ADVANCES IN MARINE BIOLOGY

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PREFACE

One of the benefits of serving as the Editor of *Advances of Marine Biology*, is the opportunity to promote the accomplishments of bright young minds that have produced excellent reviews of work in their areas of research focus. Such is the case for two chapters in Volume 76. In the chapter, *Islands in a Sea of Mud: Insights from Terrestrial Island Theory for Community Assembly on Insular Marine Substrata*, K. S. Meyer has provided an ambitious and informative treatment examining the parameters of species–area relationship and island size, degree of isolation, incidence functions, nestedness, and nonrandom co-occurrence as they relate to the biogeography of communities occupying island-like habitats in the marine environment. And in, *Patterns and Drivers of Egg Pigment Intensity and Colour Diversity in the Ocean: A Meta-analysis of Phylum Echinodermata*, E. M. Montgomery, along with her esteemed colleagues J. -F. Hamel and A. Mercier, produced an insightful review and synthesis exploring pigment intensity and colour of eggs among lecithotrophic echinoderms, including a suite of multivariate analyses testing potential relationships with key biotic and abiotic factors such as development site (parental care), egg size, egg buoyancy, adult size, phylogeny, and geographic location. Each of these two young authors conducted their research as a part of their respective doctoral research projects. This demonstrates the value of synthesized scientific reviews on several levels. First, dedicated and thorough review of the scientific literature allows the next generation of researchers to develop expertise and insight in their area of focus. Second, a well constructed review provides the scientific community with concise, accurate, and up to date syntheses of the subject matter.

This volume of *Advances in Marine Biology* contains chapters addressing two additional interesting topics in marine science. In, *Biological Conservation of Giant Limpets: The Implications of Large Size*, F. Espinosa and G. A. Rivera-Ingraham examine the effects of large size on 14 species among five genera of giant limpets (*Patella*, *Cellana*, *Scutellastra*, *Cymbula*, and *Lottia*), examining the advantages (and disadvantages) of achieving these larger sizes, while also reviewing conservation status and recommending future management strategies that might ensure the survival of threatened species. Finally, in their extensive and practical review *Advances in Biochemical Indices of Zooplankton Production*, L. Yebra, T. Kobari, A. R. Sastri, F. Gusmão, and S. Hernández-León, build upon the invaluable key reference work, the *International Council*

for the Exploration of the Sea (*ICES*) *Zooplankton Methodology Manual*, describing recent advances in biochemical methods, and providing background information including rationale, design of calibration experiments, advantages and limitations of each method, and potential suitability as proxies for *in situ* rates of zooplankton community growth and production. The authors have also produced detailed protocols of existing methods for estimating indices of zooplankton production, and outlined information relevant to the application, calibration, and development of biochemical indices for zooplankton production.

Barbara E. Curry

CHAPTER ONE

Islands in a Sea of Mud: Insights From Terrestrial Island Theory for Community Assembly on Insular Marine Substrata

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Abstract

Most marine hard-bottom habitats are isolated, separated from other similar habitats by sand or mud flats, and can be considered analogous to terrestrial islands. The extensive scientific literature on terrestrial islands provides a theoretical framework for the analysis of isolated marine habitats. More individuals and higher species richness occur on larger marine substrata, a pattern that resembles terrestrial islands. However, while larger

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terrestrial islands have greater habitat diversity and productivity, the higher species richness on larger marine hard substrata can be explained by simple surface area and hydrodynamic phenomena: larger substrata extend further into the benthic boundary, exposing fauna to faster current and higher food supply. Marine island-like communities are also influenced by their distance to similar habitats, but investigations into the reproductive biology and dispersal ability of individual species are required for a more complete understanding of population connectivity. On terrestrial islands, nonrandom co-occurrence patterns have been attributed to interspecific competition, but while nonrandom co-occurrence patterns have been found for marine fauna, different mechanisms are responsible, including epibiontism. Major knowledge gaps for community assembly in isolated marine habitats include the degree of connectivity between isolated habitats, mechanisms of succession, and the extent of competition on hard substrata, particularly in the deep sea. Anthropogenic hard substrata of known age can be used opportunistically as "natural" laboratories to begin answering these questions.

1. ISLAND BIOGEOGRAPHY AS A FRAMEWORK

Ever since MacArthur and Wilson's (1967) landmark monograph, island biogeography has been one of the most studied topics in ecology. Most scientific literature in this field has focused on terrestrial islands and habitat islands, but parasites have been studied using island theory (Kuris et al., 1980) and a number of island-like habitats in the sea have also been studied (Abele and Patton, 1976; McClain et al., 2006; Schoener and Schoener, 1981). Given that most of the seafloor is blanketed by soft sediments, any hard substratum is bound to be isolated and island-like. Habitats as diverse as stones, coral reefs, and hydrothermal vents can be considered "islands in a sea of mud" (Young, 2009). For the purposes of this review, "islands" are defined as habitats separated from similar habitats by a continuous, dissimilar habitat (i.e. stones surrounded by the soft, muddy seafloor). "Terrestrial islands" refers to islands of land surrounded by ocean; "habitat islands" refers to other isolated terrestrial or freshwater habitats that can be considered ecological islands; all isolated marine habitats are termed "island-like". The focus of this review is on hard substrata and on epibenthic megafauna, though in select cases, soft-bottom island-like habitats (i.e. cold seeps) and other size fractions of fauna are discussed. How islands and island-like habitats come to be colonized and inhabited by a developed community of fauna is known as "community assembly", here defined for marine habitats as a process including larval dispersal, recruitment, competition, facilitation, predation, and succession. Several hypotheses concerning community

assembly on islands have already been developed for terrestrial and freshwater habitats based on classical island theory (Brown et al., 2000; Fox and Kirkland, 1992; Heino and Soininen, 2005; Holdaway and Sparrow, 2006). Island hypotheses can potentially be used as a framework for understanding community assembly on isolated substrata in the sea.

"Classical island theory" is used in the context of this review to refer collectively to two previous works, those of MacArthur and Wilson (1967) and Diamond (1975a). Separately, the theories published in those works will be referred to as "island biogeography" (for MacArthur and Wilson, 1967) and "assembly rules" (for Diamond, 1975a). The publication of MacArthur and Wilson's monograph is a landmark in the history of ecology, representing a radical break from the previous focus on taxonomic descriptions in biogeography (Heaney, 2000). The theory has since been tested empirically (Simberloff and Wilson, 1969, 1970; Wilson and Simberloff, 1969), but has also been criticized (Heaney, 2000), modified extensively (Anderson and Wait, 2001; Brown and Kodric-Brown, 1977; Buckley, 1982; Losos and Ricklefs, 2010), and argued to be only applicable in a small set of cases (Haila, 1990). Nevertheless, the MacArthur–Wilson equilibrium theory continues to influence modern ecological thought for islands and habitat islands (Whittaker and Fernandez-Palacios, 2007). In fact, a new paradigm for island biogeography, the General Dynamic Model, still stands on a foundation of the classical MacArthur–Wilson theory while integrating time as a factor (Borregaard et al., 2015; Whittaker et al., 2008).

Diamond's (1975a) assembly rules, a series of hypotheses based on the distribution of New Hebrides avifauna, were immediately controversial (Connor and Simberloff, 1979; Diamond and Gilpin, 1982). However, the ensuing literature debate led to the adoption of null models as a major tool for ecological analysis (Gotelli and Graves, 1996), and over the course of several decades it also led to the development of several powerful statistical tools that are now in common practice (Gotelli, 2000; Ulrich et al., 2009). "Assembly rules" has come to refer to any apparent biotic or abiotic force structuring guilds or communities (Belyea and Lancaster, 1999), and has been used for a wide variety of fauna, including ants (Gotelli and Ellison, 2002), rodents (Brown et al., 2000), birds (Blake, 1991), and plants (Weiher et al., 1998).

Metacommunity models can also be applied to island-like marine benthic habitats (Neubert et al., 2006). This review does not address metacommunity theory specifically, but rather offers a different perspective, focusing on assembly of local communities. Comparison of the ideas presented here with

developing metacommunity theory (Leibold et al., 2004; Logue et al., 2011) may deepen our understanding of community assembly in island-like marine habitats and other systems.

Now being decades removed from the publication of the classical island theories, it is possible to view each theory through the lens of the debates and discussions that ensued. For the purposes of this review, I will distill each classical island theory to its basic elements as they have survived in the literature, and then apply this framework to island-like habitats in the sea. Five patterns found on terrestrial islands and discussed in the classical island literature are as follows:

1.1 Species–Area Relationship and Island Size

MacArthur and Wilson (1967) noted a log-linear relationship between the size of an island and the richness of its fauna, of the form $S = cA^z$ (S is species richness, A is area, c and z are constants). When a log transformation is used, the relationship becomes $\log(S) = z \log(A) + c$ (Fig. 1A). Similar patterns have been found for a variety of fauna and habitats (Connor and McCoy, 1979), leading some to note that the species–area relationship is one of the few true laws in ecology (Lawton, 1999).

1.2 Degree of Isolation

The cornerstone of MacArthur and Wilson's equilibrium theory of island biogeography is the classic figure showing immigration and extinction curves on islands of varying size and level of isolation (MacArthur and Wilson, 1967; Fig. 1B). Islands closer to a mainland were theorized to have higher immigration, while larger islands were theorized to have more possible niches and lower extinction, where the immigration and extinction curves for a particular island crossed were the equilibrium number of species for that island (MacArthur and Wilson, 1967). The basic assumption that island communities are in equilibrium is false in most cases (Heaney, 2000); therefore, for the purposes of this review, the discussion will be restricted to species diversity on an ecological timescale. To put it concisely, higher diversity is expected on insular marine habitats that are in closer proximity to other similar habitats.

1.3 Incidence Functions

Diamond (1975a) also observed a spectrum of life-history traits to exist on islands of different sizes. Small islands were inhabited only by fast-growing, generalist, "supertramp" species, while larger islands were also inhabited by

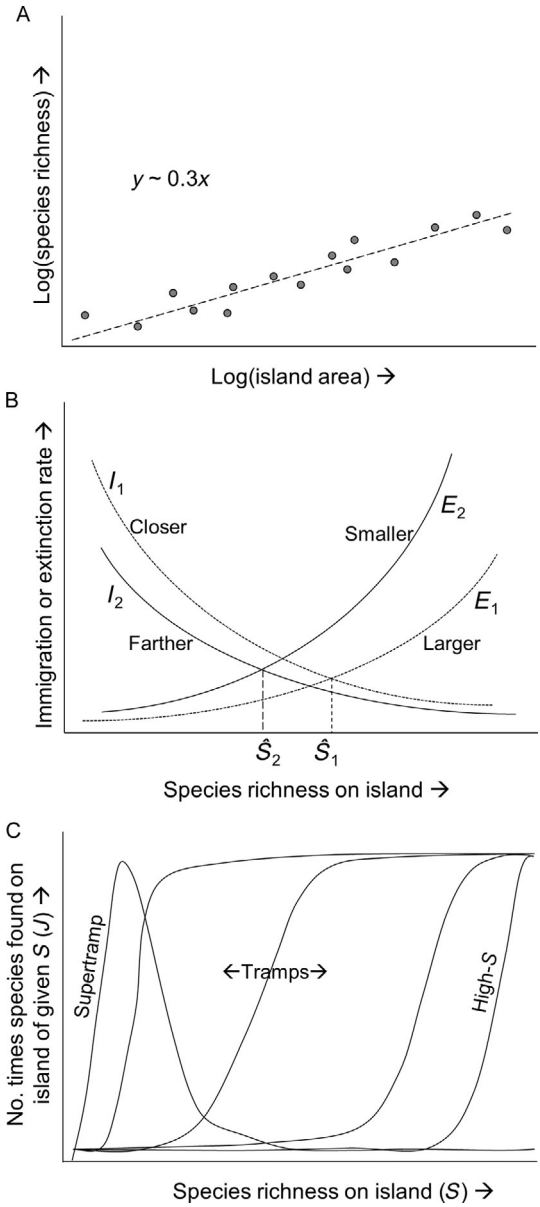

Fig. 1 Graphics associated with classical island theories. (A) Log-linear relationship between species richness on an island and the area of the island. Points are for illustration and do not depict actual data. (B) Equilibrium theory of island biogeography. Immigration and extinction curves for two islands are shown; island 1 is closer to mainland and larger. Where the curves intersect is the equilibrium number of species ($\hat{S}$) for that island. (C) Incidence functions. "Supertramp" species are generalists that primarily inhabit islands with low species richness, "tramps" are intermediate, and "high-*S*" species inhabit islands with high species richness. *Panels (A and B): After MacArthur, R.H., Wilson, E.O., 1967. The Theory of Island Biogeography. Princeton University Press, Princeton, NJ. Panel (C): After Diamond, J.M., 1975a. Assembly of island communities. In: Cody, M.L., Diamond, J.M. (Eds.), Ecology and Evolution of Communities. Belknap Press of Harvard University Press, Cambridge and London, pp. 342–444.*

long-lived, specialist, "high-*S*" species. The curve showing which sizes of islands a species is most likely to inhabit is called its incidence function (Fig. 1C).

1.4 Nestedness

Nested patterns of fauna occur when smaller or more isolated islands are inhabited by smaller subsets of fauna, and species disappear in a consistent pattern such that each subset is "nested" within the next largest subset of fauna (Fig. 2). There is some overlap in the concepts of incidence functions (see Section 1.3) and nestedness, because small subgroups of fauna (found on smaller or more distant islands) generally contain only long-dispersing, fast-growing, opportunistic species, while larger subgroups (found on larger or less isolated islands) also include long-lived, slow-growing specialists. There is also some interaction between the concepts of nestedness and succession, as young islands will be initially colonized by fast-growing opportunist species, while older islands will be inhabited by slower-growing superior competitors. However, on large islands, the fast-growing opportunist species may continue to inhabit suboptimal niches alongside the slow-growing superior competitors as succession proceeds. Nested patterns were observed by MacArthur and Wilson (1967) and subsequent authors (Blake, 1991; Kadmon, 1995; Patterson and Atmar, 1986) on islands and island-like habitats, and the nestedness of fauna has implications for conservation (Cutler, 1991).

1.5 Nonrandom Co-occurrence

Nonrandom co-occurrence of species, by far the most controversial aspect of Diamond's (1975a) theory, refers to pairs of species being found on the same island less often than expected by chance. Patterns of nonrandom co-occurrence were initially attributed to interspecific competition (Diamond, 1975a). While nonrandom co-occurrence is relatively common in various faunas (Gotelli and McCabe, 2002), the underlying mechanisms remain controversial and may include stochastic processes (Ulrich, 2004). In this review, "negative nonrandom co-occurrence" will refer to species pairs co-occurring nonrandomly less often than expected by chance, and "positive nonrandom co-occurrence" will refer to species pairs co-occurring nonrandomly more often than expected by chance. Species distribution patterns that do not significantly differ from chance will be referred to as "random co-occurrence".

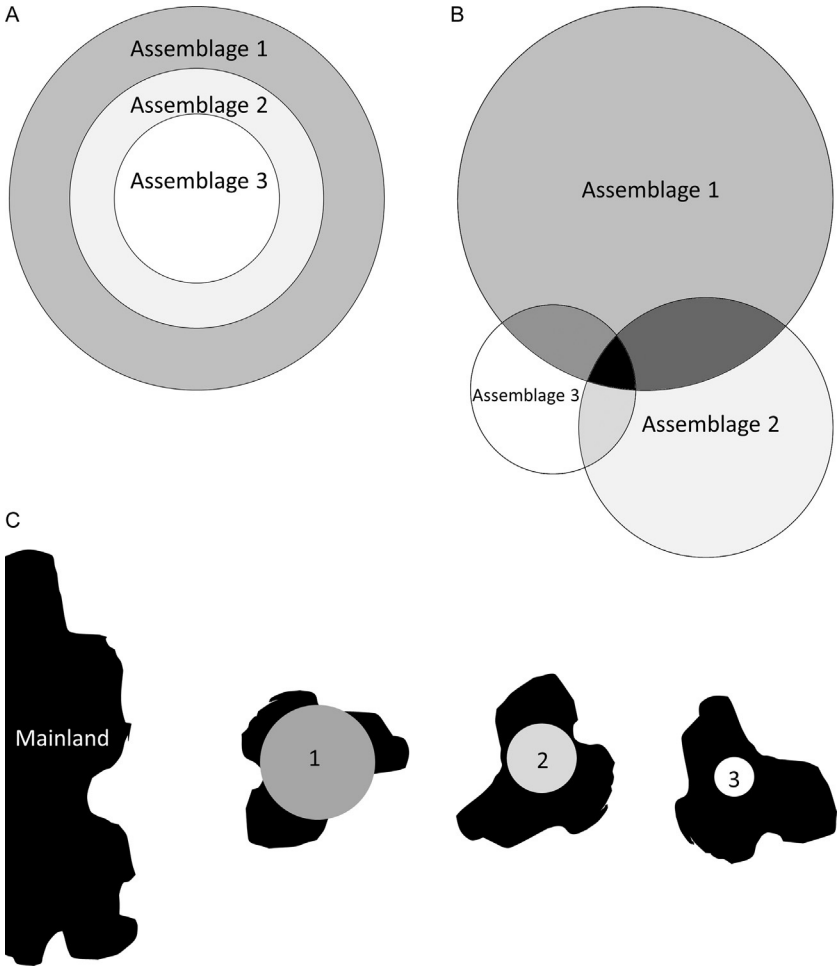

Fig. 2 (A) Venn diagram depicting nested species assemblages. Assemblage 1 contains the largest number of species, and assemblages 2 and 3 are nested within it. (B) Venn diagram depicting nonnested species assemblages. (C) How nested species assemblages may occur on islands of different distances from a mainland source population.

Throughout the remainder of this review, a modern understanding of these five basic patterns will be applied to island-like habitats in the marine environment to delineate how classical island theory can increase our understanding of insular marine habitats. The mechanisms responsible will also be discussed.

2. SUBTIDAL AND DEEP-SEA HABITATS AS ISLANDS

Most marine hard substrata are isolated and island-like, ranging in size from landscape-scale features (seamounts, Clark et al., 2010; canyons, DeLeo et al., 2010; trenches, Jamieson et al., 2010) to medium-size structures (coral reefs, Jones et al., 2009; rocky reefs, Jones, 1988; dropstones, Meyer et al., 2016) and small objects (manganese nodules, Mullineaux, 1987; sea urchin tests, Gutt and Schickan, 1998; hermit crab shells, Bałazy and Kukliński, 2013; sponge stalks, Beaulieu, 2001; water-logged plant material, Wolff, 1976, 1979). Chemosynthetic vents (Lutz and Kennish, 1993), seeps (Sibuet and Olu, 1998), and whale-falls (Baco and Smith, 2003) are also isolated and island-like (Neubert et al., 2006). Anthropogenic structures (shipwrecks, Perkol-Finkel et al., 2006; artificial reefs, Carr and Hixon, 1997; shipping containers, Taylor et al., 2014; military discard, Kelley et al., 2016; oil platforms, Gass and Roberts, 2006; even litter, Bergmann et al., 2015) are also insular habitats.

Of the island-like marine habitats listed above and discussed in this review, seamounts are the most obvious analogue for terrestrial islands. For many years, seamounts were viewed as biodiversity hotspots with high endemicity and unique evolution (de Forges et al., 2000; Hubbs, 1959; McClain et al., 2006; Rogers, 1993). Mesoscale circulation above seamounts may retain larvae and lead to genetic differentiation between populations (Mullineaux, 1994; Mullineaux and Mills, 1997). It was originally hypothesized that larval retention could lead to speciation on seamounts, but this view has shifted in recent years. The apparent endemicity on seamounts may in fact be the result of undersampling (McClain, 2007), and when samples from similar substrata, depths, and geomorphologies are compared, seamounts appear no more diverse than adjacent banks or continental slopes (Howell et al., 2010; O'Hara, 2007).

Coral reefs are well-known isolated habitats in tropical regions. Much research has been conducted to understand connectivity among coral reefs, both for corals themselves and for reef fish (Jones et al., 2009). The prevailing paradigm has shifted in the past decades from high connectivity to recruitment at natal reefs (Jones et al., 2005; Levin, 2006), but the integration of oceanographic modelling and larval biology has increased our understanding of reef connectivity (Baums et al., 2006).

Submarine canyons occur on continental margins all over the world and can also be considered island-like habitats. Because of their steep walls and

narrow basins, canyons can focus on bottom currents and internal waves, leading to turbulence and the resuspension of organic matter (Gardner, 1989). Canyons can also serve as conduits to funnel plant debris into the deep sea (Vetter and Dayton, 1999). These combined effects mean that submarine canyons have higher organic matter input and higher biomass of macro- and megafauna than the surrounding continental slope (DeLeo et al., 2010; Vetter et al., 2010). Soft-bottom infaunal and epifaunal communities in canyons are significantly different from the adjacent continental slope, with different distribution patterns in each environment (Rowe, 1971; Vetter and Dayton, 1998). Canyons often feature boulders or rocky walls that are inhabited by cold-water corals not otherwise present on the continental slope (Roberts et al., 2009). Coral community composition varies among even adjacent canyons (Brooke and Ross, 2014), and populations are genetically distinct among regions (Morrison et al., 2011).

Deep-sea trenches, located below 6000 m, are unique habitats characterized by high bottom water oxygen, high organic matter input, and extreme pressure (Jamieson et al., 2010). Though they feature primarily soft substrata, trenches could be considered island-like habitats; trench fauna show strong bathymetric zonation that at least partially isolates them from the surrounding abyssal plain (Jamieson et al., 2011). Communities in different trenches vary based on proximity to land, which influences the amount of allochthonous organic input (Gallo et al., 2015). Some hadal species are found in multiple trenches (Jamieson et al., 2013), but the extent of population connectivity among trenches is still poorly understood.

Dropstones, another type of island-like hard substratum, are defined as stones of terrestrial origin that have become frozen in a glacier, carried out to sea by an iceberg, and are deposited on the seafloor when the iceberg melts (Kidd et al., 1981; Oschmann, 1990). Dropstones are colonized by a variety of hard-bottom fauna (Oschmann, 1990; Schulz et al., 2010) and increase habitat heterogeneity where they occur (Hasemann et al., 2013; MacDonald et al., 2010). Dropstones have faunal distributional patterns similar to terrestrial islands (Meyer et al., 2016).

Hydrothermal vents have been intensively investigated since their discovery in 1977, including landmark studies of larval dispersal (Kim et al., 1994; Marsh et al., 2001), recruitment (Mullineaux et al., 2000; Van Dover and Berg, 1988), and succession (see Young, 2009). Some researchers have interpreted a deterministic course of succession at vents (Hessler et al., 1988; Shank et al., 1998), while others have interpreted a more stochastic process dependent on larval availability in the water column

(Mullineaux, 1994; Tunnicliffe et al., 1997). Similarly, cold seeps are island-like chemosynthetic habitats, most common on continental margins (Sibuet and Olu, 1998). They are colonized by some of the same genera and species as vents (Van Dover et al., 2002). Larvae of cold seep fauna may be long lived (Arellano and Young, 2009), allowing for long-range dispersal among seeps (Olu et al., 2010; Young et al., 2012).

Whale-falls are ephemeral habitats with a unique ecology (Glover et al., 2005; Rouse et al., 2009; Smith and Baco, 2003; Smith et al., 2015). After the removal of flesh, whale bones support a community of sulphophilic fauna, including bathymodiolin mussels (Lorion et al., 2009; Lundsten et al., 2010). Other organic matter-falls, including wood- and plant-falls, also represent organic islands in the deep sea, and they are colonized by numerous species of specialist xylophagid and teredinid mollusks (Heß et al., 2008; McClain and Barry, 2014). Wood-falls support anaerobic, sulphate-reducing microbes (Bienhold et al., 2013) and may even share fauna with whale-falls (Lorion et al., 2009). The species that colonize and exploit chemosynthetic vents, seeps, whale-falls, and other organic matter-falls in the deep sea are related on an evolutionary timescale, though the exact relationships are still under debate (Distel et al., 2000; Smith et al., 2015).

Each of the insular hard substrata listed here will be considered in the following sections, and the processes of community assembly for each habitat type will be framed in the context of classical island theory.

3. PATTERNS ON SUBTIDAL ISLANDS

3.1 Species–Area Relationship and Island Size

A log-linear relationship between species richness and island area is well documented, even ubiquitous, for terrestrial habitats, and various mechanisms have been proposed as explanations. These include habitat diversity, primary productivity, resistance to disturbance, equilibrium achieved through a balance of immigration and extinction, clumped distributions of species, successional development, and sampling artefacts (Connor and McCoy, 1979; Gotelli and Graves, 1996; Hill et al., 1994; MacArthur and Wilson, 1967). Similar log-linear relationships of species (morphotype) richness to area have been found for marine substrata (Abele and Patton, 1976; Huntington and Lirman, 2012; Meyer et al., 2016; Schoener and Schoener, 1981), but these proposed explanations are not satisfactory. For marine hard substrata, habitat diversity does not vary greatly with increasing

size (Abele and Patton, 1976), and extinction is rarely observed (Schoener and Schoener, 1981). For nonchemosynthetic substrata in the deep sea, primary production does not occur locally, and disturbance is relatively rare. The most parsimonious explanation for the positive species–area relationship on marine substrata is the "passive sampling hypothesis" (Connor and McCoy, 1979), which states that larger islands (substrata) are merely larger targets for dispersing propagules (Huntington and Lirman, 2012; Meyer et al., 2016). Larger substrata have higher immigration rate and "fill up" more slowly, allowing more species to accumulate over time (Schoener and Schoener, 1981). It should be noted that studies specifically addressing substratum size as a factor influencing the benthic community (Abele and Patton, 1976; Huntington and Lirman, 2012; Meyer et al., 2016; Schoener and Schoener, 1981) all deal with small, simple substrata—coral heads, settlement plates, and stones. For these substrata, habitat heterogeneity does not increase dramatically with substratum size. However, habitat heterogeneity may increase with size for more complex island-like habitats, such as seamounts, canyons, and trenches.

Another reason for the positive species–area relationship in island-like marine habitats is that larger substrata extend further into the benthic boundary layer and are exposed to faster currents for suspension feeding (Vogel, 1996). It is well documented that dense populations of suspension-feeding organisms or planktivores inhabit topographic highs such as seamounts (Genin et al., 1986), rocky reefs (Meyer et al., 2014), pinnacles (Leichter and Witman, 1997), and fjord sills (Mortensen et al., 2001), where there is greater turbulence and availability of particulate food. Even small structures, such as glass sponge stalks (Beaulieu, 2001), manganese nodules (Mullineaux, 1987), and sea urchin tests (Hétérier et al., 2008), are inhabited by suspension feeders seeking elevation higher into the benthic boundary layer. The "passive sampling hypothesis" and its corollary of higher particulate food supply on larger substrata offer parsimonious explanations for species–area relationships on subtidal and deep-sea insular substrata (Meyer et al., 2016). The species–area relationship is ubiquitous (Lawton, 1999), but the mechanisms causing this pattern in marine and terrestrial environments are not necessarily the same.

One exception should be noted for organic substrata such as wood-falls, where the higher species richness on larger substrata is more closely related to volume rather than surface area (McClain and Barry, 2014). Thus, species richness is driven by energetic content of the wood rather than larval recruitment (McClain and Barry, 2014; McClain et al., 2016). For seamounts, it

should also be mentioned that the depth range, which is directly related to seamount size, is a prominent factor influencing faunal composition. Different communities are present at different depths along the seamount, and fauna are controlled by the characteristics of water masses at each given depth (Chivers et al., 2013; Clark et al., 2010; O'Hara and Tittensor, 2010; Rogers, 1993).

If species richness and island area data are graphed in their raw form, without the traditional log transformation of each axis, the relationship is asymptotic (Meyer et al., 2016). The flattening curve may in fact indicate that only a certain number of species are capable of colonizing a given substratum, i.e., the species pool is finite (Meyer et al., 2016). Larger substrata are inhabited by a greater proportion of the available species, so the classical log-linear species–area relationship may in fact result from nothing more than a finite species pool.

3.2 Degree of Isolation

MacArthur and Wilson discussed island size and the degree of isolation as two critical factors influencing the richness of fauna (MacArthur and Wilson, 1967). An empirical test of island biogeography theory showed lower immigration rate and lower species richness on islands further away from a source (Simberloff and Wilson, 1969, 1970). For marine hard substrata, experimentally cleared patches surrounded by benthic fauna were more readily colonized by asexual growth of encrusting individuals, whereas isolated patches had to be colonized by larval dispersal and showed size-dependent diversity (Keough, 1984). Bryozoans recruited to patches of all size, whereas tunicates recruited to and dominated on large isolated patches (Keough, 1984). The effects of isolation and the extent of connectivity among island-like marine habitats depend on the dispersal capabilities of the resident fauna and can be complex.

Biogeography of marine benthos has been described (Ekman, 1953; Vermeij, 1978), leading to the designation of biogeographic provinces for vents (Tunnicliffe et al., 1998; Van Dover et al., 2002) and seeps (Baco et al., 2010; Olu et al., 2010), as well as the bathyal, abyssal, and even hadal seafloor (Watling et al., 2013). Vicariance and allopatric speciation events have also been hypothesized for seamounts and hydrothermal vents (Shank, 2010; Vrijenhoek, 2010), but our understanding of these habitats is far from complete.

The most influential paradigms for the biogeography of isolated marine habitats are the so-called island model (Wright, 1931) and "stepping-stone" or "isolation-by-distance model" (Wright, 1943). Though originally intended to describe the dispersal of individual species, these models have been reinterpreted in the literature to apply to community assembly, and it is this interpretation that will be used here. The "island model" describes the colonization of isolated habitats from a single well-mixed larval pool, while the "stepping-stone model" points to short-range larval dispersal and a positive linear correlation between genetic and geographic distances for isolated populations (Vrijenhoek, 1997). These models have been described for a variety of habitats, including coral reefs, hydrothermal vents, and cold seeps (Palumbi, 2003; Tyler and Young, 1999; Vrijenhoek, 2010).

While the so-called island model draws on the terminology of classical island theory, it does not accurately reflect the theory. Neither MacArthur and Wilson (1967) nor Diamond (1975a) ever assumed the existence of a well-mixed pool of equally dispersing propagules; rather, they discussed the dispersal potential of individual species. Species with long-range dispersal capability and fast growth were theorized to be the first successful colonists of any island, while long-lived, short-dispersing species were said to arrive later or not at all (Diamond, 1975a; MacArthur and Wilson, 1967).

The concept of stepping stones appears in the deep-sea literature with reference to whale-falls on both evolutionary and ecological timescales. In evolutionary time, it is hypothesized that sunken wood and whale carcasses served as "stepping stones" for the colonization of and adaptation to hydrothermal vents and cold seeps (Distel et al., 2000; Heß et al., 2008; Kiel and Goedert, 2006). In ecological time, it has been hypothesized that whale-falls serve as "stepping stones" to facilitate larval dispersal among vent and seep habitats (Smith and Baco, 2003). However, both ideas remain controversial, and there is not sufficient evidence to support or refute either hypothesis (Amano and Little, 2005; Levin et al., 2016; Smith and Baco, 2003).

3.3 Incidence Functions

Diamond (1975a) described "incidence functions" for the New Hebrides avifauna, using S-shaped curves to show the size of island each species was most likely to inhabit (Fig. 1C). Fast-growing opportunistic species

(supertramps) were said to inhabit all but dominate smaller islands, whereas slow-growing specialists (high-*S* species) were found exclusively on larger islands. The end result was a nested pattern of fauna, with "supertramps" and "tramps" being ubiquitous and "high-*S*" species found only on larger islands.

Incidence functions have seldom been directly sought for isolated marine habitats, but reexamination of existing data sets shows that incidence functions are generally not evident for marine substrata. For example, Schoener and Schoener (1981) found that different species dominated on settlement plates of different sizes, but the dominant species did not conform to any patterns (such as "supertramps" on small plates and "high-*S*" species on larger plates). This result does not support the existence of incidence functions for marine fauna (Schoener and Schoener, 1981). Decapod crustaceans inhabiting coral heads also occurred on various sizes of heads, without a split between species inhabiting small heads and species inhabiting large heads (Abele and Patton, 1976). For dropstones on the west Svalbard continental slope, there was no correlation between the size of a stone and the species composition on the stone (Meyer et al., 2016). Higher species (morphotype) richness was found on larger stones, but the morphotypes inhabiting each stone were randomly selected from the available species pool (Meyer et al., 2016).

