Neohelice granulata, a Model Species for Studies on Crustaceans, Volume II

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Anatomy and Physiology

Edited by

Enrique M. Rodriguez and Tomás A. Luppi

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FOREWORD

Neohelice granulata – a model species for studies on crustaceans.

Long species monographs seem to be a thing of the past, probably because few researchers have the luxury or dedication to be able to focus long-term research efforts exclusively on one species or a group of species. This up-to-date monograph on *Neohelice granulata* proves the great value that such monographs still have in current times. While in the past, species or taxon-focused monographs were typically written by single researchers who had spent their entire career investigating their favourite organisms, this monograph on *N. granulata* is a group effort gathering a total of 77 authors, primarily from Argentina. This is a gigantic achievement for the editors, who were able to shepherd such a diverse group of authors for this monograph with a total of 33 chapters. This monograph is published in two volumes, but it is a unit work, indivisible in its conception, realization and integration, evidenced by its numerous cross-references between chapters and volumes.

The result of this effort provides an overview about one of the best-studied crustacean species of the past few decades. From reading some of the chapters, it becomes evident that this is not only a collection of chapters about *N. granulata*, but these volumes are also testimony to the long-term research commitment of the Argentinian crustacean community. Collectively, they have worked over decades to investigate all aspects of the biology and life history of this intertidal crab. These volumes demonstrate the community effort put into every single chapter and becoming evident on almost any single page. At least two authors write most of the chapters, and many by more than two authors, furthermore underscoring this collaborative effort. The editors and their fellow authors are to be congratulated for pulling off this group achievement!

Each volume is divided into two sections, each with a brief introductory preface by one of the eminent scholars in the field. In the first section on life history and developmental biology, the eight chapters explore the early life of *N. granulata*, from embryonic development to larval life. The authors focus on the pelagic stages, settlement biology, and life cycles. In the section on ecology and ecosystem role of the crab, the eight chapters examine *N. granulata*'s predators, forms of food acquisition and the complexity of its mating system as well as their effects on habitat structure and species interactions. The eight chapters in the section on neurobiology cover topics ranging from sensory systems to neural integration including memory and learning. Finally, in the section on anatomy and physiology, the authors present information on the reproductive system, investment in growth and reproduction, general metabolic strategies and respiration of this fascinating crab living at the interface between air and water.

The individual chapters are all relatively concise, and they are richly illustrated, making the reading a pleasant experience. All chapters are written in clear language, making them accessible to readers from related disciplines or people interested in the general biology and ecology of crustaceans.

This work presents *N. granulata* as an ideal model species for future studies on climate change, pollution, habitat degradation, or species invasion. The distributional range of *N. granulata* is one of the areas in the world's oceans that have and will continue to experience substantial changes in seawater temperatures. Not surprisingly, *N. granulata* is increasingly confronted with invasive species, which either have been expanding their geographic ranges or which have been introduced by human activities, e.g. ship transport. For example, in large parts of its natural habitat *N. granulata* will soon be confronted with one of the most aggressive invaders around the world, the shore crab *Carcinus maenas*, which has already established a foothold in Argentinian Patagonia, and is steadily expanding its invaded range to the north. Additionally, *N. granulata* is exposed to industrial and coastal activities, and one of the chapters in the volume on endocrine disruption examines the effects of pollution on reproductive traits and success in *N. granulata*. This is the only chapter on anthropogenic impacts on this marvellous little crab from the South-West Atlantic, but future studies on human impacts will be able to build on the enormous and complete knowledge provided in this volume.

These volumes should be an inspiration for others, who might have similarly rich and integral information on their local crab species – I could easily envision several well-studied crabs that would merit a similar volume.

In the meantime, the scene is set for *Neohelice granulata* to serve as a model organism for scientists from different fields and regions of the world. This work provides researchers with all the basic knowledge needed to thoroughly examine whether and how this common intertidal crab is adapting to the changes occurring in their natural habitats.

Martin Thiel, Coquimbo, Chile

PREFACE

The idea of editing this book arose from the need to compile as much information as possible about the biology of a species that has been a model for many years for several research groups, mainly from Argentina and Brazil. Ernesto Sábato, an Argentine physicist and writer, used to say that the scientific world is evolving more and more towards specialization, being increasingly difficult or rare to find "generalists" who summarize and obtain conclusions from a large amount of specific information accumulated on each particular topic. This book does not pretend to obtain such conclusions, but to facilitate the reader to integrate the entire results obtained by most of the groups that have worked with this model species.

