ADVANCES IN MARINE BIOLOGY

Academic Press is an imprint of Elsevier
125 London Wall, London, EC2Y 5AS, UK
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA
225 Wyman Street, Waltham, MA 02451, USA
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, UK

First edition 2015

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ISBN: 978-0-12-803305-0
ISSN: 0065-2881

CONTRIBUTORS TO VOLUME 71

Elvira Alvarez
Instituto de Investigación en Medio ambiente y ciencia Marina (IMEDMAR-UCV), Universidad Católica de Valencia, Calpe, Alicante, Spain

Lorena Basso
Global Change Department, IMEDEA (CSIC-UIB), Instituto Mediterráneo de Estudios Avanzados, Esporles, Mallorca, Spain

Ricardo Calado
Departamento de Biologia & CESAM, Universidade de Aveiro, Aveiro, Portugal

Salud Deudero
Instituto de Investigación en Medio ambiente y ciencia Marina (IMEDMAR-UCV), Universidad Católica de Valencia, Calpe, Alicante, Spain

Carlos M. Duarte
King Abdullah University of Science and Technology (KAUST), Red Sea Research Center (RSRC), Biological and Environmental Sciences and Engineering (BESE), Thuwal, Saudi Arabia

José R. García-March
Instituto de Investigación en Medio ambiente y ciencia Marina (IMEDMAR-UCV), Universidad Católica de Valencia, Calpe, Alicante, Spain

Iris E. Hendriks
Global Change Department, IMEDEA (CSIC-UIB), Instituto Mediterráneo de Estudios Avanzados, Esporles, Mallorca, Spain

Miguel Costa Leal
Department of Fish Ecology and Evolution, EAWAG: Swiss Federal Institute of Aquatic Science and Technology, Center for Ecology, Evolution and Biogeochemistry; Kastanienbaum, Switzerland

Alex D. Rogers
Department of Zoology, University of Oxford, Oxford, United Kingdom

Jesse M.A. van der Grient
Department of Zoology, University of Oxford, Oxford, United Kingdom

Maite Vázquez-Luis
Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Baleares, Palma, de Mallorca, Spain

Nardo Vicente
Institut Océanographique Paul Ricard, Ile des Embiez 83140 Six Fours les Plages (France) and Institut Méditerranéen de la Biodiversité et de l'Ecologie marine et continentale (IMBE), Aix-Marseille Université, Aix-en-Provence, France

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PREFACE

Marine invertebrates play widely varying roles, sometimes as keystone species, in ocean ecosystems. Each of the three contributions to this volume focuses on a different aspect of marine invertebrate biology. The topics range from trophic ecology of benthic marine invertebrates with biphasic lifestyles to understanding regional and taxonomical variation in deep-sea meio- and macrofaunal organisms, and extend to an overview of the biology and conservation status of the pen shell, *Pinna nobilis*, in the Mediterranean Sea. An underlying theme occurring in each of these contributions is the increasingly urgent impact that anthropogenic threats, such as habitat degradation, ocean acidification and other aspects of climate change, pose to ocean ecosystems. We still do not fully understand how these threats and the loss of biodiversity, which may occur mostly as changes in local species richness, can affect key ecological processes such as ecosystem primary productivity, decomposition rates and nutrient cycling. Climate change is already having far-reaching consequences that may in part stem from shifts in abundance, distribution and phenology of marine invertebrates causing ecosystem-level effects including changes in food webs, community structure and interspecies relationships. The work in this volume underscores the need to assess our knowledge of marine invertebrates in a relatively standardized, systematic way that can allow for metadata-driven comparative analysis and encourage innovative research methodologies that will advance scientific knowledge and produce effective conservation measures.

BARBARA E. CURRY

CHAPTER ONE

Trophic Ecology of Benthic Marine Invertebrates with Bi-Phasic Life Cycles: What Are We Still Missing?

Ricardo Calado[*,1], **Miguel Costa Leal**[†,1]

[*]Departamento de Biologia & CESAM, Universidade de Aveiro, Aveiro, Portugal
[†]Department of Fish Ecology and Evolution, EAWAG: Swiss Federal Institute of Aquatic Science and Technology, Center for Ecology, Evolution and Biogeochemistry; Kastanienbaum, Switzerland
[1]Corresponding authors: e-mail addresses: rjcalado@hotmail.com; miguelcleal@gmail.com

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Advances in Marine Biology, Volume 71
ISSN 0065-2881
http://dx.doi.org/10.1016/bs.amb.2015.07.001

Abstract

The study of trophic ecology of benthic marine invertebrates with bi-phasic life cycles is critical to understand the mechanisms shaping population dynamics. Moreover, global climate change is impacting the marine environment at an unprecedented level, which promotes trophic mismatches that affect the phenology of these species and, ultimately, act as drivers of ecological and evolutionary change. Assessing the trophic ecology of marine invertebrates is critical to understanding maternal investment, larval survival to metamorphosis, post-metamorphic performance, resource partitioning and trophic cascades. Tools already available to assess the trophic ecology of marine invertebrates, including visual observation, gut content analysis, food concentration, trophic markers, stable isotopes and molecular genetics, are reviewed and their main advantages and disadvantages for qualitative and quantitative approaches are discussed. The challenges to perform the partitioning of ingestion, digestion and assimilation are discussed together with different approaches to address each of these processes for short- and long-term fingerprinting. Future directions for research on the trophic ecology of benthic marine invertebrates with bi-phasic life cycles are discussed with emphasis on five guidelines that will allow for systematic study and comparative meta-analysis to address important unresolved questions.

1. INTRODUCTION

Benthic marine invertebrate taxa commonly display complex bi-phasic life cycles that include pelagic small-sized (often microscopic), free-living and dispersive larval stages that dwell in the plankton in a pelagic environment (Thorson, 1950). Pelagic larvae spend a variable time frame in larval form, which may span from a few hours to days, weeks or even months, and experience more or less pronounced shifts in their external and internal morphology, as well as in physiology and behaviour. Afterwards, larvae undergo through an event of dramatic transition—metamorphosis. As noted by Pechenik (2006), "metamorphosis is a time of great revolution". At metamorphosis these organisms shift from a pelagic to a benthic (or bentho-pelagic) lifestyle, with a number of morphological and physiological changes taking place in a very short period of time.

Bi-phasic life cycles were once a bottleneck to marine research. Studies addressing larval biology were not usually planned to continue beyond metamorphosis, while those addressing adult benthic forms commonly overlooked embryonic and larval history of the specimens under investigation. For several decades, manuscripts simultaneously addressing larval and juvenile/adult traits of benthic marine invertebrates were rare (Pechenik, 2006).

This non-integrated perception of marine life cycles started to shift in recent decades with a number of landmark studies, which indicated that embryonic and larval history significantly influence the post-metamorphosis performance of juvenile and adult life stages and ultimately contribute to shape population size and structure (Figure 1; Allen and Marshall, 2010; Giménez, 2006, 2010; Pechenik, 1990, 1999, 2006; Pechenik et al., 1998). Additionally, issues related to maternal effects on offspring fitness have also received the attention of researchers studying benthic marine invertebrates (Marshall et al., 2008a,b), which clearly reveals that the only way to gain an in depth knowledge on these bi-phasic life cycles is by using an holistic approach addressing their pre- and post-metamorphosis history.

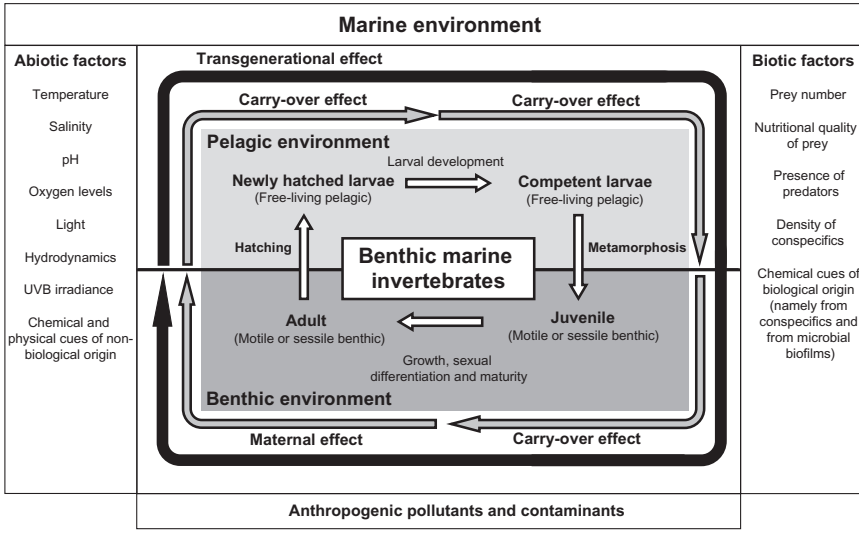

Figure 1 Schematic representation of the bi-phasic life cycle of a benthic marine invertebrate with its main life forms (adult and juvenile life forms considered in the present work may display a benthic or bentho-pelagic lifestyle). Larvae of benthic marine invertebrates may hatch from embryos: (i) brooded by the mother in more or less specialised structures, (ii) from egg masses or egg capsules laid in the substrate, or (iii) from embryos that undergo embryonic development in the plankton resulting from gamete fertilisation in the water column. Embryos from the third category (iii) are the ones first experiencing the influence of the pelagic environment, not newly hatched larvae. Larvae may display a more or less prolonged larval life relying on exogenous food sources (planktotrophy) or endogenous reserves (lecithotrophy). Competent larvae refer to larval stages already receptive to cues that may trigger metamorphosis. White arrows represent key events in the life cycle of benthic marine invertebrates. Grey and black arrows refer to phenotypic links among different life stages.

Initial estimates by Thorson (1950) on the prevalence of larvae that rely on exogenous food derived from the plankton to thrive (planktotrophic) and develop for long periods of time (weeks to months) were probably too high for benthic marine invertebrates (see Giangrande, 1997). Nonetheless, it is somehow unanimously accepted by researchers that long-term planktotrophic larval development is a feature that is more common in marine invertebrate taxa than short-term planktotrophic development, where larvae feed and dwell in the plankton for only a few hours or days. The prevalence of longer over shorter larval development times is also valid for species displaying lecithotrophic development, i.e. non-feeding larvae. Lecithotrophy refers to larvae that rely on maternal reserves to fuel larval development and do not require (or are unable) to feed on exogenous food items. Direct development, where an *imago* of the adult emerges from embryonic development without the occurrence of a free-living larval stage, is less common in benthic marine invertebrates, with some taxa even displaying dimorphic embryonic pathways in the same egg mass (see review by Dionísio et al., 2013). It is important to highlight that while the dichotomy between these two broad nutritional categories (planktotrophy vs. lecithotrophy) is certainly useful for researchers, several larval forms do not perfectly fit in either of these categories, and some studies suggest that facultative feeding is not as rare as previously assumed (e.g. Allen and Pernet, 2007).

The existence of planktotrophic larvae that may dwell in the plankton for several weeks or months allows benthic marine invertebrates to disperse to new regions and/or habitats, as well as the production of larger broods that would not be possible solely through the provision of maternal energy stores (Pechenik, 1999; Strathmann, 1985). This larval ability to survive in the pelagic environment for relatively long periods of time and, consequently, to disperse to geographically distant areas may also be displayed by embryos developing in the water column, which is paramount for increasing connectivity. Connectivity is defined as the exchange of individuals among marine populations (Cowen and Sponaugle, 2009). However, prolonged larval development may also result in larvae "over-dispersing" beyond habitats that are favourable for subsequent benthic life (Pechenik, 1999). Planktotrophic larval development is, therefore, a key life history trait that notably determines the success of juvenile and adult forms. Such development in life history evolution dates back hundreds of millions of years in numerous marine taxa and several authors have claimed the ancient nature of planktotrophy (see Nielsen, 1998, 2009), which contrasts with the repeated evolution of

lecithotrophic larvae in a number of different marine phylogenetic groups (Davidson et al., 1995; Strathmann, 1985; Wray, 1995).

Larvae of benthic marine invertebrates may hatch from embryos that result from gamete fertilisation in the pelagic environment where embryonic and larval development may also occur. Otherwise, larvae result from embryos that are brooded by females on more or less specialised structures, a feature commonly exhibited by taxa with some degree of parental care. In addition, larvae may hatch from egg masses or capsules that are laid over a variety of substrates. In any of these three early-life processes, developing embryos are exposed to a number of abiotic factors, such as shifts in temperature, salinity and oxygen, as well as to a range of contaminants and pollutants that may condition their post-hatching performance (Pechenik, 1999). While embryos brooded by females may not be as vulnerable to suboptimal environmental conditions as those present in egg masses (i.e. capsules laid in the substrate or embryos developing in the water column), brooded embryos may still be negatively affected under certain biotic and abiotic scenarios. The packing of significant numbers of embryos, which may vary from hundreds to tens of thousands, poses serious constraints to the diffusion of oxygen to the central region of those egg masses (Strathmann and Strathmann, 1995). Although energetically demanding for females, active brooding care may minimise hypoxia (Baeza and Fernandez, 2002). Exposure to hypoxia during embryonic development is known to affect not only embryos, but also subsequent larval stages. A reduced number of juveniles may emerge from broods exposed to hypoxia, with those juveniles also displaying a reduced growth and survival (Segura et al., 2014). Exposure to low salinities during embryonic development, even in embryos being brooded by females, may also have a dramatic impact on their larval development (Giménez, 2006). Nonetheless, it is worth highlighting that embryos of some benthic marine invertebrate taxa may be less prone to this type of saline stress due to the protection offered by egg capsules (Pechenik, 1982) or because the habitat occupied by ovigerous females is less environmentally variable (Bas and Spivak, 2003). Overall, it is now evident that the consequences of certain environmental fingerprints during embryonic development can be carried over to succeeding life stages (see the following sections).

The trade-off between the size and number of offspring has also been addressed by researchers studying benthic marine invertebrates, and the theoretical background supporting this trade-off can be summarised as follows: while investing in small-sized offspring may lower its viability, producing large-sized offspring may result in a lost opportunity to increase fecundity

for a particular environment (Marshall et al., 2008b). Initially, environmental factors were considered as the main drivers determining the balance in this trade-off between offspring size and number (McGinley et al., 1987). However, the scenario is certainly far more complex than previously considered. For instance, simply using size as a proxy to monitor embryo fitness and speculate on their success during larval development until settlement, as well as the trade-off between offspring size and number, is certainly not the most accurate eco-physiological trait, as size can be a poor proxy to estimate energetic content within and among marine invertebrate taxa (Moran and McAlister, 2009). Intraspecific competition is also known to play an important role with some taxa (e.g. bryozoans) displaying the ability to shift offspring size in order to maximise maternal fitness and consequently shift the phenotype of offspring over different life history stages (Allen et al., 2008). Moreover, optimal offspring size may depend on the environment experienced within and among different life stages, thus requiring researchers to survey multiple stages/events in the life cycle of these benthic marine invertebrates, such as fertilisation, larval development, metamorphosis, juvenile growth and gametogenesis (Allen and Marshall, 2014). As emphasised by the previous authors, offspring size is not a reversible trait and once the offspring is no longer dependent on its mother, no unused resources from maternal provisioning can be reclaimed, nor can mothers suppress any potential deficiencies that occurred when investing in oogenesis.

Carry-over effects—terminology commonly employed in the ecological literature—refer to features that originate in the developmental history of embryos and larvae that often stay latent for variable periods of time and re-emerge in subsequent juvenile and adult stages (Pechenik, 2006). Hence, carry-over effects are also known as latent effects. To encompass the true complexity of benthic marine invertebrate life cycles one must also take into consideration the existence of trans-generational and maternal effects that are propagated along the life cycle (Giménez, 2006; see Figure 1).

At a certain point of larval development, larvae of benthic marine invertebrates become competent and start to respond to a number of external cues that can trigger metamorphosis in habitats that are suitable for early benthic life (Hadfield et al., 2001). Metamorphosis can be defined as: "a developmental process that is preceded by a functional, free-living larval stage and results in a functional juvenile stage; it typically involves a loss of larval characters and emergence or functionalisation of juvenile characters" (Hadfield et al., 2001). We note that juveniles of some motile taxa may latter migrate to more favourable habitats for adult life. Therefore, the habitat

where competent larvae chose to settle is not necessarily one that juveniles and adults will be restricted to.

Metamorphosis is a rather rapid process in benthic marine invertebrates, as organisms are particularly vulnerable to predation during this event. Therefore, there is a "need for speed" when benthic marine invertebrate larvae undergo metamorphosis, as fast as biologically possible, as previously discussed by Hadfield (2000). Several external cues can trigger metamorphosis in competent larvae (Hadfield and Paul, 2001), and environmental conditions such as hydrodynamics, salinity, temperature and light, have been thoroughly investigated (Pawlik et al., 1991; Queiroga and Blanton, 2005; Rodriguez et al., 1993). Larval age (Howard and Hentschel, 2005) and condition (from a nutritional point of view) (Jeffs et al., 1999) may also affect larval competence. Some benthic marine invertebrate taxa may be sensitive to cues that trigger metamorphosis at significant distances from their juvenile/adult habitats and undergo remarkable migrations of several hundred kilometres (Jeffs et al., 2005). Competent larvae are also commonly dependent on the detection of chemical cues associated with the settling substratum, which are often produced by surface-associated microorganisms (Holmstrom and Kjelleberg, 1994). These cues may also originate from conspecific adults and prey (Qian, 1999; Zimmerfaust and Tamburri, 1994). The processes and cues that trigger metamorphosis in benthic marine invertebrates with planktonic larval stages in their life cycles has received attention not only from researchers working on marine ecosystem and population dynamics but also from those working on chemical ecology (Pawlik, 1992), behaviour (Burke, 1983), locomotion (Chia et al., 1984) and more applied topics such as biofouling/antifouling issues (Fusetani, 2004) and aquaculture (Roberts, 2001).

For several benthic marine invertebrate taxa, a delay in the timing of metamorphosis commonly prompts a decrease in larval condition (e.g. energetic reserves) and may even impair metamorphosis (Pechenik, 1990). Moreover, a delay in metamorphosis may negatively affect the growth performance (Pechenik et al., 1998) and enhance post-settlement mortality in young juvenile specimens (Hunt and Scheibling, 1997). It has been shown that metamorphosis does not erase embryonic or larval history and for the majority of benthic marine taxa it is certainly not a new beginning (Pechenik, 2006; Pechenik et al., 1998; Rey et al., 2015). It is therefore paramount to know the pre- and post-metamorphosis history of benthic marine invertebrates to understand the underlying mechanisms regulating population dynamics and shaping their evolutionary history. The influence

of several environmental factors on pre- and post-metamorphosis performance of benthic marine invertebrates is already well documented (Menge and Sutherland, 1987). Temperature is probably the one most commonly studied (Sanford, 2002), namely, due to the impacts that warming oceans may have in marine taxa and pre- and post-metamorphic processes (Petchey et al., 1999; Sanford, 1999). The interaction of such an important environmental factor with trophic ecology has been addressed in numerous studies focusing benthic marine invertebrates (Burgess and Marshall, 2011a; Hoegh-Guldberg and Pearse, 1995), as it is important to evaluate how swiftly a species can respond to a changing environment, as well as its effect on the diversity, abundance and quality of food items. However, as emphasised by Padilla et al. (2014), in order to gain knowledge on the role played by trophic ecology, it is important that researchers report key metadata that can allow a feasible comparison between studies. The role of metadata is highly relevant if we consider that through the use of scientific synthesis one can integrate different research studies, thus increasing the generality and applicability of the results reported in the scientific literature (Hampton and Parker, 2011). In fact, it is urgent to foster synthesis in ecology to address the global challenges of today (Mace, 2013) and capitalise on the remarkable load of scientific data already gathered over the last decades (Carpenter et al., 2009). As for other research areas, such as ocean acidification (Gattuso and Lavigne, 2009; Riebesell et al., 2009) and larval morphology studies (Clark et al., 1998), it is important to develop guidelines that clearly define the best practices of experimental design, methods, systems and data reporting for studies addressing the role of environmental and trophic interactions in the life history of benthic marine invertebrates (see Section 5 for further discussion and recommendations). Encouraging research efforts to address fundamental questions in certain areas of knowledge is a useful approach to advance our understanding on complex topics and overlooked issues (Sutherland et al., 2013, 2015). Indeed, through the use of priority setting exercises it is possible to support, in a rational way, certain research priorities and establish a solid science agenda (Kennicutt et al., 2015).

Given the significant number of studies in this broad research field, which continues to increase on a yearly basis, we decided to focus the present work on the relevance of gaining an in depth insight regarding the trophic ecology of benthic marine invertebrates along the two phases of their life. Together with several environmental factors that notably affect the performance of marine invertebrates throughout their complex life cycle (Bishop et al., 2006; Marshall and Keough, 2004), trophic interactions involving

these organisms as predators (as well as prey) are critical for energy acquisition and to successfully accomplish larval development and metamorphose into juveniles that will ultimately develop into adults and reproduce (Phillips, 2002, 2004). Planktotrophic larvae need to feed and obtain energy to fuel metabolism and prepare for metamorphosis. Once they reach their final larval stage and metamorphose, their trophic ecology continues to be a key trait to successfully obtain energy and allow them to compete for space and food in a new environment, as well as to invest energy into reproduction. The trophic ecology of males and females will notably affect the investment they are able to make on offspring quality and quantity (Marshall and Keough, 2006; Podolsky and Moran, 2006). This will consequently affect the starting point of embryos and initial larval stages that significantly rely on maternal investment and energy reserves that female allocate to offspring, regardless of larvae being lecithotrophic or planktotrophic. A better understanding of the trophic ecology for the different life stages of marine invertebrates will certainly allow researchers to better understand the role played by maternal and carry-over effects on the life cycles of these taxa.

This review will focus on benthic (or bentho-pelagic) invertebrate species that are either sessile or sedentary as adults because their benthic lifestyles differ most notably from those of their planktonic larvae. Such benthic invertebrate species compose an immense fraction of the biodiversity of marine taxa that lack "backbones" (Hickman et al., 2007). For instance, sponges (phylum Porifera), cnidarians (such as corals) (phylum Cnidaria), bryozoans (phylum Bryozoa), polychaetes (phylum Annelida), decapod crustaceans (such as caridean shrimp and brachyuran crabs) (phylum Crustacea), molluscs (such as mussels, octopus and squids) (phylum Mollusca), echinoderms (such as starfish, sea urchins and sea cucumbers) (phylum Echinodermata) display, at least for the majority of their species, planktonic larvae and benthic (or bentho-pelagic) adult forms. Because our empirical knowledge regarding the trophic ecology of these organisms relies on methods that are currently available, the tools that have been used to unravel the trophic interactions among these taxa will be critically reviewed here, discussing their advantages and disadvantages when addressing the different life stages that benthic marine invertebrates undergo in their life cycle. Highlighting the different methodological approaches for the different life stages is of utmost importance, as larvae can be several orders of magnitude smaller than their adult forms, which notably affects what they are able to eat, but also the power of the different tools currently available to accurately

assess predator–prey interactions. The suitability of these tools to deliver quantitative and qualitative data will be critically discussed, as well as the role they may play to decouple the effect of trophic ecology from that of environmental drivers on the life cycles of benthic marine invertebrates. We will also recommend a number of research topics that should be addressed in the near future by the scientific community in order to advance the state of the art on this complex and challenging research field.

2. COUPLING TROPHIC ECOLOGY AND POPULATION BIOLOGY

The larval stages of benthic marine invertebrates with bi-phasic life cycles often bear little to no resemblance to their adult forms. As they commonly display a contrasting morphology and lifestyle, the two phases of benthic marine invertebrate complex life cycles were for long studied isolated. However, at present, researchers are fully aware that a holistic approach is required to pinpoint the phenotypic and genetic linkages between these contrasting life history phases to gain a new insight on benthic marine invertebrates' ecology and evolution (Marshall and Morgan, 2011).

It has long been debated whether the larval or the adult forms of marine invertebrates with bi-phasic life cycles are the ones responsible for the regulation of population size (Young, 1990). Presently, it is acknowledged that both planktonic and benthic population regulation factors act during both phases of the life history of these marine organisms, and their regulatory effects on population dynamics can vary in space and time (Marshall and Morgan, 2011). The trophic interactions experienced by developing larvae and adult forms are one of the most important links connecting these two notably distinct phases. However, the investigation of trophic interactions of these marine invertebrates continues to pose a major challenge to researchers, even though notable methodological advances have been observed in the past decades (see Section 3). This constraint is even more pronounced when addressing trophic interactions experienced by developing larval stages (Anger, 2006). The majority of larval forms of marine invertebrates with a benthic (or bentho-pelagic) lifestyle after metamorphosis is very small compared to adults, and commonly differs in size by several orders of magnitude (>10 in some species). This makes direct observation *in situ* of potential trophic interactions (either as predators or prey) nearly impossible to achieve. Additionally, a number of pelagic larvae of benthic marine invertebrates are still extremely difficult to maintain in captivity, which impairs

the simulation of trophic scenarios in controlled settings with the ultimate goal of enhancing our knowledge on how trophic ecology may impact their life history. Nonetheless, variable degrees of success have been achieved when trying to raise these larvae to metamorphosis. A multitude of systems and apparatus have already been developed over time for stocking and culturing delicate marine larvae (Calado et al., 2008a; Greve, 1968; Hamner, 1990; Moorhead, 2015) and a remarkable range of different live prey was already tested in controlled settings; some of the most commonly employed prey in those studies, such as the branchiopod crustacean *Artemia* spp., are not natural prey of the larvae being cultured, and caution is advised when interpreting results (Calado, 2008). It is also important to note that even when results are derived from laboratory studies that try to mimic natural trophic interactions in the marine environment as closely as technically possible, extrapolations on the conditions experienced by larvae in the plankton may be misleading. Mesocosms (large volume enclosures of several m^3) may somehow minimise potential bias originating from experiments addressing the trophic ecology of the larval forms of benthic marine invertebrates with complex life cycles performed in small volumes in the laboratory, as once assembled *in situ* they entrap and keep predators and prey stocked at abundance levels similar to those that commonly occur in the plankton (Cowan and Houde, 1990; Pitta et al., 1998).

Regardless of performing trophic ecology studies *in situ* or *ex situ*, the minimal sample size of biomass required to perform certain analyses is another critical aspect that may impair research on these topics. While this bottleneck still constrains the use of some methodological approaches to study trophic interactions, significant technical breakthroughs on analytical tools used in biochemical analysis, as well as the advent of molecular methods, open good perspectives for advancing our knowledge in the near future (see Section 3). An additional challenge to understanding "the big picture" related to the trophic ecology of benthic marine invertebrates with bi-phasic life cycles is the remarkable variability of biotic and abiotic conditions that larvae may experience when developing in the plankton. These organisms can experience contrasting conditions during their daily vertical migrations in terms of temperature, salinity and food availability (due to the patchy spatial distribution of plankton) (Folt and Burns, 1999; Metaxas and Young, 1998; Queiroga and Blanton, 2005). Moreover, oceanographic processes (e.g. pre-upwelling vs. upwelling conditions) may dramatically shift, both quantitatively and qualitatively, the availability of prey for developing larvae, as well as their potential vulnerability to predation. All these features

condition and shape the survival of developing larvae to metamorphosis, as well as their success when colonising the habitats they will occupy after settlement during their juvenile and adult forms. Their larval history is unlikely to be deleted and will certainly play a key role on their post-settlement performance (Pechenik, 2006). Therefore, it is of little surprise that the role that trophic interactions play in the performance of subsequent life stages is still being unveiled. In particular, it is still poorly understood how the trophic ecology of developing larvae affect the performance of newly settled juveniles, as well as how trophic interactions of adult females affect the survival and growth of developing embryos and consequent larval stages (Gonzalez-Ortegón and Giménez, 2014).

It is acknowledged that unfavourable conditions experienced by developing embryos and larvae can negatively affect their quality and play a key role on their early post-settlement success and fitness of subsequent life stages (Pechenik, 2006). In other words, suboptimal environmental scenarios experienced during early ontogenetic stages of benthic marine invertebrates may give origin to phenotypic traits that are only displayed later in their life cycle (e.g. post-metamorphosis, at reproduction) (Giménez, 2010). Nonetheless, exceptions do occur, and metamorphosis may provide individuals with a new start in particular situations (Diederich et al., 2011). Biotic factors, namely trophic interactions, experienced during the different life stages may also give origin to latent effects that are only expressed later in their life cycle. Exposure to food deprivation and suboptimal feeding, such as low food quantity and/or quality, may indirectly impact the quality of developing embryos and directly affect the quality of developing larvae. For instance, poor food quality may condition the resources available for maternal investment. Consequently, larvae hatching from those embryos may be unable to overcome such deficiencies and eventually fail to reach the energetic thresholds that allow them to undergo metamorphosis.

The ability of several marine benthic invertebrate larvae to postpone metamorphosis has long been considered as a selective advantage that may enhance the chances of finding a suitable habitat to settle as a juvenile or even for adult life (Obrebski, 1979; Thorson, 1950). However, a delay in metamorphosis may have negative consequences for post-metamorphic fitness, such as lower juvenile survival and/or growth (Gebauer et al., 1999; Hunt and Scheibling, 1997). Such consequences are not always predictable and may vary across taxa. No significant effects on juvenile fitness were observed on planktotrophic polychaete larvae that delayed metamorphosis (Pechenik and Eyster, 1989). In contrast, planktotrophic larvae of four

tropical sea urchin species that delayed metamorphosis gave origin to juveniles that exhibited significantly lower growth rates (Rahman et al., 2014). Another study of polychaete larvae showed that a delay in metamorphosis of lecithotrophic larvae promoted a significant decrease in survival, but had no significant effect on post-settlement growth, or on the onset of sexual maturity or fecundity (Pechenik and Cerulli, 1991). In fact, the costs of delaying metamorphosis in benthic marine invertebrates with bi-phasic life cycles are rather unpredictable, with significant levels of intraspecific variability occurring on the post-settlement performance of juveniles originating from larvae that have delayed metamorphosis (Simith et al., 2013).

Intraspecific variation in larval phenotypes can affect population dynamics and it is erroneous to assume that all larvae produced by different parents from the same species display the same ability to survive and successfully give origin to a new recruit. Indeed, shifts in the quality of settling larvae may play a more relevant role on the regulation of marine populations than variations in larval supply (Burgess and Marshall, 2011b). While several studies have revealed how abiotic shifts in oceanic/coastal conditions may impact larval quality and supply (Przeslawski et al., 2015), little is still known on the synergistic effects they may have on benthic marine invertebrate larvae exposed to qualitative and quantitative variations on their trophic ecology. As highlighted by Podolsky and Moran (2006), further studies are still necessary to discriminate long-term consequences of maternal investment and carry-over effects from one life cycle stage to the next in benthic marine invertebrates and determine if these effects persist through subsequent stages, as well as if they are amplified or compensated for.

2.1 Consequences of Trophic Ecology on Life History Traits

Some topics that need further investigation to clarify the synergies between abiotic factors and trophic interactions in trait-mediated effects in marine invertebrates with bi-phasic life cycles include: (1) the role of maternal life history and how it is linked with maternal provisioning at oogenesis; (2) the dynamics of larval development under contrasting feeding scenarios and what may be the consequences to pre- and post-metamorphosis performance; and (3) how is embryonic and larval history carried over to juvenile and adult life, or in other words, how do latent effects emerge in subsequent life stages.

2.1.1 Maternal Trophic Experience and Provisioning

In general, maternal effects can be defined as the non-genetic components of the offspring phenotype (Marshall et al., 2008a). The ecological importance

of maternal effects is well recognised as it may determine the success of offspring performance (Mousseau and Fox, 1998). It is important to highlight that maternal effects may not always affect offspring fitness positively. Increased offspring fitness associated with maternal effects may be possible if mothers can predict the environment that their offspring will experience (Marshall and Uller, 2007). Indeed, under spatial and/or temporal environmental heterogeneity, a differential maternal provisioning of offspring may have, to a certain limit, a positive effect on a given phenotype if the environmental conditions to which offspring will be exposed are predictable from maternal environmental conditions/phenotype (DeWitt et al., 1998). Therefore, surveying a single reproductive event for multiple spawning species solely provides a snapshot of maternal investment into reproduction and may provide a biased perception for this trait. The study of phenological traits and environmental conditions, as well as the connection of these traits to trophic interactions, is certainly relevant to better understand maternal predictions of the environment that will be experienced by their offspring. Research on this topic is also critical in light of global climate change and its potential effects on phenology and trophic mismatches (Edwards and Richardson, 2004).