Incidence functions, or the restriction of slow-growing, long-lived species to larger insular habitats may not be a common pattern for isolated marine habitats. Nevertheless, the potential for incidence functions and the resulting nested patterns of fauna are important to keep in mind because of the implications for conservation, discussed below.

3.4 Nestedness

Nestedness is a common pattern for a variety of terrestrial and aquatic fauna, in which ever-smaller or ever-more-isolated habitats are inhabited by ever-smaller subgroups of fauna that are nested within one another (see Fig. 2A). Smaller islands have fewer resources and fewer niches, so only fast-growing opportunists can successfully establish populations. Distant islands can only be reached by long-dispersing, fast-growing opportunists, resulting in nested patterns.

For dropstones on the west Svalbard continental slope, it was hypothesized that a nearby "mainland" rocky reef served as a source of larvae to the stones, leading to a nested pattern of fauna further away from the reef (Meyer

et al., 2016). However, no nested pattern was found; instead, dropstone fauna had a clumped distribution that most likely resulted from short-range larval dispersal among the stones, with only limited influence of the rocky reef (Meyer et al., 2016).

For deep-sea wood-falls, McClain et al. (2016) found that the sets of fauna on smaller falls were nested within fauna on larger wood-falls. However, in other cases, colonization of wood-falls has been variable and stochastic (McClain and Barry, 2014; Pailleret et al., 2007).

Isolated marine habitats may experience a greater degree of connectivity driven by larval dispersal than is traditionally understood for terrestrial islands. There is also an element of stochasticity in the colonization of isolated marine habitats, because recruitment depends on larval availability, which may be temporally and spatially variable (Mullineaux et al., 2005; Siegel et al., 2008; Van Dover et al., 2001).

Save for the above examples, authors writing about isolated marine habitats usually do not test for nested patterns of fauna. However, there is a well-established methodology to detect nested faunal patterns, and a simple test could be easily incorporated into any routine data analysis (Ulrich and Gotelli, 2007). It would be interesting to look for nested patterns of faunal distribution among seamounts, coral reefs, and hydrothermal vents—habitats that currently are or may in the future be designated as marine protected areas (MPAs). The largest application of nested faunal patterns in the scientific literature in fact relates to the SLOSS (Single Large Or Several Small) debate about natural reserves and protected areas (Diamond, 1975b; Tjørve, 2010). If, according to their incidence functions, only fast-growing, opportunistic, generalist species are able to inhabit small islands or habitat islands, then small reserves would only conserve those opportunistic species, whereas a single large reserve would host niches for fast-growing generalists and long-lived specialists alike.

If nested patterns are found, they would inform the discussions about appropriate design of MPAs. Also, a better understanding of larval dispersal dynamics, connectivity, and gene flow among isolated marine habitats will improve MPA design (Levin, 2006; Shanks et al., 2003).

3.5 Nonrandom Co-occurrence

Negative nonrandom co-occurrence patterns of fauna were originally attributed to interspecific competition (Diamond, 1975a). While nonrandom co-occurrence patterns have been found for a variety of fauna,

the mechanisms responsible are various and can include stochastic processes (Ulrich, 2004).

Negative nonrandom co-occurrence was observed for encrusting fauna on dropstones, but interspecific overgrowth competition was not observed (Meyer et al., 2016). Rather, epibiontism caused positive nonrandom co-occurrence patterns for dropstone fauna (Meyer et al., 2016). Suspension-feeding species were observed on top of large hexactinellid sponges, presumably to gain an advantage in feeding. Epibiontism has been observed for suspension feeders on biotic substrata at a variety of depths and latitudes (Beaulieu, 2001; Gutt and Schickan, 1998; Hétérier et al., 2008) and is well documented for cold-water coral stands and sponge gardens (Cordes et al., 2008; Maldonado et al., 2015; Roberts et al., 2009). Epibionts may need their basibionts to different degrees—ranging from facultative to obligate relationships—and the associated species may have coevolved in some cases (Shank, 2010). How strongly epibiotic relationships affect faunal distribution remains uninvestigated for most habitats.

4. PROCESSES UNDERLYING THESE PATTERNS

4.1 Larval Dispersal

Classical island theory deals with a wide range of taxa with varying dispersal distances; how far a species disperses influences the islands it can colonize (Diamond, 1975a; MacArthur and Wilson, 1967). Similarly, for (nonchemosynthetic) marine insular habitats, empirical evidence suggests that larval dispersal patterns are taxon specific and depend on life history (Grantham et al., 2003; Jones et al., 2009; Miller et al., 2010). Short-lived lecithotrophic or brooded larvae are likely to recruit to their natal habitat (Bingham, 1992; Jackson, 1986; Marshall and Keough, 2003; Shanks et al., 2003), while long-lived planktotrophic larvae disperse much farther (Baco et al., 2016). Short larval duration and self-recruitment may be an evolutionary stable state for fauna inhabiting isolated hard substrata, as there is no guarantee of finding a suitable substratum when dispersing far away (Cowen et al., 2000; Grantham et al., 2003; Shanks et al., 2003). Self-recruitment is common for shallow-water coral reefs (Jones et al., 2005; Swearer et al., 1999) and temperate rocky reefs (Altieri, 2003; Grantham et al., 2003).

The larval biology and dispersal potential of cold-water corals have been studied for *Lophelia* and *Oculina* (Brooke and Järnegren, 2013;

Brooke and Young, 2003, 2005). Genetic studies revealed restricted gene flow for *Paragorgia arborea* and *Lophelia pertusa* (Herrera et al., 2012; Miller et al., 2010; Morrison et al., 2011). Coral stands close to one another spatially may be inbred or even clones (Brooke and Stone, 2007; Le Goff-Vitry et al., 2004; Orejas et al., 2009). However, long-range dispersal has also been hypothesized for *L. pertusa* (Gass and Roberts, 2006). Coral-associated species in deep water also show various developmental modes with a tendency towards restricted dispersal (O'Hara et al., 2008; Rowden et al., 2010).

For deep-sea hydrothermal vents, larval dispersal appears to be more influenced by local circulation patterns than by larval duration (Marsh et al., 2001). It appears that hydrothermal vent invertebrates, including tubeworms and gastropods, do not disperse away from their natal vent field, regardless of pelagic larval duration; across-ridge and reversing transport retains larvae near where they were spawned (Adams and Mullineaux, 2008; Kim and Mullineaux, 1998; Marsh et al., 2001). However, some species, including the vent crab *Bythograea thermydron*, have planktotrophic larvae that feed and disperse in surface waters (Dittel et al., 2005; Epifanio et al., 1999; Perovich et al., 2003). More isolated vents may have lower larval supply (Adams and Mullineaux, 2008), but there is at least one documented example of a vent being colonized by larvae from 100s of km away (Mullineaux et al., 2010) and genetic patterns do not necessarily fit the predictions of short-range larval dispersal models (Chevaldonné et al., 1997). Clearly, more research is needed to understand colonization patterns at isolated hydrothermal vents, as even sequential studies at the same location provide conflicting results (Hunt et al., 2004; Mullineaux et al., 2000).

The extent of larval dispersal and connectivity among isolated marine habitats is a major research question that has only begun to be answered. Connectivity is best described for shallow-water habitats such as coral reefs (Jones et al., 2005). The prevailing paradigm for larval dispersal has made a pendulum-like swing in recent decades from high levels of connectivity among habitats to mostly self-recruitment (Levin, 2006). However, genetic results do not necessarily reflect restricted dispersal and low connectivity among habitats (Adams and Mullineaux, 2008). Investigations of connectivity are largely based on population genetics (Shank, 2010), while larval biology is incompletely known or just inferred (Miller et al., 2010; Palumbi, 2003). A wide variety of techniques are available for tagging and tracking adult organisms and larvae (Cowen and Sponaugle, 2009;

Levin, 1990; Levin et al., 1993; Thorrold et al., 2002, 2007), and these techniques are just beginning to be applied in deeper water (Génio et al., 2015).

The biology of each species should be considered in connectivity studies, especially considering that pelagic larval duration for marine species ranges from minutes (Altieri, 2003) to a year or more (Arellano and Young, 2009). It is also not accurate to assume a correlation between larval duration and dispersal range, as lecithotrophs may be capable of long-range dispersal (Tyler and Young, 1999). A better understanding of connectivity among insular marine habitats will be brought about by the integration of larval biology, physical oceanography, and population genetics (Shank and Halanych, 2007). Excellent examples of integrated studies include that of Baums et al. (2006) for coral reefs and Young et al. (2012) for cold seeps.

The concept of stepping stones may be helpful for future investigations of community dynamics in insular marine habitats. Anthropogenic hard substrata, including shipwrecks, shipping containers, oil and gas platforms, fishing gear, and litter, can be colonized by sessile fauna, thereby allowing these hard-bottom organisms to inhabit new areas of the seafloor (Bergmann and Klages, 2012; Kilgour and Shirley, 2008; Ross et al., 2016; Taylor et al., 2014). Benthic adults living on an anthropogenic substratum may release larvae, which could disperse to and settle on substrata outside their native ranges, leading to gene flow or species invasion among previously isolated habitats.

4.2 Succession

A shift in the life-history traits of fauna over time was described for terrestrial islands under the terminology of r- and K-strategists by MacArthur and Wilson (1967) and under the terminology of supertramps, tramps, and high-*S* species by Diamond (1975a). In each case, the authors hypothesized that the first colonists on an island should be opportunistic, generalist species with high fecundity, long-range dispersal, and short lifespan. As more colonists arrived, the initial colonists should be outcompeted and replaced by more specialist species with lower fecundity, short-range dispersal, and longer lifespans.

Succession is best understood for shallow hard substrata at temperate latitude. Previous studies on the east coast of the United States have shown that experimental substrata (settlement plates) are first colonized by acorn barnacles and spirorbid polychaetes, followed by a community of encrusting bryozoans, ascidians, and hydroids (Dean and Hurd, 1980; Osman, 1977).

The third stage is dominated by blue mussels (Chalmer, 1982; Dean and Hurd, 1980) or one of the encrusting species from the second stage (Osman, 1977). The course of succession can also depend on season and the order in which species arrive on a substratum (Pacheco et al., 2011; Sutherland, 1974).

Acorn barnacles may enjoy early dominance on settlement plates because cyprid larvae do not require a thick biofilm for settlement (Keough and Raimondi, 1996; Todd and Keough, 1994). Barnacles have long-lived larvae capable of long-distance dispersal; they grow quickly and are poor competitors (Quinn, 1982). Therefore, their position as the first colonists of an isolated hard substratum fits the expected life-history scheme. For encrusting fauna, the first colonists are opportunists, characterized by fast growth and poor competitive ability, whereas species present later in succession are slower-growing superior competitors (Antoniadou et al., 2010; Edwards and Stachowicz, 2010; Vance, 1988). A similar shift in life-history traits has been observed on shallow (<30 m depth) shipwrecks and artificial reefs, with structures underwater for less than 20 years being dominated by opportunistic species (Carter et al., 1985; Pawlik et al., 2008; Perkol-Finkel and Benayahu, 2005) and structures underwater for more than 100 years being inhabited by long-lived superior competitors, in a community not significantly different from nearby natural reefs (Perkol-Finkel et al., 2005, 2006).

While the process of succession is well described for shallow marine hard substrata, the mechanisms responsible are less well known. Connell and Slatyer (1977) described three models of succession: inhibition, in which early colonists inhibit settlement of later species and succession is driven by the removal of early colonists by predators; facilitation, in which early colonists modify the habitat to facilitate the settlement of later colonists that ultimately outcompete them; and tolerance, in which early colonists have short lifespan and are replaced by slower-growing, longer-lived late-succession species. Different authors have attributed succession in shallow marine hard substrata to each of the three mechanisms: facilitation (Osman and Whitlatch, 1995a), tolerance (Edwards and Stachowicz, 2010), and inhibition (Lubchenco, 1983). Encrusting invertebrates recruit in higher abundance to fouling panels with already established fauna, indicating a possible facilitation mechanism (Keough, 1998; Osman and Whitlatch, 1995b; Walters et al., 1997). However, for tropical coral reefs, recruitment of fouling fauna was higher at an artificial reef, and these encrusting species may inhibit coral settlement (Perkol-Finkel and Benayahu, 2007). The mechanisms of succession may be different for sessile

and mobile fauna (Mullineaux et al., 2003). Succession on marine hard substrata can involve regular species replacements to form a stable climax community (Vance, 1988), but there is also an element of stochasticity. The first colonists to arrive and colonize a substratum impact the course of succession for the whole community (Sutherland, 1974), and even the outcome of competition can depend on circumstance, including substratum texture, orientation, and latitude (Barnes and Kukliński, 2003; Miller and Etter, 2011; Walters and Wethey, 1986).

Recruitment is lower and succession may occur more slowly on subtidal marine substrata at higher latitude (Barnes and Kukliński, 2005; Beuchel and Gulliksen, 2008; Kukliński and Barnes, 2008; Kukliński et al., 2013; Stanwell-Smith and Barnes, 1997; Stanwell-Smith et al., 1999; Svane and Lundälv, 1981). Intertidal and shallow subtidal boulders in the Arctic and Antarctic are kept at an early stage of succession because of ice scour disturbance (Barnes et al., 1996; Kukliński, 2009; Kukliński and Barnes, 2008; Kukliński et al., 2006). Eight years after a volcanic eruption in Jan Mayen, shallow subtidal communities (5–10 m) resembled old-ground (preeruption) shallow communities because both had low diversity and were dominated by opportunistic species, typical of early-succession, ice-scoured habitats (Gulliksen et al., 1980). Deeper (20–30 m) habitats were also at an early-succession stage, dominated by the bivalve *Hiatella arctica*, though these deeper communities were significantly different from more diverse, old-ground communities dominated by encrusting sponges (Gulliksen et al., 1980).

Very little is known about the process of succession on isolated marine hard substrata at greater depth. Shipwrecks found off the US east coast (~100 m depth) were dominated by species with long-lived, planktotrophic larvae and fast growth even after 100 years underwater (K.S. Meyer, unpublished data). Similarly, long-lived climax species were conspicuously absent from a shipping container in Monterey Canyon at 1281 m depth (Taylor et al., 2014). Succession may proceed more slowly on deep insular, nonchemosynthetic hard substrata because their degree of isolation makes it improbable that they will be reached by the larvae of sessile, hard-bottom species, many of which are adapted for short-distance dispersal and self-recruitment (Grantham et al., 2003).

Long-lived cold-water corals often inhabit the steep rocky walls of submarine canyons (Tyler et al., 2009), mounds, and knolls (Roberts et al., 2009). Species of *Desmophyllum*, *Primnoa*, and *Lophelia* are incredibly slow-growing and long lived, growing millimetres or less per year

(Brooke and Young, 2009; Risk et al., 2002), but the processes by which deep-water corals disperse, settle, and recruit on these substrata are largely uninvestigated. It would be interesting to know if these coral species are preceded on their substrata by any shorter-lived species that may potentially facilitate their recruitment.

For deep-sea habitats, succession has been studied at whale-falls (Smith and Baco, 2003; Smith et al., 2015). The first stage is the mobile-scavenger stage, in which large, mobile scavengers clean the flesh from the whale bones. Then, in the enrichment-opportunist stage, dense and species-rich macrofauna consume the organic material around the carcass (Baco and Smith, 2003). Finally, in the sulphophilic stage, a chemoautotrophic community thrives on the lipids in the whale bones (Smith and Baco, 2003; Treude et al., 2009). The stages often overlap (Goffredi et al., 2004), and the speed of succession depends on the presence of key mobile scavengers at the depth of the whale-fall (Dahlgren et al., 2006; Fujiwara et al., 2007). Succession at whale-falls is driven by a facilitation mechanism, as the consumers in each stage modify the carcass (i.e. removing flesh to expose the bones, eroding bone to access lipid) to facilitate colonization by the next set of organisms. However, rather than being outcompeted by their successors, the organisms in each stage ensure their own demise by consuming the resources on which they thrive (Smith and Baco, 2003; Smith et al., 2015). In this way, whale-falls are different from other isolated marine habitats because the habitat is ephemeral and is consumed by its colonists.

The species that colonize whale-falls are for the most part not whale-fall specialists (Rouse et al., 2011; Smith and Baco, 2003). The life-history traits of most whale-fall species are unknown, especially the macrofauna. Species of *Osedax*, siboglinid polychaetes, have high fecundity, continuous reproduction, and are capable of long-range dispersal—characteristics that are adaptive in an ephemeral habitat (Rouse et al., 2009). Even though *Osedax* appears relatively late in whale-fall succession, it has the life-history characteristics of an opportunistic species. However, the timing of colonization varies among species of *Osedax*, with some arriving earlier and some arriving later to a whale carcass (Braby et al., 2007; Smith et al., 2015). Whale-falls are ephemeral habitats that are consumed by their colonists; they are fundamentally different from other, longer-lasting isolated marine habitats.

Other ephemeral deep-sea habitats that are consumed by their colonists include wood- and plant matter-falls, and these too are inhabited by opportunistic species (Turner, 1973, 1977). A number of mollusks, echinoderms, crustaceans, and polychaetes use wood- or plant matter-falls as substrate,

shelter, or food (Wolff, 1979). Invertebrates inhabiting wood- and plant matter-falls are opportunists (Samadi et al., 2010; Voight, 2007; Young et al., 2013), and some mollusk species are able to live on both whale- and wood-falls (Lorion et al., 2009). The invertebrate communities on wood- or plant-falls are highly variable, even among substrata deployed at the same location for the same amount of time (Pailleret et al., 2007; Voight, 2007). However, microbial communities are highly consistent, even among different ocean basins (Palacios et al., 2009). Microbial communities vary over time as the wood is consumed (Palacios et al., 2009), and there is also a predictable shift in the invertebrate community from inferior to superior competitors over time (Voight, 2007). Succession on wood-falls may in fact resemble the process on whale-falls. The first colonists, xylophagid mollusks, create boreholes that create habitat and facilitate colonization by other species (McClain and Barry, 2014; Turner, 1977)—similar to the removal of whale flesh by scavengers, which facilitates colonization of bone-exploiting species. Wood consumption by xylophagids creates a halo of wood chips and faeces surrounding the wood-fall, where opportunistic infauna may take advantage of this organic enrichment (McClain and Barry, 2014), similar to the enrichment-opportunist stage in whale-fall succession. The process of succession on organic matter-falls differs from succession on permanent hard substrata, but evidence suggests there is a predictable succession, which is similar for wood- and whale-falls.

At hydrothermal vents in the eastern tropical Pacific, it has been hypothesized that the tubeworm *Tevnia jerichoana* provides a settlement cue to facilitate recruitment of another tubeworm, *Riftia pachyptila* (Mullineaux et al., 2000). However, in a later experiment, *R. pachyptila* settled in the absence of *T. jerichoana*, providing contradictory results (Hunt et al., 2004). It has also been suggested that *T. jerichoana* is replaced by *R. pachyptila* because of abiotic factors, namely, a decrease in hydrogen sulphide (Shank et al., 1998). Both *R. pachyptila* and *T. jerichoana* have similar egg size and developmental mode, though *T. jerichoana* has lower fecundity than *R. pachyptila* (Young, 1999). Based on the life-history traits of both species, it would not be expected for *T. jerichoana* to be a pioneer species and *R. pachyptila* to be a climax species.

The polychaete *Alvinella pompejana* has been described as a pioneer species on hydrothermal vents on the East Pacific Rise (Pradillon et al., 2005a). This species colonizes new or disturbed vent areas quickly; however, the majority of colonizing individuals likely migrated to the new area as juveniles, rather than recruiting from a larval stage (Pradillon et al., 2005a).

In fact, *A. pompejana* embryos develop best at abyssal pressure, 10–15°C, and low sulphide flow—conditions found at the periphery of their natal vent fields—meaning the embryos or larvae may not disperse very far (Pradillon et al., 2005b). An alternative interpretation is that *A. pompejana* embryos remain in a state of arrested development until they reach surface waters (Pradillon et al., 2001). If the former interpretation proves true, and *A. pompejana* develop near their natal vents and have restricted dispersal, then this species would not be expected to be a pioneer, early-succession species.

For hydrothermal vent sites at the Juan de Fuca Ridge, succession proceeds quickly, with newly-established communities resembling preeruption communities after only three years (Marcus et al., 2009). Much of this resemblance is due to the presence of *Ridgeia piscesae*, a habitat-forming tubeworm (Marcus et al., 2009; Tsurumi and Tunnicliffe, 2003; Tunnicliffe et al., 1997). While posteruption vent sites are quickly colonized by opportunistic species rare at other sites, recruitment of *R. piscesae* is far from deterministic: the species has settled at vents seven months (Tunnicliffe et al., 1997) and three years posteruption (Marcus et al., 2009). At some vent sites with low flow, *R. piscesae* dominance gave way to limpets (*Lepetodrilus fucensis*) three years posteruption (Marcus et al., 2009). However, *L. fucensis* has also been observed to opportunistically and rapidly colonize disturbed vent areas (Sarrazin et al., 1997).

It has also been observed that tubeworms decrease in abundance as mussels (*Bathymodiolus thermophilus*) dominate later in the life of a vent (Hessler et al., 1988; Shank et al., 1998). However, *B. thermophilus* has small (50 μm) eggs, indicative of planktotrophic development and continuous reproduction (Young, 1999)—characteristics of opportunistic, typically early-succession species.

It is possible that succession at hydrothermal vents does not fit the expected model of life-history traits described for terrestrial islands and observed in shallow marine habitats. Circulation patterns around vents retain larvae, so dispersal range is not necessarily related to larval duration (Marsh et al., 2001). However, more observations of hydrothermal vent communities are needed before definitive conclusions can be drawn. Larval distribution around vents is spatially and temporally variable (Mullineaux et al., 2005), and recruits can settle outside the areas occupied by adults of the same species (Mullineaux et al., 1998). An alternative explanation for the *Tevnia–Riftia–Bathymodiolus* faunal transition is that vents are merely colonized by whatever larvae happen to be in the water column at the time (Mullineaux et al., 2010), and then self-recruitment leads to the

development of dense populations. More research is needed to understand recruitment at hydrothermal vents, including repeated observations at the same sites.

4.3 Competition and Facilitation

The megafauna on marine hard substrata mostly consist of sessile suspension feeders, and while nonrandom co-occurrence patterns have been found for these fauna (Meyer et al., 2016), it is an open question how and to what extent suspension feeders compete. Overgrowth competition occurs on shallow substrata (Sebens, 1986), and the strength and hierarchical nature of overgrowth competition increases at higher latitude in both hemispheres (Barnes, 2002; Barnes and Kukliński, 2003; Barnes and Rothery, 1996). However, it has been argued on theoretical grounds that suspension feeders do not compete for food (Levinton, 1972; Lohse, 2002). In contrast to benthic food sources, which exist on a 2-D surface and are likely to become depleted, particulate food sources are constantly renewed by bottom current (Levinton, 1972; Lohse, 2002). Field and laboratory experiments have shown contradictory results: that suspension-feeding bivalves are (Cote et al., 1994) or are not affected by intraspecific competition (Olafsson, 1986). Suspension-feeding bryozoans, ascidians, and bivalves can alter flow or otherwise deplete particulate food resources, thereby affecting growth, body condition, or reproduction of individuals downstream (Dalby, 1995; Myers, 1990; Okamura, 1984; Peterson and Black, 1987, 1991). It could be expected that suspension feeders, especially erect forms, have a shading effect on downstream fauna, directing the current up and over them (Myers, 1990; Vogel, 1996). Communities of sessile suspension feeders may also be food-limited in oligotrophic waters (Svensson and Marshall, 2015).

Diamond (1975a) hypothesized that to reduce competition, species with similar niches (that consumed similar sizes of seeds) would not occur together. Interspecific competition for various sizes of seeds has also been shown to impact the distribution of desert rodents (Fox, 1981). Deep-sea sponges are known to consume particulate size fractions ranging from microbes (Pile and Young, 2006) to copepods (Vacelet and Boury-Esnault, 1995), so it would be interesting to find out whether species that have negative nonrandom co-occurrence patterns consume similar size fractions and have the potential to compete interspecifically. Sponges and ascidians consume similar size fractions of ultraplankton at tropical latitude but not at temperate latitude, where food resources are more abundant (Pile, 2005).

Competition is a ripe avenue for research in the deep sea (McClain and Schlacher, 2015). It is possible that niche partitioning exists for paralvinellid polychaetes at hydrothermal vents, as *Paralvinella pandorae* is >14× smaller where it co-occurs with *P. palmiformis*; the diets of the two species overlap (Levesque et al., 2003). Niche partitioning may also occur for hydrothermal vent gastropods and dorvilleid polychaetes at cold seeps, as stable isotope evidence shows different species consume different fractions of the available food (Govenar et al., 2015; Levin et al., 2013). Whether the species in these examples negatively nonrandomly co-occur on isolated substrata remains uninvestigated. The potential for and extent of competition for food resources among sessile suspension feeders may be important for understanding their distributions and nonrandom co-occurrence patterns.

The opposite of competition—facilitation, including commensalism—has also been shown to impact faunal distribution on island-like dropstones (Meyer et al., 2016). Given the ubiquity of epibiotic relationships in cold-water coral and sponge stands (Coleman and Williams, 2002; Cordes et al., 2008; Maldonado et al., 2015; Shank, 2010), positive non-random co-occurrence patterns are likely to be found in other habitats as well. Epibiotic relationships have also been documented for Antarctic sea urchins (Gutt and Schickan, 1998), Arctic crabs (Dvoretsky, 2012), and hermit crab shells (Bałazy and Kukliński, 2013; Bałazy et al., 2016; Barnes et al., 2007). Epibiontism may be an important mechanism for suspension-feeding organisms to achieve greater elevation in the benthic boundary layer, exposing themselves to faster water flow and higher particulate food supply (Buhl-Mortensen et al., 2010).

Large, structural organisms can provide shelter and potential protection from predation for small, mobile organisms (Buhl-Mortensen et al., 2010; Stachowicz, 2001), leading the species to positively non-randomly co-occur (Bałazy et al., 2014). Facilitation can also impact the course of succession in marine insular communities (Osman and Whitlatch, 1995b). Examples of positive biotic relationships, including facilitation, commensalism, and mutualism, abound for marine fauna (Buhl-Mortensen et al., 2010; Stachowicz, 2001), and inclusion of these positive interactions will allow for more complete ecological theories (Bruno et al., 2003). For marine hard-bottom fauna, additional research into biotic interactions, both competitive and facilitative, would enlighten the process of community assembly.

5. A DIRECTION FORWARD

Community assembly was defined at the beginning of this paper to include all steps by which an uncolonized habitat comes to be occupied by a mature community of fauna—larval dispersal, recruitment, competition, facilitation, predation, and succession. Each of these aspects of community assembly has been investigated in some insular marine habitats, but they are still incompletely understood, especially for habitats in deep water. Some similarities and differences between marine and terrestrial island-like habitats have been described above, along with how research in each environment can reciprocally inform the other and lead to a more complete understanding of the ecology of island-like habitats.

The ubiquitous log-linear relationship between species (morphotype) richness and area of a habitat may be due to nothing more than the finite nature of the species pool. Higher richness of fauna on underwater topographic highs results from the abundant particulate food source available there. These relationships are well understood and have clear corollaries between terrestrial and marine island-like habitats. However, gaps exist in other parts of our knowledge. The major gaps in knowledge of community assembly pertain to connectivity, larval dispersal, competition, facilitation, and succession on insular marine substrata.

The opportunity exists to use anthropogenic substrata deposited on the seafloor as "natural" experiments to observe community assembly on isolated marine substrata. Already the conspicuous absence of climax species from a nearby natural hard-bottom community has been noted for a shipping container underwater for seven years, indicating the container community may be at an early stage of succession (Taylor et al., 2014). Anthropogenic substrata of different known ages at similar depth and latitude could be treated as snapshots of succession at different points in time. Larval traps or settlement plates could be outplanted at these locations to observe what species are available to recruit. Population genetics of the species present could reveal which natural-substratum source population the recruits originated from. Measurements of current speed and experiments in reproductive biology could be used to build models of larval dispersal. Shipwrecks, shipping containers, oil platforms and pipelines, military discard, even litter (Bergmann and Klages, 2012; Gass and Roberts, 2006; Kelley et al., 2016; Lira et al., 2010; Taylor et al., 2014) have all been colonized by sessile and mobile hard-bottom fauna (Kilgour and Shirley, 2008;

Ross et al., 2016), and these isolated substrata can be used as an opportunity for research into community assembly, especially if the sinking date is known.

This review has focused on two classical theories of island ecology, both decades old. However, a new model of island biogeography has been proposed by Whittaker et al. (2008), which incorporates time as a factor into the MacArthur–Wilson model to acknowledge the fact that few, if any, islands are actually in equilibrium. This General Dynamic Model has great potential to explain the species richness on terrestrial islands of varying size and age (Borregaard et al., 2015). Whether it can be applicable to other island-like habitats, including marine isolated substrata, remains unevaluated. The General Dynamic Model may provide insights for the ecology and succession of seamounts and hydrothermal vents—direct island analogues with a finite lifespan.

For the most part, marine ecology has developed independently of advances in terrestrial ecological thought. Ecological theories developed for terrestrial habitats may nonetheless be applicable to marine habitats and provide new insights. Progress in the marine environment may in turn inform terrestrial ecological thought.

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CHAPTER TWO

Patterns and Drivers of Egg Pigment Intensity and Colour Diversity in the Ocean: A Meta-Analysis of Phylum Echinodermata

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Abstract

Egg pigmentation is proposed to serve numerous ecological, physiological, and adaptive functions in egg-laying animals. Despite the predominance and taxonomic

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diversity of egg layers, syntheses reviewing the putative functions and drivers of egg pigmentation have been relatively narrow in scope, centring almost exclusively on birds. Nonvertebrate and aquatic species are essentially overlooked, yet many of them produce maternally provisioned eggs in strikingly varied colours, from pale yellow to bright red or green. We explore the ways in which these colour patterns correlate with behavioural, morphological, geographic and phylogenetic variables in extant classes of Echinodermata, a phylum that has close phylogenetic ties with chordates and representatives in nearly all marine environments. Results of multivariate analyses show that intensely pigmented eggs are characteristic of pelagic or external development whereas pale eggs are commonly brooded internally. Of the five egg colours catalogued, orange and yellow are the most common. Yellow eggs are a primitive character, associated with all types of development (predominant in internal brooders), whereas green eggs are always pelagic, occur in the most derived orders of each class and are restricted to the Indo-Pacific Ocean. Orange eggs are geographically ubiquitous and may represent a 'universal' egg pigment that functions well under a diversity of environmental conditions. Finally, green occurs chiefly in the classes Holothuroidea and Ophiuroidea, orange in Asteroidea, yellow in Echinoidea, and brown in Holothuroidea. By examining an unprecedented combination of egg colours/intensities and reproductive strategies, this phylum-wide study sheds new light on the role and drivers of egg pigmentation, drawing parallels with theories developed from the study of more derived vertebrate taxa. The primary use of pigments (of any colour) to protect externally developing eggs from oxidative damage and predation is supported by the comparatively pale colour of equally large, internally brooded eggs. Secondarily, geographic location drives the evolution of egg colour diversity, presumably through the selection of better-adapted, more costly pigments in response to ecological pressure.