This book was in some way inspired by several forerunning events, at first, the workshops on "Cangrejos and Cangrejales", held in various venues. The first one took place in Buenos Aires, in June 1988, where research groups from Argentina, Brazil, and Uruguay participated; most of these groups were just beginning with their research in *Neohelice granulata*, a species characterized by the pioneer Enrique Boschi as the most conspicuous of the so-called "Cangrejal Bonaerense". The second workshop was held in Mar del Plata in November 1994 and was able to confirm the important progress of the many participant groups. In December 1996 was held the third workshop in Rio Grande, Brazil, and the fourth and last was held in Buenos Aires, in April 1999. All these meetings bolstered a fruitful exchange of information and discussion of results, also favouring the development of several joint projects between different research groups. However, the closest historical background of this book dates from the meeting that took place in Rio Grande, Brazil in April 2005, where it was proposed the publication of a book about the biology of *Neohelice*, which unfortunately did not materialize. Today we can say with pride that finally this task has been fulfilled.

This book not only intends to be a collection of information about *N. granulata* and a data source for this species, but it also aims to be a reference book for any researcher studying the biology of crustaceans, and even a reference for other disciplines in which the species serves as a representative model. The reader will save a lot of time in the search and integration of information, considering that the book concentrates in a single source practically all existing references focusing on this crab. Any researcher, who initiates a project concerned either about the environment inhabited by the crab or any other subject in which the crab is a model system, should necessarily refer to this book as a source of information. Finally, the third objective of this book is to contribute to academic teaching, both undergraduate and post-graduate, as a book for study and consultation, potentially useful in many areas of biology, anatomy, physiology, and invertebrate ecology.

Indeed, *N. granulata* is nowadays one of the most studied crab species. This species has been a very successful model for studies on ecophysiology: embryonic, larval, juvenile, and adult development; neurobiology; phenotypic plasticity; and an excellent model for understanding the ecosystem function of marine and estuarine species. On the other hand, the large body of literature produced during the last decades has been intimately linked to the training of graduate students and post-graduate researchers in Brazil, Uruguay, Argentina, and Germany, contributing to the development of cooperation projects among researchers from most of the countries above mentioned.

This book is divided into two volumes. The first volume comprises 17 chapters grouped into two main sections: "Life history traits and development" and "Inter-specific relationships, behavioural ecology and ecosystem functionality". On the other hand, the second volume comprises 16 chapters, also grouped into two sections: "Neurobiology" and "Anatomy and physiology". Both volumes bring together more than 70 authors from 6 countries. Each of the main sections is prefaced by a specialist, "Life history traits and development" by Dr. Klaus Anger, "Inter-specific relationships, behavioural ecology and ecosystem functionality" by Dr. Oscar Iribarne, "Neurobiology" by Drs. Daniel Tomsic and Arturo Romano, and "Anatomy and physiology" by Dr. Enrique Rodriguez. Each of them gives his vision about the relevance that *N. granulata* in the development of each research area. In an appendix, we include the updated list of all published papers, as an additional contribution to the edition of the different chapters.

We do not want to end this prologue without first thanking each of the colleagues who have devoted their time to the development of one or more chapters, and who have responded promptly to our demands for editing. Our thanks also to the reviewers of each chapter, as well as to those who have prefaced each section of the book. At the same time, we want to invite colleagues who work in *Neohelice,* but for some reason have not been able to contribute to this first edition, to contact us and contribute to future editions of the book.

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NEOHELICE GRANULATA BACKGROUND KNOWLEDGE

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The burrowing and semi-terrestrial crab *Neohelice granulata* Dana, 1851, (formerly known as *Chasmagnathus granulatus*) is a key South American species, endemic to the warm temperate coast of the southwestern Atlantic. This species is found along more than 4000km of the coastline of Argentina, Uruguay, and Brazil. Its geographical distribution (Fig. 1) ranges from northern Patagonia (42°25′S; Riacho San José, Chubut Province, Argentina) to southern Brazil (22°57′S; Lagoa Ararurama, Rio de Janeiro State, Brazil).