The influence that abiotic factors may have on embryos being brooded by females has been well documented (e.g. Bas et al., 2007), as well as how this may affect larval performance (e.g. Deschaseaux et al., 2011). When females brood their embryos, abiotic factors within the brooding structures may play a more important role on the conditioning of embryonic development and larval quality at hatching rather than those being experienced by the mother (Wickins et al., 1995). Nonetheless, maternal provisioning during embryogenesis may also significantly impact larval quality at hatching and potentially be carried over to late larval development or even post-metamorphosis. In benthic marine invertebrates with stenophagous feeding regimes (i.e. preying on a single or a reduced number of species), the link between suboptimal feeding scenarios and maternal provisioning to the offspring can be more pronounced, as different trophic levels display different sensitivities to climate change (Voigt et al., 2003). This linkage was addressed in a highly specialised sea slug, *Aeolidiella stephanieae*, present in tropical waters that feeds upon symbiotic anemones that harbour photosynthetic dinoflagellates from the genus *Symbiodinium*; egg masses originating from adults that were supplied bleached anemones, i.e. no longer harbouring their photosynthetic endosymbionts, displayed significantly lower levels of docosahexaenoic acid (DHA, 22:6*n*-3), which is an essential

fatty acid that plays a key role during embryogenesis (Leal et al., 2012a). Hatching success and fitness of larvae originating from these egg masses were also negatively affected (Calado, 2008, personal observation), which indicates that the effect of suboptimal diets can cascade from parental organisms to offspring. However, the consequences of maternal effects associated with poor food quality over a consecutive number of generations are yet to be determined.

Maternal provisioning may shift with female size, potentially due to the enhanced ability of larger females to secure more and/or better feeding resources. Seasonal variability may also affect the condition of dietary resources available for adults and consequently shape maternal investment, as has been described for species exhibiting larger embryos in the winter and smaller embryos in the summer (Paschke et al., 2004; Urzua et al., 2012). Contrasting biochemical profile of offspring hatching from similar sized embryos throughout the reproductive season has also been observed (Gebauer et al., 2013). Certainly more puzzling is the fact that maternal investment is not always homogenous across offspring. The existence of variable within-brood maternal provisioning in newly extruded embryos of the clawed lobster, *Homarus gammarus* (Leal et al., 2013a), opened a new research window on how maternal effects may shape offspring performance. It is well established that "not all larvae are born equal" within the same brood. However, it has been assumed that such variability is driven by parameters affecting embryonic development during the incubation period and not as early as at oviposition. At what stage of oogenesis such within-brood variability starts to occur remains an unanswered question. It appears likely that not all oocytes are "created" equal. The role that maternal trophic history may play on this variability remains to be clarified, particularly in species that may undergo gonadal maturation (e.g. oogenesis) over a period of several months (e.g. the Norway lobster, *Nephrops norvegicus*). In marine invertebrate taxa exhibiting lecithotrophic larvae, the consequences of variable maternal provisioning are even more dramatic. While lecithotrophic larvae are not entirely energetically independent from their environment (Jaeckle and Manahan, 1989; Manahan, 1990), they mostly rely on the catabolism of endogenous energy reserves to disperse and settle in a suitable habitat. Poor maternal provisioning derived from suboptimal feeding may also condition post-settlement success as larvae may be forced to metamorphose and settle more rapidly without a proper discrimination between habitats, ending up by settling in an unfavourable location (see Toonen and Pawlik (2001) and references cited within on the "desperate larvae" hypothesis).

2.1.2 Larval Development and Feeding—Consequences at Metamorphosis

The advantages of benthic marine invertebrates displaying dispersive larval forms have long been recognised (Thorson, 1950). Nonetheless, potential disadvantages have also been reported (Pechenik, 1999), such as dispersing beyond suitable habitats for later life stages. Indeed, according to the phenotype–environment mismatch theory, the dispersal of developing larvae beyond a region to which their phenotype is specifically suited may significantly reduce fitness and, ultimately, survival, even when a significant level of phenotypic plasticity is displayed by a particular taxon (Marshall et al., 2010a,b).

Extending larval duration beyond what would be "normally" expected is considered an adaptive feature exhibited by several groups of benthic marine invertebrates with bi-phasic life cycles (e.g. polychaetes, caridean shrimp, bryozoans). It is commonly assumed that this feature may provide developing larvae the advantage of "waiting" for optimal conditions to complete their larval life (e.g. reaching energetic thresholds to undergo metamorphosis, finding a suitable habitat to settle for juvenile/adult life; Pechenik, 1999). The extension of larval life in marine invertebrates may occur through the existence of extra larval stages that are not commonly recorded under optimal abiotic and trophic conditions (Anger, 1991). Larvae may occasionally pass through a series of instars where morphological differences are little or even absent, with the only exception often being an increase in larval size (Anger, 2001). Indeed, marine invertebrate larvae are able to "mark-time" through long periods of time (sometimes months; see Calado, 2008) until optimal conditions are present to reassume larval development or metamorphose. "Giant" larvae of caridean and stenopodidean shrimp, which are several millimetres larger than the "typical" last larval stage displayed by these organisms, have often been recorded in the plankton (Williamson, 1970, 1976), thus, providing evidence of a delay in metamorphosis. When analysing material collected from plankton samples, Gurney and Lebour (1941) recorded a giant stenopodidean shrimp larvae metamorphosing at a length of 21 mm. However, other larvae from the same species observed in the same plankton samples were already 31-mm long and were still in the last larval stage. If these specimens were present in the same plankton patch at the time of sampling, it may be legitimate to assume that their larval (or even embryonic) history was likely conditioning their performance. It is, however, important to note that larger larval sizes at metamorphosis may not always be synonymous to high larval quality, as often reported in the

literature (e.g. Klinzing and Pechenik, 2000; Marshall et al., 2003a). In certain marine invertebrates, particularly those displaying planktotrophic larvae that may dwell in the plankton for several months, larger sizes at metamorphosis may rather be a red flag signalling the exposure to suboptimal trophic conditions (but see below for lecithotrophic larvae). As highlighted by Fenaux et al. (1994), "any single method of testing food limitation can be misleading or inconclusive". These authors were able to gather evidence suggesting food limitation effects in echinoid larvae by performing multiple comparisons in the laboratory that allowed to check for potential laboratory bias. Juveniles originating from non-feeding larval stages (such as in the case of spiny lobsters) may display an unusually high resilience to suboptimal feeding and starvation (Limbourn et al., 2008). This feature is possible due to the high levels of energy reserves stocked prior to metamorphosis in the last larval stage, clearly providing an adaptive advantage in the transition from pelagic to benthic life. Researchers overlooking the larval history of these organisms would be unable to understand the source of such nutritional resilience.

Some newly hatched larvae are able to advance in their larval development even when deprived of exogenous food, such as by catabolising the remnants of yolk reserves originating from maternal provisioning. Nonetheless, they are also able to readily feed if provided with exogenous food (Anger, 2001). An interesting case study on the impact of suboptimal feeding during early larval life is that of larvae originating from the same brood of a caridean shrimp displaying facultative primary lecithotrophy (Calado et al., 2005a). Larvae were divided in two groups: one group was supplied with exogenous food immediately after hatching and the other was forced to catabolise its yolk reserves until it reached the next larval stage and only then supplied with the diet of the first group. Halfway through larval development no differences could be recorded in survival, larval size or larval stage duration. Nonetheless, in the following larval stages, specimens that were deprived of food immediately after hatching started to display a lower survival rate, longer larval stage duration, extra larval stages and larger larval sizes, which strongly evidences they were "marking-time" to metamorphosis. This experiment revealed that suboptimal feeding scenarios experienced by newly hatched larvae may condition their performance at metamorphosis, even when larval development may last several weeks (Calado et al., 2005a). Despite displaying larger sizes at settlement, juveniles originating from those larger sized larvae commonly exhibit poorer growth performances (Calado, 2008). The exposure of newly hatched larvae to suboptimal

feeding scenarios may also negatively impact the onset of their digestive enzymatic machinery and consequently condition larval performance (Pochelon et al., 2011), which may in turn have cascading effects on the first stages of their early benthic life. It is worth highlighting that while the effects of larval starvation are commonly evaluated, complete starvation in the plankton, with no type of food whatsoever being available, as regularly performed in laboratory trials, is unlikely to occur in the natural environment (Johnson and Shanks, 1997). Developmental plasticity commonly allows planktonic larvae to use different feeding strategies to increase their feeding efficiency even in the presence of suboptimal prey (McConaugha, 2002). Moreover, the role that background plankton (including protists and phytoplankton) plays on the trophodynamics and satiation of predatory larvae is far from being negligible (Johnson and Shanks, 1997).

The lack of suitable settlement cues that may trigger metamorphosis is commonly suggested as one of the main drivers to the occurrence of extended larval development (Pechenik, 1990). Nonetheless, as already highlighted above, developing larvae in the plankton that experience suboptimal feeding scenarios are known to develop more slowly, delay metamorphosis and often even fail to metamorphose (Pechenik et al., 2002). Lecithotrophic (non-feeding) larvae known for some benthic marine invertebrate taxa often tend to shorten their larval development and consequently decrease their time to metamorphosis, when the energetic reserves fuelling their development start to be depleted. In other words, these larvae will be more "desperate" to settle and have been shown to significantly widen their discriminating criteria to select a habitat to settle (see Botello and Krug, 2006; Toonen and Pawlik, 2001). The urgency displayed by these larvae is, therefore, regulated by the distance they disperse until finding a suitable habitat to settle, which is often correlated with the amount of time they spend in the pelagic environment catabolising their energetic reserves. Consequently, this will ultimately depend on maternal provisioning, which is mostly dependent on the trophic environment experienced by the female at oogenesis and the abiotic conditions experienced during embryonic development (Wendt, 2000). Larger non-feeding larvae that display higher quantity/quality of energy reserves are able to disperse over longer distances without jeopardising their post-settlement performance (Marshall and Keough, 2003). Still, as noted by Pechenik (1999), larger larvae may become too selective (a feature often referred to as larval "pig-headedness") and not respond to environmental cues of suitable habitats for benthic life.

As first noted by Raimondi and Keough (1990), it is reasonable to assume that most developing larvae of marine invertebrates delay metamorphosis and that significant intraspecific variation occurs in larval settlement behaviour. Nonetheless, this ability to delay metamorphosis often encompasses trade-offs with negative consequences to the larvae, the juvenile and/or the adult stage (Metcalfe and Monaghan, 2001). It is important to highlight that exceptions do occur (Pechenik and Eyster, 1989) and our understanding of the phenotypic links throughout benthic marine invertebrates ontogeny affected by predator–prey interactions is still limited. Further studies focusing on the trophic ecology of developing larvae of marine invertebrates with bi-phasic life cycles are therefore required to provide insights on this topic.

2.1.3 Post-Metamorphic Performance and Carry-Over Effects

Carry-over effects reflecting embryonic and pelagic larval history on benthic juvenile performance have been investigated in several benthic marine invertebrate taxa (e.g. bivalve molluscs, echinoderms, polychaetes, bryozoans and decapod crustaceans; e.g. Pechenik, 2006). The exposure to suboptimal abiotic and biotic conditions during embryonic, larval or adult development may have immediate effects on individuals and/or have negative effects on subsequent stages of their life cycles. This aspect is particularly relevant under current climate change scenarios, as deleterious long-term and trans-generational effects have already been demonstrated to occur in benthic marine invertebrates (Dupont et al., 2012; Przeslawski et al., 2015).

While developing individuals can experience feeding regimes that may not be nutritionally balanced, it is known that they may apparently compensate for such initial setbacks—the "grow now, pay later" trade-off discussed by Metcalfe and Monaghan (2001). Compensatory growth is a good example that illustrates how smaller sizes at metamorphosis, or poorer growth performances soon after settlement, may be overcome through, for example, trophic ecology traits (Arendt, 1997). This feature can be briefly described as the ability displayed by organisms that somehow arrested their growth when exposed to suboptimal conditions (trophic or environmental), to perform a growth acceleration under more favourable scenarios than those previously experienced (Metcalfe and Monaghan, 2001). The role played by trophic ecology on this response is paramount, because this compensatory response is commonly achieved through hyperphagia and usually occurs in a particular time frame. The relevance of this feature is such that it has been investigated in the production of some commercially important marine

invertebrates, such as shrimp (Wu and Dong, 2002) and sea cucumbers (Dong et al., 2010), to minimise food costs and simultaneously maximise growth. Nonetheless, such compensatory responses may only occur if they are not too costly to the individual and are highly dependent on the timing, i.e. when during the life cycle, and impact of the suboptimal conditions experienced. Compensatory responses may not be possible when the damages that have been inflicted to the individual are irreversible. Nutritional deficiencies at critical developmental stages in developing larvae of marine invertebrates can prompt them towards a point of no return (PNR; *sensu* Blaxter and Hempel, 1963). While this topic has already been investigated in a range of marine invertebrate taxa, such as bivalves (Moran and Manahan, 2004) and sea cucumbers (Sun and Li, 2014), it has deserved special attention in decapod crustaceans due to the number of moults that these organisms undergo during larval development and how the moulting cycle is so intrinsically linked with trophic scenarios (Anger, 2001). The time frame between fertilisation and the PNR ranges from a few days to several weeks, being dependent on maternal provisioning and the metabolic performance of larvae. Once this threshold is reached, even if larvae are allowed to develop further under optimal biotic and abiotic conditions and remain alive for a variable period of time, they will be incapable to recover from early nutritional stress, cease larval development and die. While recovery from previous exposure to suboptimal feeding can occur, it commonly comes at a cost. For example, hyperphagia may translate into the need to forage for food over longer periods, hence increasing the risk to predation in the pelagic environment. Nonetheless, it is not always easy to identify the original source of the deleterious effects being expressed on the phenotype of a developing organism—do they originate from the initial exposure to the suboptimal conditions, or are such deleterious effects the price to pay for the compensation mechanisms? As the costs for compensation may only "be paid" very late in ontogeny (Metcalfe and Monaghan, 2001), tracking the event(s) that triggered the need to set in motion such compensatory mechanisms is a challenging task that may at times be solely achieved through an experimental approach under controlled settings in the laboratory.

The impact of thermal anomalies (Jackson et al., 2014), saline stress (Montory et al., 2014; Rey et al., 2015) or both (Zimmerman and Pechenik, 1991), as well as the exposure to sub-lethal levels of contaminants and pollutants (Kimberly and Salice, 2014), and delay of metamorphosis (Marshall et al., 2003b; Pechenik, 1990) on the post-metamorphic performance of benthic marine invertebrates is well document (reviewed by

Pechenik, 2006). Moreover, trans-generational effects are also starting to be unveiled (Marshall et al., 2003a).

While the short- and long-term effects of food deprivation have already been investigated in several taxa of benthic marine invertebrates (Anger et al., 1981; Gimenez, 2002; Moran and Manahan, 2004; Olson and Olson, 1989), further studies are needed to understand the synergy of sub-optimal feeding and abnormal abiotic conditions experienced during larval development (Klinzing and Pechenik, 2000), as well as how they will affect the post-settlement performance of juvenile specimens. The timing of food supply pulses (e.g. low food levels during larval development, or a shift from high to low food availability) during larval development can affect larval size and energetic reserves. These trophic scenarios experienced during larval development can significantly impact post-metamorphosis performance, as they can promote poorer juvenile growth and higher mortality rates (Phillips, 2004).

2.2 The Need for New Insights on Trophic Ecology

As already discussed above, synergies between suboptimal feeding and other parameters that may condition the performance of different life stages of benthic marine invertebrates with bi-phasic life cycles have already started to be investigated. However, deeper knowledge is still necessary to better understand how latent effects are carried over throughout bi-phasic life cycles, interact with trophic ecology, and affect population dynamics and contribute to benthic–pelagic coupling (Kirby et al., 2007). However, the term sub-optimal feeding is simply too vague and, at present, of little help to those aiming to understand more accurately the role played by trophic ecology on the linkage of adult/embryonic/larval history. New overarching methodological frameworks are, therefore, needed to provide new ecological insights based on nutritional traits (see Section 4).

In-depth knowledge on the trophic ecology of species displaying dramatic shifts in their dietary regimes throughout their life cycle is paramount to understand how early larval life may impact post-settlement performance and vice versa. An interesting example is that of highly specialised taxa that display stenophagous feeding regimes during their adult life, while their larvae can feed on a multitude of prey in the plankton. This is the case of the harlequin shrimp, *Hymenocera picta*, a decapod crustacean whose larvae feed on a multitude of prey as the majority of other caridean shrimp larvae. However, following metamorphosis, juveniles feed solely on sea stars and will

starve to death if deprived of these prey (Calado, 2008). This occurrence brings up several questions. For example, in this scenario, can larval trophic history condition post-settlement performance when such dramatic changes in dietary regimes occur? Are there any particular groups of prey that provide key nutrients to allow the onset of such dramatic shifts post-metamorphosis? The ingestion of specific prey may have dramatic impacts on some marine invertebrates and play a key role on the shaping of populations. The ingestion of diatoms, *Cocconeis* sp., by post-larvae of a caridean shrimp inhabiting Mediterranean seagrass meadows promotes the apoptosis of the male gonad and the androgenic gland, which dramatically shifts the sex ratio of populations through the development of primary females. Moreover, this effect promoted by the ingestion of these diatoms solely occurs during a very short time frame of the species life cycle, from the 5th to the 12th day of development post-metamorphosis (Zupo and Messina, 2007; Zupo et al., 2008). It is, therefore, legitimate to question what other effects promoted by trophic interactions that shape phenotypes in benthic marine invertebrates along their life cycles may still be overlooked until researchers start using high-resolution tools to better understand if these taxa are indeed what they eat.

3. TOOLS TO ASSESS TROPHIC ECOLOGY

Our understanding of the trophic ecology of benthic marine invertebrates is inherently limited by the methods available to study predator–prey interactions. Consequently, the development of new methods and methodological approaches has been paving the way for new advances in our knowledge of marine food webs. Indeed, in recent years, significant methodological advances have been made for studying trophic interactions in the field and when using controlled settings, which has consequently opened new perspectives for trophic ecology (Bowen and Iverson, 2013; Fry, 2008; Kelly and Scheibling, 2012; Pompanon et al., 2012).

The primary tool to identify and quantify trophic interactions between species is probably direct survey performed during experiments in controlled setting or *in situ* observations using SCUBA diving or submersibles. The visual monitoring of feeding activity has been providing critical information on the trophic ecology of marine invertebrates, particularly as a direct measurement of feeding mechanisms, behaviour and foraging activities (e.g. Calado et al., 2009; Price, 1988; Smith, 2003). However, because there are limited opportunities to directly observe what benthic marine

invertebrates eat, trophic ecology investigations almost entirely rely on indirect methods. For instance, gut content analysis has been a critical method in trophic ecology that is still regularly used (e.g. Palardy et al., 2005; Pascal et al., 2015; Wilcox and Rochette, 2015). Both direct observation and gut content analysis only provide a snapshot examination of recent feeding activity. In order to have an extended view of trophic ecology, researchers have also been using biochemical methods, such as fatty acids and stable isotopes analysis, which provide time-integrated information on food ingestion and uptake (Dalsgaard et al., 2003; Fry, 2008). More recently, with the advent of molecular genetics, molecular detection of trophic interactions has also been an increasingly popular approach that has been able to provide new insights on marine food webs (King et al., 2008; Pompanon et al., 2012).

The use of the different tools outlined above to investigate the trophic ecology of benthic marine invertebrates with bi-phasic life cycles is summarised here, with particular relevance on their advantages and limitations (Table 1). As all these methods have been extensively tested and widely used across aquatic and terrestrial environments, as well as in marine invertebrates, we focus on the ecological insights that these tools have been providing rather than thorough descriptions of methodological details.

3.1 Direct Observation

Observing a predator foraging, capturing and ingesting its prey is certainly a powerful direct measurement of a trophic interaction. However, direct observation of food ingestion is mostly restricted to experiments performed in controlled settings because marine invertebrate predators are often difficult to locate and follow in their natural environment without notably disturbing them, and these constraints limit opportunities to directly observe them and assess their trophic ecology (see Table 1). Moreover, direct observation is mostly suitable when prey items are relatively large compared to the size of their predator. Otherwise it would be difficult for the observer to accurately provide a qualitative and quantitative assessment of prey ingestion. This is a notably important issue limiting the investigation of the trophic ecology of benthic marine invertebrates early-life stages, as they are often pelagic and very small (usually smaller than 1 cm), which makes it nearly impossible to make such direct observation *in situ*. Consequently, the assessment of the trophic ecology of marine invertebrates using direct observation has been mostly performed in the laboratory.

Table 1 Advantages and Disadvantages of the Different Methodological Approaches used to Investigate the Trophic Ecology of Marine Invertebrates for Qualitative and Quantitative Research

	Qualitative		Quantitative	
Method	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**
Direct observation	Direct measurement of feeding. Reliable independent estimates of diet	Difficult to use *in situ* Usually involves small sample sizes Prey identification may be difficult	May be combined with foraging activity and prey capture behaviour Accurate quantification during *ex situ* trials	Nearly impossible for small prey items Requires a continuous monitoring of feeding activity
Gut content analysis	Information on prey species composition Prey size may be estimated Large sample size is possible Relatively inexpensive to process	Invasive method Inability to detect prey without hard structures Inability to identify prey without specific-diagnosing features Information on a short dietary history False negatives if prey is rapidly digested/egested	(Same as for qualitative approaches)	Differential resistance of diagnostic hard structures to ingestion/digestion Differential retention of prey items biases diet estimates Correction factors may reduce bias, but these are not usually available
Food concentration	(Mostly used for quantitative approaches)		Useful when feeding rates are difficult to obtain *in situ*	Requires parallel controls to account for prey that sink or get trapped Cannot be used during long feeding trials with prey that rapidly reproduce Is usually limited to experiments with a single prey (or multiple prey that do not interact with each other).

Fatty acids	Widely distributed Diversity of markers in the marine environment Potentially long feeding history (depending on species and life history) Sampling location less likely to bias diet assessment	Course resolution Intermediate specificity Requires calibration coefficients May be metabolically modified by consumers	Rare prey will be identified if they have a particular fatty acid signature	Biosynthesis by consumers biases quantification Requires calibration coefficients to account for consumer metabolism Complex diet estimations for mix diets
Stable isotopes	Time-integrated assessment of feeding history Estimation of metabolised food sources Differentiation between main food components Identification of trophic level	Low prey-specific resolution Temporal, spatial and species-specific variations of isotopic signature	Labelling techniques allow quantitative assessment of specific food items	Not all prey items can be labelled Isotopic ratios do not provide quantitative estimates of food ingestion
Molecular tools	High sensitivity and specificity High taxonomic resolution Taxonomic resolution can be adjusted Can be used non-invasively (e.g. faecal material)	High sensitivity to contamination Cannot distinguish primary from secondary predation Cannibalism cannot be addressed Identification of sequences relies on reference database	High sensitivity Next-generation sequencing allows a semi-quantitative approach	Can only be employed using prey-specific markers Gene copy number may bias quantification Quantities of prey DNA in dietary samples do not always equate to biomass proportions of food consumed

Note that advantages and disadvantages for qualitative assessments also apply for quantitative approaches.

Visual observations can provide detailed information on various traits associated with trophic ecology, such as foraging behaviour, preferential feeding grounds, diel variation of feeding activity, among others (e.g. Campbell, 1984; Mather and O'Dor, 1991; Ryer, 1987). However, such information is usually more qualitative than quantitative. Quantitative data requires prolonged and/or repeated observations and must be carefully interpreted as ingestion rates may be notably affected by various abiotic factors, such as light, temperature, among others (e.g. Calado et al., 2005a; Jackson et al., 2014). Moreover, it is highly relevant to accurately mimic natural conditions in laboratory trials addressing feeding behaviour, which is often not possible due to the complexity of pelagic and benthic environments inhabited by marine invertebrates. Nonetheless, visual observations may provide new insights on feeding mechanisms that cannot be assessed using other indirect measurements, such as gut content and biochemical analyses. For instance, extracoelenteric feeding by scleractinian corals is a mechanism that has only been possible to unravel using direct observation methods (Wijgerde et al., 2011). Moreover, feeding behaviour in planktonic larvae is also an issue that can only be addressed during visual experiments in controlled settings (Calado et al., 2009; Hansen and Ockelmann, 1991; Hart, 1991), which would be impossible to assess *in situ*.

3.2 Gut Content Analysis

The analysis of gut contents can be performed using various procedures. The most popular method involves the visual analysis of gut contents and the identification and quantification of prey using diagnostic morphological remains of ingested prey that were not affected by the predator's ingestion and digestion processes (e.g. Bernárdez et al., 2000; Palardy et al., 2005; Smith, 2003; Wilcox and Rochette, 2015). Diagnostic hard structures usually include otoliths, beaks, and shells, among others, that resist to physical and chemical digestion processes. Other approaches involve the use of biochemical procedures, such as the analysis of photosynthetic pigments present in the gut (e.g. Burnett and Sulkin, 2006; Coelho et al., 2009) or gut DNA contents to identify and quantify ingested prey (King et al., 2008; Pompanon et al., 2012; see Section 3.6).

Visual identification of prey in guts provides a direct measurement of prey capture and is an important tool to generate qualitative and quantitative data on diet composition. However, it relies on the visual recognition of partially digested prey, which likely underestimates digestion times,

especially for small prey types (Nejstgaard et al., 2008). Moreover, because visual recognition relies on the presence and identification of prey species-diagnostic structures that are not affected by physical and chemical digestive processes, this method is not particularly accurate for soft-bodied prey that are quickly digested, which easily leads to false negatives. Prey without morphological characteristics that allow its accurate identification are usually underestimated. Consequently, visual identification of gut contents often leads to grouping prey items into broad categories, which may be insufficient to provide quantitative information on key trophic ecology traits, such as feeding selectivity. Careful interpretation is also needed due to secondary predation. For instance, a food item that is observed in the predator's gut may have been consumed by the ingested prey and not by the target predator being investigated. This issue is more relevant when high-sensitivity methods are used, such as molecular gut content analysis (see Section 3.6). Generally, visual identification of gut contents is more accurate and provides relatively higher taxonomic resolution when predators are larger and concomitantly feed on relatively larger prey items that are likely easier to identify with higher taxonomic resolution. For instance, adult forms of scleractinian corals prey on amphipods, crab larvae and shrimp nauplii (e.g. Palardy et al., 2008), among others, whereas shrimp may feed on fish (e.g. Burrows et al., 2001). Nevertheless, taxonomic resolution of prey items at the species-specific level is extremely difficult, and is usually limited to identifying prey up to the family level (e.g. Stevens et al., 1982). Furthermore, smaller predators will prey on small food items, such as observed for planktotrophic larvae of various invertebrate groups. This consequently limits the power of gut content analysis for identification and quantification of ingested prey. For instance, the trophic ecology of cephalopod paralarvae has been extremely difficult to assess, and the few studies that attempted to provide any new insights on what and how much paralarvae are eating, have been mostly performed in laboratory trials (reviewed by Roura et al., 2010).

Analysis of pigment gut contents has also been used to assess the diet of herbivores (e.g. Burnett and Sulkin, 2006; Coelho et al., 2009; Meyer-Harms and Harms, 1993). Microalgae eaten by marine invertebrates, particularly by pelagic early-life stages, may be difficult to be visually identified due to the lack of species-diagnosing morphological features or because their morphological traits are affected by ingestion and digestion processes. The analysis of photosynthetic pigments using high-performance liquid chromatography (HPLC) provides qualitative and quantitative information on consumed prey, especially because some of these pigments are powerful

chemotaxonomic biomarkers (Jeffrey et al., 1997). While the analysis of pigment gut contents is straightforward, fast and inexpensive, it is prone to error as pigments are especially sensitive to differential pigment breakdown. Furthermore, photosynthetic pigments are often not prey-specific (for a thorough discussion on this issue, see Nejstgaard et al., 2008).

We note that gut content analysis is a destructive method, which limits the study of the same individuals over time and, consequently, may fail to provide new insights on feeding specialisation through time. However, a non-destructive *in vivo* measurement has been developed to assess invertebrate larvae ingestion of microalgae by using chlorophyll fluorescence emitted from ingested food items through the body of live larvae using pulse amplitude modulated (PAM) fluorometry (Coelho et al., 2009). While this method is sensitive enough to estimate chlorophyll *a* present in the guts of individual crab larvae, it does not provide qualitative nor quantitative information on photosynthetic pigments with chemotaxonomic power. Consequently, although this method is certainly useful for *in vivo* measurements, it is unable to provide taxonomic information on what food items have been consumed.

Both visual and pigment analyses of gut contents only provide information on prey ingestion, and no information is available concerning digestion and assimilation, i.e., food utilisation. For instance, prey items may pass through the guts without being altered or digested (Turner, 2002). While food utilisation information is critical from a nutritional perspective, information on prey capture and ingestion is also highly relevant for a community ecology and population dynamics point of view, as predator–prey interactions notably affect community and ecosystem dynamics (Ives et al., 2005).

3.3 Food Concentration

A common method to experimentally estimate feeding rates of marine invertebrates is to assess food concentration in a closed environment before and after the predator has been placed in the experimental system (e.g. Calado et al., 2008b; Ferrier-Pagès et al., 2010; Houlbrèque et al., 2004; Pochelon et al., 2009). This method is usually known as clearance rate, because it assesses the number of prey that are cleared from the circulating water. Therefore, this method provides an indirect estimate of prey capture as it builds on the assumption that prey that disappears during incubation has been captured and ingested. While this is certainly a relevant method to obtain feeding rates from organisms in experimental chambers, it is usually limited to single-prey experiments or the analysis of a mixed prey

composition that do not interact with each other. This precaution is to guarantee that the only factor responsible for prey disappearance is food ingestion by the predator under investigation.

Experiments can be made either in laboratory settings (e.g. Houlbrèque et al., 2004; Shumaway et al., 2003) or using feeding chambers *in situ* (e.g. Ribes et al., 1998, 2000). While prey clearance rate should be a straightforward method for measuring ingestion rates, controls have to be run in parallel. Prey usually sink to the bottom of the feeding chamber or may get trapped in the water-circulation mechanism (e.g. propeller or water pump). Such prey will not be available in the water when prey concentration is assessed at the end of the experiment, and must be considered using correction factors obtained from controls. Therefore, results obtained using clearance rates may be inconsistent and should be interpreted with caution. Moreover, one other issue that is not accounted for by this method is potential dynamics of prey capture and release, which may significantly bias final clearance rates (Osinga et al., 2011).

Although clearance rates are mostly calculated using feeding chambers employed in laboratory experiments, the analysis of differences in food concentration has also been used *in situ* to assess the trophic ecology of invertebrate species. For instance, the concentration of planktonic microorganisms, such as phytoplankton, heterotrophic bacteria, ciliates and nanoflagellates, has been assessed in coral reefs and surrounding areas to assess whether such planktonic organisms are consumed by coral reef organisms, particularly corals (Fabricius et al., 1998; Houlbrèque et al., 2006). While this *in situ* depletion technique is able to provide some important data, it has to be carefully interpreted as the abundance of planktonic organisms in the water column may be affected by other factors than grazing by benthic marine invertebrates. Nonetheless, this technique is certainly important to assess the trophic ecology *in situ* of organisms that are difficult to keep *ex situ*, such as asymbiotic soft corals (Fabricius et al., 1998), and for which little information is still available.

