our electron microscopy approach was not an aimed to be a fully detailed study, but revealed that large neurons are organelle rich cells, containing higher amounts of rough endoplasmic reticulum, mitochondria, and secretory granules. The other category of neurons, smaller in size, has less amounts of cytoplasm. Finally, glial cells, even smaller sized then neurons, were identified and classified in three morphotypes, according to their

approximate geometrical shape: fusiform, roundish, and triangular. Scattered in cortex are pigmented cells, distinct by the yellowish cytoplasmic colour and by the fact that the nucleus tends to be located at the margin of the cell body. Those cells also exist in the medulla, but being seen much more rarely when compared with the cortex. We have found only unmyelinated neurites but this is a matter worth further study, namely in light of the divergent data and continuous research about the presence of early-to-recent arrangements of myelination throughout its complex evolution process (Zalc 2006; Castelfranco 2015). Still regarding the neurons cytoplasm, especially of large ones, there are vacuoles/vesicles, which appear as light roundish spaces at light and as membrane bounded bodies of varying electron-density when seen at electron microscopy. Some of these structures have large dense granules inside, much greater than the electron-dense or electron-lucent small vesicles that appear at varicosities or pre-synaptic regions of the axons. These vesicles are thought to serve multiple purposes, from packing of proteins and lipids to storage of neurohormones and neurotransmitters, and they are known to appear in various bivalves (Golding and Pow 1988; Vitellaro-Zuccarello and Biasi 1990; Siniscalchi *et al*. 2004; Meechonkit *et al*. 2010). Herein we also verified that the extent/intensity of serotonin (immunohistochemical) positivity is in visceral ganglia in maturing females and males, higher than that observed in undifferentiated animals (*i.e.*, with spent gonads). This connection with gonad progress is in line with a study of *Hyriopsis* (*Hyriopsis*) *bialata*, which show more intense staining of serotonin in the nerve fibres and termini of the visceral ganglia (Meechonkit *et al.* 2010). It can be concluded that mature *S. plana* specimens have stronger expression than undifferentiated ones, which may be connected with the role of serotonin in promoting bivalve gonad maturation and spawning. Overall, such a result strongly supports the need of further studies to get more baseline data in *S. plana*. Our work (Chapter 4) is the first to report immunoreactivity of neurotransmitters in mature (males and females) *vs*. undifferentiated *S. plana*. We show cells with consistent positivity for serotonin and dopamine in all ganglion types, while using a semi-quantification (pilot) approach of the staining to search for clues of sex-related and age-related differences.

Then, in Chapter 5 both stereological and statistical approaches allowed us to evaluate the cellularity of the nervous ganglia of *S. plana*. Herein it was firstly hypothesized that due to the key involvement of the NG in the gonad differentiation and maturation of bivalves, their microscopic morphology could also vary between sexes. Also, because each NG type seems to have specific functions in bivalves, we further theorized that the location/function of the

NG may shape its size and also cellularity. To start tackling these hypotheses quantitative studies were made to obtain the total ganglion volume and amount of cells in each ganglion, for mature (males and females) and undifferentiated animals. Our data revealed that there is no interaction between gender and ganglia type. Nonetheless the significant effect of ganglia was found in their volumes and compartments (cortex and medulla). The left cerebral and the right cerebral ganglia are much similar in size. Considering all ganglia types, the volumes increase significantly towards the pedal ganglion; which is greater than the cerebral ganglia and much smaller than the visceral ganglion. The cortex and medulla significantly follow the trends of the whole ganglia and, overall, the relative volumes (V_V) of the cortex are ≈ 60% and the medulla ≈ 40%. As to gender differences, one that is statistically valid concerns the total volume, which is greater in females than in the undifferentiated animals, with the males not differing from either of the other groups. Anyway, in whole, cortex, and medulla show significantly higher number of neurons and glia; but not of pigmented cells. The higher total number in the visceral ganglion is likely related with the direct/indirect control of a vast body area, which must be based on more neural cells. We disclosed for the first time that a small bivalve as *S. plana* has a mean total number of neural cells from 12000 (cerebral ganglia) to over 68000 (visceral ganglion). Additionally, the estimation of numerical densities [N_V (cell, containing space)] for all neural cells and in all ganglia, between females *vs*. undifferentiated specimens, was taken into consideration, and we found that undifferentiated animals have ≈ 2.3 more cells per unit of ganglionic volume when compared with either females or males; that are globally quite similar. These inferences suggest that, undifferentiated animals have a similar number of cells in their ganglia fitted into less volume. To conclude, it was estimated the glia-to-neuron ratio. As to the latter, our global data (all ganglia and gender combined) suggest a ≈ 1:1 glia-to-neuron ratio in the cortex, and when joining the medulla the ratio rise to ≈ 1:1.5. Although the factors gender and ganglia type act independently, the exact ratio depends on both of them, with cerebral ganglia having significantly lower ratios, and females showing the mean highest ratio. This are new data for malacology that makes us think about how much intricate and integrative neural networks it offers to the animal, and how it relates with unsolved issues in mollusks physiology; for instance related to nociceptive behavior, with presently at stake repercussions in animal welfare.