1. INTRODUCTION

The most primitive and widely used reproductive strategy in the animal kingdom involves the laying of eggs (Blackburn, 1999). It is exhibited by an overwhelming majority of taxa, including members of Arthropoda (insects, spiders, crustaceans), Mollusca (bivalves, gastropods), Annelida (segmented worms), Platyhelminthes (flat worms), Cnidaria (corals, sea anemones), Echinodermata (sea stars, sea urchins), and Chordata (birds, reptiles, fishes) (see Blackburn, 1999). Egg-laying can follow internal fertilization (i.e. oviparity) with or without the synthesis of a protective shell (e.g. birds); or it may involve the release of unfertilized eggs (i.e. oocytes) that are fertilized externally (i.e. ovuliparity; Blackburn, 1999; Lodé, 2012; Ostrovsky et al., 2015; Wourms, 1994), as seen in frogs, fishes, arthropods, and most aquatic invertebrates. A small number of terrestrial and aquatic animals incubate fertilized eggs for a more or less prolonged period of time before release (i.e. ovo-viviparity; Blackburn, 1999; Lodé, 2012;

Wourms, 1994). Parental investment in progeny varies according to these reproductive strategies, leading to a broad range of egg phenotypes (Blount, 2004; McEdward and Morgan, 2001; Monaghan et al., 1998; Sargent et al., 1987). Such differences in developmental nutrition are critical from an evolutionary point of view (Ostrovsky et al., 2015). While egg-laying modes are particularly diverse and taxonomically widespread in the ocean, where they first evolved, our understanding of egg phenotypes in marine animals lags behind that of terrestrial animals, especially with respect to the distribution and purpose of egg colour. Interspecific variation in egg colour is particularly widely studied in avian ecology, where trade-offs may involve crypsis, mimicry, altered protection from ultraviolet (UV) radiation, structural integrity, and sexual selection (Cassey et al., 2012; Hanley et al., 2015; Kilner, 2006; Maurer et al., 2014; Svensson and Wong, 2011). While the marine realm offers equally striking examples of brightly coloured eggs, the reason for this has been comparatively understudied, despite the fact that it may offer valuable insights into evolutionary patterns.

Cnidarians, molluscs, annelids, teleost fishes, and echinoderms are among the most notable marine taxa to possess large oocytes/eggs ranging in colour from neon pink to dark green (see Cheesman et al., 1967; Hamel and Mercier, 1996; Lindquist and Hay, 1996; McEuen, 1988). Lecithotrophic (maternally provisioned, nonfeeding, yolky) propagules are particularly colourful and often retain their colour opacity and intensity until settlement (Wray, 1996). In contrast, planktotrophic (feeding) propagules tend to be smaller and either transparent or faintly coloured (generally, coloured eggs in this group develop into transparent embryos and larvae). The relatively large size and bright colour of lecithotrophic oocytes could increase the risk of predation by visual predators in the pelagic environment due to enhanced visibility relative to planktotrophic propagules (Iyengar and Harvell, 2001; Vaughn and Allen, 2010). Despite these potential consequences, species with pelagic lecithotrophic development are common and ecologically important in temperate and polar waters, where they co-occur with planktotrophs (Marshall et al., 2012; Monro and Marshall, 2015; Pearse and Bosch, 1994). Parental provisioning among lecithotrophs has been well studied from the perspective of energetics and nutrition, whereas other features such as egg pigmentation remain poorly understood. The early origin of pigments and maternal provisioning in the animal tree of life and the link between bright eggs colours and lecithotrophy in many clades (e.g. Hamel and Mercier, 1996; Lindquist and Hay, 1996; McEuen, 1988; Ostrovsky et al., 2015) suggest as yet unresolved evolutionary patterns that warrant further investigation.

Pigments are known to play a variety of roles in biological systems, including in plants (Alkema and Seager, 1982), fishes (Losey et al., 1999), and bacteria (Soliev et al., 2011); and see Svensson and Wong (2011). Carotenoids are one of the most widespread and diverse pigment classes (Cheesman et al., 1967; Svensson and Wong, 2011); they are fundamental for internal functions such as physiology, electron transport, cell signalling, and enzymatic activity (Pereira et al., 2014; Svensson and Wong, 2011). But they also provide colouration for camouflage, sexual signals, and warning signals in animals ranging from simple invertebrates to higher vertebrates (Grether et al., 2001; Olson and Owens, 1998; Stoehr, 2006; Svensson and Wong, 2011). Animals obtain carotenoids and other pigments from their diet (Grether et al., 2001; Svensson and Wong, 2011) and modify them subsequently to generate new colours through the addition of proteins (e.g. carotenoid–protein complexes) or the overlay of multiple pigment classes, such as the stacking of carotenoids and melanin in the feathers of birds (Grether et al., 2001; McGraw et al., 2004). Yet in many species, these postmetabolic changes in pigmentation are extremely costly and reserved only for the most critical of processes (e.g. red pigments used for external body ornamentation and sexual selection in many species; Grether et al., 2001; Olson and Owens, 1998).

In oocytes/eggs, pigmentation is a product of maternal investment that imparts external colouration, prevents oxidation, and regulates cellular functions, and it is associated with toxicity in various taxa (McGraw et al., 2005; Nicola and Monroy-Oddo, 1952; Winters et al., 2014). Diet composition has been shown to affect both lipid deposition and egg yolk colour in vertebrates (e.g. chickens, *Gallus gallus*, Ferrante et al., 2011). Egg and offspring colour can be directly influenced by maternal investment in locusts (acridids), relative to specific environmental variables (Tanaka and Maeno, 2006). Brightly coloured eggs are an indicator of good maternal and offspring health in salmonid fishes (Craik, 1985), and influence male mate choice in gobiids (Amundsen and Forsgren, 2001). The yellow, red, and green eggs of lecithotrophic echinoderms exhibit toxicity and unpalatability in some Antarctic, North Atlantic, and North Pacific species (Iyengar and Harvell, 2001; Mercier et al., 2013a; Sewell and Levitan, 1992). These conspicuous colours have been proposed to act as aposematic (warning) colouration for visual predators like shrimps and fishes (Iyengar and Harvell, 2001).

While the physiological and biochemical roles of major pigments have been well studied in most animal taxa (Svensson and Wong,

2011), the ecological significance of egg colour diversity remains relatively unexplored, especially in aquatic systems and among nonvertebrate taxa. Echinodermata are well suited to phylum-wide comparisons of egg colour for a number of reasons. Representatives of this phylum thrive in nearly all marine habitats, across broad latitudinal and bathymetric ranges. Furthermore, echinoderms are deuterostomes (a developmental feature shared with vertebrates) and many species produce maternally provisioned (yolky) eggs that may be free living (pelagic or benthic) or internally/externally brooded. They also display a staggering assortment of egg colours, including yellow, red, orange, green, and black. Despite the large body of literature dedicated to reproductive strategies in echinoderms, to the best of our knowledge, the prevalence or purpose of colour diversity and intensity among their propagules is not explored beyond proposed relationships with lipid deposition and aposematic colouration (Iyengar and Harvell, 2001).

Brooding and broadcast-spawning echinoderms exist in similar habitats but possess dramatically different life-history characteristics. This raises critical questions including: Why are lecithotrophic propagules so brightly pigmented? Is egg pigmentation in the ocean randomly distributed across phylogenies, life histories and regions? While the provenance and potential role of pigmentation have been examined in various marine species (Craik, 1985; Iyengar and Harvell, 2001; Lindquist, 2002), to the best of our knowledge, no study has analysed interspecific patterns in an attempt to explain the exceptional diversity of their egg colours. The present study explores these questions by reviewing egg colour (including both pigment intensity and pigment colour) among lecithotrophic echinoderms and conducting a suite of multivariate analyses to test possible relationships with key biotic and abiotic variables, including development site (parental care), egg size, egg buoyancy, adult size, phylogeny, and geographic location.

2. STUDY OF EGG METRICS, AND BIOTIC AND ABIOTIC FACTORS

2.1 Dataset Collection

A comprehensive dataset of egg colours in lecithotrophic echinoderms from all over the world was gathered from the primary scientific literature, with complementary data obtained from internet searches and academic blogs ($N = 126$ records; Fig. 1, Appendix A). Because egg colour in marine taxa

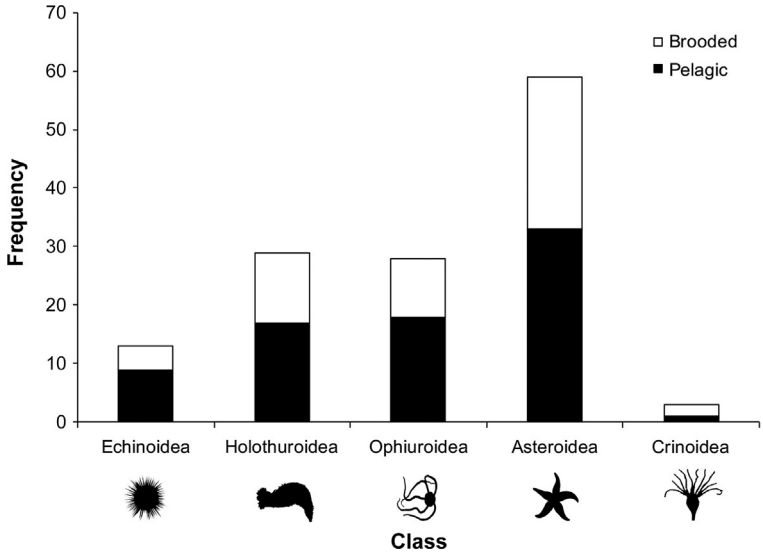


Fig. 1 Distribution of echinoderm classes and development sites (brooded/benthic vs pelagic lecithotrophy) in the dataset (Appendix A). Frequency indicates number of species (total $N = 126$ records).

is not currently considered to have clear biological or ecological value, this variable was not reported consistently in the literature. Searches were therefore conducted in a hierarchical fashion, starting with broad scale ecological papers down to reports of egg colour in developmental and species-specific papers. Keywords used for searches, included egg, oocyte, colour, pigment, spawning, and the names of known lecithotrophic species. Though comprehensive, this dataset may not include all anecdotal accounts of egg colour within larger studies. Egg diameter in the full dataset ranged from 150 to 3400 μm and adult body size from 1 to 60 cm in length (or diameter in the case of radially symmetrical animals). Geographic location and phylogeny were obtained from the World Registry of Marine Species (WoRMS, www.marinespecies.org) and the Ocean Biogeographic Information System (OBIS, www.iobis.org). As ranges of occurrence can be very broad and/or not well defined for most species, the geographic analysis centred on ocean basins instead of more precise coordinates or latitudes.

2.2 Standardization of Variables for Colour Assessment

Locally accessible echinoderm species from the North Atlantic Ocean were examined to ground truth egg colour metrics in the dataset. Coastal species

included the sea stars *Solaster endeca* (8–10 cm radius), *Henricia sanguinolenta* (2–5 cm) and *Crossaster papposus* (5–10 cm), and the sea cucumbers *Cucumaria frondosa* (10–15 cm contracted length), and *Psolus fabricii* (10–15 cm). They were collected by SCUBA dives between 10 and 20 m depth in south-east Newfoundland (eastern Canada). Deep-sea species included the sea stars *Henricia lisa* (2–5 cm radius) and *Hippasteria phrygiana* (8–15 cm); they were collected aboard the Canadian Coast Guard research vessel, *Teleost*, along the continental slope (north-east Newfoundland) between 700 and 1450 m depth. All species and individuals were housed in 375-L tanks provided with flow-through seawater at temperatures ranging from 0 to 5°C (see Mercier and Hamel, 2010). Images of eggs and embryos from natural spawning were taken with an Olympus TG-2 digital camera for in-depth colour analysis (see Section 2.3).

Where possible, egg colours listed in publications were verified with images provided in supplemental documents or through online searches. Egg colours of local North Atlantic species were confirmed from natural spawning events in the laboratory. To minimize ambiguous and/or inaccurate descriptions in the literature, egg colours obtained from the primary literature were grouped into six main families (red, orange, brown, yellow, green, and grey), and colour intensities were grouped into three categories (pale, standard, and bright). Corresponding quantitative definitions were devised, based on an analysis of egg colour images captured during the present study and those obtained through the literature, using Adobe Photoshop. Colour families were attributed a range of red ratios on the red, green, blue (RGB) additive primaries scale, whereas percent saturation was used to quantify colour intensity from pale to intense. The defined ranges are broad enough to account for any device-specific colour variations (Table 1).

Fig. 2 outlines the distributions of colour families and intensities in the primary dataset.

2.3 Hypotheses and Data Analysis

To tease out the drivers of egg colour intensity and diversity, subsets of the main dataset were examined. To be included in a subset, records had to be complete for all factors of interest and each factor combination had to be represented by a minimum of three records. Factor analysis of mixed data (FAMD) and hierarchical clustering of principal components (HCPC) were conducted using the FactoMineR package for

Table 1 Colour Families and Intensity Categories with their Corresponding Qualitative Descriptors as Found in the Scientific Literature for the Marine Species in the Present Study

Colour Family	Qualitative Descriptors	$R/(R+G)$ (%)	Colour Intensity	Qualitative Descriptors	Saturation (%)
Red	Red Pink Purple	100–75	Pale	Pale Dull Light	<50
Orange	Orange	74–55	Standard	No modifier listed	50–70
Brown	Brown Cream	74–40	Bright	Bright Intense Dark	>70
Yellow	Yellow	54–40	—	—	—
Green	Green	0–39	—	—	—
Grey	Grey White Black	—	—	—	—

Each colour family is defined by a range of red content, based on the percent ratio of red to total red and green ($R/R+G$) on the RGB scale. Intensity is defined as percent colour saturation.

R Statistical Software as per Lê et al. (2008). The FAMD analysis is similar to multivariate principle component analysis (PCA) but unlike PCA, FAMD combines both qualitative and quantitative variables, making FAMD ideal for meta-analyses of mixed variable data (Ch et al., 2010; Panneton et al., 2013).

We first tested all species in the dataset with complete records to determine general groupings based on all variables: egg colour family, egg colour intensity, egg size, development mode, ocean basin, adult size, and taxonomic class ($N=78$, Appendix B). To examine more detailed associations, we tested the hypothesis that egg colour was not randomly distributed among geographic locations, using the same subset as earlier without phylogeny as a factor (egg colour family, egg colour intensity, egg size, development mode, ocean basin, and adult size; $N=78$, Appendix B). Thereafter, we tested whether egg buoyancy correlated with egg colour and development mode, independently of geographic location ($N=56$, Appendix C). We also tested whether certain egg colours were phylogenetically linked in the four main

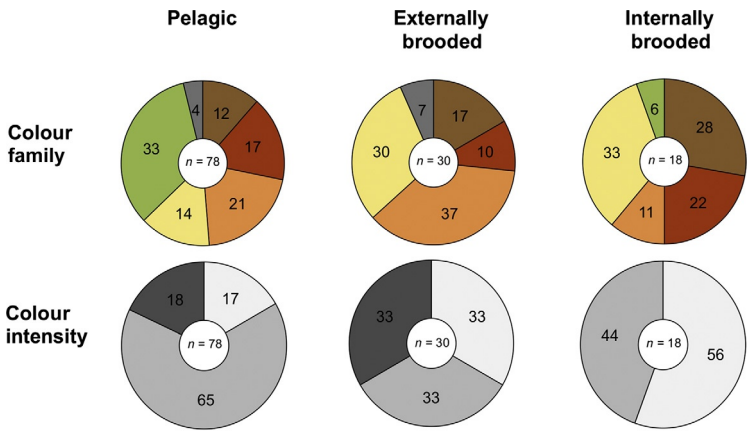


Fig. 2 Percent distribution of egg colour family and intensity in the full dataset (Appendix A) sorted by development site: pelagic, externally brooded, and internally brooded. *Shades* of egg colour families (*red*, *orange*, *brown*, *yellow*, *green*, *dark grey*) are represented as closely as possible in the upper panels. Colour intensities from *dark* to *pale* are shown on a grey scale in the lower panels. Sample size is provided in the centre of each pie (total $N = 126$ records).

extant classes: Echinoidea (sea urchins), Asteroidea (sea stars), Holothuroidea (sea cucumbers), and Ophiuroidea (brittle stars), independently of geographic location and development mode ($N = 103$, Appendix D). The HCPC trees were cut at the relative highest change in inertia, or statistical difference between the number of available groupings (Lê et al., 2008). Clusters were analysed using the proportion of individuals in each cluster possessing a nonrandom grouping of qualitative variables and/or a nonrandom mean difference from the global population among tested quantitative variables (see Lê et al., 2008). All statistical analyses were conducted at $\alpha = 0.05$.

3. DRIVERS OF EGG PIGMENTATION INTENSITY AND DIVERSITY

Five egg colours were catalogued in the entire dataset (see Figs. 2–4) with orange and yellow being the most common (comprising 25% and 20% of species, respectively), followed by roughly equal occurrences of red (17%), brown (16%), and green (16%). Only 6% of species had grey or black eggs.

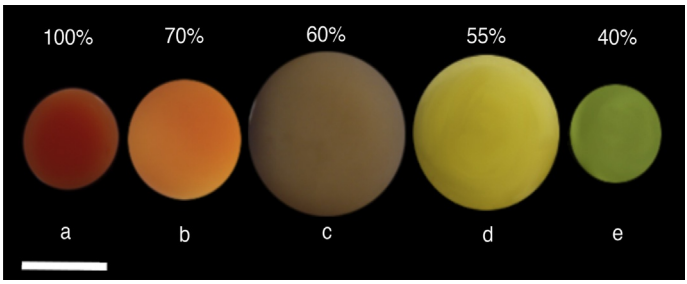


Fig. 3 Egg colour and size diversity in lecithotrophic echinoderms. (A) *Crossaster papposus* (freshly spawned egg). (B) *Cucumaria frondosa* (freshly spawned egg). (C) *Henricia lisa* (freshly spawned egg collected from under the female). (D) *Pteraster abyssorum* (from live 60-celled embryo of size/colour consistent with egg). (E) *Cucumaria miniata* (composite image from several photos, scaled to size). Percent values represent percent *red* ratio [$R/(R+G)$ ratio; see Table 1 for method]. *Scale bar* represents 500 µm. Note that egg sizes shown here do not illustrate the general relationship between egg colour and egg size in the full dataset.

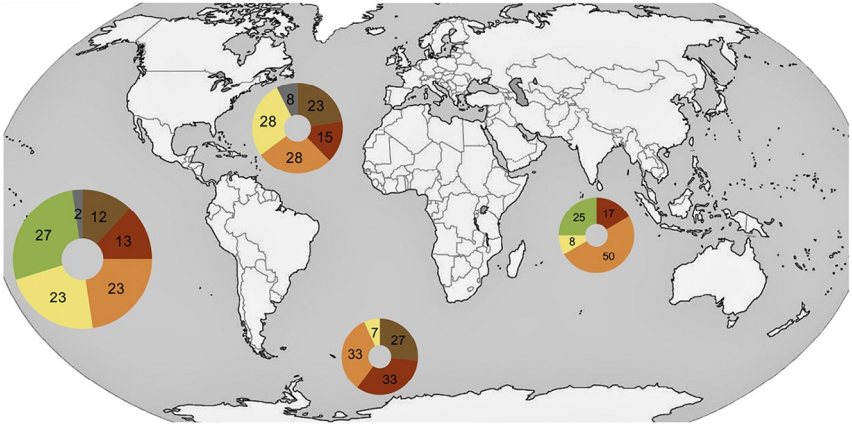


Fig. 4 Global distribution of egg colour families in lecithotrophic echinoderms. *Numbers* indicate percent (%) of species with eggs of each colour (*red*, *orange*, *brown*, *yellow*, *green*, *dark grey*) found in the corresponding ocean basin. Distribution data obtained from Ocean Biogeographic Information System (OBIS). Species with cosmopolitan distributions are included in all relevant ocean basins. Size of pies reflects relative number of records ($N=126$; Appendix A).

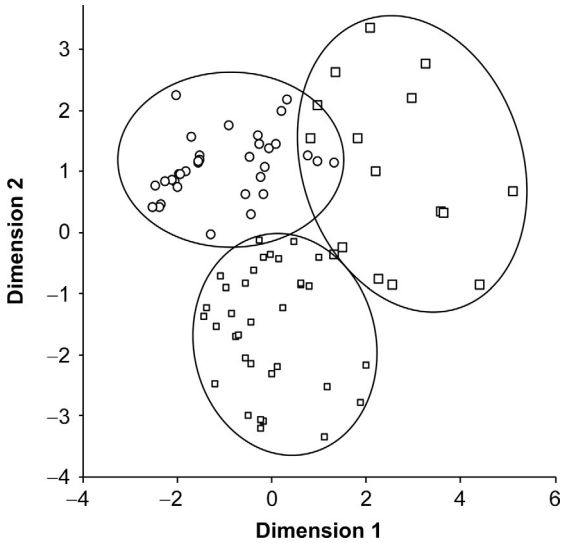


Fig. 5 Relationship among egg colour, development mode, phylogeny, and ocean basin in lecithotrophic echinoderms. Dimension 1 = factor analysis of mixed data (FAMD) component with greatest variance. Dimension 2 = FAMD component with second greatest variance. *Symbol shape* indicates developmental mode: *circle* = pelagic, *large square* = externally brooded, *small square* = internally brooded ($N = 87$, Appendix E).

3.1 Overall Patterns of Egg Colour Relative to Development Site

Egg colour was not randomly distributed in the main dataset (see Appendix E). Three main clusters emerged, corresponding to the three egg development sites tested here: pelagic, externally brooded, and internally brooded (Fig. 5; HCPC clusters, $P < 0.001$). Species with green eggs of average intensity were associated with pelagic development ($P < 0.001$). Orange egg colour was primarily associated with externally brooded development and was characterized by bright intensity ($P < 0.001$). In contrast, internally brooding species tended to have yellow and brown eggs of pale intensity ($P < 0.001$). The following sections detail the results of the multivariate analyses that further tease out the main patterns and drivers of egg pigmentation in lecithotrophic echinoderms.

3.2 Ocean Basin, Development Mode, Egg, and Adult Size

Species with red eggs generally exhibit pelagic development (HCPC, $P < 0.001$) but showed no trend in geographic distribution or adult body

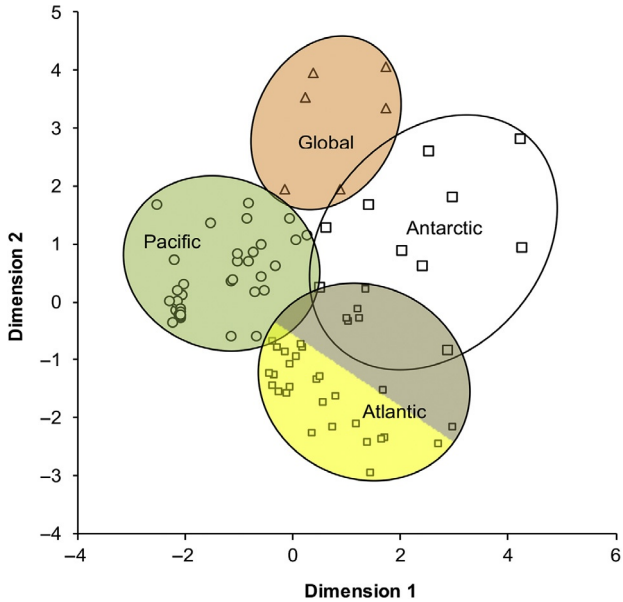


Fig. 6 Relationship among egg colour, development mode, and ocean basin in lecithotrophic echinoderms. Dimension 1 = factor analysis of mixed data (FAMD) component with greatest variance. Dimension 2 = FAMD component with second greatest variance. Colour of each ellipse reflects predominate egg colour family/ies (*orange*, *yellow*, *green*, *brown*). *Symbol shape* indicates developmental mode: *circle* = pelagic, *large square* = externally brooded, *small square* = internally brooded, *triangle* = no associated developmental mode. Test indicates associated ocean basin (global = distribution in Atlantic and Pacific ocean basins; $N = 87$, Appendix F).

size. Orange egg colour clustered with both pelagic and external-brooding development site ($P < 0.001$). Pelagic orange eggs were of average diameter and produced by species with average adults whereas externally brooded orange eggs were typically larger than average ($P = 0.005$). Orange eggs were also common in species with ubiquitous geographic distributions, independently of development mode ($P < 0.001$). In contrast, green eggs were only present in the Pacific and Indian oceans ($P < 0.001$); they were typically small in diameter ($P = 0.009$) and pelagic ($P < 0.001$) (Fig. 6).

Brown and yellow egg colours were closely linked to pale pigment intensity ($P < 0.001$); they were most common in internally brooding species ($P < 0.001$) and in the Atlantic Ocean. Brown and yellow eggs were average in size but were typically produced by species with smaller than average adults ($P = 0.004$). Grey egg colour could not be tested formally as it is relatively rare among lecithotrophic echinoderms.

3.3 Buoyancy

There is a significant relationship among egg buoyancy, egg colour, and egg development site (HCPC, $P<0.001$; Fig. 7; see Appendix G). Positive buoyancy was linked with pelagic development (HCPC, $P=0.016$) and red/orange egg colour families ($P<0.001$). Negative buoyancy was associated with externally brooded eggs ($P<0.001$) and with orange egg colour ($P<0.001$). Orange egg colour was present in both buoyancy clusters consistent with the presence of two subsets of orange eggs identified earlier with different development sites (pelagic and externally brooded). Green and yellow egg colours did not cluster with either positive or negative buoyancy.

3.4 Taxonomic Class

Overall, the full spectrum of egg colours (red, orange, yellow, brown, green, and grey) was found in four of the five extant classes of echinoderms (Fig. 8).

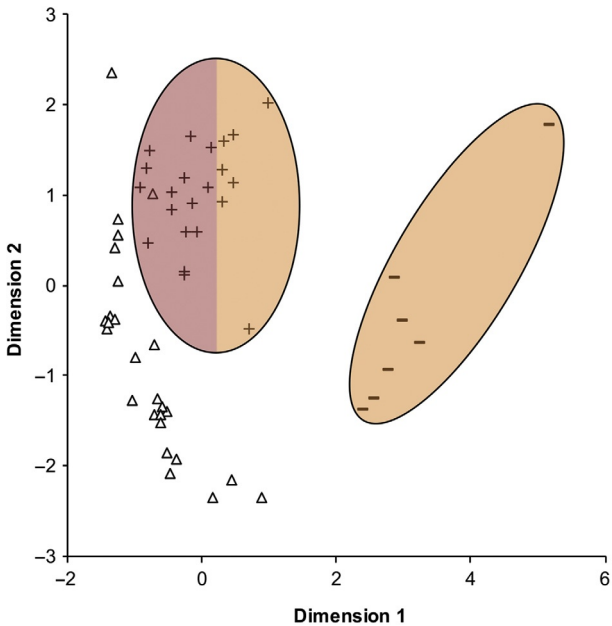


Fig. 7 Relationship between egg colour, development mode, and egg buoyancy in lecithotrophic echinoderms. Dimension 1 = factor analysis of mixed data (FAMD) component with greatest variance. Dimension 2 = FAMD component with second greatest variance. Colour of each ellipse reflects predominate egg colour family/ies (*red*, *orange*). *Symbol shape* indicates buoyancy: − = negative buoyancy, + = positive buoyancy, and *triangle* = no clustered buoyancy ($N=56$, Appendix G).

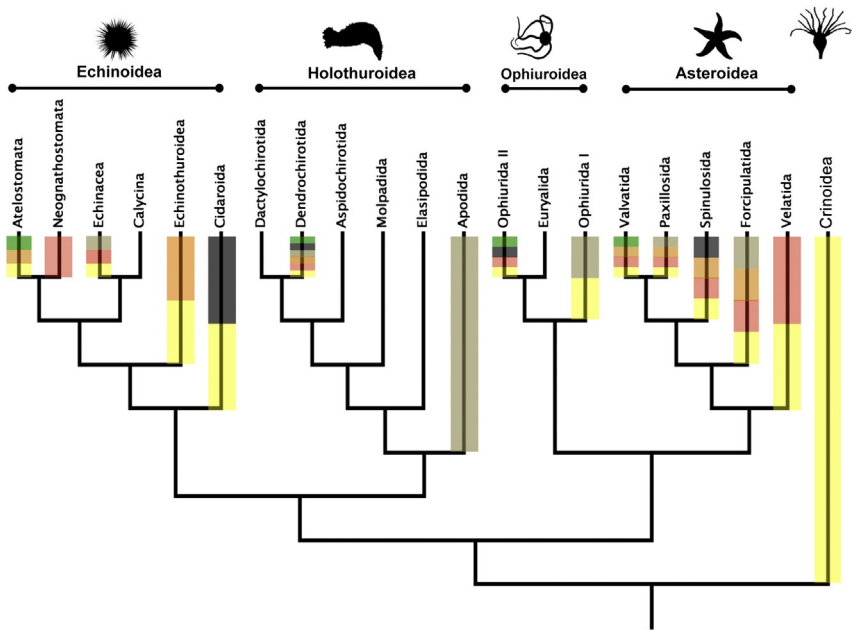


Fig. 8 Phylogenetic distribution of dominant egg colour families (*red*, *orange*, *brown*, *yellow*, *green*, *grey*) among lecithotrophic echinoderms ($N = 126$ records). Colours are ordered by first appearance from bottom (more primitive) to top (more derived). *Phylogeny modified from previously published accounts (Kerr and Kim, 2001; Kroh and Smith, 2010; Mah and Blake, 2012; O'Hara et al., 2014).*

The fifth class (Crinoidea) had only three representatives and thus could not be comprehensively analysed. Clear patterns emerged from phylogenetic distributions. Red and yellow egg colours appear early in the phylogeny, whereas the ability to produce green pigments appears among the most derived orders in Asteroidea, Echinoidea, Holothuroidea, and Ophiuroidea. The greatest egg colour diversity was found within the Valvatida, the most derived order of Asteroidea. The increase in available egg colour pigments with increasing distance from ancestral orders is also conserved within Holothuroidea, Echinoidea, and Ophiuroidea.

In addition, egg colour families were not randomly distributed across classes analysed independently of development mode and geographic location (HCPC, $P < 0.001$; Fig. 9; see Appendix H). Green eggs were most common in Holothuroidea and Ophiuroidea ($P < 0.001$), orange eggs in Asteroidea ($P < 0.001$), brown eggs in Holothuroidea ($P < 0.001$), and yellow eggs in Echinoidea ($P < 0.001$).

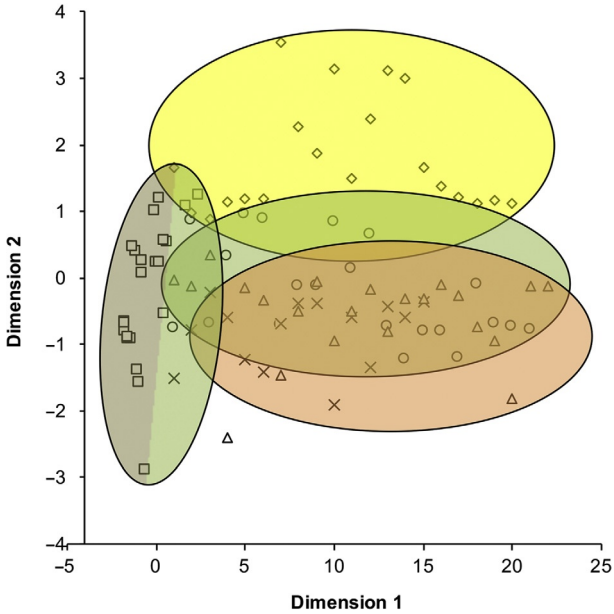


Fig. 9 Relationship between egg colour and taxonomic class in lecithotrophic echinoderms, independently of development mode. Dimension 1 = factor analysis of mixed data (FAMD) component with greatest variance. Dimension 2 = FAMD component with second greatest variance. Colour of each ellipse reflects predominate egg colour family(ies) (*orange*, *brown*, *yellow*, *green*). *Symbol shape* indicates class, *circle* = Ophiuroidea, *square* = Holothuroidea, *diamond* = Echinoidea, *triangle* = Asteroidea, *cross* = no associated phylogenetic class ($N = 103$, Appendix H).

4. DISCUSSION

The pelago-benthic life cycles that exist in the aquatic realm offer a unique framework for the study of egg phenotype evolution, one that has no parallel in terrestrial systems where the mainly studied group, class Aves (birds), relies exclusively on external brooding. The study of Echinodermata is particularly valuable in this context, given the full spectrum of reproductive strategies displayed by members of this phylum and their evolutionary closeness to higher taxa (Cameron et al., 2000; Strathmann, 2007). The present work showed that egg pigment intensities and colours are not distributed randomly across development types (illustrating site of development and level of parental care), geographic locations, and phylogenies in lecithotrophic echinoderms. In interspecific comparisons, egg colour also

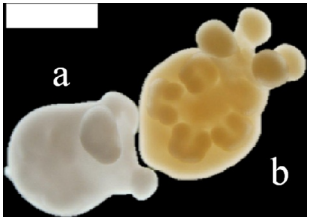
appears to be intrinsically linked with egg size and adult size, depicting contrasting life-history strategies. These findings highlight major implications for our understanding of the selective pressures and constraints that may act on egg phenotypes across evolutionary and geological time. They could also find a practical use in developing identification keys of planktonic eggs in the ocean (Appendix I).