3.4 Fatty Acid Trophic Markers

Fatty acids (FA) have been used to trace and confirm predator–prey relationships in the marine environment as they elucidate patterns of resource allocation (Dalsgaard et al., 2003; Kelly and Scheibling, 2012). This is a well-established tool for studying trophic interactions in marine systems as FA are assimilated into consumers' tissues and are subsequently transferred

up the food web. Fatty acid signatures can, therefore, be used as dietary tracers due to the diverse array of specific fingerprints present in marine organisms, particularly the high diversity of long-chain polyunsaturated FAs (PUFAs; Bell and Tocher, 2009; Budge et al., 2006). The analytical methods that have been used in the past decades to analyse the FA content of marine invertebrates usually required a total amount of biomass that was often larger than individual embryos or larvae. Consequently, researchers have been pooling individual samples to overcome this analytical constraint (Leal et al., 2012a, 2013a). While this approach provides an average-integrated signal of the individual FA profile, it loses information on each individual and, consequently, intraspecific variation. However, the latest methodological developments have provided significant advances in the amount of biomass needed for FA analysis. For instance, the study by Wang et al. (2014) used a high-resolution analysis of the FA profile of individual larvae, which represents a notable improvement. Moreover, the constant progress of chromatographic equipment holds great promise for future research on the trophic ecology of early-life stages of marine invertebrates.

The qualitative analysis of trophic interactions using FA relies on the specificity of markers to a particular resource organism. These markers are usually molecules that are only synthesised by the prey organism and that cannot be synthesised *de novo* by the predator under investigation. For example, the PUFA 18:3(*n*-3) and 18:2(*n*-6) are typically present in chlorophytes, whereas bacillariophytes usually have high values of 16:1(*n*-7)/16:0 and dinoflagellates high levels of 22:6(*n*-3), usually known as docosahexaenoic acid—DHA (Dalsgaard et al., 2003). Such diagnosing features of FA have allowed the use of this tool to identify, for example, preferential feeding habitats (e.g. Coelho et al., 2011; Soler-Membrives et al., 2011), diet composition (e.g. Alfaro et al., 2006; Hughes et al., 2005; Rosa et al., 2013), parental diets (e.g. Leal et al., 2012a; Rosa et al., 2007; Wacker and Elert, 2004) and seasonal diet variation (e.g. Baptista et al., 2012; Braeckman et al., 2012). Analysis of the FA of the target marine invertebrate, as well as their potential prey items, has also been successfully used. For instance, investigation of the trophic ecology of the spiny lobster, *Jasus edwardsii*, larvae using this approach allowed the conclusion that phyllosomes are opportunistic predators that feed on a variety of prey and that they preferentially retain specific-diet derived FA (Jeffs et al., 2004). Another study also employing FA analysis, but on a different species of spiny lobster larvae (*Panulirus cygnus*), revealed the importance that microbial food webs can play in larval nutrition, as well as how certain oceanographic conditions (cyclonic

eddies) can play a key role in the settlement and recruitment of spiny lobster populations supporting a commercially important fishery (Wang et al., 2014). Such progress on the trophic ecology of this species would hardly be possible using direct observation methods or visual gut content analysis because of the small size of microbial prey.

Information provided by FA fingerprinting may also be used for quantitative approaches, i.e. how much was eaten by the target consumer (Iverson et al., 2004; Magnone et al., 2015). However, an absolute quantification of diet composition relies on the assumption that differences in FA signatures are a function of diet rather than a reflection of predator metabolism, as well as that metabolism of the predator is predictable and quantifiable (Kelly and Scheibling, 2012; Traugott et al., 2013). Interestingly, the quantitative estimate of contents of mixed diets has been demonstrated for seabirds and marine mammals, but it relies on careful calibration even for consumers with limited ability to modify dietary FA (Iverson, 2009). Nonetheless, analysis of FA profiles has been useful for a relative quantification of dietary sources. This approach has been mostly used in controlled experiments where diet varies among treatments (e.g. Calado et al., 2005a,b; Leal et al., 2012a, 2013b). Combining FA analysis with other approaches, such as gut content and stable isotopes analysis, is also a popular approach (e.g. Alfaro, 2008; Braeckman et al., 2012; Carreón-Palau et al., 2013), as they all provide information that complement each other (Kelly and Scheibling, 2012; see Section 5.2 for further details).

3.5 Stable Isotopes

Stable isotope (SI) analysis has been broadly used to investigate marine food webs (Fry, 2008; Michener and Kaufman, 2007). This tool is very suitable for trophic ecology studies because natural isotope ratios are widely conserved between food sources and consumers, which often allows for the differentiation of the actual source of the food from other potential sources if their ratios are different (Cocito et al., 2013; Ferrier-Pagès et al., 2011; Leal et al., 2014a). Several scientific publications that summarise stable isotope ecology and its application to food webs are available and are highly recommended for further reading (Boecklen et al., 2011; Carabel et al., 2006; Fry, 2008; Layman et al., 2012; Michener and Kaufman, 2007).

Carbon (C) and nitrogen (N) have been the two elements most commonly used in food web analysis, particularly the $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios (Layman et al., 2012). The $\delta^{15}N$ signature is particularly interesting

to assess the trophic level of the target organism as this ratio exhibits a stepwise enrichment with trophic transfers. In contrast, the $\delta^{13}C$ signature varies substantially among autotrophs with different photosynthetic pathways, but changes little with trophic transfers (Fry, 2008). Consequently, this ratio is mostly used to assess original sources of dietary carbon. However, stable isotope ratios do not provide a direct characterisation of diet as does gut content analysis, nor do they allow for a quantitative assessment of trophic interactions (see Table 1). Because of the indirect nature of the data and the several sources of potential ambiguity in the interpretation of results, stable isotopes provide information that only allows the inference of feeding relationships and food-web structure (Layman et al., 2012).

To date this tool has already provided important insights on the trophic ecology of benthic marine invertebrates. For instance, herbivory by scleractinian corals has been an overlooked issue (Houlbrèque and Ferrier-Pages, 2009), and stable isotope ratios of a coral predator and their potential food sources provided evidence of such a feeding mode (Leal et al., 2014a). This finding was later confirmed by molecular gut content analysis (Leal et al., 2014b). Stable isotopes also allowed the identification of seasonal patterns in the trophic ecology of benthic invertebrates and its relation with food availability (e.g. Ferrier-Pagès et al., 2011; Leal et al., 2014c), as well as the effects of anthropogenic nutrient loads on the trophic ecology of marine invertebrates (e.g. Bode et al., 2014; Leakey et al., 2008; Tewfik et al., 2005). Additionally, stable isotopes have been particularly helpful to understand the trophic ecology of mixotrophs, such as animals living in association with photosynthetic organisms (e.g. symbiotic anthozoans, sea slugs). In these particular organisms, the role of the $\delta^{13}C$ signature is noted because photosynthetic carbon is transferred from the mutualistic algae (or sequestered chloroplast) to the animal host (e.g. Raven et al., 2001; Tremblay et al., 2013, 2015).

The use of stable isotope analysis together with other methodological approaches, such as gut content analysis and/or FA biomarkers, provides a powerful and integrated approach to address questions associated with trophic ecology (e.g. Alfaro, 2008; McKenzie et al., 2000; Spilmont et al., 2009). Gut contents provide an estimation of ingested food, whereas FA are relatively easy to metabolise and are incorporated in the organism, and SI identify food pathways within ecosystems because isotopic fractionation throughout the food web is used to differentiate assimilated food from that initially ingested. The combination of these techniques can minimise the limitations encountered when these tool are used separately (see Table 1),

and enables a thorough understanding of marine trophic networks and their implications for ecosystem functioning and evolutionary processes.

3.6 DNA-Based Methods

Molecular detection of trophic interactions is a relatively recent method in comparison to those previously summarised. The development of this tool was mostly prompted by the problems associated with prey-specific identification of gut contents and prey digestion, because DNA-based techniques allow the characterisation of feeding interactions with a high level of taxonomic resolution (King et al., 2008; Sheppard and Harwood, 2005; Symondson, 2002). While the first molecular studies addressing predator–prey interactions have been mostly focused on terrestrial invertebrates, an increasing number of studies using molecular trophic markers in the marine environment have been conducted in the past decade (Pompanon et al., 2012). Research using molecular detection of predator–prey interactions is still in its early stages in comparison to FA and SI analysis. However, it has already been used to provide new insights on the trophic ecology of marine invertebrates (e.g. Leal et al., 2014b,d, 2015; O'Rorke et al., 2014; Roura et al., 2012).

Molecular tools can be used to produce both qualitative and quantitative data on trophic interactions, with relatively high taxonomic resolution and very low amounts of sample biomass. For instance, a simple PCR approach using prey-specific primers provided new insights on herbivory by symbiotic corals and opened important questions on feeding mechanisms and prey selectivity (Leal et al., 2014b). Moreover, PCR-DGGE has been used to investigate the trophic ecology of marine suspension feeding bivalves (Maloy et al., 2009), whereas a sequencing approach provided new insights regarding the diet of planktonic bivalve larvae (Maloy et al., 2013a), lobster larvae (O'Rorke et al., 2014) and deep-sea invertebrates (Blankenship and Yayanos, 2005), among others. Sequencing of products can also be used in combination with the denaturing high-performance liquid chromatography (DHPLC) approach (Olsen et al., 2012). The use of DHPLC separates PCR amplicons based on DNA sequence differences, and sequencing such separated PCR products may provide information on the identity of prey.

Quantitative approaches, however, are still scarce as compared to qualitative assessments of diet composition of benthic marine invertebrates with bi-phasic life styles. Indeed, most of the quantitative work available thus far has been performed with copepods (e.g. Durbin et al., 2011; Nejstgaard

et al., 2008; Troedsson et al., 2009a) and other zooplanktonic organisms (e.g. Cleary et al., 2012; Frischer et al., 2014; Troedsson et al., 2009b). Nevertheless, real-time quantitative PCR has been used to quantify trophic interactions of benthic life stages. For instance, laboratory experiments with adult stages of symbiotic anthozoans provided new quantitative insights on prey ingestion and digestion (Leal et al., 2014d, 2015). Future quantitative approaches using molecular gut content analysis will depend on methodological advances to correct for cell number variability in multicellular organisms, as well as inconsistencies of gene copy number per cell/organism.

Semi-quantitative approaches using frequency data are also possible and common. Sequences can be grouped in operational taxonomic units and counted to provide quantitative information through relative abundances (Deagle et al., 2013). While it is tempting to assume that DNA sequences in the gut of sampled predators are representative of consumers' diet proportions, this assumption is rejected due to issues associated with gene copy number variability and multicellular prey. Nevertheless, the use of tissue and digestion correction factors is a creative and innovative way to successfully address this constraint and provide better estimates of ingested prey items (Thomas et al., 2014).

Even though many publications are still addressing methodological issues to identify gut contents (e.g. O'Rorke et al., 2013; Thomas et al., 2014), molecular trophic markers hold a great potential to investigate fundamental and applied aspects of marine invertebrate trophic ecology using either natural samples or experimental laboratory approaches. For instance, feeding selectivity (shifts of feeding preferences under variable environmental and/or physiological conditions of predator and/or prey species) and ontogenetic diet shifts are certainly relevant trophic ecology issues of benthic marine invertebrates with bi-phasic life cycles that remain poorly understood. However, the use of innovative molecular approaches for the investigation of trophic interactions, such as next generation sequencing, is inherently limited by the availability of reference libraries. While reference libraries with information on DNA sequences is dramatically increasing, it remains to be seen if they will be enough to identify unknown prey from gut contents, particularly from marine invertebrate larvae that prey on nano- and microzooplankton. This issue may be even more dramatic if the target predator displays a generalist diet. One way to address this limitation is to use a phylogenetic analysis on the same dataset for which gut content analysis was performed, to confirm sequence-similarity results and improve taxa identification (e.g. Hargrove et al., 2012).

4. MULTIDIMENSIONAL FRAMEWORK OF TROPHIC ECOLOGY

Trophic ecology addresses the feeding relationships among organisms in an ecosystem. This information may be used to further explore the relationships between feeding interactions and ecosystem functioning, such as food webs, nutrient recycling, community structure and productivity (Casini et al., 2008; Duffy et al., 2007; Ives et al., 2005). Besides such ecosystem-scale effects of predator–prey interactions, the information provided by research on trophic ecology can also be integrated at levels other than the organism itself, such as physiology, behaviour, morphology, life history and evolution (Marshall and Morgan, 2011). Trophic ecology has direct consequences for the multidimensional framework directly associated with feeding, particularly finding, eating, digesting, assimilating and utilising food, which involves ecological and physiological costs and compromises (Simpson and Raubenheimer, 1999). Indeed, the trophic ecology of benthic marine invertebrates with complex life cycles affects just about every facet of their life history. Therefore, research on trophic ecology is able to provide new insights on various traits that also affect population dynamics, such as maternal provisioning, larval performance and carry-over effects (see Section 2).

Complex life histories allow different developmental stages to be specialised in particular processes. For instance, marine invertebrate larvae are specialised in morphogenesis and growth, whereas adult stages are specialised in reproduction (Moran, 1994). However, different life stages are intimately linked, and the interplay of adult and larval traits determines life history differences among species (see Figure 1; Allen and Marshall, 2013; Marshall, 2008; Marshall et al., 2003a,b; Pechenik, 2006). This is especially relevant from a nutritional point of view, as organisms have to use ingested nutrients to grow or reproduce, and because energy reserves from food sources acquired in a certain life stage may notably affect organism performance at a later stage (Pechenik, 2006; Phillips, 2002). In this context, the trophic ecology of marine invertebrates with bi-phasic life cycles should be analysed using an overarching multidimensional framework that provides an integrative assessment of how nutrition affects ontogenetic development, phenotypic links throughout life history and, ultimately, evolutionary processes (Marshall and Morgan, 2011).

4.1 Phenotypic Links Across Life History

In Section 2, we discussed the various phenotypic links, including maternal investment and carry-over effects, across and within-generations of benthic invertebrates with bi-phasic life cycles. Most of these links are inevitably connected to the trophic ecology of a particular life stage, which consequently affects eco-physiological processes associated with subsequent stages. For instance, the trophic ecology of adult organisms affects offspring quality and quantity (e.g. Leal et al., 2012a; Stahlschmidt et al., 2015), and the nutrition of larvae affects the performance of juveniles and adults (e.g. Gonzalez-Ortegón and Giménez, 2014; Phillips, 2002). Phenotypic links among embryonic, larval and post-settlement stages are thus strongly connected and notably affect growth and survival (e.g. Allen and Marshall, 2013). The analysis of such nutritional-mediated links throughout life history requires a multivariate approach exploring the inter-relation among individual variables (e.g. Stahlschmidt et al., 2015) or the use of broader methodological concepts that capture the complexity of biological systems that is difficult to assess using univariate measurements alone (Raubenheimer et al., 2009; Jeyasingh et al., 2014; Sterner and Elser, 2002).

Despite the key role that nutrition has on the life history of marine invertebrate, research addressing phenotypic links across ontogeny has been mostly focused on the effects of abiotic factors (e.g. Giménez, 2011; Giménez and Anger, 2003; Przeslawski et al., 2015; Rosa et al., 2014). In fact, when addressed, nutrition often plays a secondary role in the set up of factorial experimental designs (e.g. Giménez, 2010; Gonzalez-Ortegón and Giménez, 2014). The use of factorial designs where food quantity and/or quality is an experimental treatment to assess performance of eco-physiological traits may certainly provide new ecological insights, as well as important information for grow out and production of marine invertebrates in captivity (e.g. Calado et al., 2005a,b; Leal et al., 2012b). However, such studies usually fail to identify and accurately quantify predator–prey interactions that might enhance our understanding of how the trophic ecology of larval and/or adult stages shapes the phenotypes of other life history stages.

The use of innovative methodological approaches is therefore needed to provide new insights on the trophic ecology of marine invertebrates with bi-phasic life cycles. Ultimately, new approaches will be able to address the links between trophic ecology and ecological and evolutionary consequences (Marshall and Morgan, 2011), as well as to better understand

ecosystem-scale effects associated with ontogenetic diet shifts (e.g. Schellekens et al., 2010) and its eco-evolutionary feedbacks (Matthews et al., 2011). By combining methods to investigate trophic interactions (see Table 1), one is able to minimise the weaknesses sometimes encountered with the use of such methods individually, and to achieve more robust results because of the synergies created when combining different tools. This approach will provide, for instance, high taxonomical resolution of predator–prey interactions together with energy and nutrient fluxes throughout the food web by combining molecular-based methods with FAs and SIs. A combined methodology will also provide information on ingestion and assimilation and, consequently, contribute to better identify and quantify nutrient utilisation from consumed prey (Maloy et al., 2013b; Traugott et al., 2013). It will also be critical to use this approach with experimental and/or sampling designs that allows for time-integrated sampling. This can allow the investigation of different life history traits and ultimately unravel phenotypic links throughout ontogeny mediated by trophic interactions. Additionally, this methodology will still involve the use of multivariate data analysis to integrate the multiple types of observations and ultimately detect ecologically relevant patterns, as well as which variables better explain the observed biological variability.

4.2 Phenological Responses to Food Availability

Besides using trophic ecology as an integrative trait across marine invertebrate life history, investigation of predator–prey interactions has also great potential to provide new insights on the phenology of benthic marine invertebrates with bi-phasic life cycles. Phenology is the branch of science that studies cyclic and seasonal natural phenomena, especially in relation to climate and plant/animal life. This is especially relevant from a trophic ecology point of view, as annually recurring life cycle events are often interconnected among prey and predator species. Understanding the link between phenology and the trophic ecology of marine invertebrates is also of utmost importance in view of global climate change, because the level of response from organisms to environmental changes may vary across functional groups and multiple trophic levels, which can have repercussions for food-web structures and ecosystem functioning (Edwards and Richardson, 2004). Organisms need to develop mechanisms to compensate for natural variability of food quantity and/or quality available in the environment. For instance, organisms may decrease their development rate and consequently delay

age at metamorphosis. Another alternative is to increase growth rate when food is abundant and/or in high quality to keep both mass and age at transition constant (Metcalfe and Monaghan, 2001). Moreover, time constraints imposed by seasonality affect the time available to successfully finish larval development, recruit and reproduce (Giangrande et al., 1994; Hadfield and Strathmann, 1996; Moran, 1994; Pappalardo and Fernández, 2014). These phenological traits notably interact with trophic ecology, and an integrative view of marine invertebrate nutrition throughout life history is likely to provide new insights on phenological plasticity of different life history events.

Differential shifts in the timing of resource availability and consumers may lead to trophic mismatches (Edwards and Richardson, 2004). A trophic mismatch occurs when the highest abundance of a predator species does not match with the peak of its food items. This has negative consequences for predator species, and it is particularly relevant for larval stages of benthic marine invertebrates with bi-phasic life cycles that rely on the acquisition of exogenous nutrients to grow faster and maximise survival rates (Olson and Olson, 1989). Moreover, and in view of the phenotypic links across ontogeny, trophic mismatches may have a significant role in the ecology of marine invertebrates and evolution of their life history (Marshall and Morgan, 2011; Stearns, 2000). New insights on these issues may be provided using nutrient-based multidimensional ecological frameworks.

4.3 Multidimensional Frameworks

4.3.1 Ecological Stoichiometry

Organisms are made of chemical elements, such as carbon (C), nitrogen (N) and phosphorus (P), among others. Ecological stoichiometry (ES) uses information on chemical elements to study the balance of energy and multiple elements in living systems based upon how it is affected by organisms and their interactions in ecosystems (Sterner and Elser, 2002). Ecological stoichiometry is based on two fundamental principles. First, autotrophs display larger variation in their elemental composition in response to nutrient availability, while heterotrophs use physiological mechanisms to regulate their elemental composition around taxon- or stage-specific values, defined as stoichiometric homeostasis (Sterner and Elser, 2002). Second, differences in elemental composition denote the nutritional imbalance between autotrophs and herbivorous, which may have a major effect on consumers' fitness and dynamics and therefore on the amount of energy and materials transferred up the food web (Boersma et al., 2008). Consequently, ES is a useful

framework to provide new insights on trophic relationships linking the elemental physiology of organisms with their food web interactions and ecosystem function (Hessen et al., 2013; Sterner and Elser, 2002).

Thus far, ES has been used to assess homeostasis of marine invertebrates and how it may be affected by environmental and ecosystem changes (e.g. Obermüller et al., 2013), as well as nutritional effects of feeding selectivity (e.g. Barile et al., 2004) and performance consequences of nutrient enrichment (e.g. Baggett et al., 2013). However, while ES is certainly an important tool for assessing how trophic interactions affect homeostasis and nutrient regulation, it is not a powerful approach for providing qualitative and quantitative data on the trophic ecology of a particular species with high taxonomical detail. While different prey species may show different elemental ratios (Sterner and Elser, 2002), homeostatic regulation of heterotrophs prevents linking the stoichiometric signature of a particular predator to its prey. Consequently, studies analysing elemental composition of marine invertebrates have been mostly addressing questions associated with organism condition and performance (e.g. Anger, 1998; Guerao et al., 2012; Nates and Mckenney, 2000).

Ecological stoichiometry captures the nutritional complexity in biological systems using multiple-currency frameworks (i.e. chemical elements) through the application of the laws of conservation of matter to trophic exchanges in ecosystems (Sterner and Elser, 2002). Because elements are easy to quantify, and are a common denominator relevant to all organisms and provide a link to fluxes within ecosystems, ES can be used to predict food choices, nutritional responses and functional consequences (Elser, 2006). Ultimately, this multidimensional framework provides a way to assess ecological and physiological costs and compromises associated with the trophic ecology of organisms and its integration in eco-evolutionary studies (Matthews et al., 2011). This approach thus holds great potential to understand how the nutrition of marine invertebrates with bi-phasic life cycles shapes adult populations (Marshall and Morgan, 2011).

Life history evolution is an important concept that is strongly determined by availability of resources, especially those that affect growth, survivorship, and reproduction (Stearns, 1992). Therefore, ES holds great potential to make significant contributions to the knowledge on this issue, elucidating the role of limiting nutrients in constraining consumer performance, as previously observed for freshwater systems where ES has been rapidly developing as an integrated framework (e.g. Bullejos et al., 2014; Elser et al., 1996; Jeyasingh et al., 2014; Villar-Argaiz et al., 2002). As selection acts on life

history to maximise individual fitness, the selective pressures imposed by limiting nutrients are expected to determine an organism's adaptive strategy, coupling life history with nutrient availability in order to minimise the elemental mismatch in trophic interactions throughout ontogeny.

As previously outlined (see Section 4.2), trophic mismatches are highly relevant for the phenology of marine invertebrates with bi-phasic life cycles, and are particularly relevant to address in future studies using a multidimensional nutritional framework. This is also relevant from a global climate change perspective, because trophic mismatches associated with environmental variability may notably affect the phenology of these organisms (Edwards and Richardson, 2004; Thackeray et al., 2010). A key insight that ES may provide concerns the elemental mismatch between resources and consumers, and how it can subsequently affect the nutritional demands required for animal growth (Bullejos et al., 2014). Interestingly, the bottom-up effect of poor food quality, which is usually characterised by low P content and high C:P and C:N ratios (Sterner and Elser, 2002), may propagate up the food chain because of direct stoichiometric constraints from primary to secondary and tertiary consumers (Malzahn et al., 2010). In addition, the degree of mismatch between the composition of the animal and food resource varies according to the nutrient requirements at each developmental stage (Back and King, 2013; Back et al., 2008; Pilati and Vanni, 2007; Villar-Argaiz et al., 2002). It is therefore important that future studies use a stoichiometric approach to investigate the phenotypic links across life history associated with nutritional demands of benthic marine invertebrate species. This would also provide new insights on ecosystem-scale effects, as stoichiometry traits associated with resource demand in consumers may affect the ratio and recycling rate of the nutrients used by autotrophs.

4.3.2 Nutritional Geometry

Another framework that integrates nutrition and ecology is nutritional geometry (NG). The concept of NG was developed as a tentative experimental and theoretical approach to measure the complex nutritional requirements of an animal using the following core components: organism, environment and nutrition-mediated interactions between the organism and the environment (Simpson and Raubenheimer, 1999). Nutritional geometry identifies the composition of nutritionally balanced food and assesses the trade-offs associated with variations in food quality/quantity intake. Ultimately, NG integrates feeding behaviour with physiology and

metabolism, and provides insights across levels of biological analysis, such as causation, development, ecology and evolution (Raubenheimer et al., 2009).

This methodological framework models the key relationships among relevant variables in nutritional ecology and is based on the logic of state-space geometry, where relevant variables are expressed and related to each other within a geometric space defined by relevant food components (Raubenheimer et al., 2009). This allows for the estimation of the food intake target, assessment of the consumers' current and optimal nutritional states and various performance consequences that are of potential interest (Simpson and Raubenheimer, 1999). All the information supplied by NG is key for any nutritional study, together with understanding the patterns, causes, and consequences of the trophic interactions that take place among organisms.

While this nutritionally explicit approach has been used to investigate the trophic ecology of marine copepods (Meunier et al., 2015) and fish (Raubenheimer et al., 2005; Simpson and Raubenheimer, 2001), to our best knowledge it has never been used for benthic marine invertebrates with bi-phasic life cycles. Nevertheless, as nutrition touches on just about every facet of an animal's life and it is particularly relevant for life history traits of these organisms, it is our opinion that NG holds great potential to better understand the phenotypic links among life stages and trans-generational effects (see Section 2). In view of the lack of information on the trophic ecology of these marine invertebrates, particularly regarding larval stages, NG emerges as an interesting framework because it does not require *a priori* judgements about the relative importance of different food properties or of the consumers' nutritional requirements (Raubenheimer et al., 2009). It treats all food components as equal and provides results that indicate how animals prioritise food ingestion and utilisation.

As animals grow, develop and reproduce, their nutritional requirements change both qualitatively and quantitatively. This is especially relevant for benthic marine invertebrates with bi-phasic life cycles, as ontogenetic diet shifts are very dramatic, together with the differential use of the acquired nutrients, i.e., larvae need to grow whereas adults need to reproduce. Consequently, adult males and females will also have different food intake trajectories. Nutritional geometry allows tracking of developmental shifts in food intake and investigates the mechanisms involving consumers' responses to changing nutritional needs (Simpson and Raubenheimer, 2011). Such information may also be combined with knowledge of the organisms'

foraging skills in collecting and searching for food and the energy invested in these activities that will ultimately affect the condition of compensatory feeding (Lihoreau et al., 2015). While the role of NG in providing new insights on the trophic ecology of marine invertebrates with bi-phasic life cycles remains to be tested, this overarching framework certainly holds the potential to provide a realistic perspective regarding the role that nutrients play in explaining and predicting interactions among organisms.

5. CONCLUSIONS, GUIDELINES FOR FUTURE RESEARCH AND UNRESOLVED QUESTIONS

Trophic ecology of marine invertebrates with bi-phasic life cycles is key throughout life history as it strongly determines an organism's capacity to grow, develop, metamorphose, settle, compete and reproduce. Our knowledge of the role of trophic interactions and how they affect phenotypic links across life history has been dramatically increasing over the past decades and has allowed us to understand, for instance, that parental nutrition significantly affects the success of early-life stages (Marshall, 2008) and that for most benthic marine invertebrates metamorphosis cannot be considered to be a new beginning (Pechenik, 2006). However, given the great biodiversity of marine invertebrates (WoRMS Editorial Board, 2015), together with the remarkable diversity of life histories of these organisms (McEdward, 1995; Thorson, 1950), we know surprisingly little about the ecology of many of these species and the evolution of underlying mechanisms driving such biological complexity (Marshall and Morgan, 2011). Our limited knowledge on the ecology and evolution of these invertebrates is particularly associated with the fact that most of them have pelagic larval stages and benthic adult forms, which notably affects our capacity to investigate them in natural conditions and understand the links between eco-evolutionary traits across their life history.

The study of the trophic ecology of larval and adult forms of benthic marine invertebrates with complex life cycles has benefited from the endeavours of researchers around the globe, including more recent work contributing to our knowledge of marine invertebrates in developing countries. Therefore, and in light of the extensive work here reviewed, it is our opinion that future efforts should attempt to make significant progress in particular research topics that would ultimately improve our understanding of the biology of this unique group of organisms.

In the following sections, we discuss guidelines for future research that, in our opinion, will provide new insights on trophic ecology traits in a unifying and integrated approach. The first of these guidelines calls for research on model species representative of particular taxonomic groups or certain ecological traits and life styles. The second guideline strongly recommends the use of integrated methodological frameworks addressing trophic ecology, as well as the use of complementary methods that will provide additional information using, whenever possible, the same biological sample and, consequently, will yield greater statistical power. The third guideline is aimed at espousing cooperative efforts that can lead to a unified direction for future studies, that standardises experimental ecology protocols and that takes advantage of available data and know-how from other research fields. The fourth guideline is the decoupling of the effects of trophic interactions and abiotic factors on trait-mediated effects, which is an issue that remains poorly investigated due to the confounding effects of sampling and/or experimental designs associated with the complex life histories of benthic marine invertebrates. Finally, the fifth guideline we suggest for future studies involves the assessment of eco-evolutionary consequences of ontogenetic diet shifts. This topic has been overlooked, probably because of the difficulties inherent to undertaking evolutionary-relevant studies, as well as the complexity of sampling marine invertebrates species throughout their complete life cycle and accurately and efficiently retrieve information on trophic ecology traits.

5.1 Use of Model Species

The use of model species has been advocated in a number of research fields to advance the state of the art (Hendry et al., 2013; Henry et al., 2010; Weis et al., 2008). Indeed, a large fraction of our knowledge on the trophic ecology of benthic marine invertebrates with complex life cycles derives from a small range of species that have been repeatedly investigated using experimental approaches in nature and in laboratory trials, and in some particular cases with the ultimate goal to captive breed them and achieve maximum grow out for commercial aquaculture purposes (Dionísio et al., 2013; Leal et al., 2014e; Olivotto et al., 2011). While some of the marine invertebrate species that have been recurrently investigated may not be universally considered biological models such as the common fruit fly, *Drosophila melanogaster*, stickleback, *Gasterosteus aculeatus*, or arabidopsis, *Arabidopsis thaliana*, they have certainly been relevant species for marine biology and ecology research. For instance, due to its importance for

fisheries and potential for aquaculture, the Norway lobster, *N. norvegicus*, has been investigated in a large number of studies, with considerable focus on the trophic ecology and larval biology of this species (e.g. Francis et al., 2014; Mente, 2010; Rosa et al., 2003). The same is valid for the European lobster, *H. gammarus* (e.g. Jones et al., 1997; Leal et al., 2013a; Van Der Meeren, 2005). *Carcinus maenas*, popularly known as the green crab, is another decapod crustacean that has been thoroughly investigated in field and laboratory studies (e.g. Giménez, 2006; Giménez, 2010; Wilcox and Rochette, 2015). This crab is a common littoral species, being recognised as one of the worst invasive species in some world regions (Grosholz and Ruiz, 1996). This fact prompted a large fraction of research efforts on this species, including investigations on predator–prey interactions of larval and adult stages and inter-specific competition (e.g. Calado et al., 2009; Kimbro et al., 2009), as well as ecological and evolutionary consequences of coastal invasions, particularly its disruptive role on local food webs (Grosholz, 2002).

Besides the decapod crustaceans mentioned above, other invertebrate species have also been thoroughly investigated and some of them are already referred to as model species. For instance, the Atlantic slippersnail, *Crepidula fornicata*, is a gastropod mollusc whose trophic ecology has been thoroughly investigated, as this snail feeds on phytoplankton both before and after metamorphosis (Henry et al., 2010; Padilla et al., 2014; Pechenik et al., 1998). Developing larvae capture food particles using a specialised organ that is lost at metamorphosis, but juveniles are still able to capture the same food type using ciliated gills. The bryozoan *Bugula neritina* is also an invertebrate species with a pelagic larval stage and adult benthic form that has been of great interest for marine pharmacology (Leal et al., 2012c). Efforts for captive breeding have been successful and experimental research on this species has provided new insights on, for instance, metamorphosis and cues that trigger settlement, as well as phenotypic plasticity and its links throughout ontogeny (e.g. Allen et al., 2008; Keough and Raimondi, 1995; Pechenik et al., 1998). The sea anemone *Aiptasia pallida* has been recurrently used as a model species to study photosynthetic symbiosis including the trophic ecology of mixotrophic animal (e.g. Leal et al., 2012b; Weis et al., 2008). Other invertebrate species have been thoroughly investigated, such as the sea cucumber *Holothuria scabra* (e.g. Battaglene et al., 1999; Hamel et al., 2001) and the sea slug *A. stephanieae* (e.g. Carroll and Kempf, 1990; Dionísio et al., 2013). Despite the notable advances that research on these model species have been providing, the use of standardised methodologies

together with integrated approaches to provide new insights on trophic ecology traits holds great potential to increase our understanding of the ways in which trophic interactions impact life history traits.