It is strictly associated with tidal flats in estuaries, bays, and coastal lagoons, where the halophyte cordgrasses are *Sporobolus densiflorus* (previously known as *Spartina densiflora*), *Sporobolus alterniflorus* (previously known as *Spartina alterniflora*) and *Sarcoconia perennis* form marshes located in the middle and upper intertidal zone. *Neohelice granulata* has a complex life cycle with benthic adult and juvenile stages, and a planktonic phase (four or five zoeae and one megalopa stage). Larval release from females is synchronous, and freshly hatched zoeae are exported from several parental habitats, e.g. those with low salinity waters on ebb tides. These larvae were only found in nearshore waters (marine or with moderately reduced salinities), which seem to be more favourable for development. The megalopa stage migrates from coastal waters to marine, brackish and even limnetic habitats. It is capable of swimming actively and consequently, selectively settling near their respective adult populations, while moulting to the first juvenile stage.

The habitat of *N. granulata* includes different environmental conditions. Along with its extensive and yet discontinuous geographical distribution (» 22° latitude), they inhabit areas with highly variable tidal range, salinity, temperature, sediment, and biotic factors. As a consequence, crabs must face many challenges, for instance: from a few cms up to 9m of semidiurnal tide amplitudes, with a high variance in predictability; from near 0 up to 60psu of water salinity; from soft to hard substrata (muddy and cobble beaches, respectively), from high to low-quality food, and also facing different predation pressures. Besides, these crabs live in mudflats or salt marshes placed at different intertidal levels, where adults of both sexes and juveniles are intermixed, with various emersion-submersion patterns, burrow size, shape and dynamics, physical and chemical characteristics of water, feeding habits and activity level.

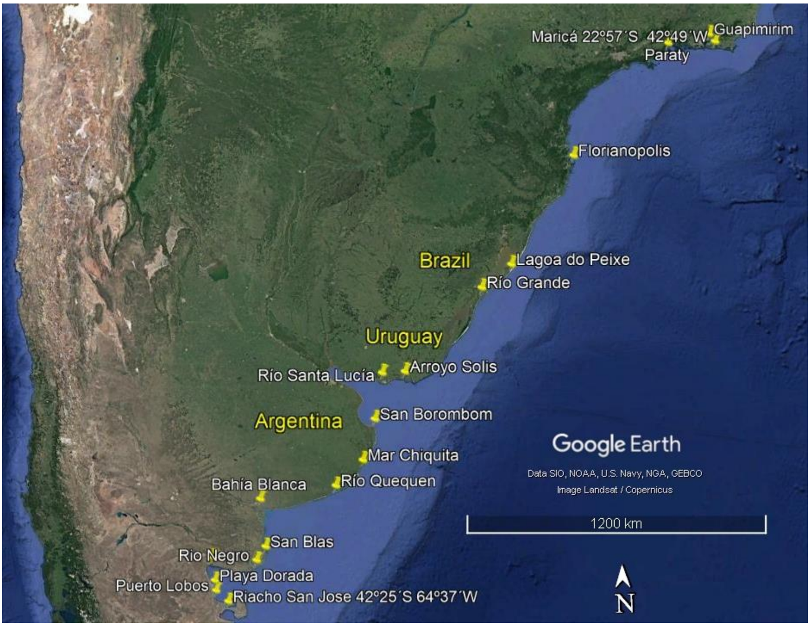

Figure 1. Locations where populations of *Neohelice granulata* have been recorded. The coordinates of the populations at the ends of the distribution are indicated.

High levels of genetic structure were detected among populations of *N. granulata* inhabiting the complete geographic range of the species, indicating that connectivity between adjacent sites may be limited to scales of hundreds of kilometres. However, such genetic disparity not always correlates with morphometric analysis; in fact, no clear geographical pattern of morphological differentiation could be observed.

Neohelice granulata digs semi-permanent burrows and is active both in air and water, given its bimodal respiration capacity. During low tides, crabs leave burrows and perform short “exploratory excursions”, but they hide in the presence of aerial predators or cannibal behaviour. Crabs are also active underwater, carrying out longer excursions, especially during high tides, and their feeding activities rise dramatically in the vegetated habitat during flooding.

Juveniles and adults feed on saltmarsh plants and sediments. The proportion of each food item in the diet will depend on location, both on an intra-populational spatial scale (mudflat vs saltmarsh), and an inter-population scale; in both cases, marked differences can exist in the content of organic matter associated to sediments, therefore influencing several life-history traits such as growth, reproduction, and cannibalism.