The informative potential of using design-based stereological methods have been extended in Chapter 6, to investigate impacts of age of volume and cellularity in two adult size-classes,

and related with the gender (males and females). The Small group (average length of 2.4 cm in either sex) has from 2 to 3 years of age, and the Big group (3.8 cm) (in both sexes as well) has from 3 to 4 years of age. However, this should be viewed as an approximate age because as the length of the animal's shell increases, the age estimation becomes more inaccurate (Green 1957). Irrespective of the lifespan range, and some uncertainty, there is no doubt that we worked with adult animals that differed in ≥ 1 year in age, and that were not extremely old. Importantly for comparative purposes regarding total numbers of neuronal cells, while older specimens were bigger and heavier, females and males of the same age did not differ in mass. As in our previous studies (viz. Chapter 5), the visceral ganglia are more voluminous, followed in size by the pedal, which was bigger than each of the cerebrals. Considering the effect of sex, and combining all animals, males have a marginally (but significantly) greater ganglionic volume, matching with a bigger cortex and medulla. Interganglionic differences were also found when looking at the relative importance of the cortex and medulla, with the visceral and the pedal ganglia having significantly greater relative volumes of the cortex (and smaller in what concerns medulla) compared with the cerebral ones. This scenario is partially in agreement with our previous study (Chapter 5), because earlier we found no differences in the relative volume compartments of visceral *vs*. pedal ganglia. These aspects are still very poorly studied, and for instance age can be one factor that influences both the relative and total volumes. Indeed, in our earlier study we used animals that have mean size values (and therefore age) that are in between those of size-cohorts used here. Our idea is supported by the fact that relative volumes found herein are significantly influenced by age, with big/older animals having greater cortical (and lesser medullar) relative volumes. Transversely to size classes, there is always a tendency for an increase in volume with more numbers of ganglion cells in bigger animals. In summary, as for gender effect, one that is statistically confirmed concerns the total volume of the ganglia, which is greater in males than in the females. As for the effect of ganglia type, visceral ganglia volumes are significantly larger than in pedal and cerebral ganglia. The cortex and medulla evidence basically similar differences' pattern. Importantly is to tackle the question of the possibility that as the animal grows the number of cells could continually increase with age in all central ganglia of this species, in particular the number of neural cells. This will request further studies with bigger and surely older animals; following the fact that body size in bivalves correlates well with age (Ridgway *et al.* 2011). Finally, facing *S. plana* abundance, wide dispersal and tolerance to a range of biotic/ abiotic

factors, and the new neural data being generated, we now view this species as one promising model not only (*e.g.*) in ecotoxicology but also for neurogenesis and age-related researches.

Estrogenic compounds, like ethinylestradiol (EE_2), are widely distributed in the aquatic system. With this is mind we know that the invertebrate nervous system is quite plastic, that steroidal estrogens seem to model diverse physiological processes on bivalves by still unclear ways, that at least some neurons control these organisms' reproductive processes, and that their neuropeptides and neurohormones may functionally interplay with other elements. In addition, but relevant in the context, estrogenic action was shown able to interfere with some mechanisms, *e.g.*, mitogen-activated protein kinases, which are known to govern cell mitosis (Canesi *et al.* 2004, 2007). They may influence the microscopic anatomy of the nervous system of bivalves. Thus in Chapter 7 we start studying the possibility of implications of EE_2 exposure namely in the size (volume) of pedal ganglion and cellularity in *S. plana.* To start testing our hypothesis that EE_2 can cause these (and other) neural impacts. Animals exposed for 5 days to EE_2 (0.05 or 5 μg/L) were compared with those from a solvent control group (0.01% ethanol). The result of the stereological approach provided that they did not show any significant differences in the selected parameters (both sexes did not differ). No significant difference was found among all treatments for the volume and cellularity (negative findings). Anyway, facing the literature background and possible modes of action of steroidal estrogens in both vertebrates and invertebrates, including bivalves, more studies are justified, viz. using more technical approaches/targets, both with acute and chronic exposures in further studies. However, we must bear in mind that our findings do not imply that other sort of structural or functional changes may not have occurred, or that longer exposure periods will not impact on the neural cell numbers. Indeed, in higher taxa we know that estrogens, viz. 17β-estradiol, do have trophic effects on a variety of brain regions and in key neurocytological aspects. The estrogenic effects attain not only neurons but also glial cells, and include functional and structural (morphometric) parameters and proliferation, both in adult and in developing brains (Fowler *et al.* 2005; McCarthy 2008), and we therefore think that more research is needed and justified to extend our tests, namely to less maturated and particularly to undifferentiated specimens. More studies on the nervous and sensory systems of *S. plana* will useful, namely bearing in mind a growing interest in using *S. plana* as bioindicator of aquatic environments.