5.2 Integrated Methodological Frameworks to Investigate Trophic Ecology

Several studies have been combining different methods to assess the trophic ecology of benthic marine invertebrates with complex life cycles. For instance, gut content analysis has been combined with SI and/or FA approaches (e.g. Alfaro, 2008; Carreón-Palau et al., 2013; McKenzie et al., 2000), and molecular trophic markers have been combined with SI (e.g. Maloy et al., 2013b). Thus, the methods reviewed in Section 3 can all be used for qualitative and/or quantitative approaches and also provide information on food integration time. We examined the relationships between the various qualitative and/or quantitative approaches and information on food integration time in an attempt to provide a guide for future trophic ecology investigations planning to combine the different methodologies (see Figure 2). The location of each method according to each axis provides information, from our point of view, on how each specific method holds the greatest potential. For instance, both prey concentration and direct observation methods provide information on what has recently been ingested (hours). However, prey concentration calculations hold greatest potential for quantitative assessments, despite their well-known limitations, whereas direct observation is an approach that is more suitable for qualitative purposes. Gut content analysis and DNA-based methods, which also rely on sampling gut contents, are positioned in between qualitative and quantitative approaches, suggesting they are useful for both approaches, although they are more accurate for qualitative assessments. However, due to the high-sensitive nature of DNA-based methods together with the longer residence time of prey DNA in predator's guts (e.g. Leal et al., 2014b), we suggest that results derived from molecular trophic approaches may provide a relatively longer time-integrated signal as compared with visual observations of gut contents. We also note that despite the use of quantitative molecular approaches still being limited, this field may be notably boosted in the near future and improve the accuracy of this method to quantify prey ingestion and digestion. Lastly, FA and SI provide the longest time-integrated information, as they are both a consequence of nutrient assimilation. However, we have pointed out that one characteristic that distinguishes the two methods is that SI has been solely used for qualitative purposes, whereas

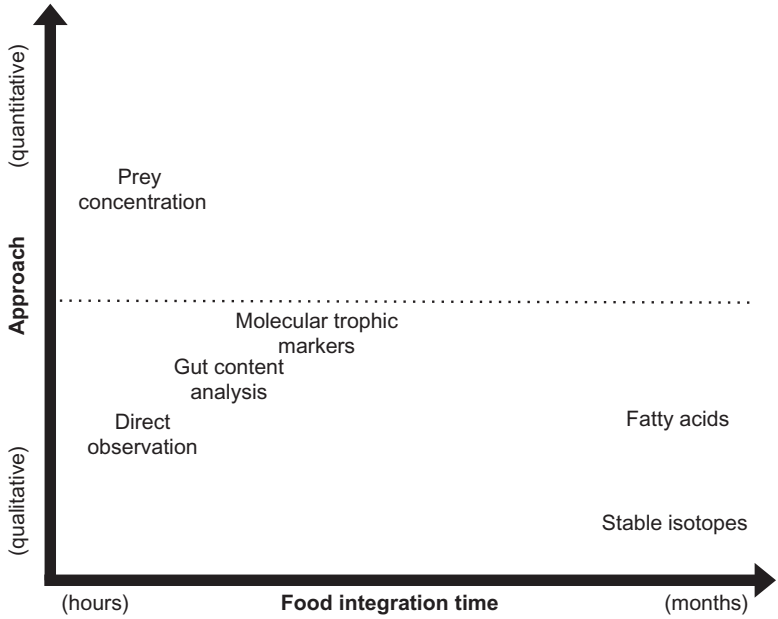

Figure 2 Graphical display of the most common methods currently used to assess the trophic ecology of marine invertebrates according to each method's suitability for qualitative and quantitative approaches (vertical axis) and according to the duration of its time-integrated signal of food consumption (horizontal axis). Dotted horizontal line represents the mid point between the two approaches, and the closer the methods are to this halfway point the most suitable they are for both qualitative and quantitatve measurements of trophic interactions.

notable advances have been made to use FA for quantitative approaches. Our intention is that this information together with our critical assessment of the advantages and disadvantages of each method will help researchers designing future sampling and experimental designs that provide more powerful empirical data on trophic interactions of benthic marine invertebrates with complex life cycles (see Figure 2 and Table 1).

While the different methods currently available have allowed important contributions to our understanding of benthic marine invertebrate ecology and physiology, integrated methodological frameworks are important to develop an overarching picture of the nutritional basis of the interactions between organisms and their environment. Ecological stoichiometry and NG hold great potential towards as a multi-currency tool for better understanding of how trophic interactions and environmental conditions affect resource use and allocation to maximise fitness. Both ES and NG

incorporate simple metrics to analyse the complexity of biological systems, but that have been rarely applied to investigate the trophic ecology of benthic marine invertebrates with bi-phasic life cycles and its ecological and evolutionary consequences. While ES and NG share fundamental similarities, they also have important differences, which have been thoroughly reviewed by Raubenheimer et al. (2009). Among the most important of these, is the fact that NG is a nutritionally explicit approach, whereas ES relates to the balance of energy and chemical elements, and the flow of matter and energy through ecosystems (Sterner and Elser, 2002). Consequently, ES measures the quantity and ratios of elements, such as carbon, nitrogen and phosphorus. In addition, other elements may also be analysed using ionomics, thus providing a powerful approach for a general exploration of poorly understood evolution-to-ecology links (Jeyasingh et al., 2014). Nutritional geometry measures macronutrients, such as protein, lipid and carbohydrate content, that are functionally relevant for the organism under investigation (Raubenheimer et al., 2009). We have discussed some of the positive and negative points identified for both themes and while we will not argue which of the frameworks should be used or which one is better, it is our opinion that both of them hold great potential for contributing to a better understanding of how trophic ecology traits may be assessed in evolutionary studies, thus integrating ecological dynamics with energy use acquisition and allocation.

5.3 Standardising Experimental Ecology Protocols and Taking Advantage of Available Data and Know-How from Other Research Fields

Different analytical methods and methodological frameworks are available for the study of ecology, but one of the biggest constraints that is still impairing our understanding of trophic ecology consequences on phenotypic links throughout life history is associated with the limitation to sample one or more life stages of benthic marine invertebrates. There is still a notable lack of knowledge of where, when and how several of these marine invertebrate species reproduce, where do these larval stages occur, when does metamorphosis take place and what are the triggers that drive habitat selection and settlement. This consequently affects the successful outcome of sampling efforts in the natural environment. Experimental biology and aquaculture therefore play an important role to advance our knowledge on these organisms with complex life cycles.

Experimental approaches have been used in the laboratory with a large number of invertebrate species. Even though our knowledge of the quality and quantity of natural food sources is scarce, some species have been successfully raised and reproduced in the laboratory using prey items that are not available in nature, such as the brine shrimp *Artemia*. This has allowed researchers to better understand larval development as well as the effects of nutrition on growth and survival (Dionísio et al., 2013; Houlbrèque and Ferrier-Pages, 2009; Leal et al., 2014e; Marshall et al., 2010a,b). The role of aquaculture research should also be acknowledged, as the use of factorial experimental designs testing different abiotic and biotic factors have notably contributed to a better understanding of how the trophic ecology of marine invertebrates is affected by environmental factors and predator–prey interactions. While ecologists and evolutionary biologists may argue that such aquaculture-based experiments fail to provide new ecological and evolutionary insights, aquaculture-derived data still holds great potential for meta-analysis studies focused on ecological and nutrient recycling issues (e.g. Czamanski et al., 2011). We particularly note that the ecological and evolutionary consequences of phenotypic and genotypic links throughout life history stages of benthic marine invertebrates are still poorly understood, and experimental ecology and evolution studies are highly encouraged (Marshall and Morgan, 2011).

Certainly, experimental biology, ecology and evolution have an important role to efficiently make progress on our understanding of the physiology, ecology and evolutionary biology of benthic marine invertebrates with complex life cycles. However, as emphasised by Padilla et al. (2014), notable insights on the role of trophic ecology may be achieved if researchers report metadata (e.g. abiotic and biotic conditions, food quality and quantity, and food provisioning) that allow realistic and accurate comparisons among experimental studies. This is critical if we consider that scientific synthesis is a realistic approach to integrate different research studies, thus increasing the scope and applicability of the results reported in the scientific literature (Hampton and Parker, 2011). Arguably, the global challenges we are currently facing create an urgent need for synthesis in ecological study (Mace, 2013), which will allow us to capitalise on the remarkable quantity and quality of scientific data already available that has resulted from enormous efforts performed over the last decades (Carpenter et al., 2009).

As most of the studies performed in the past decades have not been standardised, synthesis studies have, therefore, been difficult to perform. Consequently, we highly encourage researchers in this field to develop

and define clear guidelines for the best practices on experimental design, methods, systems and data reporting on studies addressing the role of environmental and trophic interactions in the life cycles of benthic marine invertebrates. A similar approach has been taken in other research areas such as ocean acidification (Gattuso and Lavigne, 2009; Riebesell et al., 2009) and aquaculture of some benthic marine invertebrates (Calado et al., 2003; Rocha et al., 2015), where guidelines have been suggested to standardise experiments. This approach can advance our knowledge on the biological complexity that characterises benthic marine invertebrates.

5.4 Decoupling the Effects of Trophic Interactions and Abiotic Factors on Trait-Mediated Effects

In light of the ongoing changes of the oceans of today, the scope of research on benthic marine invertebrate life cycles should urgently shift from a single to a multiple stressors approach (Byrne, 2011). In order to decouple the effects of trophic interactions and abiotic factors on different life stages, researchers are advised to simultaneously employ multiple methods that differ in their assumptions. In this way it is possible to provide a more robust conclusion than that which can be delivered by a single method when testing a null hypothesis. As experimental approaches are always prone to some type of artefact and/or rely on a number of assumptions that, more often than desired, are not tested, it is paramount to perform multiple methods simultaneously. This approach was advocated by Fenaux et al. (1994), in a study that tested food-limited growth of benthic marine invertebrate larvae. For example, when sampling competent larvae (i.e. larvae already receptive to cues that can trigger metamorphosis) from the plankton, researchers may at time collect some specimens displaying significantly smaller body sizes than others. It is therefore legitimate to question what was/were the driver(s) that promoted this trait. Did smaller larvae originate from previous larval stages that experienced suboptimal conditions during their planktonic life? Were those suboptimal conditions solely environmental, or was there any role played by trophic ecology as well? Taking simple measures of larval size does not provide enough information to recreate the larval history of these specimens, nor take into account potential mixed effects originating from environmental drivers and trophic interactions neither to decouple them (see Section 2.1.2). For these reasons, researchers should analyse collected larvae biochemically and molecularly with the ultimate goal of discovering variable fingerprints that may reflect their exposure to contrasting

trophic scenarios (e.g. using FA and/or SI analysis; Wang et al., 2014). In parallel, the enzymatic activity associated with suboptimal environmental conditions should also be assessed (e.g. heat-shock proteins and other transcriptomic tools, antioxidant enzyme activities and lipid peroxidation; Genard et al., 2011; Webster et al., 2013). If no differences are recorded, eventually it might be determined that the difference in size may have had its origin in a previous life stage. Researchers may have to go back to assess early larval life, or even to embryonic stages, to be able to understand the potential sources of the differences recorded on larval size near metamorphosis (Allen and Marshall, 2014). For these purposes, culture trials using similar sized newly hatched larvae, or larvae already exhibiting significant phenotypic differences (originating from different sized embryos within the same brood or from different broods produced by large vs. small females) can be performed *in situ* using mesocosms and *ex situ* in the laboratory. Trials should cover a range of variable environmental and trophic scenarios using standardised culture protocols (see Section 5.3). By gathering this type of data it can be possible to decouple environmental and trophic effects when surveying samples collected from the plankton. The use of complementary approaches may allow researchers to evaluate larval performance and gain a new insight on the potential role that is played by energetic reserves originating from the female (maternal investment) and those derived from planktotrophy. It will, therefore, be possible to monitor the consequence of smaller sizes at metamorphosis and to determine if size differences recorded for metamorphosing larvae are similar in young juveniles, or if differences are compensated or amplified (Podolsky and Moran, 2006). If such experimental results do not provide a reliable framework recreating the life experience of a given life stage, researchers may also have to focus their attention on the role played by parental organisms on the phenotypic traits being monitored. Some pitfalls must be avoided when performing this type of investigation, particularly when aiming to evaluate the effect of feeding regimes on maternal investment and offspring quality (see Calado et al., 2010). As is the case for larvae, biochemical and molecular tools should be employed when performing these experiments on parental organisms to control for unknown sources of bias.

It is well recognised that free-living larval stages with a prolonged life in the plankton may be particularly vulnerable to environmental and trophic stressors. However, narrowing research to the short-term responses of these life stages will unquestionably underestimate their effects on adult forms (Fischer and Phillips, 2014). As highlighted by Phillips (2004), researchers

can only perform reliable predictions on how environmental and trophic stressors experienced in different life stages affect individuals, populations and communities by integrating data from physiological and biochemical fingerprints arising from environmental and trophic experiences with developmental links along the entire complex life cycle.

5.5 Eco-Evolutionary Consequences of Ontogenetic Diet Shifts

Marine invertebrates often shift their diets throughout ontogeny. Prey-selectivity of early larval stages is strongly limited by functional morphological traits of the predator, which will limit the maximum size of the potential prey. Throughout organismal development, dietary shifts are expected for a preferential feeding on larger prey, following the assumption that prey size is positively associated with consumer body size and that larger prey size and/or higher trophic position corresponds to increased food quality (Layman et al., 2005). As early and late larval stages may occupy different planktonic habitats (Dos Santos et al., 2007; Marta-Almeida et al., 2006) and differences in planktonic prey availability are expected to occur throughout the water column (Leal et al., 2009), dietary shifts throughout larval ontogeny are also expected due to differences in food availability in different planktonic habitats. Moreover, once competent larvae settle and metamorphose, adults will feed on food items available in the benthic (bentho-pelagic) environment, which are expected to be different from those available in the pelagic environment. Dietary ontogenetic shifts from larval to adult stages are, therefore, predictable in benthic marine invertebrates with bi-phasic life cycles. However, our understanding of such dietary shifts is limited, especially because it is difficult to sample the same pool of organisms throughout their whole life cycle for accurate trophic ecology assessments. Consequently, ecological and evolutionary consequences of dietary shifts and impact of trophic mismatches associated with environmental changes have been overlooked.

Dietary shifts have important implications to the physiology of marine invertebrates, with potentially important fitness consequences. For instance, shifts from herbivory to carnivory, or even to omnivory, will modify the homeostatic balance between intake food and assimilated nutrients. Such homeostatic imbalance will have physiological costs and have potential fitness implications if the quality of consumed food items does not match the nutritional needs of the predator organism (Laspoumaderes et al., 2010; Raubenheimer et al., 2009). Hence, the degree of mismatch between the

composition of the animal and food resource may vary according to the nutrient requirements at each developmental stage. Further complexity can be added to this consumer-resource interaction if organisms have variable homeostatic capacity throughout their life history. A better understanding of these processes and their underlying mechanistic links is critical because ontogenetic driven nutritional demands share phenotypic and genetic links that have ecological and evolutionary consequences. Moreover, the effects of traits of one life history stage on another usually involve trade-offs, i.e., conflicting and complementary selection pressures across the life cycle that constrain microevolutionary change. As a phenotype may be expressed in two or more life history stages, the net selection on that phenotype will be a product of selection on multiple stages (Marshall and Morgan, 2011). Consequently, while a particular phenotype may have a selective advantage in a specific ontogenetic stage, it could also be associated with a selective disadvantage in a following stage of the life cycle. Understanding how evolutionary constraints have an effect on physiology and resilience across life history stages is, therefore, critical for developing an integrative view of the ecology and evolution of early-life and adult traits.

Besides the eco-physiological implications of ontogenetic diet shifts for the marine invertebrate organism itself, ecosystem-scale effects are also expected, particularly through modification of food web structure and length. For instance, increased food web connectivity and broad predation pressure on diverse resources are a consequence of such dietary shifts (Burress et al., 2013). Therefore, the ecological and evolutionary consequences of ontogenetic diet changes extend far beyond the organismal level and should be addressed in future studies.

5.6 Unresolved Questions

This review has highlighted the significant progress that has been made towards a more complete and integrated knowledge of the trophic ecology of benthic marine invertebrates with bi-phasic life cycles. The five guidelines we have developed for future research efforts are intended to foster new studies and unify research efforts towards a common goal—a better understanding of how trophic ecology of benthic marine invertebrates affect ecological and evolutionary processes ranging from the individual to ecosystem level.

Some key questions that remain unanswered were also raised throughout this review. It remains unclear why some early-life stage experiences

promote latent effects on juvenile and/or adult performance, whereas other biotic and abiotic affects do not have any consequence throughout their life cycle. In other words, why are some species apparently more sensitive to those experiences than others and why thus it varies even at an intraspecific level? It is therefore important to associate such biological variability to the phenotypic plasticity displayed by the different species, and understand the genetic-based mechanisms that may have an effect on resilience to environmental changes. Ultimately, it is important to address the potential evolutionary consequences of such inter- and intraspecific variability, as well as the effects on local, regional and global ecological processes.

After reading this review, we hope that readers will share our enthusiasm and fondness for the biological complexity displayed by benthic marine invertebrates with bi-phasic life cycles, as well as the many challenges that we are still facing to better understand the links between trophic ecology and ecological and evolutionary processes. A large number of mechanisms and ecological traits are now certainly clearer, but many others remain unresolved.

ACKNOWLEDGEMENTS

The present work was financially supported by FEDER through COMPETE, Programa Operacional Factores de Competitividade and by national funding through Fundação para a Ciência e Tecnologia (FCT), within the research project NO RESET PTDC/BIA-BIC/116871/2010 and UID/AMB/50017/2013.

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CHAPTER TWO

Body Size Versus Depth: Regional and Taxonomical Variation in Deep-Sea Meio- and Macrofaunal Organisms

Jesse M.A. van der Grient[1], **Alex D. Rogers**
Department of Zoology, University of Oxford, Oxford, United Kingdom
[1]Corresponding author: e-mail address: jesse.vandergrient@zoo.ox.ac.uk

Contents

Abstract

Body size (weight per individual) is an important concept in ecology. It has been studied in the deep sea where a decrease in size with increasing depth has often been found. This has been explained as an adaptation to food limitation where size reduction results in a lowered metabolic rate and a decreased energetic requirement. However, observations vary, with some studies showing an increase in size with depth, and some finding no depth correlation at all. Here, we collected data from peer-reviewed studies on

Advances in Marine Biology, Volume 71
ISSN 0065-2881
http://dx.doi.org/10.1016/bs.amb.2015.07.002

macro- and meiofaunal abundance and biomass, creating two datasets allowing statistical comparison of factors expected to influence body size in meio- and macrofaunal organisms. Our analyses examined the influence of region, taxonomic group and sampling method on the body size of meiofauna and macrofauna in the deep sea with increasing depth, and the resulting models are presented. At the global scale, meio- and macrofaunal communities show a decrease in body size with increasing depth as expected with the food limitation hypothesis. However, at the regional scale there were differences in trends of body size with depth, either showing a decrease (e.g. southwest Pacific Ocean; meio- and macrofauna) or increase (e.g. Gulf of Mexico; meiofauna only) compared to a global mean. Taxonomic groups also showed differences in body size trends compared to total community average (e.g. Crustacea and Bivalvia). Care must be taken when conducting these studies, as our analyses indicated that sampling method exerts a significant influence on research results. It is possible that differences in physiology, lifestyle and life history characteristics result in different responses to an increase in depth and/or decrease in food availability. This will have implications in the future as food supply to the deep sea changes as a result of climate change (e.g. increased ocean stratification at low to mid latitudes and reduced sea ice duration at high latitudes).

1. INTRODUCTION

Body size (weight per individual) is one of the most important properties of an organism as it is related to, and can be used to predict, many other co-varying characteristics, such as physiology, life history, and ecology (Peters, 1983). The relationship between body size and abundance can link individual- and population-level species traits with the dynamics and structure of ecological communities (Woodward et al., 2005). Furthermore, body size is a determinant of resource use as it is related to metabolism, and thus can aid in predicting resource partitioning (Brown et al., 2004). Body size relationships have been extensively studied in terrestrial environments, but less so in the oceans and in particular the deep sea (depths greater than 200 m). The deep sea is the largest ecosystem on Earth and is increasingly recognized as important in global biogeochemical cycling (Dunne et al., 2007; Giering et al., 2014). Benthic ecosystems, especially those of ocean margins, are important in carbon burial (Dunne et al., 2007) and can have remarkably high levels of biodiversity, especially considering they are generally recognized as food-limited environments (Grassle and Maciolek, 1992). Body size and temperature have been identified as having a significant influence on the metabolic rate of deep-sea organisms, as well as on aspects of life history such as longevity and population turnover (McClain and Barry, 2010) supporting the Metabolic Theory of Ecology (Brown et al., 2004).

Since the 1950s, quantitative sampling and ecological investigation in the deep sea have documented general patterns in community structure and functional ecology (Gage and Tyler, 1999). One of the most widely recognized of these patterns is the decline in community biomass and abundance, and individual body size, with increasing depth and decreasing surface-derived particulate organic matter, in other words, food (Rex et al., 2006; Wei et al., 2010a). Understanding how such patterns of community attributes and organismal traits vary both spatially and temporally is important to understanding how climate change effects, such as ocean warming, and other human impacts, like deep-sea trawling, may influence ecosystem function within these communities, ultimately with implications for predicting changes in the biological cycling of important nutrients within the Earth system.

Early work in deep-sea ecology resulted in the recognition of two trends of body size with increasing depth: dwarfism and gigantism (Berkenbush et al., 2011; Danovaro et al., 2002; Galeron et al., 2000; Jones, 1969; Pequegnat et al., 1990; Pfannkuche, 1985; Rex and Etter, 1998; Rowe et al., 1991; Schwinghamer, 1985; Shirayama and Horikoshi, 1989; Soetaert and Heip, 1989; Thiel, 1979; Thurston, 1979). Of these two, dwarfism is thought to be more common (Madsen, 1961; Pfannkuche, 1985; Rex et al., 2006; Soetaert and Heip, 1989; Thiel, 1975). Rowe and Menzel (1971) are often acknowledged as the first to have quantitatively demonstrated the trend of decreasing body size with increasing depth. Their results, however, showed no significant decrease in body size with increasing depth; rather, they accepted the hypothesis that the slope of decreasing abundance with increasing depth was different from the slope of decreasing biomass with increasing depth based on a non-significant p-value ($P = 0.13$). The trend of no change in body size with increasing depth has been supported by some studies for macrofaunal and meiofaunal organisms (Polloni et al., 1979; Shirayama, 1983; Vanhove et al., 2004), whilst other studies have found an increase in size (Alongi, 1992; Rex et al., 1999). Alternatively, parabolic patterns of body size with depth have been observed as well (McClain et al., 2005). This variety of trends may indicate that there is no general rule; rather, we should look to explain the observed differences.

The conflicting results may reflect differences in oceanographic regions. Many studies present their data and trends as a universality, rather than as a local or regional phenomenon in ecology. However, when regions differ in food availability, for example if food is vertically transported or laterally advected or is subject to resuspension in different parts of the continental slope (Dell'Anno et al., 2013), then changes in body size may not correlate

with depth. There are also potential regional variations in the efficiency of transfer of surface primary production to the deep sea (Buesseler et al., 2007).

If there is a taxonomic signal in body size, then changes in the taxonomic composition of communities as a result of changes in depth or other physical parameters (e.g. sediment grain size; Rohal et al., 2014), could also influence estimates of average community body size. Community composition is expected to differ in space and time, and this has been observed (Billett et al., 2010; Blake and Grassle, 1994; Cosson-Sarradin et al., 1998; Danovaro et al., 2010; Flach and de Bruin, 1999; Ruhl et al., 2008). Varying community composition can have contrasting influences on the nutrient recycling and carbon burial of a particular region, for example through differences in bioturbation potential (Braeckman et al., 2011; Dauwe et al., 1998).

Two size classes often studied in the benthic marine system are the meiofauna and the macrofauna. A rough distinction between the two is that macrofauna can be seen when they are lying in your hand, but are not visible in pictures of the environment; meiofauna require microscopes to see them. A more scientific distinction is the range of sieve sizes that can be used to separate them: 0.5–1 mm for macrofauna and 0.063–0.5 mm for meiofauna. These sizes are based on shallow-water ecosystems. It has been found that in the deep sea, this does not adequately capture the benthic fauna, and thus smaller sizes are used. For example, from 0.25 to 1 mm sieve meshes are used for macrofauna, while meiofauna can be determined from 0.025 to 0.5 mm meshes. An important observation in this is that the sieve sizes can overlap.

Many marine invertebrates have indeterminate growth and might be present in different size classes through different stages of their lives. Studies differ in their approach to this phenomenon: some include all organisms captured by a specific sieve size, while others look at the predetermined fauna *senso stricto* meaning that they can potentially, for example, exclude nematodes from macrofauna and polychaetes from meiofauna.

When looking at the two size classes, a bimodal distribution in the frequency-size relationship is observed. There is much debate about whether this distribution shows biological adaptation to disruptive selection for two different lifestyles, resulting in the meio- and macrofaunal peak, or whether it is a result of sampling (Bett, 2013, 2014; Warwick, 2014; Warwick et al., 2006). Many studies now confirm that the choice of sieve size can potentially influence estimates of abundance, biomass, body size, composition and biodiversity (Bett et al., 1994; Gage et al., 2002; Kaariainen and Bett, 2006; Leduc et al., 2010a; Pavithran et al., 2009).

Here, available data on abundance, biomass and body size estimates are compiled for deep-sea meio- and macrofauna to test for the influence of depth, geographical region, taxonomic structure of communities, and sampling methods on body size. The results are discussed with respect to how the community-size structure changes at the regional scale, how size varies in taxonomic groups on a global scale, and the implications of this in the context of future climate change. Recommendations for sampling strategies are made based upon our findings.

2. METHODS

2.1 Data Collection

Data were assembled from peer-reviewed studies on macro- and meiofaunal abundance and biomass, creating two datasets (see Appendix A). The oceanographic regions where the samples were taken in these individual studies were recorded. We note that the Polar Regions and southern Hemisphere are markedly under-sampled for both size classes. Few studies were available for the southern parts of the Pacific and Atlantic Oceans. The Indian Ocean is not represented in either data set. Carbon sequestration was calculated as the mean annual flux below 2000 m or the burial in sediment across time from the year 2003 to 2012 in arcGIS. The resulting data sets comprised a total of 96 published studies (3306 estimates from 46 published papers for macrofaunal data, and 5065 estimates from 66 published papers for meiofaunal data; see Appendix A).

The abundance and biomass data were used to calculate average weight per individual in both datasets (as a proxy for body size). As not all studies reported both abundance and biomass, the data sets used in the models were reduced to 601 estimates for macrofauna and 740 estimates for meiofauna. (this highlights the need to report these values in future studies).

Macrofaunal biomass was published as wet weight in all studies used in our analyses (see Appendix A). For meiofauna, various measures of biomass were used among studies: wet weight, carbon wet weight, dry weight and carbon dry weight (see Appendix A). Published conversion factors (Ankar and Elmgren, 1976; Feller and Warwick, 1988; Jensen, 1984; Wieser, 1960) have been used in this study to calculate the wet weight for meiofauna to ensure the biomass is on the same scale for all estimates.

Most studies reported values of total community abundance and/or biomass, but many also reported values for separate taxonomic groups. The highest resolutions for taxonomic data available were included in our model

to determine whether a taxonomic signal is present influencing body size change with increasing depth.

Published data on meiofaunal and macrofaunal abundance and biomass included a wide range of sampling methods, and these methods can potentially influence abundance and biomass estimates (Bett et al., 1994; Gage et al., 2002; Kaariainen and Bett, 2006; Leduc et al., 2010a; Pavithran et al., 2009). Area sampled and sieve mesh sizes used were recorded in the current study. We found that no standard sieve mesh size was used, and, indeed, the sizes used overlapped for the two size classes. For macrofauna, sieve size ranged from 0.25 to 0.5 mm, and for meiofauna it ranged from 0.032 to 0.3 mm.

Published studies differed in the actual area sampled as a result of subsampling and/or sampling device. The actual areas sampled varied between the studies, with a range of 0.003–4 m^2 for macrofauna and a range of 0.0003–0.59 m^2 for meiofauna. The actual area sampled was, more often than not, different from the standardized measure presented in the studies: for macrofauna m^2 was used, and for meiofauna 10 cm^2 was used. The actual area sampled was used in this study to explore the effect of sampling area on body size estimates. When the subsample dimension was not given in the original publication, the original core area was used for the purposes of the current work. Surface area was used as not all studies provided information about the depth of sampling.

Whether the organisms were juveniles or adults was not mentioned in the published studies used, but it was assumed data referred to adult organisms.

2.2 Statistical Analyses

Two generalized least squares models were constructed from the two datasets we compiled based on 96 published studies (see Appendix A), controlling for different variances in region, taxa, and sieve mesh size. Total community composition was used as a baseline comparison for taxonomic groups. A global average was derived from the dataset and used as a baseline for comparison of the regional effect. Standard errors (SE) and 99% confidence intervals (CI) are reported, as well as statistical significance values for analysis of variance. By using the 99% CI, a correction for multiple testing is applied. These two statistical values, SE and CI, provide the same qualitative information as *t*-tests and *F*-test, but they have the advantage that they stay closer to the biological data (Hector, 2015). The SE and CI also provide

more information than *t*- and *F*-values by showing statistical significance and the bounds of the estimated values.

All analyses were performed in the statistical program R, version 3.1.1 (R Core Team, 2014), using the *nlme* package (Pinheiro et al., 2014). The graphs were produced with the *effect* package (Fox, 2003).

3. RESULTS

3.1 Macrofauna

Average body size decreases with increasing depth ($=-7.400\times10^{-5}$ log gram, standard error of the mean $=2.040\times10^{-5}$, confidence interval of the mean $=-1.266\times10^{-4}$ to -2.125×10^{-5}, $P<0.0001$; Figure 1A). Sieve mesh size is positively correlated with average size of individual organisms collected (=2.690 log gram, standard error of the difference (SED) $=1.980\times10^{-1}$, confidence interval of the difference (CID) $=2.180$–3.200, $P<0.0001$; Figure 1B). Area sampled was not significant (Figure 1C). The carbon measure used in the model was non-significant, likely reflecting the limited use of a surface-derived carbon measure at depth.

There was a significant regional impact on the body size estimates ($P<0.0001$). All oceanographic regions show a negative shift in body size with increasing depth, but the amount of decrease varies (Figure 1D, Appendix B). For example, the Arabian Sea shows the largest shift in size ($=-5.024$ log gram, SED $=1.467\times10^{-1}$, CID $=-5.402$ to -4.646, $P<0.0001$), while the east Pacific shows the weakest ($=-2.726$ log gram, SED $=1.628\times10^{-1}$, CID $=-3.146$ to -2.307, $P<0.0001$).