4.1 Development Site Explains Pigment Intensity But Not Colour Diversity

Species with external fertilization and pelagic development have to overcome different challenges than those faced by incubating species, including exposure of eggs to sunlight and other environmental pressures (Burgess et al., 2013; Gillespie and McClintock, 2007). Whether free-spawned or brooded, lecithotrophic eggs/embryos obtain lipid reserves from maternal sources and thus do not require external nutrition during development (Falkner et al., 2013; Prowse et al., 2008). These storage lipids (i.e. wax esters in echinoderm larvae) are susceptible to oxidative stress from oxygen free radicals, metabolites, and UV radiation (Blount, 2004; Falkner et al., 2006; Villinski et al., 2002). Pelagic lecithotrophic embryos and larvae are commonly buoyant (in ~75% of species analysed here) and spend a portion of their development at or close to the ocean surface. Hence, floating propagules near the ocean surface presumably require more antioxidants, which could explain why nearly all pelagic, nonfeeding propagules in the dataset possess intense pigments, potentially a form of carotenoid, a pigment class found to have antioxidant function in other animals (Blount, 2004; Vershinin, 1999). Externally brooded propagules also possessed intense pigmentation (~65% of species), probably because they are still exposed to some level of UV radiation and environmental fluctuation in shallow benthic environments. Apart from antioxidant activity, the brighter pigmentation of externally brooded propagules may also afford cryptic colouration (matching the adult body colour) to minimize predation during their development. This pattern is well illustrated in the sea star *Trophodiscus* sp. and among brooding cidaroid urchins (Mah, 2009). In contrast, internally brooded propagules had pale brown or cream egg colours (~90% of species in dataset). This is not surprising given that internally brooded propagules are commonly not released until the feeding juvenile stage is reached, so their exposure to UV rays and free radicals is minimal during early development. Taken together, the

Table 2 Within-Species Colour Variation Is a Function of Life History and Buoyancy in Two Species of Sea Star (*Henricia lisa*, *Echinaster echinophorus*)

Species	Development Site	Egg Colour
Henricia lisa; (Mercier and Hamel, 2008a)	Brooded (−)	Pale grey (a)
	Pelagic	Bright yellow (b)
Echinaster echinophorus; (Atwood, 1973)	Pelagic (−)	Orange
	Pelagic (+)	Black



The inset photo illustrates the brachiolaria larvae of the sea star *H. lisa*. These larvae were spawned during the same event; the pale grey one (a) was brooded and the bright yellow one (b) broadcasted in the water column. Scale bar represents 1 mm. +, positive, −, negative.

lack of intense egg pigments among internal brooders strongly suggests that bright pigments provide an adaptive value for pelagic or external development.

This dichotomy between benthic/brooded and pelagic development can be exemplified within echinoderm species that possess multiple types of eggs and larvae. The deep-sea asteroid *H. lisa* broods a few eggs under its body and free-spawns the remainder. The eggs and larvae produced for brooding are pale in colour relative to the propagules produced for pelagic development in *H. lisa* (Mercier and Hamel, 2008a; see inset in Table 2). This colour difference suggests that maternal provisioning of pigments can vary across propagules of the same clutch. Evidence of deliberate alteration of larval energy reserves depending on reproductive strategy is relatively rare in echinoderms, but is common among annelids (Knott and McHugh, 2012) and opisthobranchs (Krug, 2009) with mixed modes of development (poecilogony). By increasing the concentration or type of pigments present in their free-spawned progeny, *Henricia* females may be enhancing offspring survival if such pigments are critical for lipid protection in the pelagic environment. The ability to deposit certain pigments may also be genetically controlled as seen in polychaete worms, where colour intensity variation is the result of selective pigment uptake from food (Sella and Marzoná, 1983).

In addition to pigment intensity differences, pelagic and brooded/benthic propagules also varied in buoyancy in the present study. Eggs that developed pelagically could be positively, neutrally, or negatively buoyant, whereas brooded eggs were always negatively buoyant. This is not

surprising, as it would be difficult, particularly for the external brooders, to keep floating eggs on or under the parent's body. In species with mixed types of development (like *Henricia* spp.), differing egg buoyancies could allow females to sort propagules at spawning. Positive egg buoyancy in echinoderms has historically been associated with lipid deposition by the female (Emlet, 1994). But the very large eggs seen in external brooders are not positively buoyant, despite major maternal yolk deposition (Emlet, 1994). Such variation in buoyancy may be facilitated though manipulation of lipid to protein ratios. Some sea stars (e.g. *Meridiastra* spp.) with similar egg volumes produce eggs with different buoyancies by altering the total amount of lipid present while leaving the protein levels unchanged (Prowse et al., 2008). Thus there may be an increased cost associated with production of positively buoyant eggs that explains egg size differences in species with pelagic vs brooded eggs. Overall, inter- and intraspecific variation in the intensity of egg pigmentation and egg buoyancy is clearly linked to development site in lecithotrophic echinoderms. However, these factors do not explain the broad variation in colouration seen among echinoderm eggs, suggesting that additional variables drove the evolution of egg pigment diversity in the ocean.

4.2 Why Green? The Link Between Ocean Basin and Phylogenetic Patterns of Egg Colour

Part of this puzzle is solved when geographic location is considered. The present study showed that echinoderm egg colours are nonrandomly distributed among ocean basins. Green-coloured eggs, in particular, are exclusively associated with the Indo-Pacific, independently of latitude and clime (from temperate-cold to tropical; see Mercier et al., 2013b). Presumably, species in the Pacific and Indian oceans must have been exposed to unique abiotic and/or biotic features that acted to select green egg pigments. This hypothesis is supported by evidence that green eggs may be convergent among four of the five extant echinoderm classes (the fifth, Crinoidea, being understudied). Green egg pigments are also restricted to the most derived orders of each class, inferring a more recent emergence relative to other pigments. This suggests that green pigments could require more time to emerge, consistent with the older age of the Pacific Ocean basin (Larson and Chase, 1972). The phylogenetic and spatial distribution of green eggs raises two main questions. Why did green evolve last in a

limited number of species (what functional or ecological advantages did they gain)? What unique factor(s) in the Indo-Pacific basin drove this evolution?

The nature of phytoplankton communities might be at play since they occupy the base of the food web and animals mainly obtain pigments from vegetal and microbial sources through their diet. For instance, the relative proportions of green/brown photopigments like fucoxanthin and the distribution of diatom species that harbour them (Wright and Jeffrey, 1987) differ across ocean basins, hemispheres, and geological times (Cermeño and Falkowski, 2009; Hasle, 1976). A different functional source of pigment could thus contribute to the presence of green eggs in Indo-Pacific echinoderms. However, green eggs are clearly not a passive outcome of dietary pigment availability since (1) other egg colours occur in the Indo-Pacific and (2) species with red/orange eggs may co-occur and share a similar diet with green-egg producing species. Case in point, the sea star *S. endeca* produces red eggs in both the North Pacific and North Atlantic oceans, whereas species in this genus that are confined to the Pacific (*Solaster dawsoni* and *Solaster stimpsoni*) produce bright green eggs, even though all three species occupy similar habitats and commonly feed on the same echinoderm prey (Lambert, 2000; Van Veldhuizen and Oakes, 1981).

The recent evolution of green egg pigments and their predominance in the Indo-Pacific might have been driven by a physiological or functional superiority to red and yellow pigments. Blue-green eggs are common among passerine birds, even though they are potentially conspicuous to predators while in the nest (Hanley et al., 2008; Maurer et al., 2011); the more brightly coloured eggs are suggested to reflect maternal health and are involved in communication between parents (Hanley et al., 2008; Navarro et al., 2011). Increased UV protection offered by blue-green pigments is hypothesized to explain the choice of this eggshell colour (Cassey et al., 2012; Hanley et al., 2008; Maurer et al., 2011). If green pigments also provide enhanced UV protection to pelagic lecithotrophic eggs, over time there may be a similar shift in the colour spectrum of echinoderm eggs towards the blue-green region. In our dataset, species producing green-pigmented eggs (e.g. *Cucumaria miniata*, *Cucumaria piperata*, and *Meridiastra calcar*) are commonly distributed in the intertidal and shallow-water coastal regions of the Pacific Ocean while subtidal and deep-water species of that basin maintain red, yellow, and brown egg colours. The intertidal environment is presumably associated with increased exposure

to solar radiation and predators, generating the need not only for more efficient antioxidants but also for more potent chemical deterrents. While the greater toxicity of green pigments has not been tested, green eggs were shown to be unpalatable (Sewell and Levitan, 1992). Overall, the green superiority hypothesis is attractive but still difficult to reconcile with the apparent absence of green eggs in the Atlantic basin, unless it reflects the lack of intertidally spawning lecithotrophs in this region (A. Mercier, personal observation, 2015). As far as we know, green eggs have only been reported in one lecithotrophic Atlantic species, the brittle star *Ophioderma brevispina* that can be found from the outer reefs of Central America to the temperate waters of Massachusetts, United States. However, *O. brevispina* was not listed as green-egged in our dataset because colour listings for this species are ambiguous, including 'lemon yellow', 'dark green,' and 'brown' that may vary among clutches and locations (Grave, 1916; Hendler and Tyler, 1986). If such a range in egg colours is indeed present, and not an artefact of inaccurate reporting, *O. brevispina* could represent the first parallel evolution of green egg pigments in an Atlantic echinoderm species.

4.3 Why Red and Yellow? A North Atlantic Study of Crypsis

Egg colours in lecithotrophic echinoderms from the North Atlantic Ocean typically range from light yellow/brown to red. Interestingly, red/orange oocytes from this region appear to match the background when viewed under blue light (i.e. the dominant wavelength below ~5 m in the North Atlantic; Fig. 10). As many visual predators are highly sensitive to blue-coloured objects, the absence of blue pigments might provide crypsis against predation for red and yellow eggs while in the water column (Johnsen, 2005; Umbers, 2013). The fact that red colouration is primarily associated with crypsis in the deep sea among diverse species is likely not a coincidence (Johnsen, 2005). In the North Atlantic, there are several deep-sea species with brightly pigmented eggs including: the sea star *H. phrygiana* (red-orange), the sea anemones *Hormatia* spp. (red), *Allantactis parasitica* (red) and *Urticina* sp. (orange), and the deep-sea coral *Drifa* spp. (pink) (Mercier and Hamel, 2008b, 2010; Sun et al., 2009, 2010). Relative colour matching in the deep sea is reportedly enough for crypsis against visual invertebrate and vertebrate predators (Johnsen, 2005). Background matching may also explain

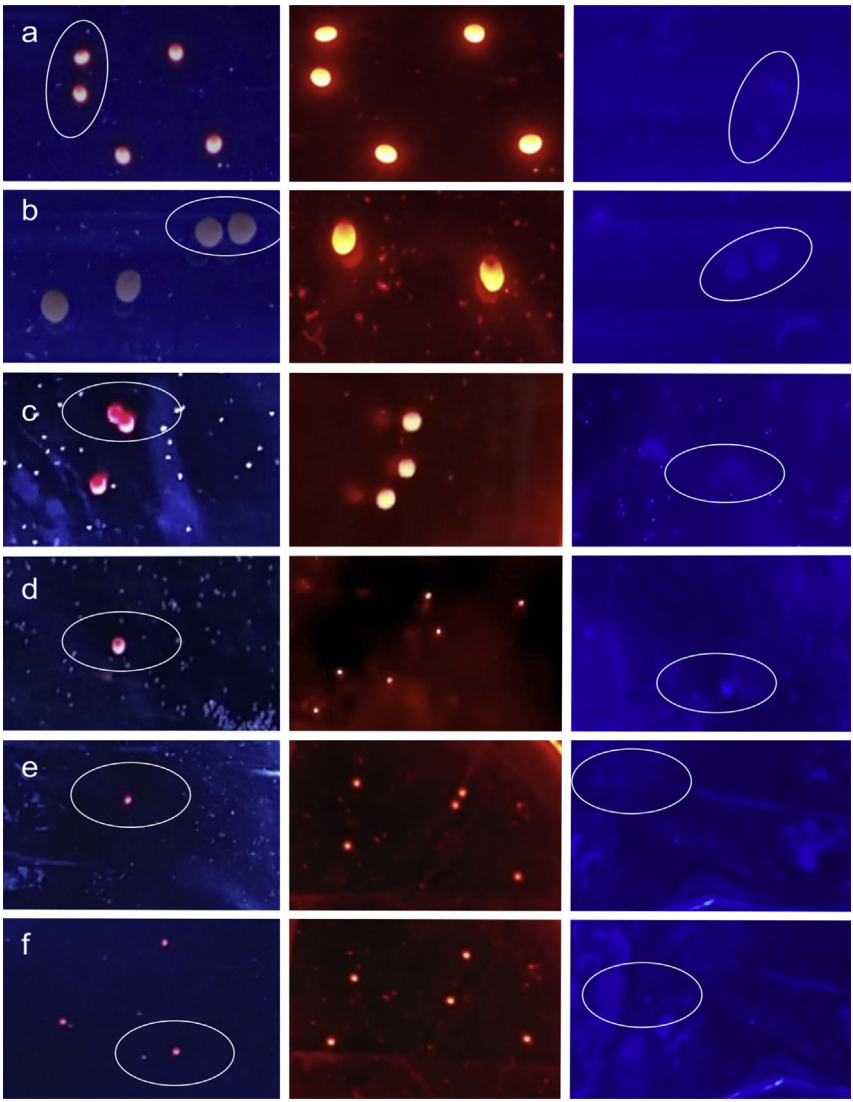


Fig. 10 Lecithotrophic echinoderm eggs viewed under various light colours/wavelengths at 300 lux: *white* (left panels), *red* (middle panels), and *blue* (right panels). Results shown for the sea stars (A) *Henricia sanguinolenta* (egg size 1.5 mm), (B) *Henricia lisa* (1.5 mm), (C) *Crossaster papposus* (0.9 mm), and (D) *Solaster endeca* (0.9 mm) and the sea cucumbers (E) *Cucumaria frondosa* (0.65 mm) and (F) *Psolus fabricii* (0.6 mm). Eggs were floating on the water surface at the time of imaging. *Circles* on the photos highlight the location of some of the eggs. Images not to scale. Methods: Freshly collected eggs were exposed successively to *white light* (present at surface, $\lambda = 440–650$),

(Continued)

the predominance of red and orange eggs among echinoderm species that release buoyant eggs in North Atlantic waters.

Similar to many biological systems, red in echinoderms eggs has historically been attributed to aposematic colouration, i.e. to signal unpalatability or chemical defences (Iyengar and Harvell, 2001; McClintock and Baker, 1997a). While some North Atlantic species with red-coloured eggs are known to deter predators with chemically defended larvae (Mercier et al., 2013a), there are several key problems associated with the aposematic colouration hypothesis that suggest it may not be the only explanation for the ubiquity of red/orange egg pigments. Firstly, chemical defence is not restricted to red or brightly coloured eggs/larvae. Case in point, *Acanthaster planci* is a tropical sea star with transparent, planktotrophic larvae that are chemically defended against fish predation (Lucas et al., 1979). Secondly, in the North Atlantic, red-coloured eggs appeared to be nearly invisible under simulated subsurface conditions in the present study (Fig. 10). Thus aposematic colouration may only be useful against certain types of visual predators. This is further confounded when predation rates by nonvisual benthic suspension feeders are considered, since the unpalatable compounds in red/orange coloured eggs also deter these types of predators (Mercier et al., 2013a). Although this mismatch raises the critical question of why unpalatable eggs would need to be visually undetectable, the specific colours evolved among lecithotrophic echinoderm eggs are clearly region-specific and nonrandom. Therefore, it is possible that the use of pigments in eggs first evolved to preserve essential lipids from oxidation, and that region-specific benefits were later actively selected for, leading to prioritization of certain pigment types (and ultimately colours) over others.

Fig. 10—cont'd *red light* (present at surface to 5 m, $\lambda = 650$ nm), and *blue light* (present at surface to 150 m, $\lambda = 440$ nm) using a Fuloon 12 V 5050 RGB light-emitting diode (LED) lamp. While wavelength varied, light intensity was standardized to near-surface values (~300 lux) typical of spring conditions when most spawning and larval development occurs. Images under each light condition were taken using an Olympus TG-2 digital camera for subsequent RBG measurements in Adobe Photoshop software. Unedited images (for a minimum of 50 eggs per species) were compared from each light condition and each tested species to assess the visibility of eggs. Data were compiled using eggs from at least two spawning events (involving >2 males and females) for species with pelagic development, and from a minimum of two clutches for brooding species.

4.4 Egg Colours in the Ocean and Beyond

The fact that egg colours in echinoderms are not randomly distributed relative to development site (position in the water column) and geographic location (ocean basin) echoes findings reported for other taxa; although, to the best of our knowledge, the scope of the present study is unparalleled (phylum-wide and worldwide). Mollusc eggs that are laid in shallow waters can be bright red, orange, or green. While this intriguing diversity was deemed worthy of further study 50 years ago (Cheesman et al., 1967), apparently the question was not pursued. The range of pigments reported in mollusc eggs exposed to solar radiation is strikingly similar to the colour palette seen here among pelagic lecithotrophic eggs in echinoderms. The presence of these specific colours in the marine environment corroborates the hypothesis that the use of certain pigments may be convergent among marine animals facing strong selective pressures to protect exposed eggs.

The association between egg colour and life history in the phylum Echinodermata is also surprisingly similar to that reported in more derived taxa such as birds (Aves, Chordata), but with a key difference. Eggs like those seen in Echinodermata consist of yolk surrounded by a thin membrane. Maternal investment is therefore consolidated into these yolk packages, inherently constraining physiological and defensive functions of pigments. The evolution of external shells in terrestrial animals like birds resulted in a two-part protection system that may be independently manipulated relative to maternal condition, sexual selection, and environmental conditions (Cassey et al., 2012; Maurer et al., 2011; Maurer et al., 2014). Like oocytes of basal echinoderms, the yolk of bird eggs is coloured yellow from maternal deposition of antioxidants such as carotenoids. In birds, it is the shell that saw an evolution of colour diversity, from pure white to blue-green, through the use of other pigments such as biliverdin (Navarro et al., 2011). White characterizes the eggshells of ancestral birds and of species that brood in cavities (i.e. not exposed to intense UV radiations), illustrating their lesser need for protective pigments (Kilner, 2006; Lack, 1958; Maldowie, 1886). This parallels the predominance of weakly pigmented (pale) eggs among internally brooding echinoderms in the present study. As for extant egg colour diversity, whether broad geographic or phylogenetic trends echoing the ones evidenced here also occur in birds is still not fully understood, as the scientific literature typically focuses on the nesting ecology of discrete species/populations and broad reviews are uncommon. In one of the rare class-wide

reviews, Kilner (2006) hypothesized that the diversity of eggshell colour and patterning in birds was largely driven by a coevolutionary arms race around brood parasitism (i.e. to make eggs more or less conspicuous, depending on the relevant need).

5. FUTURE DIRECTIONS

Taken together with results reported previously in more derived taxa, findings of the present study suggest that increasing complexity in egg colour patterns may represent an evolutionary trend in reproductive traits that emerged as animals shifted from an r-selected type of egg production, involving millions or thousands of eggs, to a K-selected model, where fewer offspring are produced. A quantitative assessment of egg colour patterns that further transcends the major boundaries in animal evolution would be a valuable step forward in deciphering the origins and adaptive value of egg colour in aquatic and terrestrial systems. For instance, primitive animals like sea anemones and corals (phylum Cnidaria) also produce brilliantly coloured, nonfeeding larvae but the ecological value of their pigments has not yet been explicitly explored. Key hypotheses to test across systems and taxa include: (1) egg pigmentation increases and diversifies as fecundity decreases, (2) pigment intensity is a function of exposure to UV and/or other sources of lipid oxidation, (3) selection of certain pigments over others is a function of ecological or energetic benefits, and (4) pigment intensity and colour can be directly manipulated by the level of maternal investment. Such findings would be invaluable to our understanding of how parental investment may be tailored to fit the needs of their offspring in egg-laying taxa. If nothing else, future studies in reproductive biology and ecological trade-offs should pay closer attention to egg colour measurements and definitions, as egg pigments clearly are more than aesthetic.

6. SUMMARY AND CONCLUSIONS

While egg-laying is widespread in the animal kingdom and marine species with maternally provisioned development produce some of the most strikingly coloured eggs, knowledge on the roles, and putative drivers of

egg pigmentation largely focuses on a small number of brooding avian taxa. Analyses and syntheses over broad taxonomic and geographic scales are lacking. The present phylum-wide study of Echinodermata exhibiting diverse life histories reveals that the colour, buoyancy, size, and development site of eggs are generally linked within reproductive and life-history strategies. Yellow emerges as the primitive egg colour and is still the most common in internal brooders, while green eggs occur only in the most derived orders of each class and are restricted to the Indo-Pacific basin. The more intense pigmentation of pelagic and externally brooded eggs compared to internally brooded eggs of similar size strongly supports the hypothesis that pigmentation is actively selected for to protect propagules against UV radiation and, possibly, visual predators. Egg colours diversified from the ancestral yellow in response to local environmental and/or ecological pressures, through a selection for better-adapted yet more costly pigments (red/orange and green), yielding defined geographic and phylogenetic colour patterns. Further quantitative assessments of egg colours and pigment types over broad scales could be used to determine whether and how fecundity and external pressures can mediate the nature and amount of maternal investment into egg pigmentation. The selective advantages of red and green pigments also need to be explored.

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APPENDICES

Appendix A.

Full dataset of lecithotrophic echinoderms used in the present study ($N = 126$). Data were collated for taxonomic grouping, embryonic development site, egg size, egg colour, egg colour intensity, egg buoyancy, adult size, and geographic distribution (ocean basin).

Class[a]	Order[b]	Species	Development Site[c]	Egg Size (µm)	Egg Colour	Egg Colour Intensity[d]	Egg Buoyancy[e]	Adult Size (cm)[f]	Ocean Basin[g]	Sources
As	For	*Anasterias antarctica*	EB	1810	Yellow	Bright	?	40	Ant	Gil et al. (2011)
As	For	*Diplasterias brandti*	EB	2870	Brown	Pale	?	110	Ant	Online image search
As	For	*Diplasterias brucei*	EB	3400	Orange	Pale	–	250	Ant	McClintock and Baker (1997a) and Pearse et al. (1991)
As	For	*Leptasterias* (*Hexasterias*) *alaskensis*	EB	?	Orange	Bright	?	160	Pac	Online image search
As	For	*Leptasterias* (*Hexasterias*) *hexactis*	EB	800	Orange	Bright	–	50	Pac	Online image search
As	For	*Leptasterias* (*Hexasterias*) *polaris*	EB	850	Orange	Pale	–	300	Pac/Atl	Hamel and Mercier (1995)
As	For	*Leptasterias aequalis* (*hexactis*)	EB	900	Yellow	Pale	?	60	Pac	Bingham et al. (2004)
As	For	*Leptasterias groenlandica*	EB	900	Brown	Pale	?	60	Pac/Atl	Mercier et al. (unpublished data), USNM 1350

As	For	*Leptasterias tenera*	EB	?	Brown	Pale	?	160	Atl	Hendler and Franz (1982)
As	For	*Smilasterias multipara*	IB	1000	Red	Regular	–	40	Pac	Komatsu et al. (2006)
As	Pax	*Astropecten gisselbrechti*	P	350	Yellow	Pale	–	80	Pac	Komatsu and Nojima (1985)
As	Pax	*Astropecten latespinosus*	P	300	Brown	Pale	–	80	Pac	Komatsu (1975) and Nojima (1982)
As	Pax	*Ctenopleura fisheri*	P	465	Brown	Pale	0	100	Pac	Komatsu (1982)
As	Pax	*Psilaster charcoti*	P	750	Red	Regular	?	300	Ant	McClintock and Baker (1997a)
As	Pax	*Trophodiscus* sp.	EB	?	Orange	Regular	?	?	Pac	Online image search
As	Pax	*Trophodiscus* sp.	EB	?	Red	Bright	?	?	Pac	Online image search
As	Pax	*Trophodiscus* sp.	EB	?	Yellow	Pale	?	?	Pac	Online image search
As	Spi	*Echinaster* (*Othilia*) *echinophorus*	P	1150	Grey	Bright	+	70	Atl	Atwood (1973)
As	Spi	*Echinaster* (*Othilia*) *echinophorus*	P	800	Orange	Regular	–	700	Atl	Atwood (1973)

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
As	Spi	*Echinaster brasiliensis*	P	1000	Brown	Bright	?	80	Atl	Nobre and Campos (2004)
As	Spi	*Echinaster graminicola*	P	850	Orange	Regular	?	80	Atl	Campbell and Turner (1984)
As	Spi	*Echinaster luzonicus*	P	1000	Red	Regular	+	150	Pac	https://charonia.wordpress.com/2016/08/25/sexual-reproduction-in-starfish/, accessed July 2015
As	Spi	*Henricia lisa*	EB	1100	Grey	Pale	–	100	Atl	Mercier and Hamel (2008a)
As	Spi	*Henricia lisa*	P	1100	Yellow	Regular	–	100	Atl	Mercier and Hamel (2008a)
As	Spi	*Henricia sanguinolenta*	EB	1000	Orange	Bright	–	150	Pac/Atl	Mercier and Hamel (2008a)
As	Val	*Aquilonastra burtoni*	P	500	Green	Pale	–	30	Ind	Achituv and Sher (1991) and James (1972)

As	Val	*Asterina gibbosa*	EB	400	Yellow	Bright	–	60	Atl	Online image search, Haesaerts et al. (2006)
As	Val	*Asterina phylactica*	EB	550	Orange	Regular	–	15	Atl	Online image search, Strathmann et al. (1984)
As	Val	*Crossaster papposus*	P	550	Red	Bright	+	300	Pac/Atl	Gemmill (1920)
As	Val	*Cryptasterina hystera*	IB	440	Green	Regular	+	12	Pac	Byrne (2005) and Dartnall et al. (2003)
As	Val	*Cryptasterina pacifica*	P	400	Orange	Regular	+	20	Pac	Dartnall et al. (2003)
As	Val	*Cryptasterina pentagona*	P	413	Orange	Regular	+	24	Pac	Byrne (2006)
As	Val	*Fromia elegans*	P	2000	Red	Regular	+	120	Ind/Pac	https://charonia.wordpress.com/2016/08/25/sexual-reproduction-in-starfish/, accessed July 2015

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
As	Val	*Fromia monilis*	P	1000	Red	Bright	+	100	Ind	Online image search; Emlet (1994)
As	Val	*Hippasteria phrygiana*	P	450	Orange	Bright	+	400	Pac/Atl	Baillon et al. (2011)
As	Val	*Iconaster longimanus*	P	1000	Orange	Regular	+	200	Ind/Pac	Lane and Hu (1994)
As	Val	*Mediaster aequalis*	P	1000	Orange	Bright	+	200	Pac	Birkeland et al. (1971)
As	Val	*Meridiastra calcar* (*Patiriella*)	P	415	Green	Regular	–	60	Pac	Byrne and Anderson (1994)
As	Val	*Meridiastra gunnii* (*Patiriella*)	P	430	Green	Regular	+	40	Pac	Byrne and Anderson (1994)
As	Val	*Meridiastra occidens*	P	400	Green	Regular	+	30	Pac	Byrne (2006)
As	Val	*Meridiastra oriens*	P	400	Green	Regular	–	20	Pac	Byrne (2006)
As	Val	*Nardoa novaecaledoniae*	P	1000	Orange	Regular	+	110	Pac	https://charonia.wordpress.com/2016/08/25/sexual-reproduction-in-starfish/, accessed July 2015

As	Val	*Nardoa tuberculata*	P	1000	Orange	Regular	+	280	Pac	https://charonia.wordpress.com/2016/08/25/sexual-reproduction-in-starfish/, accessed July 2015
As	Val	*Ophidiaster granifer*	P	600	Orange	Regular	+	100	Pac	Yamaguchi and Lucas (1984)
As	Val	*Ophidiaster granifer*	P	600	Orange	Regular	–	100	Pac	Yamaguchi and Lucas (1984)
As	Val	*Parvulastra exigua* (*Patiriella*)	EB	390	Orange	Regular	–	15	Ind/Pac	Byrne and Anderson (1994)
As	Val	*Parvulastra vivipara* (*Patiriella*)	IB	150	Orange	Regular	?	30	Ant	Prestedge (1998)
As	Val	*Perknaster fuscus*	P	1200	Red	Regular	?	300	Ant	McClintock and Baker (1997a,b)
As	Val	*Solaster endeca*	P	800	Orange	Bright	+	300	Pac/Atl	Gemmill (1912)
As	Val	*Solaster stimpsoni*	P	1000	Green	Pale	+	400	Pac	Strathmann (1987)
As	Val	*Tosia neossia*	EB	700	Orange	Regular	?	60	Ant	Online image search; Naughton and O'Hara (2009)

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
As	Vel	*Pteraster abyssorum*	IB	?	Yellow	Regular	?	80	Atl	
As	Vel	*Pteraster militaris*	IB	1400	Yellow	Regular	?	120	Pac/Atl	McClary and Mladenov (1990)
As	Vel	*Pteraster tesselatus*	P	1200	Red	Bright	+	150	Pac	McEdward and Coulter (1987)
Cr	Art	*Antedon mediterranea*	EB	200	Yellow	Regular	?	?	Atl	Barbaglio et al. (2012)
Cr	Cor	*Dorometra sesokonis*	EB	200	Yellow	Bright	?	30	Pac	Obuchi et al. (2010)
Cr	Iso	*Metacrinus rotundus*	P	350	Yellow	Regular	+	600	Pac	Nakano et al. (2005)
Ec	Cam	*Heliocidaris erythrogramma*	P	400	Orange	Regular	+	140	Ind/ Ant	Williams and Anderson (1975) and Wray (1996)
Ec	Cam	*Holopneustes purpurescens*	P	580	Brown	Regular	+	80	Pac	Morris (1995)
Ec	Cam	*Sterechinus* sp.	EB	?	Brown	Bright	?	?	Ant	Online image search

Ec	Cas	*Cassidulus mitis*		370	Yellow	Regular	0	25	Atl	Contins and Ventura (2011)
Ec	Cid	Cidaroidea	EB	?	Red	Regular	?	?	Ant	Online image search
Ec	Cid	*Phyllacanthus imperialis*	P	510	Yellow	Regular	+	80	Ind/Pac	Olson et al. (1993)
Ec	Cid	*Phyllacanthus parvispinus*	P	700	Grey	Pale	+	100	Pac	Parks et al. (1989)
Ec	Cly	*Peronella japonica*	P	300	Red	Pale	–	60	Pac	Okazaki and Dan (1954)
Ec	Ech	*Asthenosoma ijimai*	P	1200	Orange	Regular	+	130	Pac	Amemiya and Tsuchiya (1979)
Ec	Ech	*Phormosoma placenta*	P	1100	Yellow	Regular	+	120	Atl	Young and Cameron (1987)
Ec	Spa	*Abatus cavernosus*	EB	1400	Yellow	Regular	?	40	Ant	Gil et al. (2009) and Poulin and Feral (1996)
Ec	Spa	*Abatus cordatus*	EB	1300	Orange	Bright	–	30	Ant	Magniez (1983) and Schatt and Féral (1996)
Ec	Spa	*Brisaster latifrons*	P	350	Green	Regular	?	60	Pac	Strathmann (1979)

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
Ho	Apo	*Leptosynapta clarki*	IB	250	Brown	Pale	–	50	Pac	McEuen (1988) and Sewell and Chia (1994)
Ho	Den	*Athyonidium chilensis*	P	360	Brown	Pale	?	150	Ant	Guisado et al. (2012)
Ho	Den	*Cucumaria fallax* (*pallida*)	P	500	Brown	Pale	+	120	Pac/Atl	Emlet (1994) and McEuen (1988)
Ho	Den	*Cucumaria frondosa*	P	750	Orange	Bright	+	200	Atl	Hamel and Mercier (1996)
Ho	Den	*Cucumaria frondosa japonica*	P	500	Green	Regular	+	300	Pac	Tyurin and Drozdov (2002)
Ho	Den	*Cucumaria lubrica*	EB	900	Red	Regular	–	50	Pac	Engstrom (1982)
Ho	Den	*Cucumaria miniata*	P	520	Green	Bright	+	250	Pac	McEuen (1988)
Ho	Den	*Cucumaria piperata*	P	530	Green	Regular	+	120	Pac	McEuen (1988)
Ho	Den	*Cucumaria pseudocurata*	EB	1000	Grey	Bright	–	30	Pac	McEuen (1988) and Rutherford (1973)
Ho	Den	*Cucumariid* sp.	IB	800	Brown	Pale	?	10	Pac	O'Loughlin (1991)