Temperature and photoperiod are relevant environmental cues for reproduction. Ovaries develop under the control of several hormones, and marked differences among populations were noticed during the annual maturation cycles. Spawning is restricted to spring and summer in temperate sites, but ovigerous females can be found throughout the year in tropical populations. Number of eggs in each spawning, even higher than 30000, depends on crab size among other factors. The egg incubation period normally ranges from 15 to 20d, according to temperature and salinity. Fecundation is internal, and the mating system is flexible, with a combination of strategies depending on the size of crabs and type of burrows.

In temperate zones, sexual maturity is reached between one year and one year and a half, based on the time of the year when the first juvenile stage starts to grow. Longevity is estimated between two and a half and three and a half years, depending on environmental conditions and sex.

SECTION ONE

NEUROBIOLOGY

Decapod crustaceans are traditional models in neuroscience research. Investigations in crayfish, lobsters, and crabs, have provided insights of general neurobiological interest relating to matters as diverse as the identification and function of neurotransmitters, the operation of electrical synapses, the organization of neural substrates involved in behavioural decision-making, coincidence detection as a mechanism to trigger behavioural responses, the way in which social hierarchy can affect the properties of individual neurons, the neural computations underlying visuo-motor transformations, and the neural and molecular mechanisms of long-term memory formation and consolidation (Tomsic and Sztarker, 2019). These accomplishments have been feasible due to particular advantages offered by decapod crustaceans for the neurophysiological approach, such as the presence of giant neurons with powerful computational capabilities that are easily accessible for electrophysiological recording, together with the animal's strength for experimental manipulations and their readiness to behave in laboratory conditions.

In the last three decades, the crab *Neohelice* (previous *Chasmagnathus*) *granulata* has become a major protagonist among the invertebrate animals used for investigation in neuroscience. In particular, studies in *Neohelice* focused on two important areas: neurobiology of learning and memory and neural control of visually guided behaviour. The first theme was initiated and led by Professor Héctor Maldonado, who developed a variety of learning and memory paradigms in the crab that included: habituation of escape and exploratory responses, classical conditioning and operant conditioning of avoidance and appetitive behaviours, and context-signal associative memories. The neural mechanisms underlying these memories were originally studied using mainly a combination of behavioural and pharmacological methods (reviewed in Tomsic and Maldonado 2014). The investigation was later enriched and expanded by contributions of several Maldonado´s disciples, including the authors of this preface, which applied a wide variety of classical and state of the art techniques such as neuroanatomy, in vivo intracellular electrophysiology and molecular biology (reviewed by Tomsic and Romano 2013).

The second area in neurobiology where *Neohelice* is internationally well recognized as a model of study is visual processing (reviewed by Tomsic 2016). More precisely, the neural organization and computational processes underlying visually guided prey and predator behaviours in arthropods. These investigations initiated and led by professor Tomsic, encompass studies in the field and the laboratory, and include behavioural, anatomical, electrophysiological and functional imaging techniques. The anatomical studies of *Neohelice* visual system represent the most detailed description of the optic neuropils currently available in any crustacean. Electrophysiological investigations, on the other hand, led to the first account of identified neurons reflecting long-term visual memories in an arthropod.

The interest around the two themes just mentioned often drifted toward other fundamental topics in neuroscience, some of which are reflected in the different chapters contained in this section. Chapter 1 provides the reader with an introductory description of the general organization of the central nervous system of *Neohelice*, whereas Chapter 2 provides a detailed description of its visual nervous system. Chapter 3 describes the characteristics of a variety of identified individual neurons involved in different aspects of visual processing, with an emphasis on motion-sensitive cells. Chapter 4 summarizes extensive work on the cellular and molecular mechanisms that proved to be fundamentals to the formation of long-term memory. Chapter 5 presents the foremost discoveries that contributed to solving part of the debate on reconsolidation and extinction. Chapter 6 explains discoveries on the modulation of acquisition and expression of long-term memories. Chapter 7 illustrates how two different memories can compete and prevail over the other. Finally, Chapter 8 describes the use of the heart rate as a sensor for sensorial perception in the crab.

Taken together, the information contained in these chapters reflect much of what it is currently known about the neural processes underlying visually guided behaviours of crustaceans, including the cellular mechanisms subserving long-term visual memories. It is worth noting that *Neohelice granulata* is the species of crustacean in which the most compelling research on behavioural neurobiology has been and continues being done.