A significant taxonomic signal was detected ($P<0.0001$; Figure 1E, Appendix B); negative shifts in body size compared to the total community average were observed in many taxonomic groups, although most were found to be non-significant. Some taxonomic groups, like the Ophiuroidea (brittle stars) and Porifera (sponges), showed a positive shift (Ophiuroidea: $=8.518\times10^{-1}$ log gram, SED $=2.069\times10^{-1}$, CID $=3.190\times10^{-1}$ to 1.385, $P<0.001$; Porifera: $=6.283\times10^{-1}$ log gram, SED $=2.174\times10^{-1}$, CID $=6.829\times10^{-2}$ to 1.188, $P<0.001$). Most taxonomic groups did not differ from the total community average; however, taxonomic resolution can change the significance of the relationship. For example, the Phylum Echinodermata was not found to differ significantly from the total community ($=2.439\times10^{-1}$ log gram, SED $=2.083\times10^{-1}$ CID $=-2.926\times10^{-1}$ to 7.804×10^{-1}), while the Ophiuroidea seemed

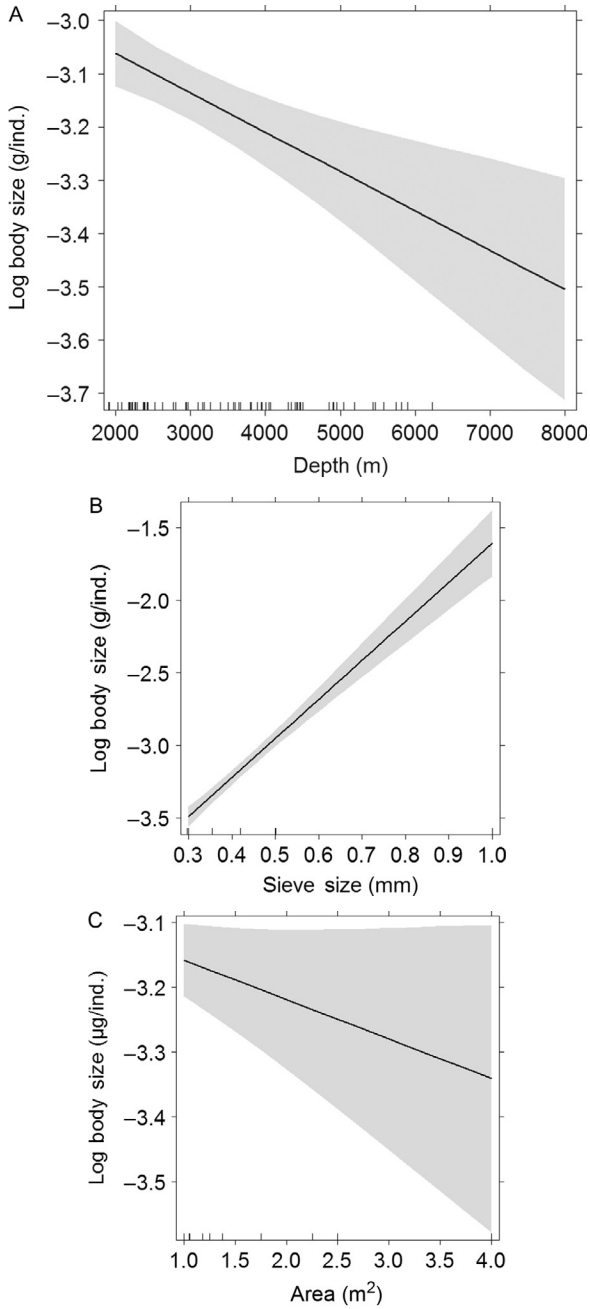

Figure 1 See legend on opposite page.

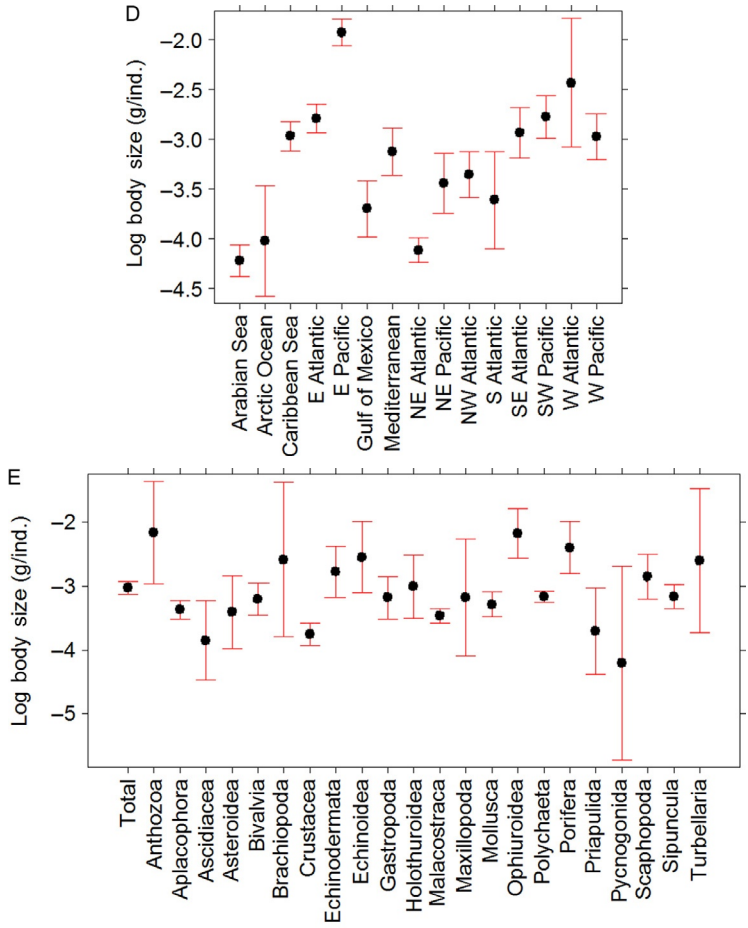

Figure 1—Cont'd Deep-sea macrofaunal body size variation in relation to depth, sampling methods, geographical region or taxonomic groups: (A) Log body size versus depth (in meters). (B) Log body size versus sieve size (in millimeters). (C) Log body size versus area sampled (in m^2). (D) Log body size versus different geographical regions. (E) Log body size versus total community and different taxonomic groups. The grey bands in (A–C) present the 95% confidence intervals, and the red (grey in the print version) bars in (D) and (E) present the standard error.

to be larger than the total average. It is possible that this pattern can hold for other groups as well when more data is collected on the taxonomic Class level rather than Phylum. Also, groups at the same taxonomic level can vary in their response: the Class Malacostraca (subphylum Crustacea) was found

to show a negative shift in body size compared to the total community, while for the Class Maxillopoda (subphylum Crustacea) no significant shift was found (Malacostraca: $=-4.409\times10^{-1}$ log weight, SED $=8.4$–6×10^{-2}, CID $=-6.574\times10^{-1}$ to -2.244×10^{-1}; Maxillopoda: $=-1.575\times10^{-1}$ log weight, SED $=4.692\times10^{-1}$, CID $=-1.366$ to 1.051, $P<0.001$).

3.2 Meiofauna

Our analyses indicated that body size decreased with increasing depth ($=-6.3\times10^{-5}$ SEM $=8.3\times10^{-6}$, CI $=-8.449\times10^{-5}$ to -4.164×10^{-5}; Figure 2A). Both sieve mesh size (Figure 2B) and area sampled (Figure 2C) were found to have a significant impact on individual body size; increasing sieve size had a positive influence on mean individual body size, while area sampled had a negative influence on mean individual body size (Sieves: $=2.342$ log weight, SED $=3.598\times10^{-1}$, CID $=1.415$ to 3.269, $P<0.0001$; area: $=-5.789\times10^{-1}$ log weight, SED $=1.261\times10^{-1}$, CID $=-9.036\times10^{-1}$ to -2.539×10^{-1}, $P<0.0001$). The carbon measure, again, was not significant.

Geographical region and taxonomic group had significant influence on body size patterns ($P<0.001$; Figure 2D and E, Appendix C). Interestingly, the shift in body size differed among different oceanographic regions. Most regions showed a negative shift in body size, and differed in the strength of the response. The largest strength of response was found in the southeast Pacific, while the weakest was in the southwest Pacific (southeast Pacific: $=-4.828$, SED $=5.57\times10^{-1}$, CID $=-6.263$ to -3.393, $P<0.0001$; southwest Pacific: $=-1.246\times10^{-1}$, SED $=3.976\times10^{-2}$, CID $=-2.270\times10^{-1}$ to -2.215×10^{-2} $P<0.0001$). Some regions, however, like the Caribbean and the northwest Atlantic, seemed to show a positive shift in body size compared to the global average (Caribbean: $=8.917\times10^{-1}$, SED $=7.383\times10^{-2}$, CID $=7.015\times10^{-1}$ to 1.082, $P<0.0001$; northwest Atlantic: $=9.908\times10^{-1}$, SED $=4.884\times10^{-2}$, CID $=8.65\times10^{-1}$ to 1.117, $P<0.0001$).

Meiofaunal taxonomic groups differed in the direction and strength of the relationship between body size and depth compared to the total community. For example, the Nematoda had a significant decrease in body size ($=-1.487\times10^{-1}$, SED $=3.157\times10^{-2}$, CID $=-2.3\times10^{-1}$ to -6.739×10^{-2} $P<0.0001$), while the Polychaeta showed an increase in body size ($=1.344$, SED $=4.797\times10^{-2}$, CID $=1.221$–1.468, $P<0.0001$).

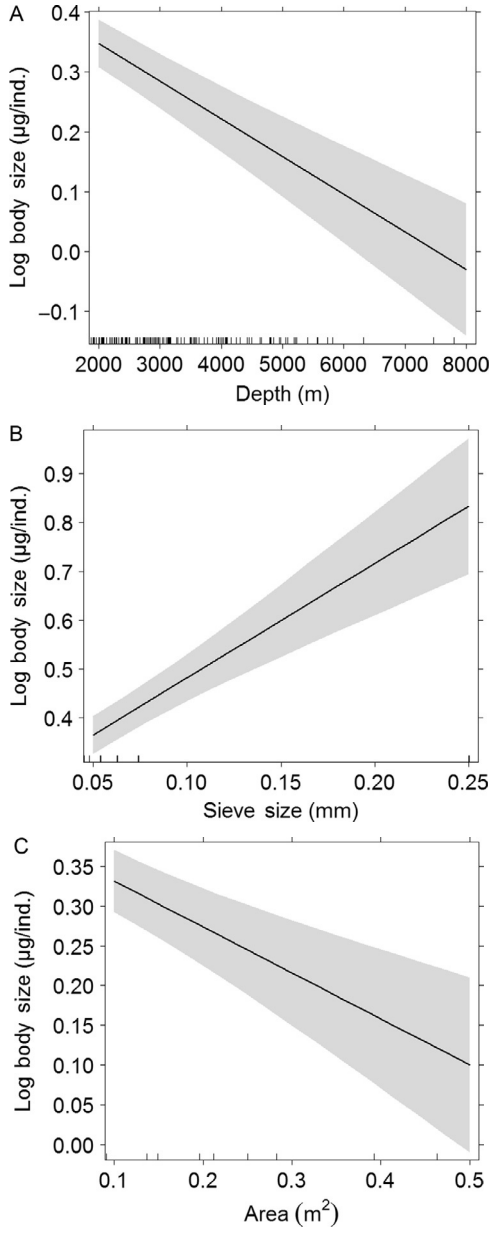

Figure 2 See legend on opposite page.

(Continued)

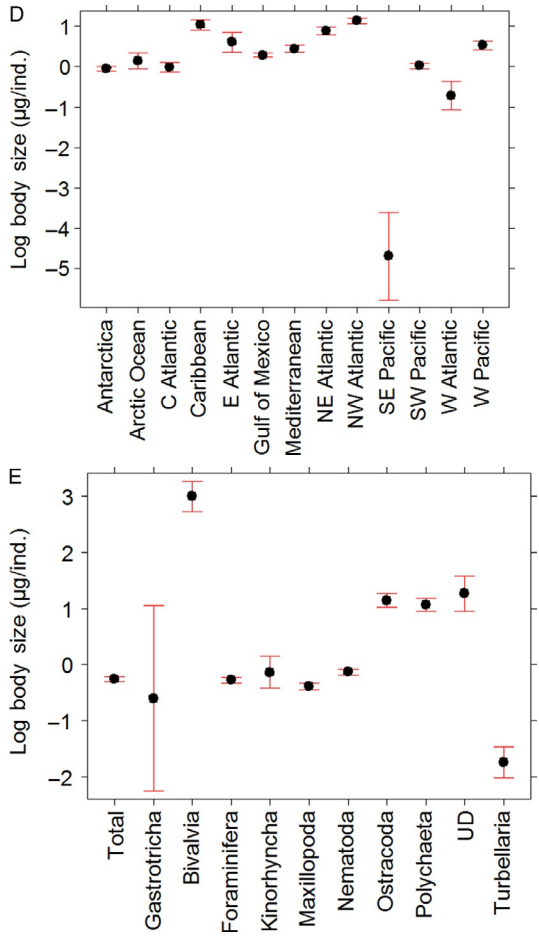

Figure 2—Cont'd Deep-sea meiofaunal body size variation in relation to depth, sampling methods, geographical region or taxonomic groups: (A) Log body size versus depth (in meters). (B) Log body size versus sieve size (in millimeters). (C) Log body size versus area sampled (in m^2). (D) Log body size versus different geographical regions. (E) Log body size versus total community and different taxonomic groups. UD, undetermined. The grey bands in (A–C) present the 95% confidence intervals, and the red (grey in the print version) bars in (D) and (E) present the standard error.

4. DISCUSSION

Dwarfism was once suggested to be the main universal trend for body size with increasing depth in the deep sea (Madsen, 1961). The models constructed here only allowed for the analyses of differences in intercepts—not

for slopes—in the correlations for regional and taxonomic effects. This choice in analysis was made based on the availability of data in the published scientific literature. The models presented here appear to be consistent with the hypothesis of a total community decrease in body size with increasing depth for both meiofauna and macrofauna (Thiel, 1975). The trend, however, can be a reflection of other depth-correlated factors that influence the change, with food availability as the proposed major factor.

4.1 Regional Effects

4.1.1 Sea Surface Primary Production

Our models indicate that there is a regional effect on the correlation of body size with increasing depth for both meio- and macrofaunal size classes, and that the responses of the two size classes can vary within the same region. The regional differences in body size could reflect variations in carbon flux. Carbon is a proxy for food resources and a form of chemical energy. It has been proposed that food availability is the main influencing factor on body size change in the deep sea (McClain et al., 2012b). The current study did not find an association between body size and carbon flux. However, it is likely that the method used has limited the detection of a correlation. The carbon flux was calculated from surface-derived carbon measures to a specific depth (below 2000 m) or burial rate. Much of the data used in the current analyses were based on samples taken at differing depths (see Appendix A). Samples at shallower depths will potentially have had higher carbon content than those from deeper depths. Another problem with this measure is the obvious time difference. Some samples were taken several decades ago, while the carbon flux measure is a mean over the last decade. Carbon flux is known to vary temporally and thus the flux calculated for the last decade can vary from the flux several decades ago. It is likely that carbon measures taken contemporaneously with samples will have a stronger correlation.

Most of the deep sea—apart from chemosynthetic environments—is dependent on sea surface primary production for food; calcareous phytoplankton, like coccolithophores, can aggregate and sink as their calcite plates form ballast (Honjo et al., 2008). Likewise, silica can be used by diatoms in exoskeletons and acts as ballast and promote sinking. Together with living and dead cells, faecal pellets, dead organisms, polysaccharide flocculates (e.g. mucus) and other organismal associations (e.g. larvacean houses), this sinking organic material forms marine snow (Buesseler et al., 2007; Honjo et al., 2008; Robison et al., 2005; Silver et al., 1978). Food availability is negatively correlated with depth as it is remineralized in the water column, mainly by

microorganisms but also by animals (Rex and Etter, 1998; Rex et al., 1999). Most is recycled in the upper layers of the oceans, resulting in a small proportion reaching the benthic system. The amount that reaches the bottom of the oceans varies regionally, with seasonality playing a role in this too (Buesseler et al., 2007).

The variation observed in the models we have produced based upon the peer-reviewed studies listed in Appendix A, agrees with previous findings that carbon can play a role in body size (McClain et al., 2012a). For example, the northwest Atlantic has a high primary production and shows that the mean meiofaunal community size is larger than the global average, while the central Atlantic, which has a lower surface primary production, has a smaller meiofaunal community size than the global mean. The Caribbean meiofaunal community may profit from the riverine runoff from the Orinoco River that increases primary production in this region (López et al., 2013), resulting in an increase in carbon flux and larger meiofaunal-community size compared to the global mean. Other larger rivers, like the Amazon are known to have an impact on the deep sea (Rex and Etter, 2010). It is also notable that the Caribbean is partially land-locked and organic material of terrestrial origin may also influence food availability in the deep sea.

Seasonality plays a role in the regional effects resulting from primary production with a change in flux during seasons at higher latitudes (Buesseler et al., 2007). It has been shown that the magnitude of the carbon flux does not determine remineralization rates, but the composition of the flux does. For example, the K2 and ALOHA time-series sites are both located in the Pacific, with K2 being present at a higher latitude with colder subarctic waters, higher nutrient concentrations and a larger seasonality flux in phytoplankton, while ALOHA is located at a lower latitude within warmer subtropical waters with low nutrient concentrations and less seasonal variation. Both sites differ in their phytoplankton composition, resulting in different ballast types that likely influence the remineralization rate of the carbon flux (Buesseler et al., 2007). In the analyses presented here, such differences were not clearly defined. Sampling coverage among the various studies included in our analyses was limited, making comparisons on a latitudinal scale difficult. Many samples have been taken in the northwest Atlantic, but fewer samples have been collected from the central West Atlantic and southwest Atlantic. The southern Hemisphere is characterized by under-sampling. It is to be expected that the benthic system in the Southern Ocean will benefit from the higher sea surface productivity that sinks in pulses linked to seasonality. Sea ice extent and duration in the Southern Ocean system is

changing, and this is likely affecting sea surface primary production. Sampling of this system is necessary now to get a baseline and to track the changes at greater depths. A more focused effort on filling in these data gaps will allow for the detection of possible patterns of seasonality with latitude and future change.

Other environmental conditions that vary by region can also influence the observed body size–depth pattern and give contrasting results with the surface primary production patterns. For example, the Arabian Sea has high primary production, yet the community size is smaller compared to the global average. This region is characterized by an extreme oxygen minimum zone (OMZ; Wyrtki, 1966). Oxygen levels are maintained in the deep sea by ocean ventilation. Microbial activity mainly influences oxygen levels, with higher activity levels resulting in a larger decrease of oxygen concentration with depth. Microorganisms use the carbon flux as food source, and their activity is correlated with depth (i.e. corresponding decrease in carbon flux with depth). Larger organisms are apparently influenced the most by the dropping levels of oxygen, resulting in standing stock changes and smaller community sizes (Gooday et al., 2009; Levin et al., 1991, 2000). Often, the community below the OMZ is more abundant and possibly larger in size as a result of reduced rates of remineralization in hypoxic water (Levin et al., 2000; Rogers, 2000). The present study found a decrease in community size for the Arabian Sea, which might well reflect the influence of oxygen. It might also reflect a hierarchy of controlling mechanisms on body size, with lower levels of oxygen resulting in smaller size even when there is a large influx of food. When oxygen levels are high, meio- and macrofauna standing stock and size likely correlate better with food availability (Levin et al., 1991).

Regional differences in meiofaunal abundance reflecting surface primary production have been observed (Lambshead and Gooday, 1990; Soltwedel, 2000; Sommer and Pfannkuche, 2000). When comparing oligotrophic sites with eutrophic sites in the Northwest Pacific, Itoh et al. (2011) found that meiofaunal organisms were smaller in the oligotrophic regions compared to the eutrophic regions with increasing depth. However, when the trenches were considered separately, no size–depth relationship was found. In a global study, Udalov et al. (2005) observed that nematode body size did not change with increasing depth in eutrophic regions, but it did decrease significantly in oligotrophic regions. Brown et al. (2001) noted that nematode body size increased with increased food supply in the central Equatorial Pacific.

Future predictions for sea surface conditions show a change in primary production in both quantity and quality, which will likely influence the

export of POC to the deep sea. In turn, this will affect remineralization and the sequestration of organic carbon by reducing benthic biomass, thereby influencing the global carbon cycle and the carbon budget (Jones et al., 2014; Smith et al., 2013; Wei et al., 2010b). The predictions are that the community-size structure will be smaller because of a decrease in biomass, but not abundance. This, in turn, can reduce energy fluxes in the system (Jones et al., 2014; Wei et al., 2010b) and possibly impact biological processes such as bioturbation leading to other effects at the community level.

4.1.2 Food Webs

Deep-sea food webs are poorly understood. There have been mixed reports for a correlation between meio- and macrofaunal abundance, biomass or body size with the availability of food, in the form of carbon flux, plant pigments or bacterial biomass. Meio- and macrofaunal abundance and biomass have been found to correlate with organic carbon or plant pigments (Alongi, 1992; Alongi and Pichon, 1988; Galeron et al., 2000; Górska et al., 2014; Johnson et al., 2007; Pfannkuche, 1985; Schaff et al., 1992; Soetaert et al., 1991; Sommer and Pfannkuche, 2000; Tietjen et al., 1989; Vanhove et al., 1995). McClain et al. (2012a) is one of the few studies that tested whether carbon flux influences body size. They found that carbon flux positively predicts body size in deep sea communities. However, many studies also reported no correlation between meio- or macrofaunal standing stock and carbon flux measures (Bianchelli et al., 2010; Clough et al., 1997; Danovaro et al., 1995; Polloni et al., 1979; Sanders et al., 1965; Shimanaga et al., 2007; Shirayama, 1983; Tietjen, 1971). Furthermore, meiofauna might not be feeding on carbon itself, but on bacterial populations that feed on the organic carbon. De Bovée et al. (1990) found a positive relation between the meiofaunal abundance and viable bacterial population size. Others have also noted strong correlations between bacterial activity and meiofaunal abundance (Danovaro et al., 2000; Flach et al., 2002; Hoste et al., 2007). Vanhove et al. (1995) did not find a correlation between meiofaunal abundance and bacteria, but they did for meiofaunal biomass. However interactions might be more complicated, with certain meiofaunal groups feeding off bacteria while others feed of the detritus (Hughes and Gage, 2004). Danovaro et al. (2000) reported no correlation of meiofaunal abundance and bacteria for the East Mediterranean, but they found one in the Northeast Atlantic and West Mediterranean. It is likely that factors influencing bacterial populations differ on a regional scale; the bacterial populations might be driven by the carbon flux from the sea surface. It is possible that the bacteria respond to the carbon flux and the meiofauna to the bacteria.

The differences among correlations between carbon and body size, abundance or biomass, can possibly be explained by another factor. Carbon is composed of labile and refractory components. It is the labile part that organisms use as food, while the refractory portion is a low-quality resource. The higher the proportion of labile carbon is present in the flux, the higher the quality of carbon. When organismal abundance and biomass are related to quality, rather than quantity, the relationships are positive, with higher quality resulting in larger numbers. This relationship is especially clear in oligotrophic regions (Berkenbush et al., 2011; Danovaro et al., 1995; Dauwe et al., 1998; Grove et al., 2006; Hoste et al., 2007; Probert and McKnight, 1993; Probert et al., 1996).

The food-web structure has to be considered, because it could explain the absence of a pattern, as found in the present study. It is possible that the "wrong" measure has been taken in this study (and potentially in others): carbon flux instead of bacterial populations, which might have shown a correlation, or that the focus has to shift from quantity (used here) to quality of carbon. Furthermore, if macrofauna and possibly meiofauna are not feeding directly on the carbon flux, there will be a lag between the influence of carbon flux on lower trophic levels and higher trophic levels as the energy is transferred through the system. A response (change in standing stock or body size) might be detected at a later stage, influenced by how quickly energy is moved through the system and how much energy is lost in this process. Thus, measures of carbon/bacteria taken at the same time as the samples can still be confounded by this lag. It is not known how quickly carbon is transferred between trophic levels in deep-sea systems.

Food is likely to be a fundamental factor affecting body size, but the question remains as to what constitutes the food source. This highlights the lack of understanding of food webs in the deep sea. With improved understanding of trophic structure in the deep sea, it will be possible to predict how communities might change when the community composition changes. This will be important in the future, for example for identifying and understanding the impacts of climate change, with changing sea surface production and increased ocean stratification.

4.2 Taxonomic Signals

There are clear taxonomic signals in the current datasets. We found that community structure can vary with depth and region, and that total community does not reflect how different taxonomic groups would respond. We found that different taxonomic groups have either a positive or negative

shift in body size compared to the total community. It is possible that this could reflect differences in functional groups or life styles. It has been shown that scavenging and non-scavenging fish differ in their change of body size with increasing depth (Collins et al., 2005), with scavenging fish becoming relatively larger and non-scavenging fish smaller with depth. It has been proposed that the predictability of food resources for fish determines the direction of selection for body size: when food resources are unpredictable in time and/or space (e.g. food falls for scavengers), organisms benefit from being larger as they can survive between finding food falls and get to resources quicker once detected (Collins et al., 2005). Food resources for non-scavengers, like filter feeders and predators, are more predictable in time and/or space and these organisms benefit from smaller sizes that function better for lower levels of food, thereby conserving energy. It is possible that similar patterns can be found in invertebrates. For example, Tietjen (1971) found that feeding styles in nematodes changed with food availability and sediment characteristics. No information is available on whether the feeding guilds differ in size, but this is conceivable. Also, a change in community composition can often be observed, depending on whether organisms colonize the sinking phytodetritus, which in turn could influence a response to change in body size (Lambshead and Gooday, 1990; Sommer and Pfannkuche, 2000). A 10-year study in the Northeast Atlantic showed that macrofaunal community structure changed in response to variation in sea surface conditions (Ruhl et al., 2008). In the northeast Pacific, the megafaunal community composition has been observed to change from a sponge-dominated community (suspension feeding) to a holothurian-dominated community (detritus feeding) in 2 years (Kuhnz et al., 2014). This was accompanied by changes in densities of organisms and community diversity, showing that the deep sea is a dynamic place. Changes in community composition and densities over time have been observed elsewhere as well (Gooday et al., 2010; Kalogeropoulou et al., 2010; Rogers, 2015; Soto et al., 2010). In the present study, taxonomic groups that were found to be larger compared to the average community size often include filter feeders. Larger filter feeders will be able to capture more food particles suspended in the water column. Larger organisms require more energy in total, but they require less energy per unit biomass. Combined with the low metabolic rate in deep-sea organisms, it may prove advantageous to be larger for certain feeding styles. For example, the taxonomic groups that tend to be smaller than the community average seem to be associated with predatory lifestyles. It should be noted that these are generalizations across

the taxonomic groups—there is still much unknown about lifestyles of deep-sea organisms, which makes comparative analyses difficult to make.

Two groups, the Bivalvia and Polychaeta seem to be larger than the community average in the meiofauna, but do not differ in size from the community average in macrofauna. This could reflect different life stages, where juveniles are present in the meiofaunal size class (and growing) and adults in the macrofaunal size class. Whether the organisms were juveniles or adults was not mentioned in the studies from which data were obtained. We suggest that it is important to incorporate this information into analyses. When the juveniles are similar in their ecology to their adult forms, the body size estimates, or any other related aspect, may be confounded by splitting them into meio- or macrofauna and treating them as two different entities, with potentially different lifestyles (Warwick, 2014). However, some taxonomic groups do have different lifestyles at varying life stages. For example, shallow-water polychaetes can switch feeding guilds depending on life stage: juvenile polychaetes from larger-bodied adult species have a macrophagous lifestyle, while the adults switch to a microphagous lifestyle. In such an instance, it is reasonable to treat them differently and put them in different size classes. This lifestyle-switch does not happen in small-bodied adult polychaete species. It is not known whether this also occurs in the deep sea, where polychaetes tend to be smaller in general (Jumars et al., 2015).

Larger organisms often have lower population sizes. A population has to be of a large enough size in order to maintain effective reproduction. If the population size is too low, organisms can suffer from Allee effects, especially in benthic invertebrates where free spawning may require sufficient concentrations of gametes to ensure effective fertilization. This mechansim may contribute to source-sink populations, with the bathyal zone containing source populations, where individuals reproduce and larvae spread across bathyal and abyssal locations. However, populations on the abyssal plain are effectively sinks, where individuals occur but are non-reproductive. Many organisms with large depth ranges seem to agree with this pattern (bivalves, gastropods, and polychaetes), although there are organisms with narrow depth ranges that are capable of maintaining sufficient population densities at deeper depths for effective reproduction and recruitment (Porifera, Isopoda, Holothuria, Echiura, and Pogonophora). Perhaps organisms with narrower depth ranges are better adapted to the environmental conditions of these depths and can adopt larger sizes over evolutionary timescales. For example, the present study found that Porifera had larger body

sizes than average community structure and this group of organisms can have restricted depth ranges (Rex and Etter, 2010).

There is also evidence that sediment grain size or type may influence the presence of some taxonomic groups (Rohal et al., 2014), therefore it is conceivable that changes in availability of substrata with depth could influence the community structure, and as a result the size structure of the overall community or individual groups. In a similar way, biogeochemistry of sediments may influence changes in body size with depth, either amongst the community in general or differentially amongst taxonomic groups depending on their physiology. An example is the response to oxygen concentration that may influence body size across the entire community or within specific groups (e.g. the extreme oxycline in the Arabian Sea; Gooday et al., 2009; Levin et al., 1991; Rohal et al., 2014).

Bianchelli et al. (2010) noted that habitat- and region-specific topographic characteristics were related to meiofaunal standing stock on a regional scale, rather than food availability. It is possible that factors on different spatial scales structure the community in different ways. Higher taxonomic resolution or functional information along with improved data on seabed composition/structure is needed to explore this further. If functional groups are found to respond differently to increasing depth (the proxy for food availability), it is possible that changes in food availability in the future (e.g. surface primary production changes through anthropogenic actions) will affect the components of the community differently, and cause a change in community structure. This in turn can affect ecosystem functions such as bioturbation that in turn impact on carbon burial rates.

4.3 Sampling Artefacts

4.3.1 Sieve Mesh Size

The results of our analyses showed that sampling method has an influence on body size, indicating that more attention to sampling design is needed, especially in sieve size choice. Sieve size was found to have a significant positive influence on mean individual body size for both meiofaunal and macrofauna. Larger sieve mesh sizes result in larger body size estimates as larger sieve sizes—used as a lower limit of the selected size—will retain larger animals, while small animals will pass through to be retained on smaller sieve sizes corresponding to meiofaunal sizes or smaller and therefore excluded from the analyses with larger size classes. This finding of the influence of sieve size is supported by previous observations (Gage et al., 2002; Kaariainen and Bett, 2006; Leduc et al., 2010a; Pavithran et al., 2009;

Schwinghamer, 1985; Shirayama and Horikoshi, 1989). Furthermore, it has been shown that the influence of sieves might be taxonomically specific, meaning it does not affect the total community in the same way (Bachelet, 1990; Gage et al., 2002). This should be another point of consideration when choosing sieves. The choice of sieve mesh size is therefore very important when studying the deep-sea benthic fauna.

The studies used in these analyses show a variety of sieve mesh sizes, especially for the meiofauna, which showed a 10-fold range. As a result, comparison of estimates across studies was confounded by the choice of sieve. Two measures might help to mitigate this problem: (1) a standardized sieve size for further studies, allowing for better comparison among studies, with one sieve size for each of these (arbitrary) size classes, or (2) the use of multiple sieves. For example, by using lower and upper sizes plus multiple sieves in between these limits would allow for better capture of the size distribution of body size and thus energy and carbon fluxes in the form of biomass. This would also allow for a better appreciation of the variation in body size and community structure and a distinction between size classes would not necessarily be needed.

4.3.2 Area and Sediment-Depth Effects

We found that the influence of area sampled on meiofauna is of importance for the investigation of changes in meio- and macrofaunal body size with depth. There are many different sample devices that can be used to sample the soft-benthic community. Our analyses found that the choice of sample device did not influence estimates, supporting previous observations (Udalov et al., 2005), although not all studies agree (Bett et al., 1994). Usually, sub-samples of the cores or grabs are taken for meiofaunal analyses, thereby reducing the surface area (and volume) that is analyzed quantitatively. From this, the data are more often than not extrapolated, which increases error. Macrofauna, on the other hand, are rarely sub-sampled. Previous researchers have suggested that the decreasing abundance of animals with depth leads to issues with small sample size whereby larger members of the community are under-sampled, skewing the data towards individuals with a smaller body size (Polloni et al., 1979). The models presented here showed that a larger sampling area resulted in larger body sizes. Sampling a larger area would increase the chance of finding rarer larger individuals. However, larger, often older, individuals might be included in the larger size classes—rather than treating groups as meio- or macrofauna *senso stricto*—thereby skewing the relationship.