Ho	Den	*Echinopsolus charcoti*	IB	1800	Yellow	Pale	?	60	Pac	O'Loughlin (2000)
Ho	Den	*Eupentacta chronhjelmi (quinquesemita)*	P	300	Green	Regular	–	60	Pac	Catalan and Yamamoto (1994)
Ho	Den	*Eupentacta fraudatrix*	P	340	Green	Regular	–	100	Pac	Kashenko (2000)
Ho	Den	*Eupentacta quinquesemita*	P	400	Green	Regular	0	100	Pac	McEuen (1988)
Ho	Den	*Neocnus* sp.	IB	600	Yellow	Regular	?	4	Pac	O'Loughlin (1991)
Ho	Den	*Pentamera populifera*	P	370	Green	Regular	0	30	Pac	McEuen (1988)
Ho	Den	*Pseudocnus (Pentactella) laevigata*	EB	1500	Brown	Regular	?	30	Ant	O'Loughlin (2000)
Ho	Den	*Pseudocnus echinatus*	P	400	Green	Regular	–	?	Ind	Emlet (1994) and Ohshima (1921)
Ho	Den	*Pseudocnus lubricus*	EB	1050	Yellow	Regular	–	50	Pac	McEuen (1988) and Rutherford (1973)
Ho	Den	*Psolidiella nigra*	EB	600	Yellow	Pale	?	40	Pac	O'Loughlin (2000)

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
Ho	Den	*Psolidium bidiscum*	P	300	Yellow	Regular	?	30	Pac	Lambert (1997)
Ho	Den	*Psolidium bullatum*	P	330	Yellow	Bright	0	25	Pac	McEuen (1988) and McEuen and Chia (1991)
Ho	Den	*Psolus chitinoides*	P	625	Red	Bright	+	75	Pac	McEuen (1988) and McEuen and Chia (1991)
Ho	Den	*Psolus fabricii*	P	500	Orange	Bright	+	200	Pac/Atl	Hamel et al. (1993)
Ho	Den	*Psolus phantapus*	P	450	Red	Regular	+	265	Atl	Baillon et al. (2011)
Ho	Den	*Squamocnus aureoruber*	IB	?	Brown	Pale	?	10	Pac	Image search
Ho	Den	*Stereoderma kirchsbergii*	P	?	Green	Regular	–	?	Pac	Ohshima (1921)
Ho	Den	*Trachythyone nina*	IB	1800	Brown	Pale	?	14	Atl	Mercier et al. (2010)
Ho	Ela	*Penilidia desbarresi*	IB	150	Brown	Pale	?	20	Atl	Gebruk et al. (2013)

Ho	Mol	*Molpadia intermedia*	P	270	Red	Pale	–	400	Pac	McEuen and Chia (1985)
Op	Oph	*Amphioplus abditus*	P	150	Grey	Bright	–	50	Atl	Hendler (1977)
Op	Oph	*Amphiura carchara*	IB	450	Yellow	Pale	?	80	Pac	Clark (1911) and Hendler and Tran (2001)
Op	Oph	*Amphiura squamata*	IB	880	Red	Pale	?	5	Atl	Byrne (1991)
Op	Oph	*Clarkcoma pulchra*	P	290	Yellow	Pale	–	120	Pac	Falkner et al. (2015)
Op	Oph	*Ophiarthrum elegans*	P	380	Green	Regular	+	13	Pac	Falkner et al. (2006)
Op	Oph	*Ophiarthrum pictum*	P	420	Green	Regular	?	30	Pac	Hendler and Meyer (1982) and Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophioderma brevispina*[h]	P	350	Brown	Regular	+	40	Atl	Hendler and Littman (1986) and Hendler and Tyler (1986)

Continued

Class	Order	Species	Development Site	Egg Size (µm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
Op	Oph	*Ophioderma brevispina*[h]	P	350	Green	Regular	+	40	Atl	Hendler and Littman (1986) and Hendler and Tyler (1986)
Op	Oph	*Ophioderma brevispina*[h]	P	350	Yellow	Regular	+	40	Atl	Grave (1916)
Op	Oph	*Ophioderma rubicunda*	P	?	Red	Regular	?	20	Atl	Hagman and Vize (2003) and Hendler and Littman (1986)
Op	Oph	*Ophiolepis elegans*	P	250	Yellow	Regular	?	50	Atl	Stancyk (1973)
Op	Oph	*Ophiomastix annulosa*	P	430	Green	Regular	?	150	Pac	Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophiomastix caryophyllata*	P	200	Green	Regular	?	10	Pac	Personal communication, Maria Byrne, University of Sydney, 2016

Op	Oph	*Ophiomastix elegans*	P	200	Green	Regular	?	10	Pac	Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophiomastix janualis*	P	200	Green	Regular	?	120	Pac	Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophiomastix marshallensis*	P	220	Green	Regular	?	?	Pac	Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophiomastix mixta*	P	335	Green	Regular	?	600	Pac	Personal communication, Maria Byrne, University of Sydney, 2016
Op	Oph	*Ophiomastix venosa*	P	500	Green	Regular	+	20	Ind/Pac	Fourgon et al. (2005)
Op	Oph	*Ophionereis olivacea*	IB	480	Orange	Regular	–	10	Atl	Byrne (1991)

Continued

Class	Order	Species	Development Site	Egg Size (μm)	Egg Colour	Egg Colour Intensity	Egg Buoyancy	Adult Size (cm)	Ocean Basin	Sources
Op	Oph	*Ophionereis schayeri*	P	240	Brown	Pale	–	150	Pac	Selvakumaraswamy and Byrne (2000)
Op	Oph	*Ophiopeza spinosa*	IB	300	Yellow	Regular	?	70	Pac	Byrne et al. (2008)
Op	Oph	*Ophioplocus japonicus*	P	300	Red	Regular	?	140	Pac	Clark (1911) and Komatsu and Shosaku (1993)
Op	Oph	*Ophiothrix oerstedii*	P	400	Brown	Pale	–	100	Atl	Mladenov (1979)
Op	Oph	*Opiolepis paucispina*	IB	480	Red	Pale	–	20	Atl	Byrne (1989)
Op	Oph	*Sigsbeia conifera*	IB	800	Red	Pale	–	10	Atl	Byrne (1991)
Op	Phyr	*Gorgonocephalus caryi*	EB	220	Orange	Pale	?	140	Pac	Patent (1970)

[a]As, Asteroidea; Cr, Crinoidea; Ec, Echinoidea; Ho, Holothuroidea; Op, Ophiuroidea.
[b]Apo, Apodida; Cam, Camerodonta; Cid, Cidaroidea; Cly, Clypeasteroida; Den, Dendrochirotida. Ech, Echinothuroida; For, Forcipulatida; Iso, Isocrinida; Mol, Molpadida; Oph, Ophiurida; Pax, Paxillosida; Spa, Spatangoida; Spi, Spinulosida; Val, Valvatida; Vel, Velatida.
[c]P, pelagic lecithotrophic; EB, externally brooded; IB, internally brooded.
[d]Refer to Table 1 for definitions of egg colour intensity.
[e]Egg buoyancy reported in the literature was categorized based on a previous comprehensive review of echinoderm larvae (Emlet, 1994). –, negative buoyancy; +, positive buoyancy; 0, Neutral buoyancy; ?, No data.
[f]Adult body size is diameter for Asteroidea, Ophiuroidea and Echinoidea, and length in Holothuroidea and Crinoidea.
[g]Ant, Antarctic; Atl, Atlantic; Ind, Indian; Pac, Pacific.
[h]Egg colour in *Ophioderma brevispina* is ambiguous due to conflicting records, it was considered brown for the purpose of analysis as this was the most common shade reported.

Appendix B.

Subset of lecithotrophic echinoderms used to test the hypothesis that egg colour is not randomly distributed among dataset variables ($N=87$). Data are shown for embryonic development site, egg size, egg colour, egg colour intensity, adult size, class, and geographic distribution (ocean basin).

Class[a]	Species	Development Site[b]	Egg Size	Egg Colour	Egg Intensity[c]	Adult Size[d]	Ocean Basin[e]
e	*Abatus cavernosus*	External	1400	Yellow	Regular	40	Ant
e	*Abatus cordatus*	External	1300	Orange	Bright	30	Ant
e	*Amphiura carchara*	Internal	450	Yellow	Pale	80	Pac
o	*Amphiura squamata*	Internal	880	Red	Pale	5	Atl
a	*Anasterias antarctica*	External	1810	Yellow	Bright	40	Ant
a	*Asterina gibbosa*	External	400	Yellow	Bright	60	Atl
h	*Asthenosoma ijimai*	Planktonic	1200	Orange	Regular	130	Pac
a	*Astropecten gisselbrechti*	Planktonic	350	Yellow	Pale	80	Pac
a	*Astropecten latespinosus*	Planktonic	300	Brown	Pale	80	Pac
h	*Athyonidium chilensis*	Planktonic	360	Brown	Pale	150	Ant
e	*Cassidulus mitis*	Planktonic	367	Yellow	Regular	25	Atl
o	*Clarkcoma pulchra*	Planktonic	290	Yellow	Pale	120	Pac
a	*Cryptasterina hystera*	Internal	440	Yellow	Regular	24	Pac
a	*Cryptasterina pacifica*	Planktonic	400	Orange	Regular	20	Pac

Continued

Class	Species	Development Site	Egg Size	Egg Colour	Egg Intensity	Adult Size	Ocean Basin
a	*Cryptasterina pentagona*	Planktonic	413	Orange	Regular	24	Pac
a	*Ctenopleura fisheri*	Planktonic	465	Brown	Pale	100	Pac
h	*Cucumaria frondosa japonica*	Planktonic	500	Green	Regular	300	Pac
h	*Cucumaria miniata*	Planktonic	520	Green	Bright	250	Pac
h	*Cucumaria piperata*	Planktonic	530	Green	Regular	120	Pac
h	*Cucumariid* sp.	Internal	800	Brown	Pale	10	Pac
a	*Diplasterias brandti*	External	2870	Brown	Pale	110	Ant
a	*Diplasterias brucei*	External	3400	Orange	Pale	250	Ant
a	*Echinaster brasiliensis*	Planktonic	1000	Brown	Bright	80	Atl
a	*Echinaster graminicola*	Planktonic	850	Orange	Regular	80	Pac
a	*Echinaster luzonicus*	Planktonic	1000	Red	Regular	150	Pac
h	*Echinopsolus charcoti*	Internal	1800	Yellow	Pale	60	Ant
h	*Eupentacta chronhjelmi*	Planktonic	300	Green	Regular	60	Pac
h	*Eupentacta fraudatrix*	Planktonic	340	Green	Regular	100	Pac
h	*Eupentacta quinquesemita*	Planktonic	400	Green	Regular	100	Pac
o	*Gorgonocephalus eucnemis*	External	220	Orange	Pale	140	Atl
a	*Henricia lisa*	Planktonic	1100	Yellow	Regular	100	Atl

Class	Species	Development Site	Egg Size	Egg Colour	Egg Intensity	Adult Size	Ocean Basin
a	*Henricia sanguinolenta*	External	1000	Orange	Bright	150	Pac/Atl
e	*Holopneustes purpurescens*	Planktonic	580	Brown	Regular	80	Pac
a	*Iconaster longimanus*	Planktonic	1000	Orange	Regular	200	Ind/Pac
a	*Leptasterias aequalis*	External	900	Yellow	Pale	60	Pac
a	*Leptasterias hexactis*	External	800	Orange	Bright	50	Pac
a	*Leptasterias polaris*	External	850	Orange	Pale	300	Pac/Atl
h	*Leptosynapta clarki*	Internal	250	Brown	Pale	50	Pac
a	*Mediaster aequalis*	Planktonic	1000	Orange	Bright	200	Pac
a	*Meridiastra calcar* (*Patiriella*)	Planktonic	415	Green	Regular	60	Pac
a	*Meridiastra gunnii* (*Patiriella*)	Planktonic	430	Green	Regular	40	Pac
a	*Meridiastra occidens*	Planktonic	400	Green	Regular	30	Pac
a	*Meridiastra oriens*	Planktonic	400	Green	Regular	20	Pac
h	*Molpadia intermedia*	Planktonic	270	Red	Pale	400	Pac
a	*Nardoa novaecaledoniae*	Planktonic	1000	Orange	Regular	110	Pac
a	*Nardoa tuberculata*	Planktonic	1000	Orange	Regular	280	Pac
h	*Neocnus* sp.	Internal	600	Yellow	Regular	5	Pac
a	*Neosmilaster georgianus*	Internal	2170	Brown	Pale	70	Atl

Continued

Class	Species	Development Site	Egg Size	Egg Colour	Egg Intensity	Adult Size	Ocean Basin
o	*Ophiarthrum elegans*	Planktonic	384	Green	Regular	13	Pac
o	*Ophiarthrum pictum*	Planktonic	419	Green	Regular	30	Pac
a	*Ophidiaster granifer*	Planktonic	600	Orange	Regular	100	Pac
a	*Ophidiaster granifer*	Planktonic	600	Orange	Regular	100	Pac
o	*Ophioderma brevispina*	Planktonic	350	Brown	Regular	40	Atl
o	*Ophioderma wahlbergii*	Internal	250	Yellow	Regular	30	Atl
o	*Ophiolepis elegans*	Planktonic	250	Yellow	Regular	50	Atl
o	*Ophiolepis paucispina*	Internal	480	Red	Pale	20	Atl
o	*Ophiomastix annulosa*	Planktonic	430	Green	Regular	150	Pac
o	*Ophiomastix caryophyllata*	Planktonic	200	Green	Regular	10	Pac
o	*Ophiomastix elegans*	Planktonic	200	Green	Regular	10	Pac
o	*Ophiomastix janualis*	Planktonic	200	Green	Regular	120	Pac
o	*Ophiomastix mixta*	Planktonic	335	Green	Regular	600	Pac
o	*Ophiomastix venosa*	Planktonic	500	Green	Regular	20	Ind/Pac
o	*Ophionereis olivacea*	Internal	400	Orange	Regular	3	Atl
o	*Ophionereis olivacea*	Internal	480	Orange	Regular	10	Atl
o	*Ophionereis schayeri*	Planktonic	240	Brown	Pale	150	Pac

Class	Species	Development Site	Egg Size	Egg Colour	Egg Intensity	Adult Size	Ocean Basin
o	*Ophiopeza spinosa*	Internal	300	Yellow	Regular	70	Pac
o	*Ophiothrix oerstedii*	Planktonic	400	Brown	Pale	100	Atl
a	*Parvulastra exigua* (*Patiriella*)	External	390	Orange	Regular	15	Ind/Pac
h	*Penilidia desbarresi*	Internal	150	Brown	Pale	20	Atl
h	*Pentamera populifera*	Planktonic	370	Green	Regular	30	Pac
a	*Perknaster fuscus*	Planktonic	1200	Red	Regular	300	Ant
e	*Phormosoma placenta*	Planktonic	1100	Yellow	Regular	120	Atl
e	*Poriocidaris purpurata*	Planktonic	1500	Brown	Pale	30	Atl
h	*Pseudocnus laevigata*	External	1500	Brown	Regular	30	Ant
h	*Pseudocnus lubrica*	External	1050	Yellow	Regular	50	Pac
a	*Psilaster charcoti*	Planktonic	750	Red	Regular	300	Ant
h	*Psolidiella nigra*	External	600	Yellow	Pale	40	Pac
h	*Psolidium bidiscum*	Planktonic	300	Yellow	Regular	30	Pac
h	*Psolidium bullatum*	Planktonic	330	Yellow	Bright	25	Pac
h	*Psolus chitinoides*	Planktonic	625	Red	Bright	75	Pac
h	*Psolus fabricii*	Planktonic	500	Orange	Bright	200	Pac/Atl
a	*Pteraster tesselatus*	Planktonic	1200	Red	Bright	150	Pac
o	*Sigsbeia conifera*	Internal	800	Red	Pale	10	Atl
a	*Solaster endeca*	Planktonic	800	Orange	Bright	300	Pac/Atl

Continued

Class	Species	Development Site	Egg Size	Egg Colour	Egg Intensity	Adult Size	Ocean Basin
a	*Solaster stimpsoni*	Planktonic	1000	Green	Pale	400	Pac
a	*Tosia neossia*	External	700	Orange	Regular	60	Ant
h	*Trachythyone nina*	Internal	1800	Brown	Pale	14	Atl

[a]a, Asteroidea; e, Echinoidea; c, Crinoidea; h, Holothuroidea; and o, Ophiuroidea.
[b]Planktonic, pelagic lecithotrophic; External, externally brooded; Internal, internally brooded.
[c]Refer to Table 1 for definitions of egg colour intensity.
[d]Adult body size is diameter for Asteroidea, Ophiuroidea and Echinoidea, and length in Holothuroidea and Crinoidea.
[e]Ant, Antarctic; Atl, Atlantic; Ind, Indian; Pac, Pacific.

Appendix C.

Subset of lecithotrophic echinoderms used to test whether egg buoyancy correlates with egg colour and development mode, independently of geographic location ($N=56$). Data are shown for embryonic development site, egg size, egg colour, egg buoyancy, and adult size.

Species	Development Site[a]	Egg Size	Egg Colour	Buoyancy[b]	Adult Size[c]
Abatus cordatus	External	1300	Orange	–	30
Aquilonastra burtoni	Planktonic	500	Green	–	30
Asterina phylactica	External	550	Orange	–	15
Asthenosoma ijimai	Planktonic	1200	Orange	+	130
Astropecten latespinosus	Planktonic	300	Brown	–	80
Clarkcoma pulchra	Planktonic	290	Yellow	–	120
Crossaster papposus	Planktonic	550	Red	+	300
Cryptasterina hystera	Internal	440	Yellow	+	24
Cryptasterina pacifica	Planktonic	400	Orange	+	20
Cryptasterina pentagona	Planktonic	413	Orange	+	24
Cucumaria frondosa	Planktonic	750	Orange	+	200

Species	Development Site	Egg Size	Egg Colour	Buoyancy	Adult Size
Cucumaria frondosa japonica	Planktonic	500	Green	+	300
Cucumaria miniata	Planktonic	520	Green	+	250
Cucumaria piperata	Planktonic	530	Green	+	120
Diplasterias brucei	External	3400	Orange	–	250
Echinaster echinophorus	Planktonic	800	Orange	–	700
Echinaster luzonicus	Planktonic	1000	Red	+	150
Eupentacta chronhjelmi	Planktonic	300	Green	–	60
Eupentacta fraudatrix	Planktonic	340	Green	–	100
Fromia elegans	Planktonic	2000	Red	+	120
Fromia ghardaqana	Planktonic	1000	Red	+	100
Heliocidaris erythrogramma	Planktonic	400	Orange	+	140
Henricia sanguinolenta	External	1000	Orange	–	150
Hippasteria phrygiana	Planktonic	450	Orange	+	400
Holopneustes purpurescens	Planktonic	580	Brown	+	80
Iconaster longimanus	Planktonic	1000	Orange	+	200
Leptasterias hexactis	External	800	Orange	–	50
Leptasterias polaris	External	850	Orange	–	300
Mediaster aequalis	Planktonic	1000	Orange	+	200
Meridiastra calcar (*Patiriella*)	Planktonic	415	Green	–	60
Meridiastra gunnii (*Patiriella*)	Planktonic	430	Green	+	40
Meridiastra occidens	Planktonic	400	Green	+	30
Meridiastra oriens	Planktonic	400	Green	–	20
Metacrinus rotundus	Planktonic	350	Yellow	+	600

Continued

Species	Development Site	Egg Size	Egg Colour	Buoyancy	Adult Size
Nardoa novaecaledoniae	Planktonic	1000	Orange	+	110
Nardoa tuberculata	Planktonic	1000	Orange	+	280
Ophiarthrum elegans	Planktonic	384	Green	+	13
Ophidiaster granifer	Planktonic	600	Orange	–	100
Ophidiaster granifer	Planktonic	600	Orange	+	100
Ophioderma brevispina	Planktonic	350	Green	+	40
Ophiolepis paucispina	Internal	480	Red	–	20
Ophiomastix venosa	Planktonic	500	Green	+	20
Ophionereis olivacea	Internal	480	Orange	–	10
Ophionereis schayeri	Planktonic	240	Brown	–	150
Ophiothrix oerstedii	Planktonic	400	Brown	–	100
Parvulastra exigua (*Patiriella*)	External	390	Orange	–	15
Phormosoma placenta	Planktonic	1100	Yellow	+	120
Phyllacanthus imperialis	Planktonic	510	Yellow	+	80
Pseudocnus echinatus	Planktonic	400	Green	–	40
Psolus chitinoides	Planktonic	625	Red	+	75
Psolus fabricii	Planktonic	500	Orange	+	200
Psolus phantapus	Planktonic	450	Red	+	265
Pteraster tesselatus	Planktonic	1200	Red	+	150
Sigsbeia conifera	Internal	800	Red	–	10
Solaster endeca	Planktonic	800	Orange	+	300
Solaster stimpsoni	Planktonic	1000	Green	+	400

[a]Planktonic, pelagic lecithotrophic; External, externally brooded; Internal, internally brooded.
[b]Egg buoyancy reported in the literature was categorized based on a previous comprehensive review of echinoderm larvae (Emlet, 1994). –, negative buoyancy; +, positive buoyancy; 0, neutral buoyancy; ?, no data.
[c]Adult body size is diameter for Asteroidea, Ophiuroidea and Echinoidea, and length in Holothuroidea and Crinoidea.

Appendix D.

Subset of lecithotrophic echinoderms used to test whether certain egg colours are phylogenetically linked in the four main extant classes ($N = 103$). Data are shown for taxonomic class, egg size, egg colour, and adult size.

Class	Species	Egg Size	Egg Colour	Adult Size[a]
Echinoidea	*Abatus cavernosus*	1400	Yellow	40
Echinoidea	*Abatus cordatus*	1300	Orange	30
Ophiuroidea	*Amphiura carchara*	450	Yellow	80
Ophiuroidea	*Amphiura squamata*	880	Red	5
Asteroidea	*Anasterias antarctica*	1810	Yellow	40
Asteroidea	*Aquilonastra burtoni*	500	Green	30
Asteroidea	*Asterina gibbosa*	400	Yellow	60
Asteroidea	*Asterina phylactica*	550	Orange	15
Echinoidea	*Asthenosoma ijimai*	1200	Orange	130
Asteroidea	*Astropecten gisselbrechti*	350	Yellow	80
Asteroidea	*Astropecten latespinosus*	300	Brown	80
Holothuroidea	*Athyonidium chilensis*	360	Brown	150
Echinoidea	*Cassidulus mitis*	367	Yellow	25
Ophiuroidea	*Clarkcoma pulchra*	290	Yellow	120
Asteroidea	*Crossaster papposus*	550	Red	300
Asteroidea	*Cryptasterina hystera*	440	Green	12
Asteroidea	*Cryptasterina hystera*	440	Yellow	24
Asteroidea	*Cryptasterina pacifica*	400	Orange	20
Asteroidea	*Cryptasterina pentagona*	413	Orange	24
Asteroidea	*Ctenopleura fisheri*	465	Brown	100
Holothuroidea	*Cucumaria fallax* (*pallida*)	500	Brown	120
Holothuroidea	*Cucumaria frondosa*	750	Orange	200
Holothuroidea	*Cucumaria frondosa japonica*	500	Green	300

Continued

Class	Species	Egg Size	Egg Colour	Adult Size
Holothuroidea	*Cucumaria lubrica*	900	Red	50
Holothuroidea	*Cucumaria miniata*	520	Green	250
Holothuroidea	*Cucumaria piperata*	530	Green	120
Asteroidea	*Diplasterias brandti*	2870	Brown	110
Asteroidea	*Diplasterias brucei*	3400	Orange	250
Asteroidea	*Echinaster brasiliensis*	1000	Brown	80
Asteroidea	*Echinaster echinophorus*	800	Orange	700
Asteroidea	*Echinaster graminicola*	850	Orange	80
Asteroidea	*Echinaster luzonicus*	1000	Red	150
Holothuroidea	*Echinopsolus charcoti*	1800	Yellow	60
Holothuroidea	*Eupentacta chronhjelmi*	300	Green	60
Holothuroidea	*Eupentacta fraudatrix*	340	Green	100
Holothuroidea	*Eupentacta quinquesemita*	400	Green	100
Asteroidea	*Fromia elegans*	2000	Red	120
Asteroidea	*Fromia ghardaqana*	1000	Red	100
Ophiuroidea	*Gorgonocephalus eucnemis*	220	Orange	140
Echinoidea	*Heliocidaris erythrogramma*	400	Orange	140
Asteroidea	*Henricia lisa*	1100	Yellow	100
Asteroidea	*Henricia sanguinolenta*	1000	Orange	150
Asteroidea	*Hippasteria phrygiana*	450	Orange	400
Echinoidea	*Holopneustes purpurescens*	580	Brown	80
Asteroidea	*Iconaster longimanus*	1000	Orange	200
Asteroidea	*Leptasterias aequalis*	900	Yellow	60
Asteroidea	*Leptasterias groeanlandica*	900	Brown	60
Asteroidea	*Leptasterias hexactis*	800	Orange	50
Asteroidea	*Leptasterias polaris*	850	Orange	300
Holothuroidea	*Leptosynapta clarki*	250	Brown	50

Class	Species	Egg Size	Egg Colour	Adult Size
Asteroidea	*Mediaster aequalis*	1000	Orange	200
Asteroidea	*Meridiastra calcar* (*Patiriella*)	415	Green	60
Asteroidea	*Meridiastra gunnii* (*Patiriella*)	430	Green	40
Asteroidea	*Meridiastra occidens*	400	Green	30
Asteroidea	*Meridiastra oriens*	400	Green	20
Holothuroidea	*Molpadia intermedia*	270	Red	400
Asteroidea	*Nardoa novaecaledoniae*	1000	Orange	110
Asteroidea	*Nardoa tuberculata*	1000	Orange	280
Asteroidea	*Neosmilaster georgianus*	2170	Brown	70
Ophiuroidea	*Ophiarthrum elegans*	384	Green	13
Ophiuroidea	*Ophiarthrum pictum*	419	Green	30
Asteroidea	*Ophidiaster granifer*	600	Orange	100
Asteroidea	*Ophidiaster granifer*	600	Orange	100
Ophiuroidea	*Ophioderma brevispina*	350	Brown	40
Ophiuroidea	*Ophioderma wahlbergii*	250	Yellow	30
Ophiuroidea	*Ophiolepis elegans*	250	Yellow	50
Ophiuroidea	*Ophiolepis paucispina*	480	Red	20
Ophiuroidea	*Ophiomastix annulosa*	430	Green	150
Ophiuroidea	*Ophiomastix caryophyllata*	200	Green	10
Ophiuroidea	*Ophiomastix elegans*	200	Green	10
Ophiuroidea	*Ophiomastix janualis*	200	Green	120
Ophiuroidea	*Ophiomastix mixta*	335	Green	600
Ophiuroidea	*Ophiomastix venosa*	500	Green	20
Ophiuroidea	*Ophionereis olivacea*	400	Orange	3
Ophiuroidea	*Ophionereis olivacea*	480	Orange	10
Ophiuroidea	*Ophionereis schayeri*	240	Brown	150
Ophiuroidea	*Ophiopeza spinosa*	300	Yellow	70

Continued

Class	Species	Egg Size	Egg Colour	Adult Size
Ophiuroidea	*Ophiothrix oerstedii*	400	Brown	100
Asteroidea	*Parvulastra exigua* (*Patiriella*)	390	Orange	15
Asteroidea	*Parvulastra vivipara* (*Patiriella*)	150	Orange	30
Holothuroidea	*Penilidia desbarresi*	150	Brown	20
Holothuroidea	*Pentamera populifera*	370	Green	30
Asteroidea	*Perknaster fuscus*	1200	Red	300
Echinoidea	*Phormosoma placenta*	1100	Yellow	120
Echinoidea	*Phyllacanthus imperialis*	510	Yellow	80
Holothuroidea	*Pseudocnus echinatus*	400	Green	40
Holothuroidea	*Pseudocnus laevigata*	1500	Brown	30
Holothuroidea	*Pseudocnus lubrica*	1050	Yellow	50
Asteroidea	*Psilaster charcoti*	750	Red	300
Holothuroidea	*Psolidiella nigra*	600	Yellow	40
Holothuroidea	*Psolidium bidiscum*	300	Yellow	30
Holothuroidea	*Psolidium bullatum*	330	Yellow	25
Holothuroidea	*Psolus chitinoides*	625	Red	75
Asteroidea	*Psolus fabricii*	500	Orange	200
Holothuroidea	*Psolus phantapus*	450	Red	265
Asteroidea	*Pteraster militaris*	1400	Yellow	120
Asteroidea	*Pteraster tesselatus*	1200	Red	150
Ophiuroidea	*Sigsbeia conifera*	800	Red	10
Asteroidea	*Smilasterias multipara*	1000	Red	40
Asteroidea	*Solaster endeca*	800	Orange	300
Asteroidea	*Solaster stimpsoni*	1000	Green	400
Asteroidea	*Tosia neossia*	700	Orange	60
Holothuroidea	*Trachythyone nina*	1800	Brown	14

[a]Adult body size is diameter for Asteroidea, Ophiuroidea and Echinoidea, and length in Holothuroidea and Crinoidea.

Appendix E.

Clusters from factor analysis of mixed data (FAMD) as shown in Fig. 5—Testing all factors.

Cluster	Factor		*P*-Value
I	Egg colour	Green	<0.001
	Egg intensity	Standard	<0.001
	Egg size	<Mean	=0.025
	Adult size	>Mean	=0.017
	Phylogeny		
	Ocean basin	Pacific	<0.001
	Development site	Planktonic	<0.001
II	Egg colour	Yellow	=0.002
		Brown	<0.001
	Egg intensity	Pale	<0.001
	Egg size		
	Adult size	<Mean	=0.003
	Phylogeny	Ophiuroidea	=0.02
	Ocean basin	Atlantic	<0.001
	Development site	Internal	<0.001
III	Egg colour	Orange	<0.001
	Egg intensity	Bright	<0.001
	Egg size	>Mean	<0.001
	Adult size		
	Phylogeny	Asteroidea	=0.003
	Ocean basin	Global	<0.001
		Antarctic	<0.001
	Development site	External	<0.001

Appendix F.

Clusters from factor analysis of mixed data (FAMD) as shown in Fig. 6—Testing geographic location.

Cluster	Factor		*P*-Value
I	Egg colour	Green	<0.001
	Egg intensity	Standard	<0.001
	Egg size	<Mean	=0.009
	Adult size		
	Ocean basin	Pacific	<0.001
	Development site	Planktonic	<0.001
II	Egg colour	Yellow	=0.001
		Brown	<0.001
	Egg intensity	Pale	<0.001
	Egg size		
	Adult size	<Mean	=0.004
	Ocean basin	Atlantic	<0.001
	Development site	Internal	<0.001
III	Egg colour	Orange	<0.001
	Egg intensity	Bright	<0.001
	Egg size		
	Adult size	>Mean	=0.02
	Ocean basin	Global	<0.001
	Development site		
IV	Egg colour		
	Egg intensity		
	Egg size	>Mean	<0.001
	Adult size		
	Ocean basin	Antarctic	<0.001
	Development site	External	<0.001

Appendix G.

Clusters from factor analysis of mixed data (FAMD) as shown in Fig. 7—Testing egg buoyancy.

Cluster	Factor		*P*-Value
I	Egg colour	Orange	=0.0011
	Development site	External	<0.001
	Buoyancy	Negative	<0.001
	Egg size	>Mean	=0.005
	Adult size		
II	Egg colour	Red	<0.001
		Orange	<0.001
	Development site	Planktonic	=0.016
	Buoyancy	Positive	=0.023
	Egg size		
	Adult size		

Appendix H.