References

Bullock, T.H. & Horridge, G.A. (1965) *Structure and function of the nervous systems of invertebrates*. W. H. Freeman & Co, London.

Castelfranco, A.M., & Hartline, D.K. (2015) The evolution of vertebrate and invertebrate myelin: a theoretical computational study. *Journal of Computational Neuroscience* 38, 521-538.

Croll, R., Boudko, D. & Hadfield, M. (2001) Histochemical survey of transmitters in the central ganglia of the gastropod mollusc *Phestilla sibogae*. *Cell and Tissue Research* 305, 417 - 432.

Fowler, C.D., Johnson, F. & Wang, Z. (2005) Estrogen regulation of cell proliferation and distribution of estrogen receptor-alpha in the brains of adult female prairie and meadow voles. *Journal of Comparative Neurology* 489, 166-179.

Golding, D.W. & Pow, D.V. (1988) The new neurobiology – ultrastructural aspects of peptide release as revealed by studies of invertebrate nervous systems. In: Thorndyke, M.C. &and Goldsworthy, G.J. (Eds.) *Neurohormones in Invertebrates*. Cambridge University Press. pp. 7-18.

Green, J. (1967) Activities of the siphons of *Scrobicularia plana* (da Costa). *Journal of Molluscan Studies* 37, 339-341.

McCarthy, M.M. (2008) Estradiol and the developing brain. *Physiological Reviews* 88, 91-124.

Meechonkit, P., Kovitvadhi, U., Chatchavalvanich, K., Sretarugsa, P. & Weerachatyanukul, W. (2010) Localization of serotonin in neuronal ganglia of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialata*. *Journal of Molluscan Studies* 76, 267-274.

Orrha, L. & Muller, M. C. M. (2005) Morphology of the nervous system of Polychaeta (Annelida). *Hydrobiologia* 535, 79-111.

Ridgway, I., Richardson, C.A. & Austad, S.N. (2011) Maximum shell size, growth rate, and maturation age correlate with longevity in bivalve molluscs. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 66A, 183-190.

Richter, S., Loesel, R., Purschke, G., Schmidt-Rhaesa, A., Scholtz, G., Stach, T., Vogt, L., Wanninger, A., Brenneis, G., Doring, C., Faller, S., Fritsch, M., Grobe, P., Heuer, C., Kaul, S., Moller, O., Muller, C., Rieger, V., Rothe, B., Stegner, M. & Harzsch, S. (2010) Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary. *Frontiers in Zoology* 7, 1-29.

Roth, G. & Dicke, U. (2013) Evolution of Nervous Systems and Brains. In: Galizia, C.G. & Lledo, P.-M. (Eds.), *Neurosciences - From Molecule to Behavior*, Springer-Verlag Berlin Heidelberg, pp. 19-45.

Siniscalchi, A., Cavallini, S., Sonetti, D., Sbrenna, G., Capuano, S., Barbin, L., Turolla, E. & Rossi, R. (2004) Serotonergic neurotransmission in the bivalve *Venus verrucosa* (Veneridae): a neurochemical and immunohistochemical study of the visceral ganglion and gonads. *Marine Biology* 144, 1205-1212.

Vitellaro-Zuccarello, L., De Biasi, S. & Amadeo, A. (1990) Immunocytochemical demonstration of neurotransmitters in the nerve plexuses of the foot and the anterior byssus retractor muscle of the mussel, *Mytilus galloprovincialis. Cell and Tissue Research* 261, 467-476.

Zalc, B. (2006) The acquisition of myelin: a success story. In: Chadwick, D.J. & Goode, J. (Eds), *Purinergic Signalling in Neuron-Glia Interactions*, No. 276 Wiley, Chichester. pp. 15-25.