Some researchers have viewed the meio- and macrofaunal size classes as arbitrary, not reflecting any meaningful biological division (Bett, 2013, 2014; Thiel, 1975), while others have suggested that the size divisions represent optimal sizes for different lifestyles (Warwick, 2014; Warwick et al., 2006). Meiofauna predominantly live in interstitial habitats whilst macrofauna utilize sediment as a whole (Schwinghamer, 1985). Given the results concerning the influence of sampling area of the present study, it is possible that the differences observed between the two size classes result from under-sampling of large meiofauna and small macrofauna. While larval stages might well show different lifestyles compared to adults, juveniles and adults might not show such large differences; they can show similar adaptations to similar environmental conditions, thus eliminating the need to categorize them into different size classes.

Depth in the sediment was excluded in the analyses presented here, as not all studies provided information on the sampling depth within the sediment. This is important, as depth penetration can vary as a result of food availability. For example, Sommer and Pfannkuche (2000) noticed that sediment penetration by nematodes in the Arabian sea was deeper when more carbon was available. Dauwe et al. (1998), however, showed that the deepest macrofaunal depth penetration in the North Sea was dependent on intermediate quality of organic carbon. When the organic carbon was of higher quality, the penetration depth was less. When the organic carbon consisted of mainly refractory carbon, the macrofauna penetrated deepest, but were also smaller. Further, Shirayama (1983) showed that size-depth trends can be restricted to certain layers in the sediment, thereby indicating again the importance of sampling at various layers in the sediment. Macrofauna may influence the depth penetration of meiofauna by bioturbating the sediments, which allows deeper oxygen and carbon penetration in the sediments, and thus creating niches that can be occupied by meiofauna (Braeckman et al., 2011). Importantly, Leduc et al. (2010b) showed that meiofaunal estimations of abundance and biomass are influenced by depth penetration. Core penetration can be affected by sediment characteristics as well, thereby potentially influencing estimates of standing stock.

Knowledge gaps

- The distribution of body size change in deep-sea organisms is not well understood. Although many studies suggest food availability is a major controlling factor of body size in deep-sea organisms, there are a very limited number of studies that have demonstrated this, and these studies

have lacked resolution on the regional scale (where there is much variation). Furthermore, other factors like oxygen availability are likely to play a major role in body size control in certain geographic areas. More focused efforts on the effects of oxygen and other biogeochemical parameters on body size are needed, and could provide a hierarchical framework of environmental factors controlling body size.

- Sampling effort is unequal for many regions, with the Southern Hemisphere being massively under-sampled. The North Atlantic has been more sampled than the North Pacific. This limits comparisons, and potentially generalizations, for trends such as abundance, biomass and body size between oceans and latitudes. More sampling is required in the areas for which we are currently lacking data.
- Community compositional change with depth and region is not well studied. Often organisms are grouped in classes, going from specific size classes to perhaps taxonomical super Orders. Without data on taxonomical Class level or (super) Order level, community composition change is harder to study thereby we are potentially missing shifts in community structure and potentially functioning. Whether body size has an effect on community function, and the potential extent of effects of individual body size on community composition remain unclear.
- Knowledge of the food-web structure in the deep sea is lacking. Generalizations are made about lifestyles of organisms without proper study. A better understanding of how carbon is transferred through the trophic system is needed. Stable isotope research can inform this topic.

5. CONCLUDING REMARKS

The analyses presented here confirm the previously suggested trend that meio- and macrofaunal body size decreases with increasing depth at the global scale. When the data are partitioned at the regional scale, there is variation in the trend, likely reflecting the difference in food availability in different areas. Furthermore, at a global spatial scale, different taxonomic groups respond in different ways, with some groups showing a positive shift, negative shift or no difference from the community mean. These results could reflect differences in life history characteristics, physiology and/or life styles. Understanding patterns of body size in deep-sea communities will allow for understanding and prediction of patterns in abundance, diversity, food-web interactions, human impacts (Hildrew et al., 2007), and climate change effects. For example, there is often a right skew toward larger sizes

in fished areas, thereby influencing body size estimates (Brown et al., 1993; Maurer et al., 1992). Climate change will likely lead to changes in global surface primary productivity as well as changes in the flux of particulate organic carbon to the seabed (Gregg et al., 2003; Tunnicliffe et al., 2003). Deep-sea organisms will respond differently to this change in food supply, depending on their life history characteristics, resulting in changes in community structure. This, in turn, can influence remineralization and carbon burial in the deep-sea sediments. The deep sea is already showing signs of being affected by climate change (Balmaseda et al., 2013; Llovel et al., 2014; Touratier and Goyet, 2011). To understand the future effects of climate change and to implement mitigating measures, it is important to know how community structure responds to varying regional factors.

ACKNOWLEDGEMENTS

We like to thank Dr. Peter Long, James Martin research fellow, University of Oxford for supplying carbon availability data, Oliver Ashford, Ocean Research and Conservation Group, University of Oxford, and anonymous reviewers for their valuable and multitude of comments on the manuscript.

APPENDIX A. SOURCE REFERENCES FOR MEIO- AND MACROFAUNAL ABUNDANCE AND BIOMASS

Meiofauna:

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APPENDIX B. PARAMETER ESTIMATES WITH VARIABILITY MEASURES RELATED TO MACROFAUNAL BODY SIZE

	Mean Body Size[a]	Standard Error	Confidence Interval 0.5–99.5%
Depth	-7.400×10^{-5}[b]	2.040×10^{-5}[c]	-1.266×10^{-4} to -2.125×10^{-5}[d]
Sieve Size	2.690	1.980×10^{-1}	2.180–3.200
Area Sampled	-6.094×10^{-2}	3.462×10^{-2}	-1.501×10^{-1} to 2.8239×10^{-2}
Arabian Sea	−5.024	1.467×10^{-1}	−5.402 to −4.646
Arctic Ocean	−4.822	2.890×10^{-1}	−5.566 to −4.077
Caribbean Sea	−3.771	1.340×10^{-1}	−4.117 to −3.426
East Atlantic	−3.593	1.382×10^{-1}	−3.949 to −3.237

Continued

	Mean Body Size	Standard Error	Confidence Interval 0.5–99.5%
East Pacific	-2.726	1.628×10^{-1}	-3.146 to -2.307
Gulf of Mexico	-4.500	1.504×10^{-1}	-4.888 to -4.113
Mediterranean	-3.929	1.407×10^{-1}	-4.291 to -3.566
Northeast Atlantic	-4.915	1.110×10^{-1}	-5.201 to -4.629
Northeast Pacific	-4.243	1.832×10^{-1}	-4.715 to -3.771
Northwest Atlantic	-4.159	1.723×10^{-1}	-4.603 to -3.716
South Atlantic	-4.414	2.680×10^{-1}	-5.104 to -3.724
Southeast Atlantic	-3.738	1.996×10^{-1}	-4.252 to -3.224
Southwest Pacific	-3.579	1.511×10^{-1}	-3.968 to -3.190
West Atlantic	-3.234	3.662×10^{-1}	-4.178 to -2.291
West Pacific	-3.776	1.638×10^{-1}	-4.198 to -3.354
Anthozoa	8.616×10^{-1}	4.152×10^{-1}	-2.079×10^{-1} to 1.931
Aplacophora	-3.484×10^{-1}	9.497×10^{-2}	-5.931×10^{-1} to -1.038×10^{-1}
Ascidiacea	-8.310×10^{-1}	3.248×10^{-1}	-1.668 to 5.773×10^{-3}
Asteroidea	-3.867×10^{-1}	2.977×10^{-1}	-1.154 to 3.802×10^{-1}
Bivalvia	-1.759×10^{-1}	1.420×10^{-1}	-5.417×10^{-1} to 1.900×10^{-1}
Brachiopoda	4.413×10^{-1}	6.205×10^{-1}	-1.157 to 2.040
Crustacea	-7.289×10^{-1}	9.506×10^{-2}	-9.738×10^{-1} to -4.841×10^{-1}
Echinodermata	2.439×10^{-1}	2.083×10^{-1}	-2.926×10^{-1} to 7.804×10^{-1}
Echinoidea	4.755×10^{-1}	2.921×10^{-1}	-2.769×10^{-1} to 1.228
Gastropoda	-1.577×10^{-1}	1.794×10^{-1}	-6.199×10^{-1} to 3.045^{-1}
Holothuroidea	1.608×10^{-2}	2.605×10^{-1}	-6.548×10^{-1} to 6.870×10^{-1}
Malacostraca	-4.409×10^{-1}	8.406×10^{-2}	-6.574×10^{-1} to -2.244×10^{-1}
Maxillopoda	-1.575×10^{-1}	4.692×10^{-1}	-1.366 to 1.051

	Mean Body Size	Standard Error	Confidence Interval 0.5–99.5%
Mollusca	-2.638×10^{-1}	1.037×10^{-1}	-5.310×10^{-1} to 3.426×10^{-3}
Ophiuroidea	8.518×10^{-1}	2.069×10^{-1}	3.190×10^{-1} to 1.385
Polychaeta	-1.383×10^{-1}	6.305×10^{-2}	-3.007×10^{-1} to 2.409×10^{-2}
Porifera	6.283×10^{-1}	2.174×10^{-1}	6.829×10^{-2} to 1.188
Priapulida	-6.838×10^{-1}	3.507×10^{-1}	-1.587 to 2.195×10^{-1}
Pycnogonida	-1.185	7.778×10^{-1}	-3.188 to 8.189×10^{-1}
Scaphopoda	1.733×10^{-1}	1.892×10^{-1}	-3.139×10^{-1} to 6.606×10^{-1}
Sipuncula	-1.375×10^{-1}	1.153×10^{-1}	-4.346×10^{-1} to 1.596×10^{-1}
Turbellaria	4.240×10^{-1}	5.777×10^{-1}	-1.064 to 1.912

[a]All macrofauna body size values are in log gram.
[b]Indicates the mean, all else represent the differences between the mean.
[c]Indicates the standard error of the mean, all else represent the standard error of the difference.
[d]Indicates the confidence interval of the mean, all else represent the confidence interval of the difference.

APPENDIX C. PARAMETER ESTIMATES WITH VARIABILITY MEASURES RELATED TO MEIOFAUNAL BODY SIZE

	Mean Body Size[a]	Standard Error	Confidence Interval 0.5–99.5%
Depth	-6.300×10^{-5}[b]	8.300×10^{-6}[c]	-8.449×10^{-5} to -4.164×10^{-5}[d]
Sieve Size	2.342	3.598×10^{-1}	1.415–3.269
Area Sampled	-5.789×10^{-1}	1.261×10^{-1}	-9.036×10^{-1} to -2.539×10^{-1}
Antarctica	-1.946×10^{-1}	3.722×10^{-2}	-2.905×10^{-1} to -9.87×10^{-2}

Continued

	Mean Body Size	Standard Error	Confidence Interval 0.5–99.5%
Arctic Ocean	-1.698×10^{-3}	9.917×10^{-2}	-2.571×10^{-1} to 2.537×10^{-1}
Central Atlantic	-1.560×10^{-1}	6.816×10^{-2}	-3.316×10^{-1} to 1.957×10^{-2}
Caribbean	8.917×10^{-1}	7.383×10^{-2}	7.015×10^{-1} to 1.082
East Atlantic	4.687×10^{-1}	1.295×10^{-1}	1.350×10^{-1} to 8.024×10^{-1}
Gulf of Mexico	1.420×10^{-1}	4.058×10^{-2}	3.749×10^{-2} to 2.466×10^{-1}
Mediterranean	3.003×10^{-1}	5.262×10^{-2}	1.647×10^{-1} to 4.358×10^{-2}
Northeast Atlantic	7.482×10^{-1}	6.688×10^{-2}	5.759×10^{-1} to 9.204×10^{-1}
Northwest Atlantic	9.908×10^{-1}	4.884×10^{-2}	8.650×10^{-1} to 1.117
Southeast Pacific	−4.828	5.570×10^{-1}	−6.263 to −3.393
Southwest Pacific	-1.246×10^{-1}	3.976×10^{-2}	-2.270×10^{-1} to -2.215×10^{-2}
West Atlantic	-8.512×10^{-1}	1.866×10^{-1}	−1.332 to -3.706×10^{-1}
West Pacific	3.829×10^{-1}	5.564×10^{-2}	2.396×10^{-1} to 5.262×10^{-1}
Gastrotricha	7.433×10^{-1}	6.446×10^{-1}	-9.170×10^{-1} to −2.404
Bivalvia	2.123	1.403×10^{-1}	1.761–2.484
Foraminifera	-1.229×10^{-1}	3.359×10^{-2}	-2.095×10^{-1} to -3.639×10^{-2}
Kinorhyncha	1.159×10^{-1}	1.350×10^{-1}	-2.320×10^{-1} to 4.637×10^{-1}
Maxillopoda	3.564×10^{-1}	5.790×10^{-2}	2.072×10^{-1} to 5.055×10^{-1}
Nematoda	-1.487×10^{-1}	3.157×10^{-2}	-2.300×10^{-1} to -6.739×10^{-2}
Ostracoda	1.429	6.044×10^{-2}	1.273–1.584
Polychaeta	1.344	4.797×10^{-2}	1.221–1.468
Undetermined	1.382	9.687×10^{-2}	1.132–1.631
Turbellaria	−1.734	1.461×10^{-1}	−2.110 to −1.358

[a]All meiofaunal body size values are in log microgram.
[b]Indicates the mean, all else represent the differences between the mean.
[c]Indicates the standard error of the mean, all else represent the standard error of the difference.
[d]Indicates the confidence interval of the mean, all else represent the confidence interval of the difference.

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CHAPTER THREE

The Pen Shell, *Pinna nobilis*: A Review of Population Status and Recommended Research Priorities in the Mediterranean Sea

Lorena Basso*,[1], Maite Vázquez-Luis[†], José R. García-March[‡], Salud Deudero[‡], Elvira Alvarez[‡], Nardo Vicente[§], Carlos M. Duarte[¶], Iris E. Hendriks*

*Global Change Department, IMEDEA (CSIC-UIB), Instituto Mediterráneo de Estudios Avanzados, Esporles, Mallorca, Spain
[†]Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Baleares, Palma, de Mallorca, Spain
[‡]Instituto de Investigación en Medio ambiente y ciencia Marina (IMEDMAR-UCV), Universidad Católica de Valencia, Calpe, Alicante, Spain
[§]Institut Océanographique Paul Ricard, Ile des Embiez 83140 Six Fours les Plages (France) and Institut Méditerranéen de la Biodiversité et de l'Ecologie marine et continentale (IMBE), Aix-Marseille Université, Aix-en-Provence, France
[¶]King Abdullah University of Science and Technology (KAUST), Red Sea Research Center (RSRC), Biological and Environmental Sciences and Engineering (BESE), Thuwal, Saudi Arabia
[1]Corresponding author: e-mail addresses: lorena@imedea.uib-csic.es; lorena_basso@libero.it

Contents

Advances in Marine Biology, Volume 71
ISSN 0065-2881
http://dx.doi.org/10.1016/bs.amb.2015.06.002

Abstract

The pen shell *Pinna nobilis* (also known as the fan mussel) is an endemic bivalve of the Mediterranean Sea. Threatened by human activities, it has been listed as an endangered and protected species under the European Council Directive 92/43/EEC since 1992. The ecological role of this species is of importance because it filters and retains large amounts of organic matter from suspended detritus contributing to water clarity. In addition, as a hard substrate in the soft-bottom seafloor, it provides a surface that can be colonized by other (floral and faunal) benthic species. Here, we provide an overview of all available published studies on the pen shell, compiling available data and summarizing current knowledge on the conservation status and viability of populations over the full range of the Mediterranean Basin. Additionally, we discuss the different practices in applied methodology and identify gaps and new research areas in order to render conservation programmes of the species more effective.

1. INTRODUCTION

The pen shell *Pinna nobilis* (Linnaeus, 1758), reaching a size of up to 120 cm (Zavodnik et al., 1991), is the largest bivalve of the Mediterranean Sea (where it is endemic), and ranks amongst the largest in the world. It occurs at depths between 0.5 and 60 m, mostly on soft-bottom areas overgrown by seagrass meadows, but also occasionally on bare sandy substrate and maërl beds (García-March et al., 2002; Katsanevakis, 2007b; Zavodnik et al., 1991). *P. nobilis* is long-lived, with a maximum reported age of 27 years (Galinou-Mitsoudi et al., 2006; García-March et al., 2011). It has been a valued resource for human exploitation since Egyptians and Romans developed high-value fabric from its byssus threads, so-called "sea silk", to which only the wealthier social classes had access as reported by Plinius (Historia Naturalis, Liber IX). Other cultures, such as the Islam, collected nacre shells for the production of buttons, jewelry items or knife handles in the XIX century. As a food source, the pen shell has been of common use in traditional cooking in some Mediterranean regions (Greenwald, 1996; Katsanevakis et al., 2011). The pressure on pen shell populations, however, has never been as high due to human interference as it is at present. Problems affecting this species are caused by habitat degradation, illegal trawling, coastal construction, boat anchoring, illegal extraction and pollution (Sureda et al., 2013a,b) the latter mainly affecting eggs and latter larval stages (Hendriks et al., 2013; Katsanevakis, 2007b, 2009; Rabaoui et al., 2007; Richardson et al., 1999; Vázquez-Luis et al., 2015). All of these anthropogenic and environmental threats have contributed to accelerating the decline of populations of this species in the Mediterranean Basin. This

decline has in turn led to the pen shell's listing as an endangered and protected species under the European Council Directive 92/43/EEC (EEC, 1992) and in the ANNEX II of Barcelona Convention. The species is also under protection by local law in all European Union Mediterranean countries.

The ecological role of *P. nobilis* is of importance as it filters large amounts of detritus and retains a high percentage of its organic matter (Trigos et al., 2014a), contributing to water clarity. It also provides a hard type of substrate in soft-bottom areas, thus increasing the variety of environments and providing a surface that can be colonized by other (floral and faunal) benthic species. Because of its commercial and ecological value, this large bivalve has attracted the attention of many scientists over the years, resulting in a wealth of literature in various languages (i.e. English, French, German, Italian, Spanish). However, a comprehensive review of the biology and ecology of this unique and endangered species has been lacking.

Here, we aim to (1) provide an overview of all available scientific literature on the pen shell that has been published in peer-reviewed journals; (2) summarize current knowledge, providing evidence of the conservation status and viability of populations throughout the Mediterranean Basin; (3) identify best practices in the research methodology applied and (4) identify gaps and promote new research areas to render conservation programmes of the species more effective.

2. METHODS

Published reports on *P. nobilis* were derived from the Web of Science (http://portal.isiknowledge.com/), Science Direct (http://www.sciencedirect.com) and Google Scholar (http://scholar.google.com). This search was conducted using the keywords "*P. nobilis*", "*pen shell*" and "*fan mussel*" in different combinations. Only research published in peer-reviewed scientific journals was retrieved, as this is considered to guarantee the reliability of data throughout the review process prior to publication. In this analysis, we did not consider data for which the measurement method was not reported (and which sometimes yielded disparate densities like those reported in Porcheddu et al., 1998; Rabaoui et al., 2010 (both 56 ind/100 m^2, each), Galinou-Mitsoudi et al., 2006 (80 and 130 ind/100 m^2), De Gaulejac and Vicente, 1990 (200 and 600 ind/100 m^2) and Catsiki and Katsilieri, 1992 (500 ind/100 m^2). The final database from these combined searches performed during December 2014 contained a total of 130 references, which were classified in particular research areas (Appendix). "Population study" was dominant the sector with the majority of references

(25%), followed by "Ecology" (19%), "Biology" and "Morphology" (12%), "Growth" (10%), "Bioaccumulation", "Interspecific relationships" and "Genetic" (5%), "Reproduction/Recruitment" and "Transplantation" (3%).

With extracted data from these papers, we performed a meta-analysis to identify the likely spatial patterns of *P. nobilis* distribution. We used the reported mean density and, if available, all reported data (min–max density and all transect measurements). Statistical analysis was applied to categories with more than one observation and density reports for which the measurement method was not reported were excluded (Table 1). The spatial pattern of *P. nobilis* abundance was analysed with a two-way ANOVA (fixed factors *Mediterranean ecoregions* and *habitat type*) and a General Lineal Model with covariant *depth*.

3. BIOLOGY AND CONSERVATION STATUS OF *P. NOBILIS* IN THE MEDITERRANEAN BASIN

3.1 General Description

P. nobilis is a Pteriomorphian bivalve mollusc that is thought to have inhabited the Mediterranean Sea since the end of the Miocene (Gómez-Alba, 1988). The species is common in *Posidonia oceanica* meadows, where it lives with the tapered anterior third of the shell buried in the substratum for anchoring. Attachment, as is the case for many other Pteriomorphia, is achieved by byssus threads, which are glued to pebbles, maërl, sand, small pieces of hard biodetritic material, and roots and rhizomes of *P. oceanica* (García-March, 2005). The shell and soft tissues of Pinnids show such distinctive features that they deserved the classification of the group in a single superfamily with only one family and three genera: Superfamily Pinnoidea, Family Pinnidae, genera Pinna, Atrina and Streptopinna. The triangular shape of Pinnids and their anysomiarian condition (i.e. the reduction of the anterior adductor muscle with respect to the posterior adductor muscle) are a consequence of the adaptation to tethering to the substrate by byssus threads. Their large size is owed to the great posterior extensions of mantle and shell, probably as a consequence of the semi-infaunal habitat in soft substrates (Yonge, 1953; Figure 1).

3.1.1 Shell Microstructure

3.1.1.1 Periostracum

The periostracum is the outermost proteinaceous layer of the shell of bivalves and is quickly eroded after its formation in *P. nobilis*. In addition

Table 1 Comparison of Pen Shell, *Pinna nobilis*, Densities Recorded in the Mediterranean Sea

Mediterranean Ecoregions	Country (Sites)	Sampling Year	Habitat Type	Density (ind/100 m^2)	Depth Min	Depth Max	References
Adriatic Sea	Croatia (Mljet National Park)	1998	*Cymodocea* sp.	20	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet National Park)	1998	*Cymodocea* sp.	19	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet National Park)	1998	*Cymodocea* sp.	4	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet National Park)	1998	*Cymodocea* sp.	3	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet Natinal Park)	2000	*Cymodocea* sp.	9	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet Natinal Park)	2000	*Cymodocea* sp.	2	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet Natinal Park)	2000	*Cymodocea* sp.	13	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia (Mljet Natinal Park)	2000	*Cymodocea* sp.	17	5	15	Siletic and Peharda (2003)
Adriatic Sea	Croatia		*Cymodocea* sp.	9	10	20	Zavodnik et al. (1991)
Adriatic Sea	The Island of Mljet (Malo.A)	1998–2001	*Cymodocea* sp.	17	0	15	Peharda et al. (2002)
Adriatic Sea	Italy (Venetia Lagoon)	2006–2012	*Zostera marina*	1200[a]	–	–	Russo (2012)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0	0	1	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	1.48	1	3	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	4.74	3	5	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	2.17	5	7	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	1.35	7	9	Katsanevakis (2005)

Continued

Table 1 Comparison of Pen Shell, *Pinna nobilis*, Densities Recorded in the Mediterranean Sea—cont'd

Mediterranean Ecoregions	Country (Sites)	Sampling Year	Habitat Type	Density (ind/100 m^2)	Depth Min	Depth Max	References
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	1.88	9	11	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	3.34	11	13	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	2.63	13	15	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0.59	15	17	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0.08	17	19	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0.04	19	21	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0.06	21	23	Katsanevakis (2005)
Aegea Sea	Greece (Lake Vouliagmeni)	2004	Unvegetated	0	23	32	Katsanevakis (2005)
Aegea Sea	Greece (Golf the Geras)	1986–1987	–	500[a]	–	–	Catsiki and Katsilieri (1992)
Aegea Sea	Greece (Thermaikos Gulf)	2004	–	130	2	3	Galinou-Mitsoudi et al. (2006)
Aegea Sea	Greece (Thermaikos Gulf)	2004	–	80	2	3	Galinou-Mitsoudi et al. (2006)
Aegea Sea	Greece (Lake Vouliagmeni)	–	Unvegetated	0.45	5	6	Katsanevakis (2007b)
Algero Provencal Basin	Italy (Gulf of Oristano)	2006–2007	*P. oceanica*	3.3	2	6	Addis et al. (2009)
Algero Provencal Basin	Italy (Gulf of Oristano)	2006–2007	*P. oceanica*/ *Cymodocea* sp.	3.9	2	6	Addis et al. (2009)
Algero Provencal Basin	Italy (Gulf of Oristano)	2006–2007	Unvegetated	11.6	2	6	Addis et al. (2009)

Algero Provencal Basin	Spain (Moraira Bay)	1998–2002	*P. oceanica*	6	6	6	García-March et al. (2007a)
Algero Provencal Basin	Spain (Moraira Bay)	1998–2002	*P. oceanica*	10.3	13	13	García-March et al. (2007a)
Algero Provencal Basin	Spain (Alicante)	1995–1996	*P. oceanica*	4	4	14	Richardson et al. (1999)
Algero Provencal Basin	Spain (Alicante)	1995–1996	*P. oceanica*	30	4	14	Richardson et al. (1999)
Algero Provencal Basin	France (Port-Cors)	1969–1979	*P. oceanica*	1[a]	–	–	Moreteau and Vicente (1982)
Algero Provencal Basin	Moraira Bay (East Spain)	1993–2003	*P. oceanica*	10.3	10	15	García-March et al. (2011)
Algero Provencal Basin	Italy (Golfo of Aranci)	–	*Caulerpa prolifera*	56[a]	8	11	Porcheddu et al. (1998)
Algero Provencal Basin	France (Port-Cros)	–	–	1.93	–	–	Vicente et al. (1980)
Algero Provencal Basin	France (Port-Cros)	–	–	1	10	12	Combelles et al. (1986)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	4.8	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	1.4	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	10	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	1.8	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	1.7	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Spain (Balearic Islands)	2007–2010	*P. oceanica*	8.8	5	6	Hendriks et al. (2013)
Algero Provencal Basin	Italy (Gulf of Oristano)	2007	*P. oceanica*	0.3	2	5	Coppa et al., 2010

Continued

Table 1 Comparison of Pen Shell, *Pinna nobilis*, Densities Recorded in the Mediterranean Sea—cont'd

Mediterranean Ecoregions	Country (Sites)	Sampling Year	Habitat Type	Density (ind/100 m^2)	Depth Min	Depth Max	References
Algero Provencal Basin	Italy (Gulf of Oristano)	2007	Unvegetated	3.8	2	5	Coppa et al. (2010)
Algero Provencal Basin	Italy (Gulf of Oristano)	2007	Unvegetated	0.1	2	5	Coppa et al. (2010)
Algero Provencal Basin	Spain (Balearic Islands)	2012–2013	*P. oceanica*	0	10	10	Vàzquez-Luis et al. (2014)
Algero Provencal Basin	Spain (Balearic Islands)	2012–2013	*P. oceanica*	37.3	10	10	Vàzquez-Luis et al. (2014)
Algero Provencal Basin	Spain (Balearic Islands)	2012–2013	*P. oceanica*	0	20	20	Vàzquez-Luis et al. (2014)
Algero Provencal Basin	Spain (Balearic Islands)	2012–2013	*P. oceanica*	29.3	20	20	Vàzquez-Luis et al. (2014)
Ionian Sea	Italy (Gulf of Taranto)	2004–2005	*P. oceanica*/ *Cymodocea* sp.	0.001	3	16	Centoducati et al. (2007)
Ionian Sea	Italy (Gulf of Taranto)	2004–2005	*P. oceanica*/ *Cymodocea* sp.	0.007	3	16	Centoducati et al. (2007)
Ionian Sea	Italy (Messina)	1986	–	6.89[a]	10	20	Giacobbe and Leonardi (1987)
Thyrrhenian Sea	France (Dina Bay)	–	*Cymodocea* sp.	600[a]	0.5	1	De Gaulejac and Vicente (1990)
Thyrrhenian Sea	France (Dina Bay)	–	*Cymodocea* sp.	200[a]	1	4	De Gaulejac and Vicente (1990)
Thyrrhenian Sea	Tunisia (Tabarka)	–	*P. oceanica*/ *Cymodocea* sp.	0.02	0	6	Rabaoui et al. (2008b)

Thyrrhenian Sea	Tunisia (Negro Cape)	–	*P. oceanica*	2	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Sidi Mechreg)	–	*P. oceanica*	1	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Echaâra)	–	*Cymodocea* sp.	14	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Njila)	–	*Cymodocea* sp.	17	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Menzel Jemil)	–	*Ulva*	3	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Oued Tinja)	–	*Cymodocea* sp.	0	0	6	Rabaoui et al. (2008b)
Thyrrhenian Sea	Tunisia (Sidi Rais)	–	*P. oceanica*/ *Cymodocea* sp.	13	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Port Prince)	–	*P. oceanica*	7	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Ghar El Melh Lagoon)	–	*Cymodocea* sp.	0	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Tunis North Lagoon)	–	*Ruppia*	0	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Marsa Beach)	–	*P. oceanica*/ *Cymodocea* sp.	0	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Hammamet Beach)	–	*P. oceanica*/ *Cymodocea* sp.	0.03	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Kelibia Beach)	–	*P. oceanica*	0	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Khniss)	–	*Cymodocea* sp.	13	0	6	Rabaoui et al. (2008b)

Continued

Table 1 Comparison of Pen Shell, *Pinna nobilis*, Densities Recorded in the Mediterranean Sea—cont'd

Mediterranean Ecoregions	Country (Sites)	Sampling Year	Habitat Type	Density (ind/100 m^2)	Depth Min	Depth Max	References
Tunisian plateau	Tunisia (Teboulba)	–	*P. oceanica*/ *Cymodocea* sp.	15	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Stah Jaber)	–	*Cymodocea* sp.	20	0	6	Rabaoui et al. (2008b)
Tunisian plateau	Tunisia (Gulf of Gabes)	2008–2009	*P. oceanica*/ *Cymodocea* sp.	56	0	6	Rabaoui et al. (2010)
Tunisian plateau	Tunisia (Gulf of Gabes)	2008–2009	*P. oceanica*/ *Cymodocea* sp.	0	0	6	Rabaoui et al. (2010)

[a]Densities for which measurement method was not reported.

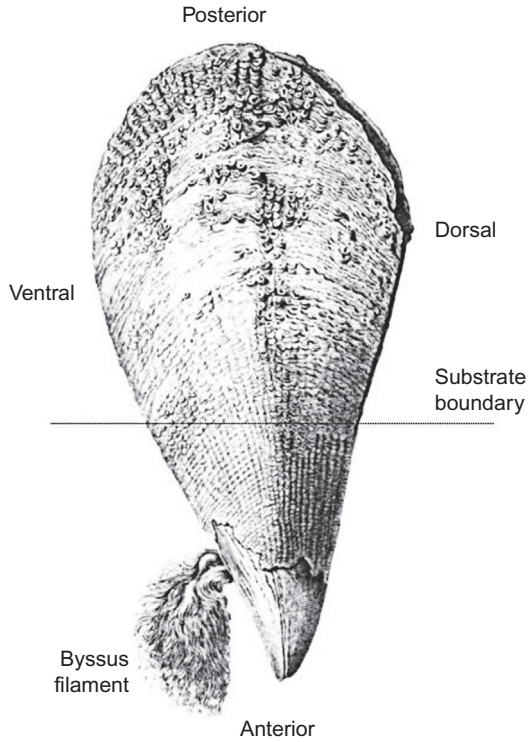

Figure 1 General drawing of an adult individual pen shell, *Pinna nobilis*, as it is commonly observed, with the tapered anterior third of the shell buried in the sediment and attached to the substratum by byssus threads. *Redrawn from Czihak and Dierl (1961).*

to this early layer, three other microstructures can be observed, of which the most prominent and evident layers are the outer calcitic layer of simple prisms (also called regular simple prismatic layer) present in the whole shell, and the inner nacreous layer formed by unusual row-stack nacre (Marin et al., 2011). The inner nacreous layer is present only in the anterior part of the shell, starting approximately at the point of insertion of the posterior adductor muscles. A third layer of aragonitic irregular simple prismatic microstructure, the myostracum, can be observed under the adductors and also forms part of the duplivincular ligament as the ligamental ridges. The first ligamental ridge is not only structurally identical to the myostracum, but also continuous with it (García-March et al., 2008).