Clusters from factor analysis of mixed data (FAMD) as shown in Fig. 9—Testing phylogenetic class, independently of development mode.

Cluster	Factor		*P*-Value
I	Egg colour	Green	<0.001
	Class	Ophiuroidea	<0.001
	Egg size	<Mean	<0.001
	Adult size		
II	Egg colour	Green	<0.001
	Class	Holothuroidea	<0.001
	Egg size		
	Adult size		

Continued

Cluster	Factor		*P*-Value
III	Egg colour	Brown	<0.001
	Class	Holothuroidea	=0.036
	Egg size		
	Adult size		
IV	Egg colour	Yellow	<0.001
	Class	Echinoidea	<0.001
	Egg size		
	Adult size		
VI	Egg colour	Orange	<0.001
	Class	Asteroidea	<0.001
	Egg size	>Mean	=0.002
	Adult size		

Appendix I.

General key to lecithotrophic echinoderm eggs/early embryos.

1a. Propagules are greenish and yolky_____ Planktonic (Pacific/Indian)

1b. Propagules are reddish/yellowish and yolky_____2

2a. Propagules are positively buoyant_____ Planktonic

2b. Propagules are neutral or negatively buoyant _____ 3

3a. Large adult size (>10 cm) and/or small egg size (<1 mm)_____ Planktonic

3b. Small adult size (<10 cm) and/or large egg size (>1 mm)_____4

4a. Propagules are brightly coloured or match adult colour _____ External Brooder

4b. Propagules are pale in colour _____ Internal Brooder

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CHAPTER THREE

Biological Conservation of Giant Limpets: The Implications of Large Size

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Abstract

Patellogastropods, also known as true limpets, are distributed throughout the world and constitute key species in coastal ecosystems. Some limpet species achieve remarkable sizes, which in the most extreme cases can surpass 35 cm in shell length. In this review, we focus on giant limpets, which are defined as those with a maximum shell size surpassing 10 cm. According to the scientific literature, there are a total of 14 species across five genera that reach these larger sizes. Four of these species are threatened or in danger of extinction. Inhabiting the intertidal zones, limpets are frequently affected by anthropogenic impacts, namely collection by humans, pollution and habitat fragmentation. In the case of larger species, their conspicuous size has made them especially prone to human collection since prehistoric times. Size is not phylogeny-dependent among giant limpets, but is instead related to behavioural traits instead. Larger-sized species tend to be nonmigratory and territorial compared to those that are smaller. Collection by humans has been cited as the main cause behind the decline and/or extinction of giant limpet populations. Their conspicuously large size makes them the preferred target of human collection. Because they are protandric species, selectively eliminating larger specimens of a given population seriously compromises their viability and has led to local extinction events in some cases. Additionally, sustained collection over time may lead to microevolutionary responses that result in genetic changes. The growing presence of artificial structures in coastal ecosystems may cause population fragmentation and isolation, limiting the genetic flow and dispersion capacity of many limpet species. However, when they are necessitated, artificial structures could be managed to establish marine artificial microreserves and contribute to the conservation of giant limpet species that naturally settle on them.

1. INTRODUCTION

The order Patellogastropoda includes the true limpets and constitutes the most primitive group of present-day gastropods (Nakano and Ozawa, 2007). Traditionally, five families have been recognised: Patellidae, Nacellidae, Acmaeidae, Lottiidae and Lepetidae. However, genetic studies only distinguish the families Patellidae, Nacellidae and Lepetidae as monophyletic groups. The family Acmaeidae is composed of two clades, Acmaeinae and Pectinodontinae, the first of which has been included in the family Lottiidae, while the second has been elevated to the rank of family (Pectinodontidae). Finally, the group *Patelloida profunda* has been recognised as being the earliest group in the order, and it has been proposed to be assigned

to both a new genus (*Eoacmaea*) and a new family (Eoacmaeidae). Therefore, we may currently consider the families Patellidae, Nacellidae, Lepetidae, Lottiidae, Pectinodontidae and Eoacmaeidae (see Nakano and Ozawa, 2007).

Limpets are found in very diverse habitats, not only do they colonise coastal rocky substrates (Raffaelli and Hawkins, 1996), but they can be encountered on seaweed of the order Laminariales (kelp, *Laminaria* spp. and *Saccorhiza polyschides*, for example), on corallinaceous algae, seagrasses (e.g. *Zostera* spp.), the shells of other molluscs, and they are associated with hydrothermal vents as well as other benthic regions (see Branch, 1981; Nakano and Ozawa, 2007).

Limpets are considered key species in rocky coastal ecosystems as they maintain the composition and structure of the community and, when their habitats disappear, cause a cascade effect that affects the structure of the communities in which they live (Raffaelli and Hawkins, 1996). For example, they control barnacle numbers by removing the juveniles in their feeding passes, while at the same time they limit the growth of macroalgae, which could completely impede barnacle recruitment (Branch, 1981). By employing these mechanisms, the limpets maintain a mosaic of open spaces in the intertidal zone that enables many species to coexist, thus contributing to increased marine biodiversity on rocky shores. For this reason, limpets may be the most important group of grazers in temperate rocky intertidal zones (e.g. Burgos-Rubio et al., 2015).

Here, we focus on the giant limpets. For practical purposes, "giant limpets" are defined as referring those species with a maximum shell size surpassing 10 cm in length (confirmed either by direct observation in the field or by a minimum of two different references in the scientific literature). Using these criteria, we have recorded a total of 14 species among five genera: *Patella*, *Cellana*, *Scutellastra*, *Cymbula* and *Lottia*. In this review, we provide a brief overview of current knowledge regarding the biology and ecology of these species. We examine their common characteristics and discuss the costs and benefits of achieving their, sometimes, extraordinary sizes. Finally, we review the main conservation threats to these species, and future management strategies that might ensure the survival of the most threatened species.

2. GIGANTISM IN LIMPETS

Gigantism is not uncommon in invertebrate species (Vermeij, 2012), and there are many factors and evolutionary forces that favour

organisms to attain extreme large sizes. For instance, high oxygen availability and the absence of constraints to access food resources are some advantages of extreme size (Vermeij, 2012). Certain limpet species can potentially grow to achieve significantly conspicuous dimensions. Referred to as giant limpets in the scientific literature, these species have several common characteristics (Creese et al., 1990). For example, these larger limpet species are often territorial and show specialised trophic behaviours. The size of giant limpets, together with their presence in high densities in easily accessible coastal zones at low tide, has determined their intensive use in ecological studies, becoming "guinea pigs" in research focusing on, among other subjects, adaptations to the harshness of their environment (e.g. desiccation) and the bioenergetics of growth (Lindberg, 2007).

As is the case for many invertebrate species, maximum size in limpets is known to be correlated with gradients of primary productivity (Bosman et al., 1987; Vermeij, 1978, 1980). This correlation has been shown for the giant limpet *Patella ferruginea* (the ribbed Mediterranean limpet), which attains its record size (11.4 cm) in Ceuta Harbor, North Africa, where primary production is higher than in comparison with nearby waters (Espinosa et al., 2008a). However, South Africa, with highly productive waters (up to 800 g C m^{-2} $year^{-1}$) due to the upwelling zones associated with the Benguela Current (Mann, 2000; Walsh, 1988), hosts the majority of giant limpets. The ability of some of the South African limpets (particularly those belonging to the genus *Cymbula*) to consume large drifting algae may have contributed to the evolution of large size in these herbivores (Estes et al., 2005). The Northeast and Northwest Pacific also host exceptionally large-bodied species of molluscs, and the maximum size of giant limpets has been observed in these basins (313 mm in the Northeast Pacific Ocean, 210 mm in the Northwest; see Vermeij, 2012). Still, gigantism in limpets is not solely associated with distribution. The maximum size of these limpets has increased over geological time, from the Early Miocene to the present, in the North Pacific Ocean, South African waters, and the Northeast Atlantic Ocean (the areas where many giant limpets occur).

It might be noted that a positive feedback occurs between grazers and primary producers, in the sense that herbivores with high metabolic demands exert strong selection on prey species performing higher biomass turnover on them. Such selection allows herbivores to become larger and

more abundant where production and consumption remain stable for extended periods of time (Vermeij, 2012). Otherwise, large body size becomes disadvantageous when food supply is reduced or becomes unstable (e.g. if access to food is restricted by extreme conditions). As a consequence, regions that have historically had low extinction rates are the most favourable for the evolution of very large size (Vermeij, 2012).

As we have described, gigantism confers organisms with a series of biological particularities, lending them, for example, increased capacity to structure the ecosystem, greater interspecific competition and increased resistance to pressure from predators. Creese et al. (1990) pointed out that this is also the case for giant limpets, as there is much more intense inter- and intraspecific interaction. Nevertheless, large size also makes them more vulnerable and the frequent target of human collection because they are a large biomass that is often easily accessible.

The maximum sizes of the 14 species included in this review are included in Fig. 1 and Table 1. There is no apparent phylogenetic connection between the maximum size that animals can attain, and giant limpets that are found across lineages. It is interesting to note that when comparing maximum length with mean size within a genus, there is a similarity among phylogenetic lineages (see Fig. 1). Nevertheless, within the genus *Scutellastra*, the two largest limpets ever recorded: *Scutellastra kermadecensis*, with a maximum recorded size of 174 mm, and the largest known limpet, *Scutellastra mexicana*, with a maximum size of 355 mm. While the genera *Patella*, *Cellana* and *Lottia* have only one giant species each, the genus *Cymbula* has the largest proportion of giant species. Rather than being associated with phylogeny, gigantism in limpets seems to be more strongly associated with behavioural traits. Nonmigratory and territorial taxa attain larger size in comparison with migratory and nonterritorial taxa (Lindberg, 2007). In fact, the status of largest-sized within a guild is retained briefly and phylogenetic usurpation of this top status is the rule (Vermeij, 2012). In fact, a trait (in this case a large size), with supposed adaptive advantages, is very much contingent upon the identity of the clades with that trait or the sources of selection favouring the trait, since the results of the evolution are more predictable than the actors in the evolutionary theatre (see Vermeij, 2006, 2010).

Below, we briefly review current knowledge regarding the biology and ecology of these giant limpets, providing insight regarding their characteristics and behavioural traits.

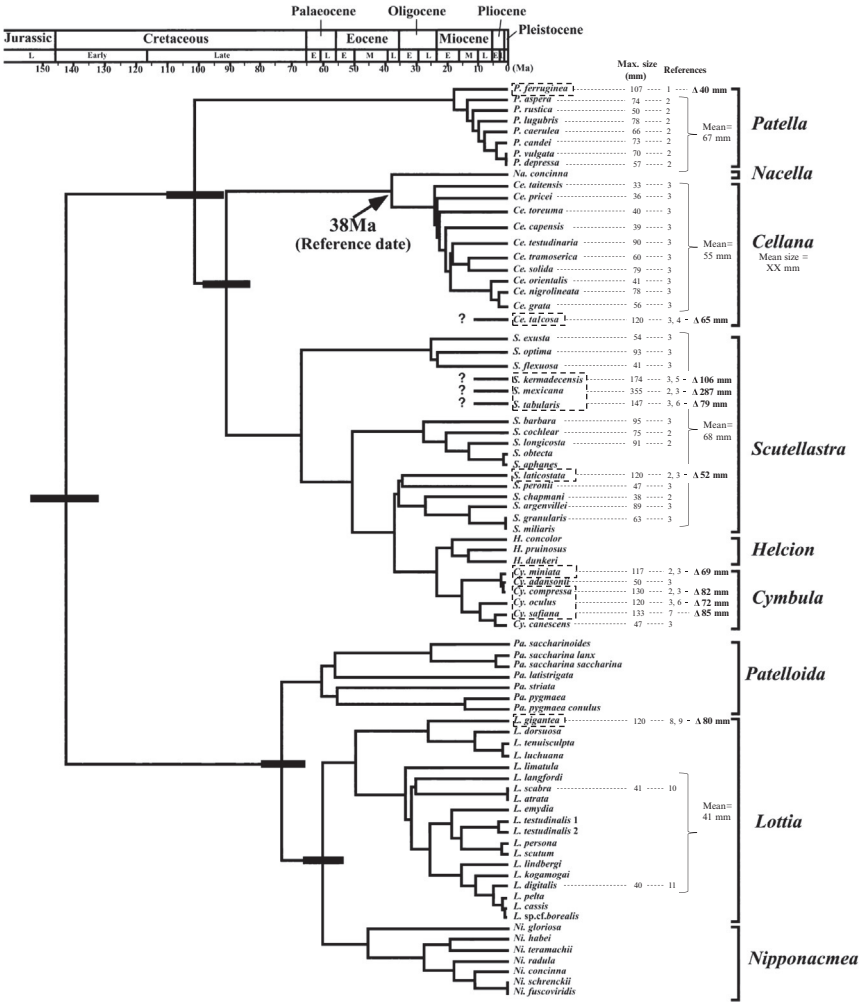

Fig. 1 Phylogeny of limpets of the order Patellogastropoda. *Interrogation marks* indicate species that were not present in the original study by Nakano and Ozawa (2007), but that have been included here for the purpose of this review. *Dotted boxes* indicate species that have been documented to reach 10 cm in shell length (see Table 1). References: (1) Rivera-Ingraham et al. (2011a), (2) Christiaens (1973), (3) Powell (1973), (4) Kay (1979), (5) Lindberg (2007), (6) Jerardino and Navarro (2008), (7) Kilburn and Rippey (1982), (8) Rivera-Ingraham et al. (2011b), (9) G.M. Branch, University of Capt Town, personal communication (2 July 2015), (10) Fenberg and Rivadeneira (2011), (11) Erlandson et al. (2011), (12) Shanks et al. (2014) and (13) Hahn and Denny (1989). *n.a.*, no available information. *Adapted from Nakano, T., Ozawa, T., 2007. Worldwide phylogeography of limpets of the order Patellogastropoda: molecular, morphological and palaeontological evidence. J. Moll. Stud. 73, 79–99.*

Table 1 List of Giant Limpet Species

Species	Distribution	Maximum Size (Source)	Status	Legal Document
Cellana talcosa	Hawaii	1065 mm (Powell, 1973); 120 mm (Kay, 1979)		
Cymbula compressa	South Africa	1175 mm (Powell, 1973); 130 mm (Christiaens, 1973)		
Cymbula granatina	South Africa	>100 mm (G.M. Branch, personal communication, 2015)		
Cymbula sanguinans	South Africa	117 mm[a] (Christiaens, 1973; Powell, 1973)		
Cymbula safiana	Western Africa, Mediterranean	133 mm (Rivera-Ingraham, 2010; Rivera-Ingraham et al., 2011b)	Species in danger or threatened	Barcelona Convention 1993
			Species strictly protected	Annex II Bern Convention 1995
			Species endangered	Red book of invertebrates of Andalusia
Cymbula oculus	South Africa	120 mm (Jerardino and Navarro, 2008; Powell, 1973)		
Lottia gigantea	Pacific North America	>100 mm (Fenberg and Rivadeneira, 2011); 120 mm (Erlandson et al., 2011)		
Patella ferruginea	Mediterranean	105 mm (Espinosa et al., 2009a); 107 mm (Rivera-Ingraham et al., 2011a)	Species strictly protected	Annex II Bern Convention 1979
			Species of European interest with strictly protection requirements	Annex IV of Habitats Directive (92/43/CEE)

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Table 1 List of Giant Limpet Species—cont'd

Species	Distribution	Maximum Size (Source)	Status	Legal Document
			Species in danger or threatened	Annex II Barcelona Convention 1996
			Species in danger of extinction	National List of Endangered Species, law of 9 July 1999, BOE number 302
			Species in danger of extinction	Andalusian List of endangered species, Law 8/2003 of 28 October, BOJA number 218
Scutellastra argenvillei	Namibia and South Africa	100 mm (Kensley, 1973); 104 mm (Kilburn and Rippey, 1982)		
Scutellastra barbara	South Africa	100 mm (Kensley, 1973); 100 mm (Kilburn and Rippey, 1982)		
Scutellastra kermadecensis	Kermadec Islands, New Zealand	>130 mm (Schiel et al., 1986); 136 mm (Lindberg, 2007); 174 mm (Powell, 1973)		
Scutellastra laticostata	Australia	107 mm (Christiaens, 1973); >110 mm (Powell, 1973)		
Scutellastra mexicana	Mexico	158 mm (Lindberg, 2007); 350 mm (Christiaens, 1973); 355 mm (Powell, 1973)	Special protection	Mexican Red List (NOM-059-SEMARNAT-2010)
Scutellastra tabularis	South Africa	127 mm (Lindberg, 2007); 1475 mm (Jerardino and Navarro, 2008)		

[a]Described in the original publication as *Cymbula* (*Patella*) *miniata*, and considered here to be *Cymbula sanguinans* (G.M. Branch, personal communication, 22 July 2015).

3. DISTRIBUTION

3.1 Geographic Range

The distributions of giant limpet species are shown in Fig. 2. These species are found to coexist only in the Mediterranean region, and in South Africa. Species diversity is highest in South Africa. Giant limpets are distributed worldwide (see Fig. 2). The Pacific Ocean hosts representatives of giant limpets belonging to all genera, with the exception of *Cymbula* and *Patella*. Two species are restricted to islands: (1) *Cellana talcosa*, which is found throughout

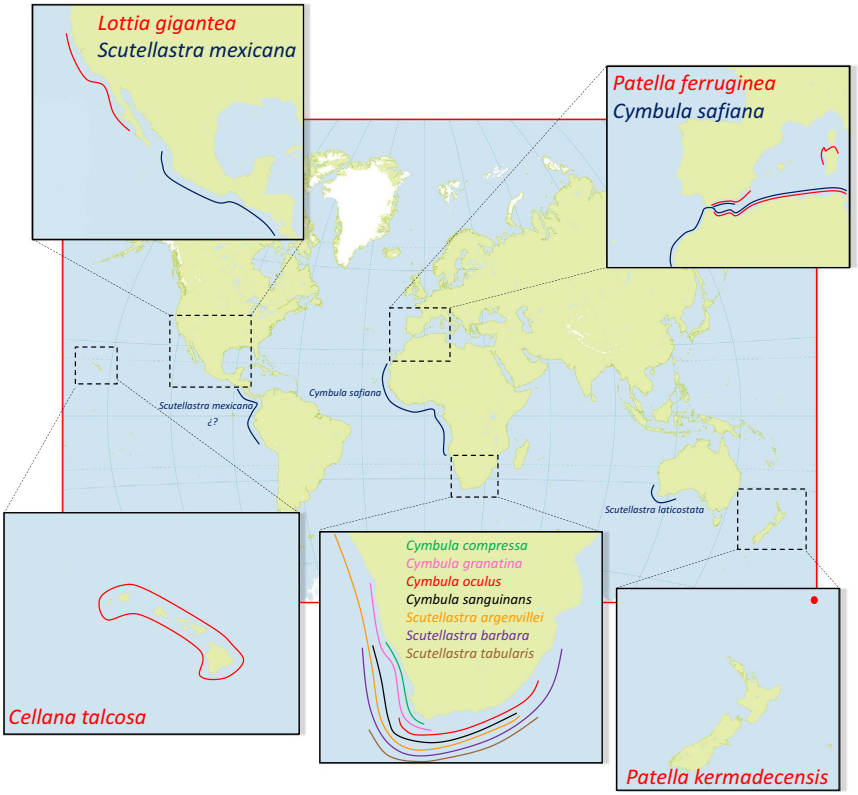

Fig. 2 Map of the giant limpet distribution. Ranges of geographical distribution were obtained from Bird et al. (2007) (*Cellana talcosa*); Powell (1973) (*Cymbula compressa* and *C. miniata*); Christiaens (1973) and Boukhicha et al. (2015) (*Cymbula safiana*); Powell (1973) and Whittington-Jones (1997) (*Cymbula oculus*); Fenberg and Rivadeneira (2011) (*Lottia gigantea*); Espinosa et al. (2014) (*Patella ferruginea*); Bustamante et al. (1995) (*Scutellastra argenvillei* and *Cymbula granatina*); Powell (1973) and Ridgway et al. (1999) (*Scutellastra barbara*); Fleming (1973) and Powell (1973) (*Scutellastra kermadecensis*); Powell (1973) (*Scutellastra laticostata*); Powell (1973) and Ríos-Jara et al. (2006) (*Scutellastra mexicana*); and Powell (1973) (*Scutellastra tabularis*).

the Hawaiian Islands and is locally endemic along with the species *C. exarata* and *C. sandwicensis*, and (2) *Scutellastra kermadecensis*, found only in the Kermadec Islands, a group of 11 islands and islets located 750 km northeast of New Zealand and that make up the largest and most isolated marine reserve in that country (Eddy, 2009). *Scutellastra kermadecensis* is often considered to be endemic to the Kermadec Islands; however, fossil evidence of this species along the New Zealand coasts suggests that it is secondary endemism (meaning that the present distribution of *S. kermadecensis* is restricted to these islands due to the disappearance of the species from its original range) (Fleming, 1973).

Species diversity is highest in South Africa. More than half of the giant limpet species are concentrated along the South African coasts. The bearded limpet, *Scutellastra barbara*, is distributed throughout the east and west coasts of South Africa, between Port Nolloth and Umpangazi (Koch, 1949; Powell, 1973).

Most of the giant limpets found in South Africa belong to the genus *Cymbula*. The granite limpet, *Cymbula granatina* (Fig. 3), is present from Cape Frio in the west, to Cape Infanta in the south, coinciding with the distribution of the kelp *Laminaria pallida*, which forms the main part of their diet. However, other populations are located between Tweepad and the Olifants River (Bustamante et al., 1995). *Cymbula oculus* ranges along the eastern coast (characterised as having warmer waters) as far south as the Cape of Good Hope (Branch, 1971; Whittington-Jones, 1997). We note that *C. sanguinans*, as was also the case for *C. compressa*, was included in the complex *Patella miniata*, prior being designated as a species in the genus *Cymbula* (Ridgway et al., 2000). This highly complicates the task of discerning which of the previous studies refer to *C. miniata*, and which refer to *C. sanguinans*, as they are closely related species. *Cymbula sanguinans* is distributed from Port Nolloth in the west, to Qolora on the Transkei coast in the east (Christiaens, 1973; Powell, 1973). In their work, these authors refer to *C. miniata* as a species that can reach 117 mm, but because *C. miniata* does not surpass a size of 100 mm (G.M. Branch, University of Cape Town, personal communication, 22 July 2015), we can only assume that the authors were describing the distribution range of *C. sanguinans*.

The only giant species of the genus *Cymbula* that is present outside of South African coasts is the Black, or Safi limpet, *Cymbula safiana* (Fig. 3). This species is currently found between Angola (Gofas et al., 1988) and Tunisia (Boukhicha et al., 2015), and has been recorded in the Macaronesian archipelagos of the Azores (Droüet, 1858), the Canary Islands (Nordsieck and García-Talavera, 1979), and Cape Verde (Rolán, 2005). It is remarkable that individuals present on the Atlantic coasts rarely grow to be more than 6 cm (Poppe and Goto, 1991), but individuals located in the Mediterranean region

Fig. 3 Giant limpets of the genus *Cymbula*: *Cymbula compressa* (photograph, C. Griffiths), *Cymbula granatina* (photograph, G.M. Branch), *Cymbula oculus* (photograph, G.W. Maneveldt) and *Cymbula safiana* (photograph, G.A. Rivera-Ingraham).

reach significantly larger sizes (Rivera-Ingraham et al., 2011b). It is in these areas that its distribution overlaps with that of the highly endangered ferruginean limpet, *Patella ferruginea*. This species is endemic to the western Mediterranean, where it has been exploited since the Quaternary (Espinosa et al., 2009a). Along with *C. safiana*, it is the largest limpet species in the Mediterranean, and its distribution ranges from the Strait of Gibraltar to the Strait of Sicily. However, the largest populations are found in certain

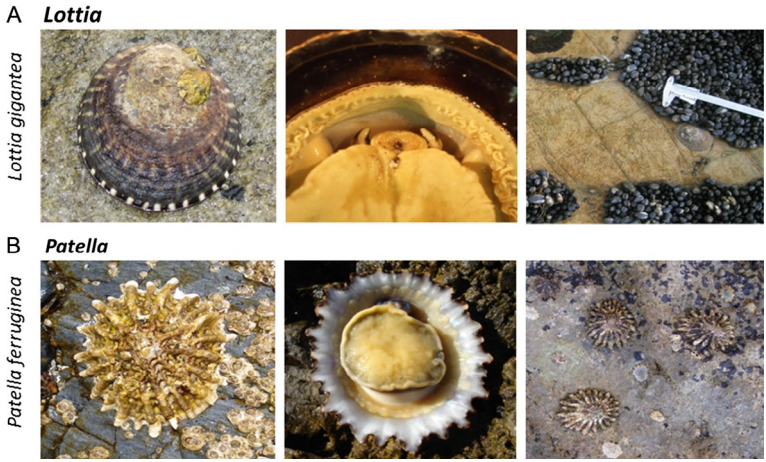

Fig. 4 Giant limpets: (A) Genus *Lottia*: *Lottia gigantea* (photographs of shell and habitat, P. Fenberg; photograph of foot, D. Lindberg). (B) Genus *Patella*: *Patella ferruginea* (photographs, G.A. Rivera-Ingraham).

North African enclaves (Ceuta, Melilla, Chafarinas Islands, Habibas Islands, and the island of Zembra), having practically disappeared from the European coasts, with the exception of small populations in Andalusia (Southern Iberian Peninsula) and islands such as Corsica and Sardinia (Espinosa et al., 2014).

On the continental Pacific coast, the owl limpet, *Lottia gigantea* is probably the best studied giant limpet (Fig. 4A). *Lottia gigantea* is distributed all along the rocky coasts from northern Washington to the coasts of Baja California, Mexico (Fenberg and Rivadeneira, 2011; Morris et al., 1980). They are particularly abundant south of San Francisco (Harley and Rogers-Bennett, 2004) and have been reported to reach their greatest abundances in San Diego, California (Fenberg and Rivadeneira, 2011). In these areas, *Scutellastra mexicana* is also present. It is, by far, the largest limpet in the world, and while once thought to be extinct, some populations are still present along the coasts of mainland Mexico (see Bastida-Zavala et al., 2013; Esqueda et al., 2000; Ríos-Jara et al., 2006; Riosmena-Rodríguez et al., 2005). The largest populations are apparently in certain inaccessible areas, such as the Islas Marías (J.L. Carballo, Universidad Autónoma de México, personal communication, 2010), a group of three islands located in the Pacific Ocean, approximately 100 km from the Mexican mainland (Fig. 5).

3.2 Preferred Substrate

There is a general lack of knowledge regarding substrate preference in giant limpets, though there is some information on the ecology of several species (see Table 2A). In some cases, substrate selection is apparently associated with

Fig. 5 Giant limpets of the genus *Scutellastra*: *Scutellastra argenvillei* (photograph of shell, C. Griffiths, photographs of the animals in their habitat, G.M. Branch), *Scutellastra kermadecensis* (photographs, M. Francis), *Scutellastra mexicana* (photographs, J. L. Carballo) and *Scutellastra tabularis* (photograph, C. Griffiths).

Table 2A Comparison of Distribution and Life History Traits Among Giant Limpet Species

	Distribution			Life-History Traits				
Species (Common Name)	**Geographic Range**	**Preferred Substrate**	**Intertidal Distribution/ Exposure to Wave Action**	**Maximum Density (individual per m^2)**	**Maximum Size (mm)**	**Growth Rate (mm/ year)**	**Seasonal Effects on Growth Rate**	**Longevity (Years)**
Cellana talcosa (Opihi)	Hawaiian Islands	Basaltic rocks	From the calcareous algal zone to shallow subtidal (<10 m) (Kay et al., 2006; Magruder and Kay, 1983)/high (Bird, 2006)	n.a.	1065 (Powell, 1973); 120 (Kay, 1979)	Decreases with size (Kay et al., 2006). Estimation of 51 mm/year	n.a.	n.a.
Cymbula compressa (kelp limpet)	South Africa (Port Nolloth to Danger Point) (Powell, 1973)	Stipes of *Ecklonia maxima* (Branch, 1975a; Maneveldt and Frans, 2001)	Infratidal (Langejans et al., 2011), sublittoral (Choat and Black, 1979)/n.a.	1 (per stipe) (Branch, 1974a)	1175 (Powell, 1973); 130 (Christiaens, 1973)	n.a.		Generally matches algal longevity (Muñoz and Santelices, 1989)
Cymbula granatina (granite limpet)	South Africa (Cape Frio to west of Cape Infanta) (Bustamante et al., 1995)		Mid–low intertidal/low (Bustamante et al., 1995)	288 (Bustamante et al., 1995)	>100 (G.M. Branch, personal communication, 2015)	Decreases with age. Thirty-five for first year (Branch, 1974a). Sixteen for the first year in areas of high density, reaching 40 in exploited areas (Eekhout et al., 1992)		5–6 (Branch, 1974a)

Cymbula oculus (goat's eye limpet)	Eastern coast of South Africa (Branch, 1971; Whittington-Jones, 1997)	Flat surfaces (Branch, 1976)	n.a./low (Branch and Marsh, 1978)	1 (Branch et al., 1988)	120 (Jerardino and Navarro, 2008; Powell, 1973)	Decreases with age, 56 for first year, 14 for second, 9 for third year (Branch, 1974a)	No (Branch, 1974a,b)	2–3 (Branch, 1974a)
Cymbula safiana (black limpet)	Africa (Angola to Tunisia) (Gofas et al., 1988) and South of Spain (Rivera-Ingraham, 2010)	Flat and smooth substrates (Rivera-Ingraham et al., 2011c)	Intertidal to shallow subtidal (<5 m) (Renault and Moueza, 1971)/high (G.A. Rivera-Ingraham, personal observation, 2010)	20[a] (Rivera-Ingraham et al., 2011b)	133 (Rivera-Ingraham, 2010; Rivera-Ingraham et al., 2011b)	Decreases with age, e.g., 30.5 for animals <4 mm and 3.36 for animals >80 mm (Rivera-Ingraham, 2010)	Higher in summer and lower in winter months (Rivera-Ingraham, 2010)	Unknown
Cymbula sanguinans	South Africa (Port Nolloth to Qolora) (Christiaens, 1973; Powell, 1973)	n.a.	n.a./exposed rocky shores (Lasiak, 2006)	n.a.	117[a] (Christiaens, 1973; Powell, 1973)	n.a.	n.a.	n.a.
Lottia gigantea (owl limpet)	Pacific coast of North America (Washington State and Baja California) (Fenberg and Rivadeneira, 2011; Morris et al., 1980)	Vertical substrates (Denny and Blanchette, 2000)	Upper to mid-intertidal zones (Kido and Murray, 2003)/ n.a.	>64[a] (Kido and Murray, 2003)	>100 (Fenberg and Rivadeneira, 2011); 120 (Erlandson et al., 2011)	12.1 (Kido and Murray, 2003)	Greatest during spring and Early summer (Kido and Murray, 2003)	8.5 (Kido and Murray, 2003)

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Table 2A Comparison of Distribution and Life History Traits Among Giant Limpet Species—cont'd

	Distribution			Life-History Traits				
Species (Common Name)	**Geographic Range**	**Preferred Substrate**	**Intertidal Distribution/ Exposure to Wave Action**	**Maximum Density (individual per m^2)**	**Maximum Size (mm)**	**Growth Rate (mm/ year)**	**Seasonal Effects on Growth Rate**	**Longevity (Years)**
Patella ferruginea (ferruginean limpet)	Western Mediterranean (notably north African coasts) (Espinosa et al., 2014)	Preferentially granitic rocks (Guerra-García et al., 2004) with irregular surfaces (Rivera-Ingraham et al., 2011a)	Intertidal/ medium to high (Guerra-García et al., 2004)	35[a] (Rivera-Ingraham et al., 2011a)	105 (Espinosa et al., 2009a); 107 (Rivera-Ingraham et al., 2011a)	Decreases with age, e.g., 13.9 mm for animals <25 mm or 5 mm for animals >50 mm (Rivera-Ingraham et al., 2011f)	Lowest during autumn and highest during summer (Rivera-Ingraham et al., 2011f)	>35 (Espinosa et al., 2008a)
Scutellastra argenvillei (argenvillei limpet)	Atlantic coasts of South Africa (Eekhout et al., 1992)	n.a.	n.a./moderate (Bustamante et al., 1995)	388 (Bustamante et al., 1995)	100 (Kensley, 1973); 104 (Kilburn and Rippey, 1982)	Decreases with age: average of 16 during the first year of growth (maximum values of 23–28) (Eekhout et al., 1992)		20 (Eekhout et al., 1992)
Scutellastra barbara (bearded limpet)	South Africa (Port Nolloth to Umpangazi) (Koch, 1949; Powell, 1973)	Juveniles: *Haliotis midae* shells (Branch, 1975a)	Intertidal (coralline and *Balanus* belts) (Branch, 1971)/ n.a.	Low densities (Branch, 1975a)	100 (Kensley, 1973); 100 (Kilburn and Rippey, 1982)	n.a.	n.a.	n.a.