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CHAPTER ONE

CENTRAL NERVOUS SYSTEM OF THE CRAB *NEOHELICE GRANULATA*: A NEUROANATOMICAL DESCRIPTION.

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Introduction

We present a study of the CNS of *Neohelice granulata* performed at both the neuronal cluster and the neuropil level, describing eyestalk ganglia, the thoracic ganglion, and the central brain, with special focus on the latter. This characterization is based on the functional study of the crab´s nervous system during two main phases: visual information processing, and storage of memory. The nervous system delineation uses classical histological techniques as well as immunohistochemistry to characterize cellular clusters and neuropils. This is the first general description of *N. granulata* nervous system as a whole, at neuropil and cellular level. Few representations of the central nervous system of *N. granulata* have been published besides the general scheme published by Bond-Buckup et al. (1991).

To make available a more detailed and complete picture of the CNS of *N. granulata*, we compiled the results of previous research on the immunohistochemical and physiological origin. Each of the major ganglia was delineated then, as well as the nerves that connect ganglia, including the eyestalk ganglia (EG), the central brain (CBr), and the thoracic ganglion (TG). A "modular" and an anatomical segmentation is retained among Eumalacostraca; this significant feature allows us to trace the sensory inputs from the various receptor systems to the neuropils (Sandeman et al. 2014). The brain of the Malacostracans has significant areas dedicated to processing inputs from visual, chemical, and mechanical receptor systems. Some of these neuropils (e.g., olfactory lobe, accessory lobe) have structural features and connectivity characteristics that made them identifiable, as compared to different Malacostracan species (Krieger et al. 2012). A "ground pattern" has been proposed for the decapods (Sandeman and Scholtz 1995), which can be used as a template for *N. granulata* because common neuropils can be identified. These maps and nervous system representations would allow us to make comparisons with other invertebrate models, establishing analogies/homologies with previously described neuroanatomical descriptions of different species of decapods, (e.g. Sandeman et al. 1992).

The nomenclature that was used for the construction of the present scheme coincides with the one proposed in the literature (Sandeman et al. 1992, Richter et al. 2010). We have a particular interest in one of the central ganglia of the CNS, the CBr since it is postulated as an important nervous centre for sensory processing and multimodal integration required for memory storage. Evidence for these functions came from neuroanatomical and comparative data indicating multimodal integration in the CBr (Sandeman 1982, Utting et al. 2000); it has also been shown that the CBr is extremely important for learning and the formation of memories (e.g. Freudenthal et al. 1998, Freudenthal and Romano 2000, Hepp et al. 2016).