3.1.1.2 Prisms

A comprehensive description of *P. nobilis* prisms can be found in Marin et al. (2011). Here, we indicate the remarkable peculiarities of this microstructure.

P. nobilis prisms are unusually large (up to 1 mm) and the large amount of protein enveloping them confers a flexible nature to the outer layer when wet. Despite their crystallographic simplicity, each prism behaving as a simple crystal, *P. nobilis* prisms are far from simple. The prisms are formed by complex substructures that superimpose at different scales. Remarkably, all of these units exhibit exactly the same optical orientation of their three axes, which explains the "monocrystal" appearance of each single prism (Marin et al., 2011).

3.1.1.3 Nacre

The nacre in *P. nobilis* is also unusual because it shows a particular stacking with consecutive tablets arranged in staggered rows. The nacre of *P. nobilis* is defined as "row-stack nacre" and has been described as a "nacreous structure in which mutually parallel elongate tablets show vertical stacking in vertical sections perpendicular to their length axes, and brick wall and/or stair step stacking in vertical sections parallel to their length axes" (Carter and Clark, 1985; Marin et al., 2011; Wise, 1970). Close observation of these layers in thin sections of a radial cut of the shell, shows that the nacre is deposited in at least two steps in the inner side of the shell. Firstly, a first nacre layer (n1; sensu García-March and Marquez-Aliaga, 2007; García-March et al., 2011) is deposited covering the calcite. A second nacre layer with the same characteristics is deposited covering the myostracum in the anterior part of the shell, growing in the posterior direction with ontogeny (n2; *sensu* García-March and Marquez-Aliaga, 2007; García-March et al., 2011). This n2 layer covers and hides previous records observable in the inner side of the shell.

3.1.1.4 Myostracum

The myostracum of the posterior adductor muscle scar (PAMS) is a continuous thin mineral layer covering the nacre where the muscles attach to the shell. In fact, it is observed in the dorsal lobe of the inner side of an empty valve except where it is covered by n2. In a radial section view, the myostracum starts at the most posterior tip of the last PAMS always covering the n1 layer at the inner side of the shell. The myostracum forms the innermost layer for approximately half the extension of the dorsal nacre lobe. Then, it is covered by the n2 and is imbibed within progressively thicker n2 and progressively thinner n1 in anterior position. The calcite is abraded in the anterior portion of the shell until this layer disappears completely. Eventually, the nacre below the calcite is also abraded, leaving the

myostracum in contact with the exterior in the anterior portion of the shell. At the tip of the valve, the myostracum is also abraded leaving n2 in contact with the environment in the outer side of the shell (García-March and Marquez-Aliaga, 2007; García-March et al., 2007a). As a consequence, the myostracum crosses the anterior portion and extends partly into the inner side of the shell. The myostracum is also partly embedded within the nacre, and partly in contact with the exterior on the outer side of the shell. The myostracum is used as a tracer to detect growth records obscured by n2 and to evaluate the degree of abrasion of a shell in its anterior portion.

3.1.1.5 Transitions Between Prisms and Nacre

The transition between nacre and calcite is described as abrupt and marked by an intermediate organic chitino-proteinaceous layer (Cuif et al., 1985; Marin et al., 2011). Because the nacreous layer in *P. nobilis* is deposited in about one-half of the shell from approximately the youngest PAMS to the anterior tip of the shell, it has been suggested that the mineralization fronts of nacre and of prisms are distant from each other. This would mean that, "the mantle epithelial cells that secrete the ionic and macromolecular components for nacre deposition are physically remote (several centimetres in a 2-year-old specimen) from those that initiate the prisms at the shell edge" (Marin et al., 2011). The formation of nacre tongues (nt) in *P. nobilis*, however, seems to contradict this idea and to depict a more intricate and complex structure of secreting mantle tissue and to support shell growth. Nacre tongues are annual, wedge-like thickenings of the nacreous inner shell layer, visible in radial sections passing through the posterior adductor myostracum. The shape of the nacre tongue varies with ontogeny and is related to shell growth rate. In adults, as shell growth is resumed after a winter break, a new nacre tongue is developed slightly interior and posterior to the previous tongue, separated from it by a gap occupied by prisms from the calcitic outer shell layer (Carter et al., 2012). The deposition of prisms growing on the gap implies the capacity of *P. nobilis* for the nucleation of new prisms far from the mantle edge. These prisms often have an angle of formation slightly different to the other prisms, probably owing to the fact that their formation originates independently origin of their formation from the rest of prisms. Other examples of nucleation far from the mantle edge and calcite deposited below the nacre are observed in the ventral edge of the shell in the axis separating the dorsal and ventral nacre lobes, and in the ligament of *P. nobilis*. These growth fronts form clear interdigitations between nacre and calcite. Both microstructures are deposited at different

time periods in the nacre tongues (García-March et al., 2011). However, in the ventral edge, the axis and ligament are likely to be deposited simultaneously and the interdigitations would be the result of slight dorso-ventral displacements of the mantle or the nucleation areas for calcite and nacre formation.

3.1.2 The Ligament

The ligament shows peculiarities with respect to microstructure deposition, particularly with reference to the nacre and the aragonitic irregular simple prismatic layer forming the ligamental ridges and the myostracum (García-March et al., 2008). The ligament of *P. nobilis* is also atypical because it is hard and mineralized, and its function is holding the valves together. Gaping is produced by flexion of the posterior part of the shell, while the ligament remains immobile. This condition is probably an adaptation to the semi-infaunal habitat of this species reducing sediment incorporation into the pallial cavity. The ligament of *P. nobilis* is duplivincular opisthodetic, similar to its Pterineid ancestors. Multiple couplets (up to four) of outer lamellar sublayer (LL) and inner fibrous layer (FL) are repeated and grow continuously in the posterior direction with ontogeny. The first-formed adult couplet in *P. nobilis* is covered dorsally by a more posterior extended, non-mineralized layer, the extended lamellar layer (ELL) situated between LL1 and the dorsal calcite, which in turn attaches to the calcitic prismatic outer shell layer in both valves. The ELL is attached to the calcite and where ELL and LL1 coincide, the former is virtually continuously attached to the latter. The ELL is observed from a wedge between nacre and calcite close to the posterior terminus of the first LL to the most posterior terminus of the ELL itself. Within the wedge, the calcitic outer shell layer interdigitates with the nacreous shell layer over a distance not exceeding 1 cm (García-March et al., 2007a). In the first-formed adult couplet, LL1 attaches to the nacreous middle shell layer and FL1 attaches partially to the nacreous layer and partially to an aragonitic irregular simple prismatic ligamental ridge (LR1) *sensu* Carter (1990), or pseudonymph. As in the other pinnoideans, the ligament layers are continuous between the two valves. The initial ligamental ridge is physically continuous with the early formed myostracum, which has the same aragonitic irregular simple prismatic microstructure. With continued shell deposition, however, this initial prismatic pseudonymph is cut off from the pallial myostracum as it becomes covered internally by the nacreous inner shell layer (García-March et al., 2007a).

3.1.3 Byssus Threads

The byssus system of *P. nobilis* is a complex structure specialized for fixation to the substrate. A fully developed adult byssus can be formed of 20,000–30,000 filaments protruding from the animal and attached to the substrate by adhesion plates. The most remarkable feature of the byssus system is the occurrence of two different filament types of ovoid section and smooth surface: the thick and the thin filaments. Thick filaments protruding from the animal (100–150 mm long × 0.04 mm thick) are formed by the fusion of 4 thin filaments (50–10 mm long × 0.01–0.02 mm thick). The thin filaments are laterally fused in pairs. Two of them form a junction where the other two fit to form the thick filament. This singular feature of *P. nobilis* byssus threads enables that every thick filament be attached in four different points, two within each posterior pedal retractor muscle (García-March, 2005). The strength and elasticity of these filaments is presently unknown.

As in other bivalves, attachment to the substrate is accomplished by adhesion plates. In *P. nobilis*, these plates have the shape of an arrow head and measure between 0.2 and 0.5 mm. Fixation usually occurs beneath the anterior tip of the shell on the ventral surface. The filaments form separate groups, decreasing in number with distance from the animal, and diverge in all directions. In this way, the structure widens with increasing depth in the substrate. There appears to be a preference for selecting many different particles for adhesion instead of attaching the plates to a single large structure. Plates may, for example, be attached to several sand grains, glueing them together. Some filaments are also attached to the shell of the animal itself, either on the outer or on the inner surface. Attachment is a complex process where filaments may be fixed in lines or the plates attached to other filaments. Furthermore, previous to attachment, the foot crawls among the particles effectively tying the filaments to the substrate, increasing the adhesion strength. A tied filament will continue to remain attached even if the adhesion plate is lost (García-March, 2005).

3.1.3.1 Function of the Byssus of *P. nobilis*

The byssus system of *P. nobilis* is strongly adapted to substrate fixation and is of little utility for displacement. Contrary to what happens with other bivalves, such as the blue mussel, *Mytilus edulis*, the high number of filaments and the attachment strategy of *P. nobilis* imply that the animals stay sessile reinforcing continuously, and by all means possible, the fixation to the substrate. Furthermore, the entire byssus needs up to 6 months for its regeneration (Cerruti, 1938, 1939; Mihailinovic, 1955). It is improbable that an

animal would shed the filaments voluntarily to move a few metres, leaving itself vulnerable to predators and strong hydrodynamics for such a long period of time (García-March, 2005).

3.1.4 The Large Muscles

The posterior adductor muscle and the posterior foot retractor are so close in proximity that they appear one muscle. The foot retractor muscles are only called "foot" retractors to homologize the names with those of other bivalve species, but are actually byssus-retractors. The posterior adductor muscle is composed of dark and light phases. The light phases (blocking muscles) are slower in contracting but can hold more force (12 kg/cm^2) and serve to keep the shell closed, for hours if needed. The dark phases (work muscles) can contract rapidly but are not as strong (0.5 kg/cm^2). The percentage of dark phases is larger, as a relatively large amount of water needs to be displaced rapidly upon sudden closure; an animal of 60 cm shell length, with an average opening of 1 cm, needs to eject approx. 400 cm^3 water upon closure. A relatively small blocking muscle can keep the shell hermetically closed. The colour difference is caused by the content of reserve compounds, with the lighter blocking muscles using more reserve energy because their effort lasts longer (Czihak and Dierl, 1961).

3.1.5 The Gill

In the adult animal, the outer and inner leaf consists of up- and down-ward moving gill filaments, which are connected in a complicated way, so that the gill seems compact. The organization of these filaments creates a space inside the gill, flowing into a large quadratic space and finally in the upper mantle cavity. The outer gill filaments are especially rich in glandular cells with big acidophilic secretory granules, which produce the mucus that covers the gill and composes the mucosal net that captures and filters particles (Czihak and Dierl, 1961).

3.1.6 Reproductive Organs

Due to the protected status of the species, few studies of *P. nobilis* reproductive organs have been conducted (see De Gaulejac et al., 1995a,b,c). The structure of the spermatogonium, spermatocyte, spermatidics and spermatozoon is similar to that described for other bivalves of the subclass Pteriomorpha (De Gaulejac et al., 1995c), in spite of some anomalies that were observed at different stages of spermatogenesis (De Gaulejac et al., 1995c). The general process of oogenesis is similar to that of other bivalves

such as *Atrina pectinata* (Fang and Qi, 1988), *M. edulis* (Albertini, 1985; Pipe, 1987), *Brachidontes virgiliae* (Bernard et al., 1988) and *Pecten maximus* (Dorange and Le Pennec, 1989). Ultrastructural stages of female gametogenesis are described and illustrated in De Gaulejac et al. (1995b).

3.1.7 Blood Vessels

The heart: the atria are thin-walled pyramid-shaped bags that are crossed by a muscle braid. They laterally flow into a thick-walled ventricle, also pierced by a muscle braid. The large circulatory system has two routes, one from the heart through the gill and back, and the other from the heart through the body to the kidney system in the gills and back. The small circulatory system goes from the heart through the mantle and back. The blood does not coagulate and contains amoebocytes and erythrocytes with nuclei. Haemoglobin is not present, but it is possible that copper atoms (Cu) in heamocyanin act as the blood colouring agent. Manganese (Mn) is present in high proportions and may function as respiratory pigment in the compound akroglobulin (Czihak and Dierl, 1961). In *P. nobilis*, haemolytics participate in the regulation of metabolism via storage and degradation of glycogen, and also contribute to the detoxification process by the sequestration of mineral elements in their lysosomes (Henry et al., 1992). Moreover, these haemolytics are characterized by a bacterian phagocytic activity, which suggests their role in antibacterial defense (Henry et al., 1992).

3.2 Biology and Ecology

3.2.1 Feeding

Feeding ecology of *P. nobilis* has been investigated using stable isotopes (Alomar et al., 2015; Cabanellas-Reboredo et al., 2009b, 2010; Kennedy et al., 2001), stomach content analysis (Davenport et al., 2011) and fatty acid composition (Najdek et al., 2013). *Pinna nobilis* inhabiting seagrass beds rely on particulate and sedimentary organic matter, seagrass leaves and their epiphytes for food (Cabanellas-Reboredo et al., 2010). Davenport et al. (2011) found size differential feeding in *P. nobilis*. Although *P. nobilis* is a filter feeder, it seems to preferentially ingest detritus (95% of ingested material), phytoplankton, micro- and mesozooplankton and pollen grains. This might depend on the specific region though, as Alomar et al. (2015) did show that the diet of *P. nobilis* is related to the pelagic rather than the benthic compartment, with phytoplankton the highest contributing food source. Differential feeding is exhibited by consumption of high number of harpacticoid copepods in all size classes (Davenport et al., 2011). Recently, fatty acids analysis

performed on digestive gland and abductor muscle tissues showed that small *P. nobilis* were associated with a detrital food chain, characterized by saturated and branched fatty acids, while diets of medium and large individuals had a larger proportion of polyunsaturated fatty acids (Najdek et al., 2013). This reflects that small individuals feed within the benthic boundary layer with high detritus concentrations. These results agree with stomach contents analyses conducted by Davenport et al. (2011). Interestingly, *P. nobilis* retains a high proportion of ingested organic matter (OM) from detritus ($48.01 \pm 13.69\%$ of filtered OM irrespectively of shell size) and this could be the main source of OM for the species (Trigos et al., 2014a). Enhanced pools of suspended matter within *Posidonia* canopies increase the food supply to *P. nobilis*, which may provide an explanation for their close association with seagrasses as habitat (Duarte et al., 1999).

3.2.2 Growth

Growth marks are observed in the dorsal nacre lobe. Externally they form the PAMS and internally the nacre tongues. A fully formed nacre tongue is the result of two cycles of deposition occurring between late summer and early winter, but corresponding to 2 consecutive years. When nacre growth is resumed in midsummer after the winter growth break, it starts anterior to the previously deposited nacre completing the previous nacre tongue and increasing its width. The new nacre continues to grow in posterior direction until December, forming a new wedge of nacre with a myostracum intrusion when the annual growth cycle ends. Young individuals are an exception to this rule because they do not show winter growth breaks (Katsanevakis, 2007b; Richardson et al., 1999) and hence do not form nacre tongues. The real annual increment corresponding to a full uninterrupted growth period of nacre, includes the material deposited at the base of the wedge of the previous nacre tongue to the wedge of the new nt. (García-March et al., 2011). The study of growth of *P. nobilis in situ* has been addressed in early published work on the species, but logistical problems arising from slow growth of adults (lengthy time span required to conduct studies) and low population density have been difficult to overcome. Long-term growth monitoring has been conducted on two populations located in Alicante (Spain) over 5 years of repeated measurements (García-March et al., 2007a), where shell dimensions were estimated using modified callipers to reduce measurement errors (García-March et al., 2002). An alternative, aggregated approach involved the use of modal class progression analysis from the size frequency distribution of a dense *P. nobilis* population in Lake Vouliagmeni, Greece (Katsanevakis, 2005).

In an attempt to circumvent, the time span and effort needed to monitor the growth of *P. nobilis* in the field, another approach involving sclerochronology reconstructions based on the register of the PAMS from empty shells was pursued (Combelles et al., 1986). The characteristic growth ring shape of the PAMS was used to back calculate the distances of the PAMS to the anterior end of the shell to total shell height using a linear relationship. The high correlation and the low residuals confirmed a high degree of confidence in the calculation of total shell height at the moment of PAMS deposition. Initially, the periodicity of formation of growth rings was mathematically estimated as approximately every 4.35 time units (i.e. months). However, as oxygen stable isotopes showed that each growth ring is formed annually (Richardson et al., 1999), this produced a considerable bias in the growth parameters, underestimating age and overestimating growth rates. Thus, growth of *P. nobilis* has been estimated from the back-calculation of the distances of the PAMS to the anterior end of the shell to total shell length and using age-at-length data, taking into account that the first (juvenile) growth record is unrecorded in the PAMS. However, this method is not accurate because not only the first but also several growth rings observed in juvenile shells are not homologous to those observed in an adult or an old individual; an undetermined number of growth rings are obscured by nacre deposited on them during ontogeny (García-March et al., 2007a). Furthermore, in the oldest specimens, up to seven growth records may be deposited one below the other in the posterior end of the dorsal nacre lobe. These records would be considered as a single year using the PAMS. García-March et al. (2011) described a new method to estimate growth using this inner record of the shell, where all records can be observed. The new method requires five steps: (i) preparation of thin sheets of shell material containing the growth records, (ii) identification of the growth records and measurement of the distances to the anterior part, (iii) back-calculation of total sizes corresponding to each growth record, (iv) estimation of missing records and (v) calculation of growth parameters using length-at-age data. This process enables the establishment of the growth parameters of a population using empty shells in the laboratory.

Determination of individual growth in the field is a technique that can only be applied over lengthy time scales and requires so much effort that it is not generally applicable. Since the method using PAMS of empty shells (Combelles et al., 1986) is not fully reliable for determining the age of an individual (Richardson et al., 1999), we recommend using the inner record method for the estimation of age-length relationships in empty shells (García-March et al., 2011). Although this method requires a slightly complicated methodology, it is the most reliable to date.

3.2.3 Reproduction and Recruitment

The pen shell is a successive hermaphrodite, with an asynchronous gamete maturation (De Gaulejac et al., 1995b,c), which avoids self-fertilization. Sexual maturity is reached by 2 years of age (Richardson et al., 1999), with gametogenic development occurring from March to June followed by a succession of alternate spawning and fast gametogenesis from June to August (De Gaulejac, 1995). The pen shell, *P. nobilis*, has a dispersal phase with pelagic larvae; a possible weak spot for the population because early life stages of marine organisms, and in particular eggs and larvae, are considered the most vulnerable to environmental extremes (Przeslawski et al., 2005). Adverse conditions are likely to affect vulnerable life stages first, so the timing of spawning matches optimal environmental conditions required to maintain viable populations (Todd and Doyle, 1981). The timing and duration of the reproductive cycle is controlled by an interaction of environmental factors and bivalve endogenous factors (Sastry, 1979). This phase in the life cycle of the pen shell has not been well studied (Katsanevakis, 2007b), and nothing is known about the mortality of its larvae. There is also very little information available regarding juvenile *P. nobilis*. Due to this limited knowledge and the associated uncertainties, little is known about the dispersal capacity of the species. Spawning is thought to occur in the late summer and early fall (De Gaulejac, 1993). Around the Balearic Island, western Mediterranean, spawning is thought to be generally restricted to a peak period between the last week of August and the first week of September (Cabanellas-Reboredo et al., 2009a). Larval duration for the genus *Pinna* has been estimated to be a maximum of 10 days (Butler et al., 1993), but this estimate has not yet been confirmed. Species-specific information about behaviour and larval duration is still lacking.

3.2.4 Gaping Activity

Far from being open all of the time, *P. nobilis* follows marked activity rhythms in gaping activity. The main patterns are shell opening during the day, and shell closing during the night. Night opening also occurs when the moon is in the sky and sufficiently (more than 50%) illuminated. In summer/autumn full moon nights, individuals may keep the gap closed for ca. 12 h. Furthermore, storms with strong hydrodynamics affecting the seabed also alter gaping activity, reducing the maximum time the shell remains open and promoting shell closing. Simultaneously studied individuals show synchronized behaviour, indicating that all members of the population respond to the same stimuli (García-March et al., 2008). *Pinna nobilis* has been

suggested, therefore, as a possible biomonitor of environmental conditions. In addition, *P. nobilis* responds to the proximity of threats, like fish or human swimmers, by closing their shells. Sensorial capacities of *P. nobilis*, triggering their gapping behaviour, may involve sensing pressure (and therefore, changes in hydrodynamic fields), as well as sensitivity to changes in light (García-March et al., 2008).

3.2.5 Hydrodynamics and Shell Orientation

Hydrodynamics seem to be a determinant factor in the ecology of this species. Size distribution, spatial distribution, shell orientation and growth have all been related directly or indirectly to this factor. Hydrodynamics affect population parameters by influencing food availability and reducing survival by dislodging and killing individuals due to wave action.

Slow bottom currents with high food content influence shell orientation (Combelles et al., 1986). Eighty percent of 27 individuals situated between 20 and 26 m depth at La Palud, Port-Cros, France, showed a common orientation in the direction of the slope, presumably caused by food availability. Recently, modelling studies in a sheltered population have confirmed that bottom current direction and speed affect shell orientation by improving food availability (Coppa et al., 2013).

Orientation preference has been observed in a population located at 6 mm depth on an exposed shore in Alicante, Spain (García-March et al., 2007b). The common shell orientation (80%) was N–S, which also followed the slope direction. A deeper water population living at 13 m depth in the same area had no preferential shell orientation. There were significant differences in hydrodynamic drag acting in the two populations. Orientation of the shallow 6 m populations exposed a minimum surface area of individual *P. nobilis* and provided a lower drag coefficient. Observations of growth in both populations showed that individuals living at 6 m depth grew to a smaller maximum size than those at 13 m depth. Hydrodynamic stress, excessive re-suspension of sediments due to wave action and the stress derived of wave energy reaching the seabed more often at 6 than at 13 m was thought to cause this size discrepancy (see García-March et al., 2007b).

There are many factors modulating the effects of hydrodynamics on *P. nobilis* (Hendriks et al., 2011), which complicate the establishment of clear cause-effect directionality, except for the most obvious cases. Seabed microtopography can change wave direction, bottom current or shelter locally some individuals leaving them generally unaffected by hydrodynamic pressures. Seabed composition can modulate the degree of sediment

re-suspension and the stress supported by individuals due to wave action. The presence of a *P. oceanica* meadow and the distribution of the individuals, within it or at the meadow edge, can also affect the final influence of hydrodynamics and can influence growth and survival. All these factors interact simultaneously and add to or diminish effects in a complex mode depending on local ecological features (Hendriks et al., 2011).

Indeed, the vulnerability of *P. nobilis* to drag forces, with their broad surfaces protruding vertically from the sediment, may be the primary cause of their close association with *P. oceanica* meadows as habitat. *Posidonia oceanica* meadows dissipate wave energy and attenuate flow (Hendriks et al., 2007), thereby reducing the drag experienced by *P. nobilis* living within their canopy (Hendriks et al., 2011). Moreover, *P. oceanica* canopies are very effective at trapping particles from the flow (Hendriks et al., 2007), thereby providing an environment with enhanced food supply for *P. nobilis*. In addition, the dense network of robust rhizomes and roots formed by *P. oceanica* meadows provide a substrate, where *P. nobilis* can anchor themselves through attachment of their byssus threads. *Pinna nobilis* also become anchored to the substrate via compression of the basal part of the shells, embedded within the *P. oceanica* matte, as the individuals grow. Moreover, *P. oceanica* meadows may provide refugia from predators, as the animals, particularly the more vulnerable juveniles, are relatively cryptic within the dense seagrass canopies. Last, the capacity of *P. oceanica* canopies to trap particles from the flow (Hendriks et al., 2007) also facilitates the settlement of *P. nobilis* larvae.

3.2.6 Epibionts and Commensals

Few studies have evaluated the natural epibiontic community associated with *P. nobilis*. Giacobbe (2002) found 119 species of mollusks, within 41 shells, including mobile fauna, associated with the bivalve. Individuals in that study were collected along 4 km of coastline and at different depths (10–20 m) in the Central Mediterranean Strait of Messina. Also in the Strait of Messina, Cosentino and Giacobbe (2007) recorded 69 species of mollusks among individuals collected from a broad range of depths (from 10 to 20 m depth and from 25 to 50 m depth). Rabaoui et al. (2009) counted 146 species (76 sessile and 70 motile, in 150 shells), among samples collected from five different *P. nobilis* populations located along the northern and eastern Tunisian coastline (separated by several hundreds of kilometres). The epibiontic community was dominated, in terms of species numbers, by mollusks (39.73%), followed by annelids (16.44%), crustaceans (15.07%), ascidians (7.53%), sponges (6.85%), cnidarians (6.16%), echinoderms (4.79%) and bryozoans (3.42%). Addis et al. (2009) conducted an epibiontic study of

P. nobilis situated in three different habitats in the Gulf of Oristano, Sardinia, western Mediterranean: *P. oceanica* meadows, mixed meadows of *P. oceanica* and *Cymodocea nodosa*, and estuarine unvegetated bottoms ($n = 10$ right valves with heights greater than 50–60 cm length). They found a total of 16 taxa and 3 morphological categories, with different assemblage patterns because some species were found exclusively in each environment. Shells of *P. nobilis* were colonized by epibionts in estuarine unvegetated habitats, primarily by filamentous dark algae. Filamentous dark algae were also found in meadows of *P. oceanica* and mixed meadows of *P. oceanica* and *C. nodosa*, but in lower quantities. Encrusting coralline red algae occurred in meadows of *P. oceanica* and mixed meadows of *P. oceanica* and *C. nodosa*, but were not observed in unvegetated habitats.

Two species of crustaceans live inside *P. nobilis*; *Pontonia pinnophylax* and *Nepinnotheres pinnotheres*, however, this relationship is still poorly understood. Richardson et al. (1997) examined 11 *P. nobilis* and one *P. rudis* for the presence of shrimp, *P. pinnophylax*, six (~55%) of the shells contained shrimps, typically occurring in adult pairs, but occasionally solitary male individuals were found. In a lagoon in Bizerte, Tunisia, Rabaoui et al. (2008a) observed that *P. nobilis* were occupied by *P. pinnophylax* in 23.3% of the samples and the associated shrimp population was dominated by males (63.6%), while the pea crab, *N. pinnotheres*, was observed in 56.7% of the cases and the sex-ratio of the associated crab population was also male biased. Rada and Milat (2009) sampled four specimens of *P. nobilis* from the southern Adriatic (Croatia). *Pontonia pinnophylax* were associated with their *Pinna* host in pairs, with an occupancy rate of 100%. Cabanellas-Reboredo et al. (2010) sampled 24 individuals of *P. nobilis* over seagrass beds at 7–10 m depth. The pen shells hosted the shrimp *P. pinnophylax* in 54.2% and *N. pinnotheres* in 8.3% of the samples. *Pontonia pinnophylax* pairs (female and male) were present in 33.33% and a solitary shrimp in 20.8% of the samples. Both guests were present in 4.2% of the *P. nobilis* samples. All of these observations accorded a relationship of largest commensals in the largest pen shells.

In conclusion, the taxonomic community associated to the megafaunal bivalve *P. nobilis* revealed a high biodiversity both in terms of specific richness and of taxonomic relatedness. These basibiontic bivalves increase the local spatial heterogeneity and favour the settlement of benthic species.

3.3 Population Dynamics

3.3.1 Density

Obtaining reliable estimates of *P. nobilis* population abundances can be time-consuming and problematic. These populations are often difficult to reach

due to limitations in diving time, and access. Even though methods including quadrants, transects and evaluations in concentric circles may yield a rough population estimate, these methods currently do not account for differences in detection success between size classes, caused by different visibility between small and cryptic (often deeply inserted in sand) and large and prominent *P. nobilis* individuals, especially those living in seagrass meadows with leaves generally covering small- and medium-sized individuals. Traditional models rely on the hypotheses that all individuals are equally likely to be captured. If not corrected, unequal "catchability" leads to biased estimates of abundance (Pollock et al., 1990). For the pen shell, Hendriks et al. (2011) demonstrated that the probability of detection of individuals in *P. oceanica* meadows is positively associated with shell size, but that this association is similar across sites within a region (at the same depth of 5 m). More sophisticated methodology such as straight transects, including line-distance sampling (Katsanevakis, 2007a), permits the calculation of errors in density estimates but does not necessarily correct for the probability of encountering an individual. However, techniques routinely used in terrestrial environments, such as capture-mark-recapture (CMR) models, have been proposed as a way to improve population estimates for *P. nobilis* (Hendriks et al., 2012a). Capture-mark-recapture estimates are based on multiple observations of marked individuals and are sometimes used to estimate animal abundance (Seber, 1982; Williams et al., 2002), so that CMR models often include a set of parameters that account for the observational process, such as detection failures (Schwarz and Anderson, 2001; Williams et al., 2002), which are expected to be a problem for organisms living hidden in seagrass meadows.

Pinna nobilis occurs over a range of depths and substrates, but has only been studied intensively in a few zones within the Mediterranean Basin (Figure 2). We found that a total of 24 scientific papers reported population density of *P. nobilis* based on a total of 77 observations (see Table 1). There were significant differences in population density among habitats ($F_4 = 12$, $p < 0.01$), Mediterranean ecoregions ($F_5 = 2$, $p < 0.05$) and depth ($X^2_1 = 36$, $p < 0.05$). The average population density was 9.78 ± 2.25 ind/100 m^2 (mean $\pm$ SE) and varied between 0 and 130 ind/100 m^2 (see Table 1).

Among Mediterranean ecoregions, the Aegean Sea had the highest mean density of 14.30 ± 9.14 ind/100 m^2 ($n = 16$; $\pm$SE), followed by Adriatic sea with 11.30 ± 2.17 ind/100 m^2 ($n = 10$; $\pm$SE), the Tunisian plateau with 10.09 ± 5.10 ind/100 m^2 ($n = 11$), Algero Provencal Basin with 7.90 ± 2.16 ind/100 m^2 ($n = 23$; $\pm$SE), Thyrrhenian sea 6.25 ± 2.52 ind/100 m^2 ($n = 8$) and last the Ionian sea with 0.004 ± 0.004 ($n = 2$). Pen shells

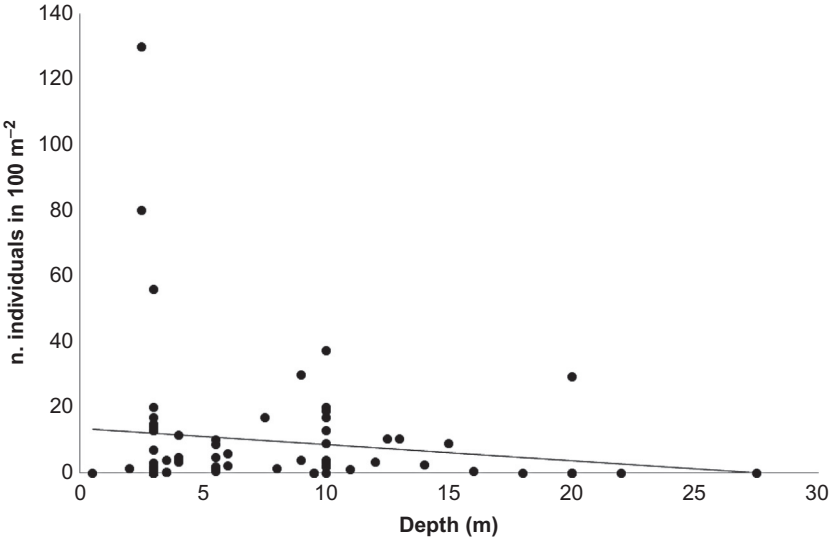

Figure 2 Pen shell, *Pinna nobilis*, population density (individuals per 100 m^2) along a depth gradient. Note the lack of studies on population density over 30 m of depth.

were most frequently observed in *P. oceanica* beds with 27% of the reports and an average of 8.06 ± 2.35 ind/100 m^2, while average densities in *Cymodocea* meadows were the highest with averages of 11.06 ± 1.82 ind/100 m^2. We found a decreasing trend in the number of individuals with increasing depth, with higher densities in the first 10–12 m (see Figure 2).