Scutellastra kermadecensis	Kermadec Islands	n.a.	Lower intertidal and shallow subtidal (<3 m) (Schiel et al., 1986)	21 (Schiel et al., 1986)	>130 (Schiel et al., 1986); 136 (Lindberg, 2007); 174 (Powell, 1973)	n.a.	n.a.	n.a.
Scutellastra laticostata	Australia (Powell, 1973)	Rocky coasts	Lower intertidal and shallow subtidal/high (Scheibling and Black, 1993)	n.a.	107 (Christiaens, 1973); >110 (Powell, 1973)	n.a.	n.a.	n.a.
Scutellastra mexicana (Mexican limpet)	Pacific coast of Mexico (e.g. Bastida-Zavala et al., 2013; Esqueda et al., 2000; Ríos-Jara et al., 2006; Riosmena-Rodríguez et al., 2005) and Islas Marías (J.L. Carballo, personal communication, 2010)	n.a.	Lower intertidal (Esqueda et al., 2000) to shallow subtidal (J.L. Carballo, personal communication, 2010)/high (Esqueda et al., 2000)	6 (Esqueda et al., 2000)	158 (Lindberg, 2007); 350 (Christiaens, 1973); 355 (Powell, 1973)	n.a.	n.a.	n.a.
Scutellastra tabularis	South Africa (http://www.marinespecies.org)	Substrates covered by *Ralfsia* (Branch, 1975a)	n.a.	n.a.	127 (Lindberg, 2007); 1475 (Jerardino and Navarro, 2008)	n.a.	n.a.	n.a.

[a]Data per linear metre of coastline.
n.a., to the authors' knowledge, no data are available.

access to food sources. *Cymbula compressa* constitutes a remarkable example of this, since this nonmigratory species lives and feeds off stipes of *Laminaria* (Lindberg, 2007) or *Ecklonia maxima* (Branch, 1975b; Maneveldt and Frans, 2001). *Scutellastra tabularis*, although they display more generalist feeding patterns, shows a clear preference for substrates covered by *Ralfsia* (Branch, 1975b). The typical pattern is that, for more generalist grazers, physical substrate parameters are stronger factors in determining substrate preference. Among them, substrate microtopography is probably one of the key parameters determining limpet recruitment (Martins et al., 2010). *Cymbula oculus*, for example, colonises relatively flat surfaces, making it easier for individuals to traverse significant rock surfaces (Branch, 1976). Conversely, other generalists such as *Patella ferruginea* show a preference for heterogeneous and irregular substrates (Rivera-Ingraham et al., 2011a). Such topographically complex substrates favour recruitment processes, but in homing species, as for *P. ferruginea*, has a profound effect on the maximum size individuals can attain (Rivera-Ingraham et al., 2011a). This is the case for *Cymbula safiana*, in which juveniles recruit in the upper intertidal zone on substrates with a medium to high rugosity (similar to *P. ferruginea*), whereas the adults are found separated from juveniles and are consistently located near the lower intertidal limits on significantly smoother surfaces (Rivera-Ingraham et al., 2011b,c).

As for many benthic invertebrates, substrate inclination is another important factor determining recruitment. Though it does not affect some giant limpet species such as *P. ferruginea* (Rivera-Ingraham et al., 2011e), other species (such as *Lottia gigantea*) are frequently found on vertical substrates (Denny and Blanchette, 2000), and this physical orientation could aid in flushing waste away from animals (Abbott, 1956).

Specific adaptations are also found in some species in connection with substrate preferences. Hence, *Scutellastra barbara* juveniles commonly colonise *Haliotis midae* shells, which has been interpreted as a behaviour directed towards protection against other herbivores. *Cellana talcosa* has been observed to have a preference for basaltic rocks (Bird et al., 2007), though the reasons for this preference have not been studied.

We conclude that there are no common patterns regarding giant limpet substrate preference, and that traits are as diverse as they are for smaller-sized limpet species.

3.3 Intertidal Distribution/Exposure to Wave Action

Among the limpet species considered in this review, some show a wide bathymetric distribution. *Cellana talcosa* is distributed from lower intertidal and shallow subtidal areas to a maximum depth of 10 m (Magruder and Kay, 1983),

making them accessible to pelagic predators (Bird, 2011). *Cymbula safiana* also has a wide vertical distribution, appearing from the upper intertidal zones (recruitment area) to depths of up to 5 m (Renault and Moueza, 1971; Rivera-Ingraham et al., 2011b,c). However, there are other species with a much more restricted vertical distribution, such as *Scutellastra kermadecensis* (found from lower intertidal to a maximum of 3 m deep) (Schiel et al., 1986), *S. laticostata*, *S. mexicana* and *Patella ferruginea*, the latter being strictly confined the middle to upper intertidal zone (Espinosa, 2006; Rivera-Ingraham, 2010).

The presence of limpets in the intertidal environment means that they are exposed to wave action. Limpet tenacity in adhering to the substrate can largely determine distribution in relation to wave exposure. In general, giant limpets exhibit a high tenacity that allows them to settle on wave-beaten shores. Species such as *C. talcosa* (Bird, 2011), *S. kermadecensis* (M. Francis, National Institute of Water and Atmospheric Research, New Zealand, personal communication, 2015), *S. laticostata* or *P. ferruginea* are known to prefer wave-beaten rocky coasts (Espinosa, 2006; Scheibling and Black, 1993). However, the highly mobile *C. oculus* is an exception and holds less tenaciously to substrates. It is likely more adapted to areas with less wave action (Branch and Marsh, 1978).

4. LIFE HISTORY TRAITS

4.1 Density

Average densities of giant limpet species are extremely variable (see Table 2A). While many species occur at very low densities (<1 individual per m^2), others achieve remarkable values. *Scutellastra argenvillei* and *Cymbula granatina* can reach densities of 338 and 288 individuals per m^2, respectively (Bustamante et al., 1995). Thus, these species constitute two of the most important South African intertidal invertebrate grazers (Eekhout et al., 1992). Interestingly, both of these species feed on tide-drifting kelp (Bustamante et al., 1995), a factor that may explain the high biomasses that these species can attain within their habitats (see Section 6).

4.2 Size

Variability in giant limpet size is not particularly high, considering that the majority of the species are only slightly larger than 10 cm (see Table 2A). Nevertheless, some species, such as *Scutellastra kermadecensis* and *S. mexicana*, represent extreme cases of gigantism and attain lengths of 174 and 355 mm, respectively.

4.3 Growth Rates

As noted above, many giant limpet species have restricted distributions or are present in areas that are difficult to access, which often makes monitoring tasks particularly difficult. There are nevertheless data on growth rates for several species. Growth rates can attain high values (>50 mm/year) for some species (e.g. *Cellana talcosa*, Kay et al., 2006; *Cymbula oculus*, Branch, 1974a). However, as for any other organism, these values will be clearly impacted by factors affecting metabolic rates. Age is a clear example of this, and thus, limpet growth rates decrease with increasing size. Giant species are, without surprise, not an exception, but for some species such as *Cymbula safiana*, this decrease between recruits and adults can be as large as one order of magnitude (Rivera-Ingraham, 2010).

Most of the physiological studies of giant limpets have been undertaken on *C. oculus*. Analyses have demonstrated the metabolic rate of this species to be relatively high, leading it to have growth rates between 30 and 60 mm per year (Branch et al., 1988). Branch et al. (1988) also showed that when individuals endure periods of fasting or inhabit zones with a high density of individuals, *C. oculus* significantly reduces its metabolic rate (recorded as a reduction in respiration rates), which causes a decrease in growth rates. Thus, Branch et al. (1988) described this species as metabolically "exploitive" as opposed to other "conservationist" species that have lower basal metabolic rates and are adapted to living at high population densities. Water temperature (resulting from either geographic location or from season) can also be a key factor influencing metabolic and growth rates. Populations of *Patella ferruginea*, located in warmer water areas, showed higher growth rates than those located in colder waters, and growth rates were higher in summer (Rivera-Ingraham et al., 2011f).

Branch (1979a) analysed the energetics of *Cymbula granatina*, showing how this species has a clear ontogenetic adaptation in terms of respiration rates: smaller animals, which are present in the lowest areas of the shore and are thus immersed for longer periods than they are exposed, respire faster in water than they do in air; on the other hand, larger animals, which are located higher in the intertidal, and therefore exposed more time than they are submerged, show the opposite pattern. Moreover, Branch (1979a) showed that the basal respiration rates of *C. granatina* were higher than those of other species occurring in South Africa (e.g. *Scutellastra cochlear*, *Cymbula granularis* or *C. oculus*). Given that there was sufficient food availability, the high basal metabolic rate of *C. granatina* was interpreted by Branch (1979a), to be part of an exploitative strategy in which high growth rates and high

reproductive output lead to the enormous biomasses *C. granatina* can attain. However, and as it can be inferred from Table 2A, there is a general trend in giant limpets for higher growth rates to be associated with lower longevity.

4.4 Longevity

To date, there are no reliable methods for ageing limpets (e.g. using shell analyses). Thus, estimating longevity implies going through the extremely time-consuming task of monitoring marked specimens through long periods of time to obtain reliable growth rate data for developing accurate age estimates. For this reason, longevity estimates are only available for relatively well-studied species (see Table 2A). We can differentiate between relatively short-lived species, which includes *Lottia gigantea* (8.5 years), the goat limpet *Cymbula oculus* (2–3 years) and longer-lived species (e.g. *Patella ferruginea*, living over 30 years). It is however surprising that *Cymbula granatina* and *Scutellastra argenvillei*, which are similar species in terms of distribution, density and feeding habits, largely differ in life expectancy (5–6 years and 20 years, respectively). We may hypothesise that, as it for many animal species (Pörtner, 2006), high longevity is associated with extreme-sized limpets, though so far, this is not supported by current scientific data.

5. REPRODUCTION

5.1 Size of Sexual Maturation

Size of sexual maturation is highly relevant information, especially when considering exploited populations and when needing to develop conservation strategies and/or management programmes. Human exploitation is unregulated for many of the species that we are considering here (see Section 7), and there is no information on size at sexual maturity for more than half of them. Three species appear to reach sexual maturation around 20 mm in length (*Cymbula safiana*, *Lottia gigantea* and *Patella ferruginea*), whereas three other species achieve maturity around 40 mm in length (*Cellana talcosa*, *Cymbula granatina* and *Scutellastra argenvillei*) (see Table 2B). Reported growth rates for these species (see Table 2A) indicate that, in all cases, these sizes are achieved within the first or second year of life. It seems an advantage to reach maturation at lower sizes in order to increase reproductive output and dispersion capabilities. However, among limpets with an early age of sexual maturity, *Patella ferruginea* shows a clear regression in its geographical range (Espinosa et al., 2014).

Table 2B Comparison of Reproductive and Behavioural Traits Among Giant Limpet Species

	Reproduction					Behaviour			
Species (Common Name)	**Size at Attainment of Sexual Maturity (mm)**	**Spawning**	**Reproductive Strategy/Size at Sex Change (cm)**	**Sex Ratio (F:M)**	**Diet**	**Homing/ Algal Gardener**	**Territorialism/ Aggressive Behaviour**	**Adult-Juvenile Segregation**	**Migration**
Cellana talcosa (Opihi)	35–40 (Kay et al., 2006); 45 (Bird, 2011)	1 Summer event: May–September; 1 winter event: November–January (Kay et al., 2006)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cymbula compressa (kelp limpet)	Unknown (Muñoz and Santelices, 1989)	Possibly winter (Branch, 1974b)	n.a.	n.a.	Superficial epithelial layers of kelp (Heller, 2015)	Yes (Choat and Black, 1979; Lindberg, 2007)/n.a.	Yes/yes (Choat and Black, 1979; Lindberg, 2007)	Yes (Heller, 2015)	Yes, as it ages it migrates down the stipes of kelp (Heller, 2015)
Cymbula granatina (granite limpet)	40–50 (Eekhout et al., 1992)	Main event in June and minor event in December (Branch, 1974b)	Protandric hermaphrodite (Branch, 1974b)/n.a.	0.82 (Branch, 1974b)	Generalist grazer, eating algae, diatoms, lichens or spume (Branch, 1974b) but also drifting macroalgae such as *Laminaria pallida* (Bustamante et al., 1995) and *Ecklonia maxima* (Heller, 2015)	Poorly or temporarily developed (Branch, 1975b)/n.a.	No (Branch, 1975b)/n.a.	Yes (juvenile present in the lower areas) (Branch, 1979a)	Yes, larger animals occurring higher (Branch, 1979a)

Cymbula oculus (goat's eye limpet)	n.a.	Main spawning in September Partial spawning in April (Branch, 1974b)	Protandric hermaphrodite (Branch, 1974b)/n.a.	0.43 (Branch, 1974b)	Generalist grazer (Branch, 1976) eating algae, diatoms, lichens or spume (Branch, 1974b)	No (Branch, 1975a)/n.a.	No (Branch et al., 1988)/yes (Branch, 1979b; Whittington-Jones, 1997)	Yes (juvenile in irregular surfaces) (Branch and Newell, 1978)	Yes (adults towards smoother and higher surfaces) (Branch, 1975b; Branch and Newell, 1978)
Cymbula safiana (black limpet)	<20 (Rivera-Ingraham, 2010)	One main event in February, one minor event in May (Rivera-Ingraham, 2010)	Protandric hermaphrodite (Rivera-Ingraham, 2010)/ >3 cm (Rivera-Ingraham, 2010)	0.49 (G.A. Rivera-Ingraham, personal observation, 2010)	Algal biofilm (Rivera-Ingraham, 2010); generalist (Burgos-Rubio et al., 2015)	Yes (G.A. Rivera-Ingraham, personal observation, 2010)/algal gardener (Branch, 1975b)	Yes (Rivera-Ingraham, 2010)/n.a.	Yes (Rivera-Ingraham et al., 2011c)	Yes (adults towards smoother and lower surfaces) (Rivera-Ingraham et al., 2011c)
Cymbula sanguinans	n.a.	n.a.	n.a.	n.a.	*Hildenbrandia rubra* (G.M. Branch, personal communication, 22 July 2015)	n.a.	Yes/yes (G.M. Branch, personal communication, 22 July 2015)	n.a.	n.a.
Lottia gigantea (owl limpet)	<21 (Wright and Lindberg, 1982)	1 Winter event: January–February (Daly, 1975)	Protandric hermaphrodite (Wright and Lindberg, 1982)/ according to social environment (Wright, 1989)	0.38–0.5 (Kido and Murray, 2003)	Algal biofilm within territory (Stimson, 1973)	Yes (Fisher, 1904; Wells, 1917)/n.a.	Yes (see Schroeder, 2011; Shanks, 2002)/ yes (Shanks, 2002)	n.a.	n.a.

Continued

Table 2B Comparison of Reproductive and Behavioural Traits Among Giant Limpet Species—cont'd

	Reproduction					Behaviour			
Species (Common Name)	**Size at Attainment of Sexual Maturity (mm)**	**Spawning**	**Reproductive Strategy/Size at Sex Change (cm)**	**Sex Ratio (F:M)**	**Diet**	**Homing/ Algal Gardener**	**Territorialism/ Aggressive Behaviour**	**Adult-Juvenile Segregation**	**Migration**
Patella ferruginea (ferruginean limpet)	20–30 (Espinosa et al., 2006a)	1 Event during November–December (Frenkiel, 1975)	Protandric hermaphrodite (Espinosa et al., 2009b; Frenkiel, 1975)/ according to social environment (>4 cm) (Rivera-Ingraham et al., 2011d)	0.04–0.31 (Rivera-Ingraham et al., 2011d)	Generalist (Burgos-Rubio et al., 2015)	Yes (Espinosa et al., 2008b)/ no (Espinosa et al., 2008b)	No/no	No (gregarious pattern) (Espinosa et al., 2006b; Rivera-Ingraham et al., 2011e)	No
Scutellastra argenvillei (argenvillei limpet)	40–50 (Eekhout et al., 1992)	June–July (Branch, 1974b). One single winter spawning according to Eekhout et al. (1992)	Unknown/ little size difference between sexes (Branch, 1974b; Eekhout et al., 1992)	0.87 (Branch, 1974b)	Diatoms and kelp (Bustamante et al., 1995; Heller, 2015)	Well-developed scar (Branch, 1971)/n.a.	n.a.	Yes (Vermeij, 1972)	Yes (adults towards lower levels) (Stephenson et al., 1940)

Scutellastra barbara (bearded limpet)	n.a.	May–June (Branch, 1974b)	n.a./little size difference between sexes (Branch, 1974b)	1.18 (Branch, 1974b)	n.a.	Poorly defined scar often random orientation on scar (Branch, 1971)/n.a.	Yes (Branch, 1971; Branch et al., 1992)/n.a.	No (Branch, 1975a)	n.a.
Scutellastra kermadecensis	n.a.	n.a.	Protandric hermaphrodite (Creese et al., 1990)/n.a.	0.33 (Creese et al., 1990)	n.a.	Yes (Schiel et al., 1986)/ n.a.	n.a.	n.a.	n.a.
Scutellastra laticostata	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Yes (Lindberg, 2007)/yes	n.a.	No (Scheibling and Black, 1993)
Scutellastra tabularis	n.a.	n.a.	n.a.	n.a.	*Ralfsia* algae (Branch, 1975a)	Yes (Branch, 1975a)/algal gardener (Branch, 1975b)	Yes (Branch, 1975b)/yes (Branch, 1975b)	n.a.	n.a.

Note: *Scutellastra mexicana* was not included here because no information was available.
n.a., to the authors' knowledge, no data are available.

5.2 Spawning

In the marine environment, the condition of being an external spawner determines sperm concentration as a limiting factor in fertilisation, particularly for animals living in habitats with strong water flow (Levitan and Petersen, 1995). For some species, individuals separated by only a metre or less are unable to cross-fertilise (Denny and Shibata, 1989; Grosberg, 1991). For broadcast spawners, the turbulent ocean environment into which the gametes are released can strongly influence fertilisation rates. Fluid motion can transport and mix the gametes together, facilitating commingling between sperm and eggs, or it can have a detrimental effect by causing rapid dilution and prevention of sperm–egg encounters (Babcock et al., 1994; Denny and Shibata, 1989; Pennington, 1985).

The time of the year at which spawning occurs may be of additional importance. According to Branch (1974a), spawning in winter may be an advantage to maximise larval and recruit survival. In fact, all the giant limpets considered here spawn during winter, although some species showed secondary spawning events throughout the year (see Table 2B). However, these species show significant variability regarding the time required for larval development. While for *Cellana talcosa* the larvae are able to settle three to four days after fertilisation (Bird et al., 2007), in species such as *Patella ferruginea* this period can extend to as much as 15 days (Guallart et al., 2013a).

5.3 Reproduction Strategy/Size at Sex Change

Within the order Patellogastropoda, protandry (sequential hermaphroditism, maturing as a male and changing sex to female during the life history) is a common reproductive strategy (Branch, 1981). The available information supports that this is indeed also the case for giant species. In addition, intertidal and shallow subtidal are patchy habitats with strong ecological gradients at small scales (Little and Kitching, 1996). According to Allsop (2003), the general pattern is that such small patchily distributed habitats favour more sex change. In contrast, larger and more homogeneous habitats result in less sex change. From this perspective, giant limpets that live in such patchy habitats are expected to show high degree of sex change. There are, however, some particularities to some of the giant species we are discussing. For example, *Patella ferruginea* shows also a reversal sex change and females can turn again to males (Guallart et al., 2013b). Species may also differ in how this sex change occurs. While for some species, such as *P. ferruginea*, this process occurs during reproductive resting states (Frenkiel, 1975; Renault and Moueza, 1971), for others such as *Cymbula safiana*, which

has mature gonads throughout the year, the process passes through a phase of simultaneous hermaphroditism (Rivera-Ingraham, 2010).

Some giant limpets, such as *Cymbula oculus*, have a relatively fixed timing of sex change (Branch and Odendaal, 2003), while in others the size at which sex change occurs is highly variable and determined by external factors. Density and sex ratio have been shown to influence the timing of sex change within the order (see Borges et al., 2016). Hermaphrodites can respond to group size and adjust their sexual investment accordingly (Schleicherova et al., 2005). In general, protandric limpets change sex at smaller sizes under depletion of females and lower densities, and this has been experimentally shown, by means of field manipulations, in species such as *Patella vulgata* (Borges et al., 2016). Within giant limpets, to our knowledge, only evidence of social control of sex change has been observed, though indirectly, in *P. ferruginea* (Rivera-Ingraham et al., 2011d), which explains the high variability of the size at which sex change occurs in this species. However, there is a severe lack of information regarding the cues allowing hermaphrodites to assess their social environment (Schleicherova et al., 2005). The field of chemical ecology will likely provide interesting findings that will allow a better understanding of how species perceive their environment, setting the basis for addressing conservation and management challenges regarding giant endangered limpet species such as *P. ferruginea*.

5.4 Sex Ratio

According to theoretical predictions, sex ratio is biased towards the sex that individuals reach upon first reproductive maturation (see Charnov, 1982). This has been demonstrated for fish and crustaceans (Allsop, 2003), and the results summarised in Table 2B show that once again giant limpets (as is true of limpets in general) are not an exception. McCarthy et al. (2008) pointed out that higher male to female ratios are common in limpets. Giant limpets, particularly larger individuals, are especially prone to human collection (see Section 7). Thus, it seems interesting to note that, in situations where sex ratios are strongly biased towards males, the viability of species suffering from strong harvesting pressure (such as *Patella ferruginea*) may be especially threatened (Rivera-Ingraham et al., 2011d).

6. BEHAVIOUR

6.1 Diet

Although limpet foraging has been the topic of extensive scientific research, information about diet composition is scarce, probably due to the difficulty

of the proper identification of gut contents (Burgos-Rubio et al., 2015). Branch (1981) pointed out that most limpets are generalist grazers, feeding on any microflora or detritus available on the rock surface. Yet, some species specialise on particular macroalgae. Even though most of the species analysed are generalist grazers, others show trophic dependence with specific algae such as *Cymbula compressa* on kelp, *C. sanguinans* on *Hildenbrandia rubra*, or *Scutellastra tabularis* on *Ralfsia* sp.

Some of the most remarkable feeding behaviours in Giant Limpets are exhibited by some of the species occurring in South Africa. Bustamante et al. (1995) described how *Cymbula granatina* lifts its shell ("mushrooming" behaviour) and the anterior part of its foot, while secreting large amounts of mucus that enables it to trap the drifting kelp fronds on which it feeds. *Scutellastra argenvillei*, at sizes larger than 40 mm, also feeds on drifting kelp. In *C. granatina*, limpets acquire a mushrooming posture, and when a kelp frond is washed under the limpet shell and touches the foot, the animal responds by very quickly dropping the shell downwards in order to pin the frond (Bustamante et al., 1995). The characteristic serrated edge of their shells as well as the presence of "teeth", along with wave action, helps to cut the kelp fronds, facilitating their consumption. However, it is of special interest that this species shows "collective feeding", and several animals can feed from a kelp frond trapped by one individual. This lack of intraspecific competition likely permits the enormous densities observed for this species, and this is without any density-related effects on shell size as occurs for *C. granatina* (Bustamante et al., 1995) or other intertidal invertebrate grazers.

6.2 Homing/Algal Gardener

Homing to a fixed scar is a well-known behaviour in limpets and is often considered as a mechanism to reduce desiccation (Branch, 1981). If this is indeed the reason, it is logical to think that (1) small specimens would not home, (2) territorial limpets would home rigidly and (3) homing would be more frequently shown by high-shore species and high-shore specimens (Branch, 1981). In general, all of the above are applicable for the giant limpet species considered here. There are, however, some interesting exceptions, including *Patella ferruginea*, wherein individuals with clumped distribution patterns are frequently observed (Espinosa et al., 2006b). In addition, even though they are not territorial, they do display a rigid homing behaviour, even at smaller sizes (Espinosa et al., 2008b). *Cymbula oculus* constitutes another interesting exception, since animals display a rather flexible homing behaviour: whereas juveniles have no home scars and are very mobile, the larger adults have only

temporary home scars, as they never remain in the same place for more than 2 weeks, as described by Branch (1975b). Branch (1975b) even comments on the fact that individuals of this species actively choose their location on the basis of the environmental conditions, so that on hotter days, the individuals are able to actively move from a zone of exposed rock and settle in an intertidal well where they can be partially submerged.

6.3 Territorialism/Aggressive Behaviour

When diet and spatial patterns overlap within a given habitat, competition emerges strongly in limpets (Branch, 1981). In fact, competition can affect the distribution of one or both competitors, where one species intrudes into the centre of the range of another and excludes it from that area. Thus, territoriality introduces another dimension of competition among limpets (Branch, 1981).

Among giant limpets, it is usual to observe territorialism and an aggressive behaviour aimed to defending territory (see Table 2B). For instance, the highly territorial *Cymbula compressa*, living on the stipes of *Laminaria*, behaves aggressively towards intruders that could also feed off the kelp (Lindberg, 2007). Alternatively, *C. oculus*, which is not a territorial species and is found in very low densities (approximately 1 individual per m^2) (Branch et al., 1988), shows aggressive behaviour towards possible predators. Branch (1979b) showed that, although the smallest individuals of this species (<30 mm) usually flee quickly from predators (at speeds of 3–7 mm/s), adults react quite differently. In the presence of a predator such as the whelk *Thais dubia*, individuals raise the section of their shell closest to the predator, letting it fall back down, often cutting off part of the gastropod's foot. Another interesting detail about *Cymbula oculus* was highlighted by Whittington-Jones (1997), who suggested a possible symbiotic relationship between this species and the red algae *Gelidium pristoides*, which preferentially colonises the shell of this limpet. The aggressive behaviour of *C. oculus* prevents other grazers, with the exception of juvenile *Scutellastra longicosta*, from feeding off these algae on their shell.

Territorialism is not unusual in giant limpets: the limited space in the intertidal habitat together with the high densities of these relatively enormous individuals could have resulted in the need of acquiring territorial behaviour and the adoption of specific feeding patterns. In order to ensure access to sufficient food resources, the area of the territory is proportional to size of the individual defending it (Rivera-Ingraham, 2010; Stimson, 1970). It is also interesting that, among the territorial species, there are some cases for which the chlorophyll concentration within the areas being defended by

these individuals is greater than in adjacent zones. For this reason, Rivera-Ingraham (2010) hypothesised that this species could be an "algal gardener", as other authors previously suggested for *Lottia gigantea* (see Schroeder, 2011; Shanks, 2002).

Many algal gardeners have aggressive behaviours as does *Cymbula sanguinans*. Being a territorial species, these limpets defend gardens specifically located on the encrusting red algae *Hildenbrandia rubra* (G.M. Branch, University of Cape Town, personal communication, 22 July 2015). Moreover, *L. gigantea* defends its territory fiercely, and when faced by an invasion, *L. gigantea* responds by smashing its shell against the intruder, causing it to detach (Shanks, 2002). The reason for this territorial behaviour is, in the opinion of Stimson (1970), because larger individuals have a bigger radula and need to defend a territory that allows the growth of sufficiently thick microalgae in order to be able to graze properly. Some other algal gardeners, such as *Scutellastra tabularis*, defend their territory aggressively, but only during high tide (Branch, 1975b).

Changes in behaviour due to interspecific competition have also been reported in giant limpets. Due to an invasion in South Africa, of the Mediterranean mussel, *Mytilus galloprovincialis* (which is composed up to 74% of intertidal mussel beds in some areas), *Scutellastra argenvillei* has experienced intense competition for space. This invasion caused large demographic changes in *S. argenvillei* populations, and in areas dominated by *M. galloprovincialis*, the species no longer formed characteristic belts, and in turn showed a patchy distribution (Ruiz Sebastian et al., 2002). This intense mussel–limpet competition has also influenced an important behavioural change in *S. argenvillei*, which showed a reduced homing behaviour as well as an increased mobility, likely due to decreased access to kelp and an increased dependency on alternative food sources. The interaction is significantly facilitated by wave exposure because substrates with intense wave actions are more favourable for *M. galloprovincialis* domination over *S. argenvillei* (Steffani and Branch, 2003a,b). In addition, *Scutellastra barbara* shows high behavioural plasticity, wherein east and south coast individuals are more territorial than those present along the highly productive west coast of South Africa (Branch et al., 1992).

6.4 Adult/Juvenile Segregation

Several giant limpet species show segregation between adults and juveniles, probably as a way to avoid intraspecific competition and to maximise the

survival of recruits in the early stages after recruitment events. Nevertheless, the habitat where juveniles can be found depends on the species considered. For *Cymbula granatina*, juveniles are present in the lower areas of the intertidal, whereas for *Cymbula safiana*, juveniles recruit on higher levels than where adults are located, migrating downwards when they grow (see Table 2B). Independent of tidal height, topography of the substrata is also a main factor for consideration. Among giant limpets, juveniles of both *Cymbula oculus* and *C. safiana* recruit in small structures and irregularities on rocky surfaces, and adults migrate to smoother rocky zones (Branch and Newell, 1978; Rivera-Ingraham et al., 2011c).

6.5 Migration

Branch (1975b) suggested that upward migration reduces intraspecific competition, shifting larger individuals above the range of recruits. In fact, there is an extensive literature that correlates limpet body size with height on the shore, which indicates that this may be due to an upward migration (see Branch, 1981). The giant limpets *Cymbula granatina* and *C. oculus* show this pattern. Downward migration of adults has also been suggested for giant limpets *Cymbula compressa*, *C. safiana* and *Scutellastra argenvillei* (see Table 2B).

7. THREATS

The distribution of intertidal organisms, located in the interface between the aquatic and terrestrial environments, is affected by both biotic (competition, availability of food and predation) and abiotic (notably temperature, salinity and desiccation) factors. These factors are often natural aspects of the marine environment (Raffaelli and Hawkins, 1996), but intertidal organisms are also profoundly affected by anthropogenic sources (see Thompson et al., 2002). Of course, the accessibility of the intertidal ecosystem makes highly susceptible to anthropogenic impacts. In this section, we discuss collection by humans, pollution and habitat fragmentation, which are generally the main threats affecting intertidal species, and we elaborate on how these specifically affect giant limpets.

7.1 Collection

Since prehistoric times, human beings have exploited limpets, which have complemented the diet of many human populations by offering an important quantity of animal biomass for a minimum of capture effort (see Branch

and Odendaal, 2003; Espinosa et al., 2014; Garcia and Smith, 2013; Keough et al., 1993; Lasiak, 1993). Humans preferentially select larger individuals as they are more conspicuous and offer a greater biomass than do small ones, which results in a decrease in the average size of the exploited populations (see Garcia and Smith, 2013). In the case of limpets, which have external fertilisation and higher fertility with increased size, this selective collection can significantly decrease the reproductive capacity of the populations by up to 150 times (Branch and Odendaal, 2003). The reduction in population density of molluscs subjected to harvesting may be as much as 90% (see Keough et al., 1993), which can lead to a marked Allee effect (Branch and Odendaal, 2003; Espinosa et al., 2009a). Because limpets fertilise externally, they must be present at a minimum density to guarantee a sufficient concentration of gametes in the water column that will enable fertilisation (Hockey and Branch, 1994).