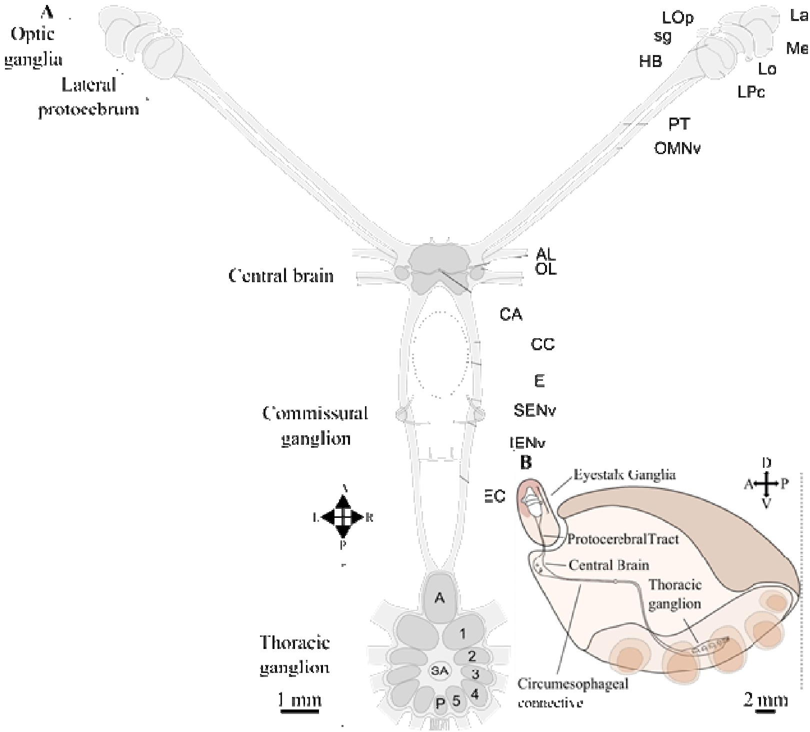

Figure 1.1. A. General organization of the *Neohelice granulata* central nervous system. The optic neuropils (La, lamina; Me, medulla; Lo, lobula; Lop, lobula plate), sinus gland (sg) and the lateral protocerebrum (LPc) are contained in the eyestalk. The protocerebral tracts (PT) and the oculomotor nerves (OMNv) project from the eyestalk ganglia to the central brain. The central artery (CA) marks the midline. In this ganglion, two conspicuous structures, the olfactory lobes (OL), are discernible in regular dissections, adjacent to the accessory lobe (AL). The posterior nerves, termed circumesophageal connectives (CC), surround the esophagus (E) and reach the commissural ganglia, which is connected to the stomatogastric ganglia by the superior and inferior oesophagal nerves (SENv and IENv, respectively). The CC are linked by the postesophageal commissure (EC) and end in the thoracic ganglion, where five pairs of symmetrical limb-associated neuropils are identified (from 1 to 5), as well as two non-bilateral neuropils, A and P. The centre of the thoracic ganglion is demarcated by the sternal artery (SA). B (inset). Diagram of the crab body containing the central nervous system. Lateral

view, excluding the chelae and walking limbs, showing the EG located in the eyestalks, the CBr situated medially in the anterior ventral side, and the CC running in a rostrocaudal direction to the commissural ganglion, and then toward the most ventral surface of the cephalothorax, ending in the TG. The insertions of limbs are represented with dotted lines. The crosses indicate positions: A, anterior; P, posterior; L, Left; R, right; D, dorsal and ventral. Modified from Hepp et al. (2013).

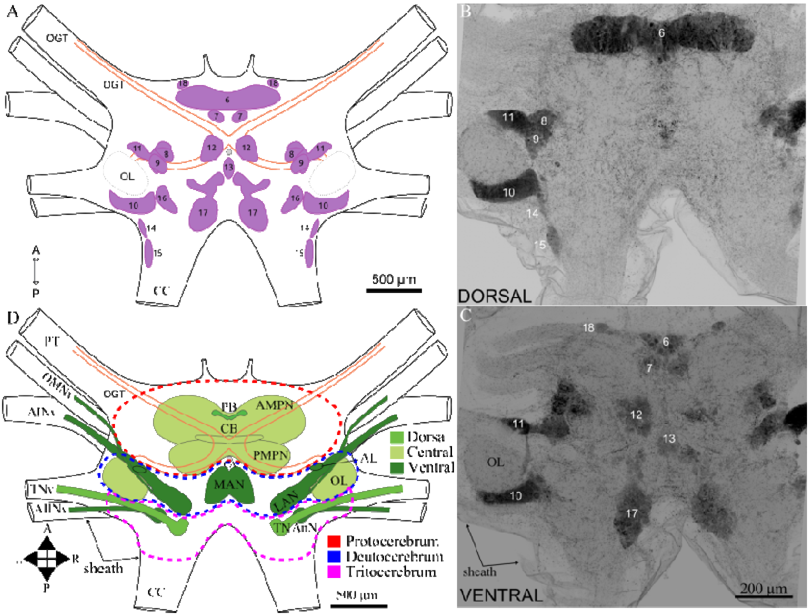

Figure 1.2. A. Map of the central brain (transverse view, flat representation). Localization of its clusters (groups of somas), numbered from 6 to 17, from anterior to posterior and according to the nomenclature reported by the literature. A. The olfactory globular tract (OGT) and the olfactory lobules (OL) were plotted. B and C show representative extended focus images of the whole CBr stained with post-fixation propidium iodide, which allows characterizing the cytoarchitecture and cell morphology of the identified clusters; dorsal (B) and ventral (C) extended focus views are displayed. D. Map of the CBr with conspicuous neuropils detailed. AMPN, anterior medial protocerebral neuropil; PMPN, posterior medial protocerebral neuropil; PB, protocerebral bridge; CB, central body; MAN, medial anterior neuropil; LAN, lateral antenna neuropil I; TN, tegumentary neuropil; AnN, antenna II neuropil; OL, olfactory lobe; AL, accessory lobe (accessory neuropil). Main connectives: OGT, olfactory globular tract; OMNv, oculomotor nerve; AINv, antenna I nerve; TNv, tegumentary nerve; AIINv, antenna II nerve.