Figure 3 shows the results of the meta-analysis of *P. nobilis* population density in the Mediterranean Basin (individuals per 100 m^2). There are few observations of population density deeper than 30 m, while some of the data was collected in limited (or very specific) areas and there are differences among the methodological approaches used that may have influenced the results of our analysis.

Capture-mark-recapture combined with data augmentation can supply reliable estimates corrected for all size classes (Hendriks et al., 2012a,b). However, this methodology is time-consuming and reduces the area that can be surveyed compared to the same effort of normal transects. Considering the generally low pen shell population densities, and thus the need to sample large areas to account for local variability, the CMR technique may not be feasible for larger scale studies. However, the correction factor for populations at the same depth are similar, independent of season (and thus leaf length within seagrass meadows). Therefore, for an island-sized

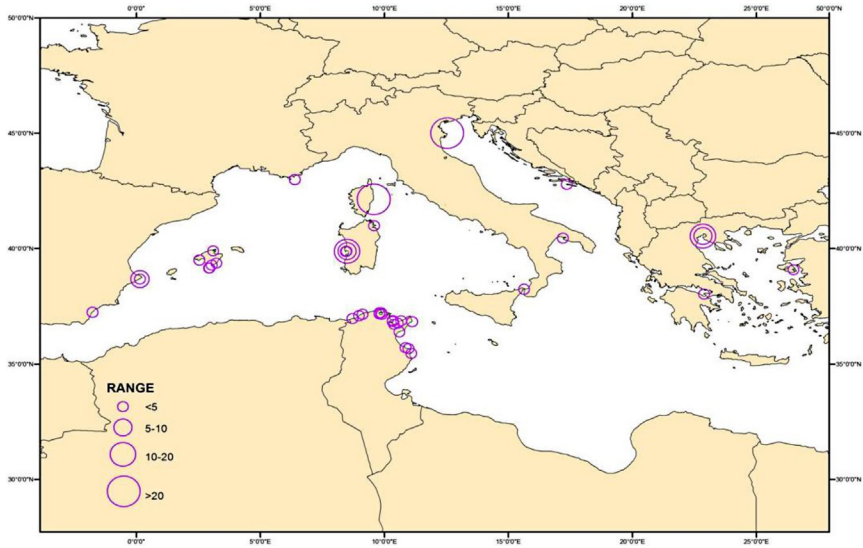

Figure 3 Map of pen shell, *Pinna nobilis*, population density in the Mediterranean Basin (individuals per 100 m^2). Compiled from information gathered from the database used in this study (including only English language and peer-reviewed scientific literature).

survey scale, we suggest a hybrid approach; where an intensive study using CMR on a smaller spatial scale is used to supply correction factors for observation error per size class for observations over much larger scales with less time-consuming methods like normal transects.

As is true for many species, recruitment is a key component of the population dynamics of *P. nobilis*. Hence, the assessment of recruitment is particularly important to understand population dynamics and to make predictions regarding future population status. To capture post-larvae, larval traps with mesh-bags (Cabanellas-Reboredo et al., 2009a) have proved their efficiency and should be used preferentially for assessment. Capturing larvae from the water-column primarily, provides insight into larval supply before various pressures on benthonic juveniles and adults (predation, dislodgement, etc.) are present. Due to the low density and low visibility of benthonic individuals, vast areas would have to be sampled to get to a significant number of individuals, possibly causing damage to established populations. Sampling larval stages circumvents the challenges associated with sampling established populations and is far less likely to damage the population, as only a fraction of the pelagic larvae would have been able to successfully recruit into the population (McEdward, 1997).

4. GENETIC STRUCTURE

The ability of a species to survive future environmental changes may depend on the genetic variation of their populations. Knowledge of the amount of genetic variability and distribution in space and time is therefore crucial for a correct diagnosis of the conservation status and viability of populations and the threats to their survival (Escudero et al., 2003; Frankham, 1995). There are few studies assessing the population genetics and connectivity patterns of *P. nobilis* (but see Katsares et al., 2008; Rabaoui et al., 2011; Sanna et al., 2011, 2013, 2014). These studies used two mitochondrial (cytochrome *c* oxidase subunit I, COI and 16S genes) and two nuclear (18S and 28S) markers. Recently, Gonzalez-Wanguemert et al. (2015) reported the isolation and development of ten highly polymorphic microsatellites for *P. nobilis*, which will provide a higher resolution for population-genetic studies. Mitochondrial DNA (mtDNA) markers, COI and 16S ribosomal DNA (16S) demonstrated the lack of genetic variability of four Aegean populations (Katsares et al., 2008; Table 2). A high level of haplotypic diversity was found for the COI gene, whereas the 16S gene showed a lower level of variability. Even though high values of intra-population haplotypic diversity were found, the results revealed a lack of genetic structuring among the Aegean populations. Rabaoui et al. (2011) described five populations from the northern, eastern, and southern Tunisian coasts using the same mitochondrial COI marker (Katsares et al., 2008). A North-East decreasing gradient of genetic variability was found among these populations, which was explained by the hydrodynamic regime of the areas analysed. Even so, the phylogenetic analyses and population-genetic statistical analyses did not support any genetic differentiation related to morphometric variations, probably due to phenotypic plasticity associated with some specific environmental factors like substrate type and hydrodynamics, without certainly excluding the possibility of a genetic component. Therefore, mtDNA COI sequences have not provided evidence for a differentiation pattern among Tunisian coastline populations. Hence, *P. nobilis* populations are thought to be structured in two large, homogeneous groups of populations, one spanning two Mediterranean marine ecoregions (the western Mediterranean and Ionian Sea), which genetically diverges from the other, consisting of the Adriatic subpopulation and those from the Aegean Sea and Tunisian coasts (eastern Mediterranean; Sanna et al., 2013). New specific primers for COI (see Table 2) showed a genetic discontinuity between

Table 2 Primers Used in Amplifying and Sequencing Mitochondrial Ribosomal 16S and Mitochondrial Cytochrome *c* Oxidase Subunit I (COI)

Primer Name	Primer Sequence (5′–3′)	References
Primer COI		
COI-F	TGATAGGGGTTCCGGATATG	Katsares et al. (2008)
COI-R	GAAAGTGCCCGGTAACAAAA	Katsares et al. (2008)
L	GGTTGAACTATHTATCCNCC	Sanna et al. (2013)
H	GAAATCATYCCAAAAGC	Sanna et al. (2013)
Primer 16S		
16S	CGCCTGTTTATCAAAAACAT´	Palumbi (1996)
16Sbr	ACGTGATCTGAGTTCAGACCGG	Palumbi (1996)
L	TGCTCAATGCCCAAGGGGTAAAT	Sanna et al. (2013)
H	AACTCAGATCACGTAGGG	Sanna et al. (2013)
Microsatellites		
Pn 2.1	GGAACTGCACTCGATGACG	Gonzalez-Wanguemert et al. (2015)
Pn 2.2	ACAGGAAAATTAGAACTTAGGAACG	Gonzalez-Wanguemert et al. (2015)
Pn 3.2	TGCCCTTTGTGTCATTATTTCG	Gonzalez-Wanguemert et al. (2015)
Pn 3.3	TGGCCCTGAACAGTAGGTG	Gonzalez-Wanguemert et al. (2015)
Pn 3.4	ACAGTGCTTTCATATGTCGGG	Gonzalez-Wanguemert et al. (2015)
Pn 3.5	TCATGTCTATGTCAAATGAACTCG	Gonzalez-Wanguemert et al. (2015)
Pn 3.6	CGGACTTTGTCAGTATGATCGG	Gonzalez-Wanguemert et al. (2015)
Pn 4.2	ATTCCCGCAAATCCATCGC	Gonzalez-Wanguemert et al. (2015)
Pn 4.3	ACAGTGCCATGCTATGTTGC	Gonzalez-Wanguemert et al. (2015)
Pn 5.2	TTCATACCGATGAGCCAAATG	Gonzalez-Wanguemert et al. (2015)

P. nobilis from the Adriatic Sea and those from the rest of the Mediterranean Sea, which may result from the semi-enclosed nature of the Adriatic Sea, which represents a well-defined phylogeographic region within the Mediterranean (Patarnello et al., 2007). In addition to their peculiar geographic position, samples from Adriatic Venetian Lagoon are considered "peripheral isolate" (Frey, 1993). The presence of only one point mutation separating the Aegean and Tunisian samples from those from Sardinia, Corsica, Elba Island, Sicily and the Venetian Lagoon, evidenced a common origin of *P. nobilis* populations in the Mediterranean Sea (Sanna et al., 2013). The low genetic variability among populations suggests that pelagic larval dispersion of the species, which is influenced by hydrogeography, leads to high gene flow and high connectivity among populations (Katsares et al., 2008).

Use of a common genetic methodology for determining population structure is necessary to facilitate comparisons among and between regions. Microsatellite markers have permitted greater resolution of genetic variation of populations. These markers are potentially useful because they are abundant, highly polymorphic, and provide information about the state of specific loci, facilitating a number of population-genetic inferences (Bruford and Wayne, 1993; Schlötterer and Pemberton, 1994). Accordingly, we recommend using the microsatellites isolated and developed by Gonzalez-Wanguemert et al. (2015), instead of mitochondrial (COI and 16S genes) and nuclear (18S and 28S) markers for future genetic studies on connectivity and dispersal patterns of the species.

5. THREATS

5.1 Climate Change

The Mediterranean Sea is a rapid warming region (Giorgi, 2006) and is warming at more than twice the rate of the global ocean. Hence, there is concern that climate change may impact of Mediterranean marine organisms (Benoit and Comeau, 2005; Boudouresque, 2004; Coll et al., 2010; Coma et al., 2009). Indeed, many habitat-forming species, such as soft corals (Kuffner et al., 2014; Rodolfo-Metalpa et al., 2008, 2010) and *P. oceanica* (Jordá et al., 2012) are vulnerable to warming, as is evidenced by their substantial decay during heat waves (Marbà and Duarte, 2010). Predicted temperature changes in the Mediterranean, combined with shifts in food availability caused by changes in planktonic blooms, changes in local hydrodynamics and disruption of the settlement habitat acting on the most

vulnerable stage of *P. nobilis* might present additional threats to the already endangered populations. A study of recruitment patterns of *P. nobilis* over 5 consecutive years (2009–2014) around the island of Mallorca at several sites, showed high temporal (annual) and spatial variability in recruitment peaks and indicated that the number of post-larval recruits is related to temperature, wind stress, and Chl*a*. (Hendriks pers. obs.; data collected between June and October each sampling year from 2009 to 2014). This could potentially influence populations of this emblematic species through disruption of recruitment under global change scenarios. Phenological (timing) shifts are consistent with predicted effects of climate warming (Parmesan and Yohe, 2003; Root et al., 2003). The synergism of rapid temperature rise and other stresses could easily disrupt the connectedness among species.

Experimental evidence from a laboratory study has demonstrated that warming induces a decrease in juveniles survival rate (Basso et al., 2015a). However, there is still limited understanding of the thermal niche of *P. nobilis* as well as of its temperature dependence in terms of key processes, such as growth, metabolism or survival across their life cycle. Hence, it is not possible, as yet, to formulate predictions on the direct effects of warming on the *P. nobilis*, although available experimental evidence suggests severe impacts (Basso et al., 2015a). As the expansion of hypoxic zones in coastal areas and warming are stressors that are likely to co-occur in the future, Basso et al. (2015b) showed resistance of juveniles to hypoxia and interaction with warming for short exposures. However, *P. nobilis* is a high oxygen consumer, reaching average consumption values of up to 12.0 ± 3.9 mg O_2 h^{-1} at 20 °C (Trigos et al., 2014b), which could cause quick depletion of oxygen in for instance shallow coastal lagoons. Another direct effect of climate change is decreased seawater pH (ocean acidification). Atmospheric carbon dioxide concentrations (pCO_2) are predicted to increase to 900 ppm by the end of the century (Meinshausen et al., 2011; Peters et al., 2012). Direct impacts of raised atmospheric CO_2 are a reduction of ocean pH (Caldeira and Wickett 2003; Cao and Caldeira, 2008; Orr et al., 2005) and the ocean's heat uptake (Meehl et al., 2007; Reid and Beaugrand, 2012), where the primary direct consequence is ocean de-oxygenation (Bopp et al., 2002; Keeling et al., 2010; Plattner et al., 2002). Under this changing scenario, the physiological performance of marine bivalves such as metabolism and calcification processes could be negatively affected (Kleypas et al., 1999; Kroeker et al., 2013; Riebesell et al., 2000). In fact, *P. nobilis* is likely to be particularly vulnerable to ocean

acidification, as it supports the fastest shell growth reported for any bivalve (Richardson et al., 2004). However, the experiments of the impact of ocean acidification on pen shell juveniles conducted so far, did not show a negative influence of ocean acidification on the performance of juveniles of this species (Basso et al., 2015a,c). Survival, growth, metabolic rate and shell structure of *P. nobilis* juveniles did not seem to be affected at pH levels expected by end of century relative to present levels (7.7 pH; Basso et al., 2015a,c). The robustness of *P. nobilis* to ocean acidification is not surprising because juveniles grow embedded in the sediment, where high respiration rates are expected to lead to high CO_2 levels and low pH, which suggests they should be able to deploy mechanisms to cope with low pH (Hendriks et al., 2014). Of note, acidification experiments are typically conducted with the organism isolated from the ecosystem (Hendriks and Duarte, 2010), which may not adequately represent the environments that coastal organisms experience either presently or in the future. In fact, coastal habitats are characterized by high pCO_2/low pH and carbonate chemistry variability (Duarte et al., 2013; Hofmann et al., 2011) with orders of magnitude more variability than the scales of ocean acidification predicted for the end of the century (Aufdenkampe et al., 2011). The protective role of *P. oceanica* would, therefore, also help this species cope with ocean acidification. In addition, *P. nobilis* may be also vulnerable through indirect effects of climate change, affecting ecosystem constituents upon which it depends. In particular, its close dependence on *P. oceanica* meadows, which are highly vulnerable to current levels of warming (Marbà and Duarte, 2010) and are predicted to decline greatly with further warming (Jordà et al., 2012), renders *P. nobilis* vulnerable due to loss of its habitat.

5.2 Invasive Species

Global change is promoting the establishment of invasive species worldwide. New invasive and established macroalga represent a threat to biodiversity, and additionally can impact sessile species. Understanding interactions among native and invasive species is crucial, for instance, the invasive macroalga *Lophocladia lallemandii* can alter the potential food sources of *P. nobilis* and its associated fauna. Mixing models of ^{15}N and ^{13}C stable isotopes from invaded versus non-invaded *P. nobilis* shells, showed that food sources are different for invaded individuals, especially with regard to ^{15}N (Cabanellas-Reboredo et al., 2010). Those changes exerted by the invasive

L. lallemandii could be related to the physiological effects of the bioactive alkaloids, lophocladines.

In addition to changes in the food source of invaded *P. nobilis* shells, the antioxidant responses of individuals colonized by *L. lallemandii* were also found to have changed (Box et al., 2009). Enzymes indicating antioxidant response (SOD, CAT, GPX, GST) and markers of oxidative damage (TR, MDA) were present at both gills and the digestive gland, indicating that invasive species induce a biological stress and oxidative damage in the *P. nobilis* invaded by macroalgae. This can be a significant problem if *P. nobilis* is forced to ingest alternative food. For example, in the Cabrera National Park, a marine protected area (MPA) in the Balearic Islands, north-western Mediterranean, *L. lallemandii* covered 49.37% of the *P. nobilis* population and *Caulerpa racemosa*, another invasive algae, 1.38%, with increasing percentages with depth (20 m compared to 10 m; Vàzquez-Luis et al., 2014). This occurrence of invasive algae reduced the diversity index of native species community (Banach-Esteve et al., 2015).

5.3 Contaminants

Sessile organisms such as *P. nobilis* cannot escape from terrestrial or marine inputs of contaminants; however, few studies have addressed this issue to date (Sureda et al., 2013a,b). In one instance, bioaccumulation of polycyclic aromatic hydrocarbon levels have been observed in gill tissue after 1 and 6 months of exposure, with an increase in the antioxidant enzymes even 1 year after an oil spill (Sureda et al., 2013a). Oxidative damage in lipids increased, though reverted to normal levels after 1 year. Moreover, environmental pollutants might exert inmunotoxical effects on *P. nobilis*. These increased activities of antioxidant enzymes were only detected at impacted locations, while no differences on oxidative damage markers were observed in individuals from pristine and polluted waters.

5.4 Habitat Loss and Boat Anchoring

Posidonia meadows, the primary habitat of *P. nobilis*, have experienced widespread degradation across the Mediterranean (Marbá et al., 2014), consistent with trends for seagrass worldwide (Waycott et al., 2009). Recruitment, as well as hydrodynamic protection of juveniles (Hendriks et al., 2011), could be negatively affected by the on-going degradation and fragmentation of *Posidonia* meadows. Such degradation could therefore negatively affect population dynamics of *P. nobilis*. Marbá et al. (2014) estimated that between

13% and 50% of seagrass area of *P. oceanica* in the Mediterranean Basin appear to be lost, and that the remaining meadows of the Mediterranean may have thinned shoot density by 50%. Loss of habitat is likely to lead to a decline of *P. nobilis* by affecting the recruitment of larvae, the mortality of juveniles to predators and the survival of young adults, more exposed to hydrodynamic forcing.

Damage by anchors is one of the causes of decline of *P. oceanica* meadows (Montefalcone et al., 2008) and is likely, therefore, to also impact on *P. nobilis* indirectly. However, *P. nobilis* is highly vulnerable to direct anchor damage, which is an important driver of impacts to its populations (Hendriks et al., 2013). This has been confirmed recently with a study using mimics for the bivalve shells that demonstrated a three times higher effect compared to control areas without anchoring (Vázquez-Luis et al., 2015).

5.5 Food Web Alterations

Common octopus, *Octopus vulgaris*, are reported to be the main predators of *P. nobilis* (Fiorito and Gherardi, 1999; García-March et al., 2007a). Octopus have also been reported to prey upon *P. nobilis* adults, by manipulating pen shell valves to open, and subsequently preying upon the exposed animal (Fiorito and Gherardi, 1999). Other predators include the gastropod, *Hexaplex trunculus* (Zhakama-Sraieb et al., 2011), and the sparid fish, *Sparus aurata* (Addis et al., 2009), which imposes a particularly high mortality on juveniles. Predation pressure on *P. nobilis* may have increased through a cascade of food web impacts, where the dusky grouper, *Epinephelus marginatus*, the main predator of octopus in the Mediterranean (Aronson, 1991; Renones et al., 2002) has declined greatly due to commercial and recreational overfishing (Marino et al., 2001). Octopus populations are believed to be predator-controlled, and we suggest that diminishing pressure on octopus populations may have led to increased predation pressure on *P. nobilis*.

5.5.1 Summary

Whereas it is impossible to estimate the magnitude of the decline of *P. nobilis* due to a lack of background information, available evidence, largely anecdotal, points at major declines, at least as severe, but possibly more than that of *P. oceanica* (Marbá et al., 2014), its primary habitat. *Pinna nobilis* is exposed to a number of cumulative stresses that are responsible for its decline and cause concern for its future status. Among those threats, the loss of *P. oceanica* habitat, may possibly be the largest driver, followed by damage

from anchors, and food web alterations leading to increased predatory pressure along with, in the past, fishing pressure. Contaminant burden may also affect this species. Whereas conservation measures should have reduced some of these pressures, particularly those most directly derived from human activities, such as fishing pressures and anchoring damage, the effectiveness of current protection measures is questionable and current practices need be reviewed. This is particularly important because the number of threats are likely to increase in the future, particularly through climate change, as the organisms appear to be vulnerable to warming and because, most importantly, *P. oceanica* is predicted to accelerate its decline with further warming (Jordá et al., 2012).

6. RESEARCH PRIORITIES

Currently, a number of knowledge gaps preclude the formulation of effective conservation strategies for *P. nobilis* and prevent forecasts of their future status taking into account the predicted trajectories of human pressures.

Studies on population density and the conservation status of this endangered species should be evaluated in different habitats, in different regions of the Mediterranean Basin and at different time scales (in particular, long-term studies are essential) to identify probable, common or peculiar, sources of mortality within the Mediterranean Basin. Observing the population density map of *P. nobilis* (Figure 4), we see that research efforts are concentrated in a few Mediterranean areas, causing gaps in our knowledge of population density and health on a basin scale. Monitoring efforts, using standardized techniques and coordinated across the Mediterranean Basin, are needed to assess the trends of the species, and resolve the rates of decline, which are currently unknown at the basin scale.

Additional studies on age and growth are recommended to obtain more information about the resilience of the *P. nobilis* populations in different environments. Analysing the inner record from empty shells could lead to the construction of an "age map" with corresponding environmental data in order to assess common environmental factors, pinpointing those sites where the healthiest populations are located (with presence of old individuals and high densities), to ultimately define the best conditions for pen shell survival. This database could be useful not only to preserve the species but also to promote the recovery and conservation of marine ecosystems by design of marine reserves.

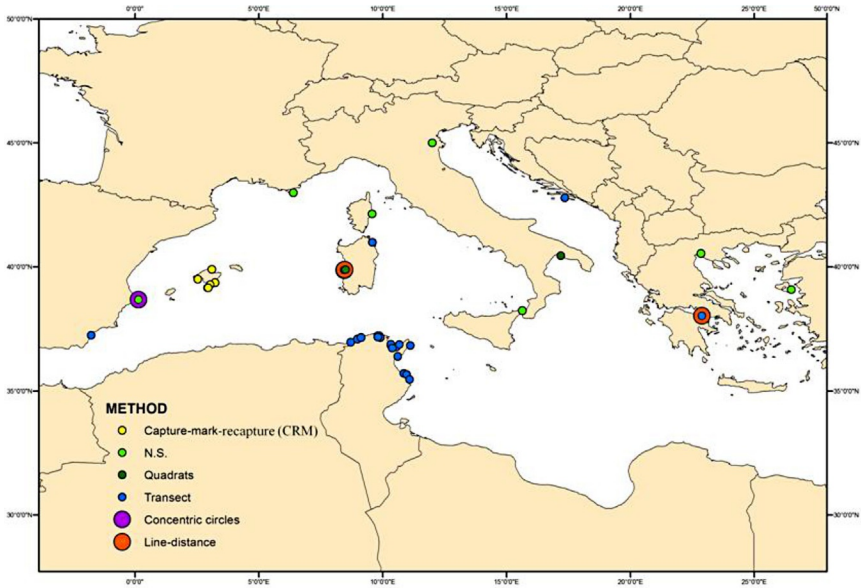

Figure 4 Map including the methodology used to measure pen shell, *Pinna nobilis*, population density at each location. Compiled from information gathered from the database used in this study (including only English language and peer-reviewed scientific literature). N.S., not specified.

Despite the conservation importance of *P. nobilis*, few studies have investigated population genetics in this species. The general picture drawn by these few studies reveals the occurrence of a West-East genetic break located eastward of the Siculo-Tunisian Strait, and also provides evidence of a further genetic break in this area in the North-South direction. In this context, part of the range of the species remains unsampled. Studies on genetic variation of a population may be fundamental to understanding how the species might respond to climatic stress over on ecological time scale (Reusch et al., 2005). Therefore, there is an urgent need to increase our knowledge regarding genetic variation *P. nobilis* populations, to evaluate the degree of connectivity and identify source and sink populations using state-of-the-art genetic techniques that will allow us to set effective conservation targets for our target species. Concerning connectivity studies, regulatory mechanisms for *P. nobilis* population sizes, such as density-dependent adult mortality or density-dependent recruitment, could be defined as knowledge gaps with potential for future research. These two compensatory mechanisms potentially regulate the abundance of the species, and overrule the control of

abundance by the supply of recruits. Moreover, the thresholds in adult density for successful reproduction have not been assessed. Local population size and density may interact and affect processes like fertilization success. Further, decreasing densities can result in lower reproductive success because the costs of low fertilization outweigh any benefits of reduced intraspecific competition. At the very least, variation in fertilization success is likely to have important repercussions for the subsequent recruitment of individuals back into populations. Several reproductive traits of the pen shell are still unknown. The proportion of energy allocated to reproduction (i.e. concentration of sperm, size and number of eggs, weight gonads) usually changes with either body size or age of individuals and can be influenced by a changing environment.

Recent rapid changes in the Earth's climate have altered ecological systems around the globe. Warming, ocean acidification, decreasing oxygen concentration and high pollution concentration have been linked to changes in physiology, phenology, species distributions, interspecific interactions and disturbance regimes. Understanding the responses of different life stages of *P. nobilis* may allow us to predict future changes and may help to improve the knowledge regarding its biological and ecological traits, its inter- intraspecific relationships (particularly with new invasive species) and to develop conservation and management goals and strategies for promoting the sustainability of this Mediterranean species.

ACKNOWLEDGEMENTS

This work was supported by the project "Estado de conservación del bivalvo amenazado *Pinna nobilis* en el Parque Nacional del Archipiélago de Cabrera" (024/2010), and partly funded from the European Community's Seventh Framework Programme (FP7/2007–2013) under Grant Agreement No. 287844 for the project "Towards Coast to COast NETworks of marine protected areas (from the shore to the high and deep sea), coupled with sea-based wind energy potential" (COCONET). L.B. was supported by JAE pre-DOC fellowship and I.E.H. by a JAE-DOC fellowship (CSIC, Spain). We thank Carlo Mazzotta for his help.

APPENDIX

English language and peer-reviewed studies involving *Pinna nobilis* classified in mean research areas, in alphabetical order. *Asterisks denote references in supplementary material (http://dx.doi.org/10.1016/bs.amb.2015.06.002).

References	Ecology	Biology	Morphology	Population Studies	Growth	Bioaccumulation	Reproduction/ Recruitment	Interspecific Relationships	Transplantation	Genetic
Abderhalden (1908)*		x								
Acarli et al. (2011)*					x					
Addis et al. (2009)*	x		x	x	x					
Alomar et al. (2015)		x								
Banach-Esteve et al. (2015)								x		
Barbera et al. (1996)*				x						x
Basso et al. (2015a)	x	x								
Basso et al. (2015b)	x	x								
Basso et al. (2015c)	x	x								
Boccoloni et al. (1988)*						x				
Box et al. (2009)	x									
Butler et al. (1993)	x									
Cabanellas-Reboredo et al. (2009a)	x									
Cabanellas-Reboredo et al. (2009b)							x			
Cabanellas-Reboredo et al. (2010)	x							x		
Calapaj and Ongaro (1971)*						x				
Caronni et al. (2007)*									x	
Carter and Clark (1985)			x							
Catsiki and Katsilieri (1992)				x		x				

References	Ecology	Biology	Morphology	Population Studies	Growth	Bioaccumulation	Reproduction/ Recruitment	Interspecific Relationships	Transplantation	Genetic
Czihak and Dierl (1961)			x							
Centoducati et al. (2007)				x						
Cerruti (1938)				x						
Cerruti (1939)				x						
Chessa et al. (1994)*				x						
Combelles et al. (1986)				x						
Coppa et al. (2010)				x						
Coppa et al. (2013)			x	x						
Corriero et al. (1985)*	x									
Corriero et al. (1987)*	x									
Cosentino and Giacobbe (2007)	x									
Cosentino and Giacobbe (2008)*	x									
Cuif and Raguideau (1982)*			x							
Cuif and Raguideau (1983)*			x							
Cuif and Raguideau (1985)			x							
Dauphin (2002)*		x								
Dauphin (2003a)*			x							
Dauphin (2003b)*		x								
Davenport et al. (2011)*	x	x								

De Gaulejac and Vicente (1990)	x			x						x
De Gaulejac (1993)	x									
De Gaulejac (1995)							x			
De Gaulejac et al. (1995b)			x				x			
De Gaulejac et al. (1995c)			x				x			
Entrop and Faber (1980)*									x	
Freitas et al. (2005)*		x								
Galinou-Mitsoudi et al. (2006)				x						
García-March (2005)				x						
Garcia-March and Ferrer (1995)*					x					
García-March and Marquez-Aliaga (2007)			x							
García-March et al. (2002)					x					
García-March et al. (2007a)				x	x					
García-March et al. (2007b)				x	x					
Garcia-March et al. (2008a)			x							
Garcia-March et al. (2008b)			x							
García-March et al. (2011)					x					
García-March et al. (2011)*	x									
Ghiretti et al. (1972)*						x				
Giacobbe and Leonardi (1987)*				x						
Giacobbe (2002)	x									

References	Ecology	Biology	Morphology	Population Studies	Growth	Bioaccumulation	Reproduction/ Recruitment	Interspecific Relationships	Transplantation	Genetic
Gonzalez-Wanguemert et al. (2015)										x
Harmelin (1977)*	x									
Hendriks et al. (2011)		x								
Hendriks et al. (2012)*					x					
Hendriks et al. (2012a,b)				x						
Hendriks et al. (2013)				x						
Henry et al. (1992)		x								
Hignette (1979)*		x								
Hignette (1983a)*					x				x	
Hignette (1984)*		x				x				
Jakson et al. (1953)*			x							
Jebalis et al. (2014)*						x				
Katsanevakis (2005)				x						
Katsanevakis (2007a)				x						
Katsanevakis (2007b)					x					
Katsanevakis (2009)				x						
Katsanevakis et al. (2009a)*	x			x						
Katsanevakis et al. (2009b)*				x						
Katsanevakis et al. (2011)										

Katsares et al. (2008)								x
Kennedy et al. (2001)	x							
Kennedy et al. (2001)*	x					x		
Kozul et al. (2011)*					x			
Lavini (1835)*		x						
Laganà et al. (2014)*	x	x						
Marin et al. (1994)*		x						
Marin et al. (2000)*		x						
Marin et al. (2003)*								x
Marin et al. (2011)		x	x					
Marin and Luquet (2005)*		x						
Mathew et al. (1996)*		x						
Mihailinovic (1995)*			x				x	
Moreteau and Vicente (1980)*			x					
Moreteau and Vicente (1982)*				x				
Najdek et al. (2013)	x							
Peharda et al. (2002)				x				
Peharda and Vilibić (2008)*	x							
Porcheddu et al. (1998)				x				
Pujol (1967)*		x						
Rabaoui et al. (2007)					x			

References	Ecology	Biology	Morphology	Population Studies	Growth	Bioaccumulation	Reproduction/ Recruitment	Interspecific Relationships	Transplantation	Genetic
Rabaoui et al. (2008b)								x		
Rabaoui et al. (2008a)*	x			x						
Rabaoui et al. (2009)	x							x		
Rabaoui et al. (2010)				x						
Rabaoui et al. (2011)										x
Rabaoui et al. (2011)*			x		x					
Rada and Milat (2009)								x		
Richardson et al. (1997)								x		
Richardson et al. (1999)				x	x					
Richardson et al. (2004)*					x					
Russo (2012)	x			x						
Sanna et al. (2011)										x
Sanna et al. (2013)										x
Sanna et al. (2014)										x
Siletic and Peharda (2003)				x						
Sureda et al. (2013a)						x				
Sureda et al. (2013b)						x				
Templado (2001)*				x						
Tlig-Zouari (1993)*	x	x		x						

Trigos et al. (2014a)	x									
Trigos et al. (2014b)		x								
Vàzquez-Luis et al. (2014)	x									
Vàzquez-Luis et al. (2014)*				x						
Vázquez-Luis et al. (2015)				x						
Vicente et al. (1980)				x						
Vicente (1990)	x			x						
Vicente et al. (1991)*				x						
Wolf et al. (2012)*			x		x					
Zavodnik (1967)*	x									
Zavodnik et al. (1991)				x						
Total information (%)	19	12	12	25	10	5	3	5	3	5

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