Due to their large size, giant limpets have been a preferred target for collection, which has affected some species since the Pleistocene (Espinosa et al., 2014). Moreover, eliminating the larger specimens from these populations seriously compromises their viability, even leading to their extinction in a few decades (Rivera-Ingraham et al., 2011f). For example, in *Patella ferruginea* a growth increase of 2 cm results in the female being 10 times more fertile (Espinosa et al., 2006b). Some species are able to modify the size at which they undergo sex change as a function of population parameters. In *P. ferruginea* populations with a greater number of large individuals, the change in sex from male to female occurs at larger sizes, while in populations with few large specimens the sex change occurs when the individuals are much smaller (Rivera-Ingraham et al., 2011d). These adaptive strategies allow populations that are subjected to collection to reproduce, but to a lesser degree since small females contribute much less to the global reproductive effort. In addition, although they were unable to verify it for the giant limpet species *Lottia gigantea*, Fenberg et al. (2010) suggested that sustained collection over time may lead to microevolutionary responses and result in genetic changes.

Collection by humans has been cited as the main cause for the decline and/or extinction of giant limpet populations, such as *P. ferruginea* in the Mediterranean Sea (Coppa et al., 2012; Espinosa et al., 2009a, 2014). This species used to be abundant throughout the Mediterranean Basin (Laborel-Deguen and Laborel, 1991a) but has been relegated to very localised enclaves at the present time (Espinosa et al., 2014). The main threat to the species has been its use as food, fishing bait and for decorative purposes (Laborel-Deguen and Laborel, 1991a,b; Ramos, 1998; Templado and

Moreno, 1997), causing a decrease in its average size in places subjected to anthropogenic pressure as opposed to inaccessible areas (see Guerra-García et al., 2004).

Patella ferruginea has apparently been consumed by humans throughout its range since prehistoric times and is very common in numerous deposits, including in the Palaeolithic of the Mousterian period in Algeria (Neanderthal 70,000–32,000 years ago) (Caton-Thompson, 1946), the Palaeolithic and Neolithic of Gibraltar, Neolithic cooking sites in continental France, the Phoenician epoch in Ceuta as well as during the Middle Ages both in Ceuta and in Gibraltar (see Laborel-Deguen and Laborel, 1991b). Chamorro-Moreno (1988) demonstrated the use of *P. ferruginea* as a nutritional resource in certain areas such as Ceuta, in the Strait of Gibraltar, based on several deposits dating from the 10th to the 16th century, and suggested that the observed size decrease was a consequence of selective harvesting by humans over centuries. Similarly, Laborel-Deguen and Laborel (1991b) implicated collection by humans in the disappearance of specimens greater than 40 mm.

Scutellastra mexicana is another example of human pressure on giant limpets. It is currently very rare and is endangered, at least in part, as a result of commercial overexploitation since the 1970s, when its meat was eaten and the shell was used for crafts (Ríos-Jara et al., 2001). The species was once distributed along the American Pacific coast from Mexico to Peru and is now locally extinct in certain zones on the mainland Mexican coast (Brian, 2000; Fleming, 1973; Ridgway et al., 1998). Brian (2000) indicated that the species has not been seen in the Baja California area since 1972, and that no living specimens were found in six consecutive field campaigns in the region. The species has been cited in archaeological studies that analysed the use of marine shellfish in the Postclassic period (1100–1350 C.E.) in various deposits of Pacific Mexico, from Sinaloa to Michoacán, an area where the species has been recorded in recent times. Deposits analysed indicated that the shells of this species were used for bracelets and earrings. *S. mexicana* also appeared in offerings from the Pyramid of the Feathered Serpent in the Xochicalco deposit (600–900 C.E.) located in Mexico's interior (Gómez-Gastelum, 2007). These data indicate a historical presence of the species in the Mexican Pacific, and centuries of it being used by man, which provides us with a general view of the pressure that has impacted it throughout time.

Thus, human populations affected the coastal fishing grounds, and giant limpet species, long before the appearance of commercial and technological

fishing. Although collection by humans has not pushed *L. gigantea* to near extinction, its average population size has decreased in many regions. Erlandson et al. (2011) indicated that populations of this species significantly reduced in size during the Holocene, beginning 6500 years ago, and this trend was accelerated 4400 years ago. *Cellana talcosa* has also been the object of sustained collection through time by local Hawaiian populations (Twaddle, 2011), and *Cymbula oculus* and *C. miniata* have been widely harvested in South Africa (Lasiak, 2006).

7.2 Pollution

Many pollutants, especially those that float, have a direct impact on sessile organisms, particularly limpets (Chan, 1973; Le Hir and Hily, 2002; Suchanek, 1993). Limpet species are habitually used as bioindicators, as they accumulate heavy metals (e.g. Hamed and Emara, 2006; Pérez-López et al., 2003) and aromatic hydrocarbons (e.g. Glegg and Rowland, 1996; Glegg et al., 1999; Kennicutt et al., 1992), mainly in their soft tissues (e.g. Cravo and Bebianno, 2005; Lobel et al., 1982), but also in their shells (Cravo et al., 2004; Foster and Chacko, 1995) where the toxic substances may be stored in such a way that their harmful effects on the viability of the individuals are reduced.

Although the physiological response of limpets to pollution has been studied using some species of the genera *Cellana* (e.g. Chelazzi et al., 2004), *Nacella* (e.g. Ansaldo et al., 2005; Najle et al., 2000; Weihe et al., 2010) and *Patella* (e.g. Bini et al., 2008; Brown et al., 2004; De Pirro et al., 2001; Marchán et al., 1999; Nuwayhid et al., 1980), this is not the case for giant limpet species. Studies examining giant limpet physiology and the effects of pollutants on these species have been lacking. However, there are exceptions, Rivera-Ingraham et al. (2013) drew conclusions about the impact of metal accumulation in the tissues of *Cymbula safiana* in zones of the Bay of Gibraltar in relation to differing levels of anthropogenic influence. Individuals of *C. safiana* from the most impacted areas contained important quantities of Cr and Mn, but at a subcellular level. These individuals also presented greater antioxidant activity (especially with regard to catalase). Rivera-Ingraham et al. (2013) demonstrated that the species is relatively tolerant to this kind of impact, and by increasing its antioxidant defences, *C. safiana* can withstand the detrimental effects of the free radicals produced when accumulating these heavy metals in its tissues. Espinosa and Rivera-Ingraham (2016) examined the effects of pollutants on *Patella ferruginea* and found that unlike in *C. safiana*, individuals located inside Ceuta Harbour

(more exposed to pollutants and physical alterations of the environment than individuals in other areas of the Strait of Gibraltar) have less catalase activity than that of individuals found in less accessible zones with greater renewal of the water column. Individuals of *P. ferruginea* recovered from the harbour had a greater level of cell damage (measured as 3/7 caspase activity) (Espinosa and Rivera-Ingraham, 2016). At a subcellular level, this demonstrates the difference in tolerance between these two species in resisting the harmful effects of these pollutants.

The majority of studies analysing the pollution tolerance of giant limpet species have employed ecological approaches. If we compare *C. safiana* and *P. ferruginea* by analysing their distribution patterns, we might come to the same conclusions about the contamination tolerance of these two species. Taking the Strait of Gibraltar, where both species are present, as a model, we can see that in the Bay of Gibraltar, a highly industrialised zone affected by numerous types of anthropogenic impact, there are large populations of *C. safiana*, whereas *P. ferruginea* is rarer (Espinosa et al., 2005). However, along the North African coasts of Ceuta, which are considerably less industrialised and where the water is of a higher environmental quality than in the Bay of Gibraltar, we find one of the most important present-day populations of *P. ferruginea*, where it has been estimated that there are around 44,000 individuals (Rivera-Ingraham et al., 2011a). However, we recognise that differences in environmental or other factors (e.g. suitable habitat, historical or current collection) could also account for the disparity in population sizes in these regions.

Another clear example of using an ecological approach to study pollution tolerance is the work of Espinosa et al. (2007). These authors evaluated the tolerance of different limpet species to the presence of urban wastewater outfalls by recording variations in abundance and size for each species according to the distance from a pollutant source. Their results indicated that *C. safiana* is a more tolerant species than *P. ferruginea*, being found in areas next to the outfall point, whereas *P. ferruginea* only appears in areas removed from the emission source. This is in agreement with the observations made by Rivera-Ingraham et al. (2013), who concluded that *P. ferruginea* is relatively sensitive to increases in turbidity, ammonium and phosphate, as well as to the reduced O_2 levels in the water column that characterise this type of emission. Supporting this hypothesis, Tlig-Zouari et al. (2010) analysed the distribution of *P. ferruginea* along the Tunisian coastline and found that *P. ferruginea* is present in greater densities in coastal zones with a higher environmental quality. Many authors have suggested that the pollution, along

with other factors, has been partially responsible for the enormous decrease in this species since the beginning of the 20th century, which have driven *P. ferruginea* to near extinction (Espinosa, 2006; Laborel-Deguen and Laborel, 1991a; Rivera-Ingraham, 2010).

Recently, a study carried out by Reinecke et al. (2012) showed the bioaccumulative capacity for Cd of *C. oculus*, leading them to suggest this species as a possible tool for biomonitoring. The work of Schoeman (2007) suggests that this species may be capable of actively regulating the quantity of heavy metals (specifically Cd) present in its tissues by, for example, transporting these metals to the shell to avoid the harmful of effects of having them in the soft tissues. These results, combined with analysis of the integrity of its lysosomal membrane, indicate that *C. oculus* is relatively sensitive to Cd exposure compared with other species in the area, such as *Scutellastra longicosta* and *Cymbula granatina* (Reinecke et al., 2008; Schoeman, 2007).

7.3 Artificial Substrates and Habitat Fragmentation

In the current socioeconomic framework of constant human population growth, increasing pressure on the coastal environment, and climate change, habitat degradation (including construction of artificial structures) in the littoral domain is expected to increase in the coming decades (Bulleri and Chapman, 2010). Artificial substrates introduced to the marine environment may cause a loss of biodiversity inasmuch as many species will not settle there, or will do so in lower densities than on natural substrates (Bulleri and Chapman, 2010; Chapman, 2006; Vaselli et al., 2008). For some species of limpets, a decrease in reproductive capacity has been recorded for populations that settle on artificial substrates in comparison to those found on natural substrates (Moreira et al., 2006). Fauvelot et al. (2009) recorded lower genetic diversity in *Patella caerulea* populations established on artificial substrates when compared to diversity measured in populations settled on natural substrate. These findings indicate that the growing presence of artificial substrates in coastal ecosystems may cause population fragmentation and isolation, limiting the genetic flow and dispersion capacity of many limpet species.

However, artificial structures can also contribute to the conservation and management of limpet species. For example, the giant limpets *Patella ferruginea* and *Cymbula safiana* settle in high densities on breakwater structures (Rivera-Ingraham et al., 2011a,b). Paradoxically, *P. ferruginea*, which is listed as an "endangered species" in the Spanish Catalogue of Endangered Species, is the most abundant species in certain artificial coastal facilities in the Strait

of Gibraltar, and *C. safiana* is considered "vulnerable" in the Andalusian Catalogue of Endangered Species. It is possible that these coastal structures could be properly managed for the conservation of certain species, establishing a kind of "no take–no entry zone" (see Section 8). Artificial substrates can be optimised to increase their ecological benefits, and so that they resemble natural substrates as far as possible. For example, some studies found an increase in limpet recruitment on artificial substrates that contained experimental holes to increase the heterogeneity of the substrate (Martins et al., 2010).

8. CONSERVATION RECOMMENDATIONS

8.1 *In Situ* Conservation: Marine Protected Areas

Establishing marine protected areas (MPAs) is a key factor in maintaining biodiversity. However, as discussed by Edgar et al. (2014), protected areas often do not reach their full potential due to various factors, especially a lack of effective protection to halt illegal fishing.

Among giant limpets, only *Patella ferruginea*, *Cymbula safiana* and *Scutellastra mexicana* are listed as endangered or threatened species, and no effective protection mechanisms are in place to prevent the disappearance of these species. In general, there are few cases where the establishment of MPAs has truly and directly benefited the populations of giant limpet species. Branch and Odendaal (2003) analysed the effect of MPAs on South African *C. oculus* populations and found that collecting is the main factor decimating the populations of this limpet species. Populations located within the boundaries of the MPAs differed from those outside in various aspects. Collecting affects larger individuals, which are the most conspicuous and sensitive to harvesting by humans. Because the majority of species were protandric hermaphrodites, those populations protected from the impact of collection had more balanced sex ratios, and the average size of adults was significantly greater (Branch and Odendaal, 2003). For the same reason, the protected populations had greater densities, and the growth and survival rates were significantly higher than those recorded in unprotected areas (Branch and Odendaal, 2003). Lasiak (2006) analysed the effect of the Dwesa Marine Reserve, also in South Africa, on populations of limpet species. Similar to Branch and Odendaal (2003), *Cymbula oculus* displayed the greatest densities and biomass in the interior zones of the MPA and was practically absent in unprotected zones in the immediate vicinity. This was due to the fact that *C. oculus* is one of the most exploited limpet species in the area

(Hockey and Bosman, 1986; Lasiak, 1991), because for other, less frequently exploited species, like *Scutellastra tabularis* and *S. barbara* (Hockey and Bosman, 1986), Lasiak (2006) observed that the differences were less significant than those recorded for *C. oculus*. This underscores the fact that human collection is one of the most important impacts to giant limpets.

Although the status of *Lottia gigantea* is not recognised in any conservation document, the problem of its overexploitation has led local governments to ban commercial fishing and restrict the recreational catches of this species. The many MPAs declared within its range mean that the effect of these areas on *L. gigantea* populations can be evaluated. For example, Kido and Murray (2003) compared limpet populations in MPAs established in different time periods. The earliest established MPAs (>26 years before commencement of the study) had *L. gigantea* populations with greater numbers and also larger average individuals than those declared more recently (three years before the start of the study), demonstrating the benefits of implementing MPAs and managing collection rates.

For *P. ferruginea*, the beneficial effects of MPAs are evident in numerous cases. For example, Bazairi et al. (2004) recorded and analysed a large population of *P. ferruginea* in Cala Iris, a small islet within the Al Hoceima National Park, Morocco. In adjacent areas, this species was not very abundant, but there was an average density of 0.24 individual per m on the islet (Bazairi et al., 2004). Another example can be found in the Tavolara-Capo Coda Cavallo Marine Reserve, northeast of Sardinia, where *P. ferruginea* is found only in the zones catalogued as category "A" (no entry, no take) (Ceccherelli et al., 2005). Tlig-Zouari et al. (2010) and Espinosa et al. (2014) showed that the populations of *P. ferruginea* located within the MPA around the island of Zembra, Tunisia, were found in significantly larger average sizes than in other areas, which, although also having good environmental quality, are easily accessible by humans.

The case of *Scutellastra kermadecensis* may also be an example of the beneficial effect of controlling limpet collection by humans, because although it is now restricted to the Kermadec Islands, archaeological remains show that this species used to be found along the New Zealand coastline where it was collected as a food resource (Creese et al., 1990; Fleming, 1973). On the basis of this evidence, it is possible to conclude that the current distribution of *S. kermadecensis* may be due to the remoteness and inaccessibility of the islands where it is currently present.

When designing marine reserves to protect benthic species whose life cycle involves a pelagic phase, it is important to include the protection of

adjacent areas, located within the larval distribution limits for the target species, in this way creating a network of areas that favour an extended range of those species. Gene flow between populations is necessary to increase the genetic diversity and avoid bottlenecks. The case of *P. ferruginea* is particularly remarkable in relation to MPA design, because this species settles on a relatively specific type of substrate, with a specific topographic complexity (Espinosa et al., 2011; Rivera-Ingraham et al., 2011a). Seawalls of many commercial ports and sports marinas and beach breakwaters in the western Mediterranean Sea are composed of rocks of the nature and topographic complexity required by *P. ferruginea*, and in zones where this species is present, individuals tend to settle on these structures (Guallart et al., 2013c; Guerra-García et al., 2004; Rivera-Ingraham et al., 2011a). This has led to the concept of "artificial marine microreserves" (AMMRs) being proposed (García-Gómez et al., 2011), for which already-existing seawalls that house well-established communities would be set up as reserves. This type of reserve, with considerably more limited in dimensions and easy access, would be easy to effectively protect from poaching. This type of planning would enable the implementations of a system of microreserves that would allow genetic exchange between populations located in the AMMRs, favouring the future of various threatened species (García-Gómez et al., 2015).

8.2 *Ex Situ* Conservation

Typically, *in situ* conservation measures must be complemented with *ex situ* conservation actions (e.g. captive breeding), for later reintroduction of specimens into populations below the viable limit, or to increase the range of a species (Espinosa et al., 2010). Often, captive breeding and the development of techniques to transfer specimens are undertaken together in order to maintain the wild populations of threatened species (IUCN, 2012).

8.2.1 Captive Breeding

There is little information on the captive breeding of threatened giant limpets; in fact to our knowledge, trials and breeding programmes have only been implemented in the species *Patella ferruginea*. The first captive breeding trials described the trochophore and preveliger larval phases and involved a very low fertilisation rate and limited viability (48 h) of the larvae obtained (Espinosa et al., 2010). However, obtaining larvae in captivity suggested the possibility of completing the cycle and obtaining juveniles that could be used in future reintroduction programmes. Guallart et al. (2013a) completed the

life cycle of the species, with individuals reaching the juvenile phase 15 days after fertilisation and kept captive-born specimens for more than a year. Nevertheless, the successful rearing of individuals to the juvenile stage was limited (Guallart et al., 2013d), making it difficult to implement captive-bred reintroduction programmes. Regardless, this conservation measure may still have promise (Espinosa et al., 2010; Guallart et al., 2013a,d).

Nonetheless, reintroduction of specimens from individuals obtained by means of captive breeding offers, for many species, a much lower success rate than does reintroduction using wild individuals (Griffith et al., 1989). For this reason, it is necessary to evaluate the success of future reintroductions of giant limpet species using captive-bred and wild specimens, for assessment of the most feasible approaches for potential use in the reintroduction of individuals to populations of threatened or endangered giant limpet species.

8.2.2 Transplantation

Transplantations could be used to increase current distributional range of vulnerable species and could also increase population numbers for those that are below the viable limits. In both cases, it is necessary to identify and eliminate the causes of the disappearance and/or decline of the populations. If this is not the case before initiating any transferral process, management programmes must be implemented to eliminate the threats, among which pollution and collection are the most significant.

For the species *Patella ferruginea*, transplantations have been made, but there is no established method that guarantees high survival rates. To present, transplants have been limited to capturing specimens in donor zones, detaching them from the substrate and reintroducing them in receiving zones. Laborel-Deguen and Laborel (1991c) transferred 222 specimens from Corsica to Port-Cros National Park, near Marseilles, with a mortality rate of 50% after the first day, 75% after one year and 90% after two years. Espinosa et al. (2008a) transplanted 420 specimens in Ceuta with mortality rates of around 50% in the first days after the transplantation. Guallart et al. (2014) transplanted 10 established specimens from the Chafarinas Islands and from captive breeding, to the Islas Hormigas, and documented a mortality rate of 40% at 24 h. For another method in which individuals settled on the rocks of the artificial breakwaters of the Chafarinas Islands were transferred together with their substrate (entire rocks) and relocated at the same bathymetric level as that at which they were originally situated, the mortality rate reached 46.6% after three months (Paredes-Ruiz et al., 2014). Espinosa

et al. (2006a) proposed the use of cages as a method to concentrate reproductive individuals and to carry out transfers.

Another possibility for the reintroduction or bolstering of populations is the transplantation of juveniles, and the work of Guallart et al. (2013d) indicated that juvenile *P. ferruginea* obtained through captive breeding could be used in such programmes, in agreement with the National Strategy for Species Conservation (MMAMRM, 2008). However, the number of individuals obtained to date does not allow programmes of this type to be put into practice on a large scale, while the process is not optimised (see Guallart et al., 2013d). Another potential method is obtaining juveniles from wild populations using plates impregnated with gamma-aminobutyric acid (Rivera-Ingraham et al., 2011f), a metamorphosis inducer for the larvae whose presence has recently been discovered in the tissue of the feet of adult *P. ferruginea* specimens (Rivera-Ingraham et al., 2015). According to Rivera-Ingraham (2010), it may be possible to extract up to 18% of recruits annually from a dense population without jeopardising its viability, although this depends on the demographic parameters of each donor population, as well as the time series those parameters are based on.

In the case of *Cymbula safiana* the results are similar, with little success seen for specimen transplantation. On the coast of Granada, Spain, a transplantation of individuals of this species was made using cages, although mortality rose to 50.2% after one week and 88% after the first month (see Rivera-Ingraham, 2010). To our knowledge, there is no further information on transplanting species of giant limpets, and the results available indicate that it is a complex technique that requires further research to define protocols that ensure high survival rate; otherwise, it cannot be considered a tool for the conservation management of endangered species.

This is, therefore, a field open to applied research, in which progress is necessary. MPAs, marine artificial microreserves, captive breeding and reintroduction/transplantation may all play a role in the protection and conservation of these valuable giant limpet species.

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CHAPTER FOUR

Advances in Biochemical Indices of Zooplankton Production

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Abstract

Several new approaches for measuring zooplankton growth and production rates have been developed since the publication of the ICES (International Council for the Exploration of the Sea) Zooplankton Methodology Manual (Harris et al., 2000). In this review, we summarize the advances in biochemical methods made in recent years. Our approach explores the rationale behind each method, the design of calibration experiments, the advantages and limitations of each method and their suitability as proxies for *in situ* rates of zooplankton community growth and production. We also provide detailed protocols for the existing methods and information relevant to scientists wanting to apply, calibrate or develop these biochemical indices for zooplankton production.

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1. INTRODUCTION

Quantifying marine ecosystem function is essential for a complete understanding of system-wide responses to climate change and anthropogenic forms of perturbation (e.g. Boyce et al., 2010; Edwards and Richardson, 2004; Walther et al., 2002). Zooplankton communities are of particular interest because they play a central role in the flow of material and energy passing from primary producers, to animals at higher trophic levels (Lalli and Parsons, 1993). Specifically, the efficiency by which primary production is transferred to zooplankton production represents a critical measure of ecosystem function because it determines the amount of energy available to higher trophic levels in the pelagic (Blanchard et al., 2012). A calculation of transfer efficiency (*TE*) demands knowledge of both phytoplankton (*PP*) and zooplankton production rates (*ZP*) (i.e. $TE = ZP/PP$; Lindeman, 1942). As an introduction to our discussion of the use of biochemical indices of zooplankton production, we provide a brief background relative to the development of measurements of zooplankton growth and production rates.

1.1 Definition of Zooplankton Production

Zooplankton production (often inaccurately referred to as secondary production—zooplankton production is just one component of secondary production) is defined as the increase in biomass of a population over a specific period of time, regardless of its fate, and can be generalized as the sum of all growth (somatic and reproductive) plus biomass losses over that period (Downing and Rigler, 1984; Omori and Ikeda, 1984; Winberg, 1971; Zaika, 1973). Hence,

$$ZP = (B_{t1} - B_{t0}) + M, \tag{1}$$

where ZP is the zooplankton production, B_{t1} and B_{t0} are the biomasses at final and initial time, respectively, and M is the biomass lost to mortality. Using simple models, Kimmerer (1987) showed that the instantaneous zooplankton production rate could be calculated as the product of total biomass and the weight-specific growth rate of a given population. Consequently, all methods developed for the estimation of zooplankton production require the measurement of both biomass and weight-specific growth rate (hereafter referred to as growth rate) (Huntley and Lopez, 1992).

1.2 Biomass or Standing Stock

Biomass is among the most frequently reported biological measurements for oceanographic studies that census zooplankton (see Hays et al., 2005). Estimates of zooplankton biomass are themselves the object of studies that attempt to relate zooplankton variation to physico-chemical oceanographic conditions. However, since zooplankton estimates are also necessary for the calculation of production rate, we briefly discuss the various estimation methods currently in use (see Beers, 1974; Omori and Ikeda, 1984; Postel et al., 2000). Zooplankton biomass can be estimated using either direct or indirect methods (Fig. 1). Direct methods include mass estimates for single zooplankton individuals taken from samples, or for bulk (often size-fractionated) zooplankton. The first step toward direct biomass assessment is usually sample weighing. Weight can be measured as wet weight (samples blotted dry) or dry weight (samples dried in an oven or lyophilized). The weight of samples combusted in a muffle furnace yields ash-free dry weight. More specific expressions of biomass can also include elemental (carbon, nitrogen, phosphorus, hydrogen) or biochemical/macromolecular measurements such as protein, carbohydrate, chitin, lipids, adenosine triphosphate (ATP), and total energy content.

From the perspective of zooplankton production assessments, some authors have proposed to divide the measured weight into two categories: structural and non-structural (Carlotti and Hirche, 1997; McLaren, 1986). Structural weight consists of components making up the bulk of the body, such as the haemolymph, cuticle and muscle, and is assumed to be of low variation for individuals of the same size (Carlotti and Hirche, 1997). Non-structural weight is composed of the most variable body components, such as the gut, gonads and metabolic reserves (see Carlotti and Hirche, 1997). In copepods, lipids are linked to energy storage and can contribute significantly to individual weight. This is particularly important for some polar and subpolar copepods known to accumulate large amount of lipids in their body (e.g. Kobari and Ikeda, 1999, 2001; Kobari et al., 2003; McLaren, 1986; Miller et al., 2000; Shoden et al., 2005). Indirect biomass estimates are based on the size or volume measurements that are converted to biomass using general or specific relationships or conversion factors. Size (or volume) is typically measured for preserved or *in situ* zooplankton samples, using automated tools (see Fig. 1). Biomass of samples collected with plankton nets can be estimated indirectly in the laboratory by means of settling or displacement volume, by individual zooplankton identification and measurement under the microscope, or

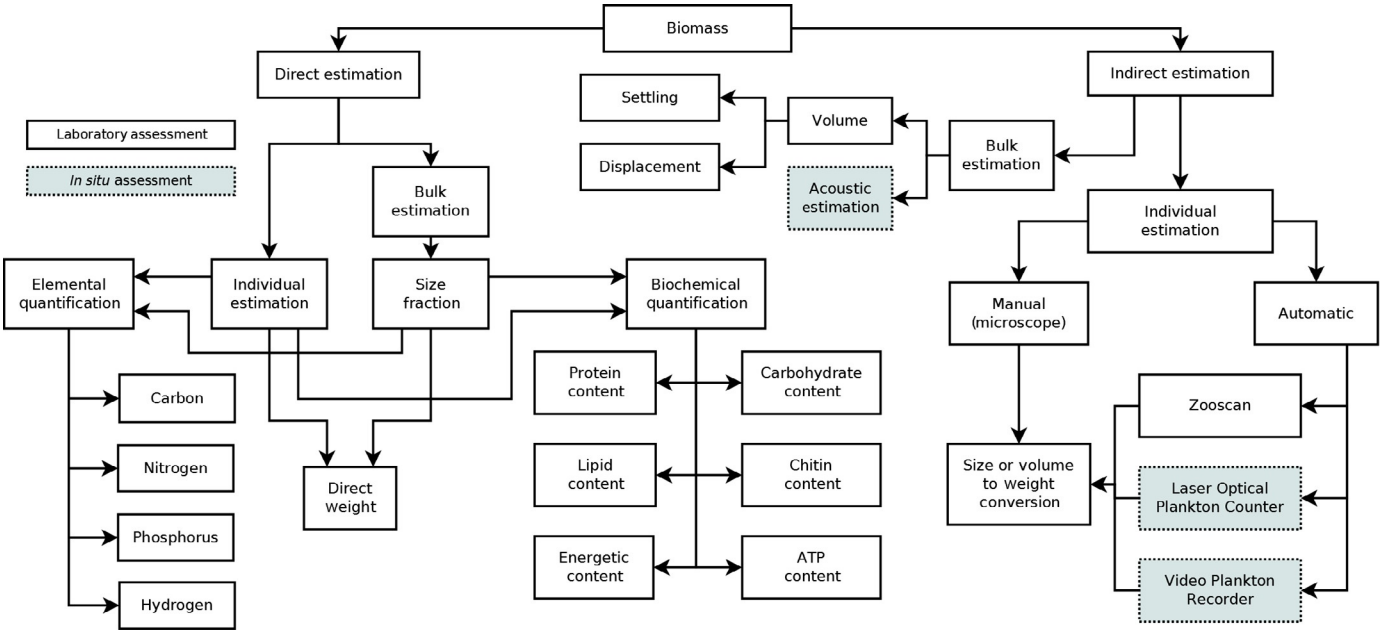

Fig. 1 Categorization diagram of zooplankton biomass estimation methods.

by image analysis using devices such as ZooScan (Gorsky et al., 2010). Zooplankton biomass can also be determined *in situ* from sound scattering using echo sounders (e.g. Lavery et al., 2007; Powell and Ohman, 2012; Stanton et al., 1994), or with optical particle imaging systems such as the Laser Optical Plankton/Particle Counter (LOPC; Herman et al., 2004), Video Plankton Recorders (Davis et al., 2005), and holographic imaging (Jaffe, 2008).

Indirect zooplankton biomass techniques present several advantages over direct biomass approaches: (1) they allow fast and easy biomass determination, as the laboratory labour involved in sample analysis is either quick (e.g. volumetric methods and ZooScan) or not necessary (e.g. LOPC), allowing continuous assessments of biomass in both space and time; (2) organisms can be preserved for future analysis also providing some taxonomic information, while the direct methods (e.g. dry weight) destroy the samples; (3) biomass determinations using *in situ* approaches are thought to be less sensitive to avoidance and less selective than direct approaches because they do not depend on discrete samples collected with plankton nets (Wiebe and Benfield, 2003), which are recognized for being selective and for promoting avoidance (Clutter and Anraku, 1968). For these reasons, the use of methods such as ZooScan (or ZooImage) and LOPC to estimate zooplankton biomass is becoming more common in the scientific literature.

Nevertheless, direct biomass estimates also have important advantages over indirect procedures: (1) differences in condition can introduce significant errors into biomass estimated from morphometrics, length–weight regressions or volume of an organism (Hirst and McKinnon, 2001; Miller, 2008) and thus, to avoid the introduction of significant errors, indirect biomass assessment techniques must be calibrated with a direct biomass assessment. For example, Garijo and Hernández-León (2015) compared direct (dry weight) and indirect (ZooImage) methods, validating the use of the latter; (2) given the differences between structural and non-structural weight components, direct procedures are necessary for determining them; (3) since direct estimates of biomass have been in use for decades, these facilitate long-term trend analysis of zooplankton biomass (e.g. Siokou-Frangou et al., 2010; Steinberg et al., 2012); and (4) direct biomass assessments allow for the measurement of multiple biomass indicators in the same sample, such as weight (e.g. wet and dry weight), elemental (e.g. C and N content) and macromolecular composition (e.g. proteins).

1.2.1 Biomass Assessments Associated With Biochemical Indices

Zooplankton biomass assessment is crucial, as it may vary in the field by two to three orders of magnitude, thus exerting a strong influence on production rate estimates (Huntley and Lopez, 1992). In zooplankton production studies, biomass is measured and/or expressed in the same terms in which growth is estimated, and because these measurements are intrinsically linked with biogeochemical cycles in the oceans, biomass is frequently expressed in terms of elemental composition (especially C and N) per unit volume. Also, zooplankton production studies report biomass for the same level or organization (e.g. individual or guild) for which production is measured. For instance, in traditional zooplankton production procedures (see Section 1.3.1), biomass is often reported at the species level because growth measurements are also made at the species level. On the other hand, some biochemical indices can be applied to either individual or bulk zooplankton, and thus bulk biomass estimates are also used. Another important factor to consider when assessing biomass is that some biochemical indices are sensitive to organism condition. Therefore, the biomass estimators used alongside these indices must also be sensitive to organism condition. Indirect methods rely on size or volume to biomass conversion factors and equations, assuming that weight will not vary in animals of the same size or volume, hence not sensitive to changes in individual condition. From the perspective of zooplankton production, the ability to detect changes in organismal condition is crucial for obtaining reliable production estimates since animals of the same size can have different weights and biomass production depending on the overall condition of the organism. The weight of crustacean zooplankton is known to vary among individuals of the same size depending on various factors. For instance, weight changes during the intermoult and interclutch periods, during pre- and postreproductive stages or during lipid accumulation (Hirst and McKinnon, 2001; Miller, 2008). These changes can only be detected using direct measurements of weight, elemental or macromolecular composition of organisms.

Since biochemical indices are linked with the production of macromolecules associated with growth, biomass is also expressed in terms of these macromolecules. For instance, protein content has been used as biomass estimator in biochemical indices using protein synthesis as a proxy of growth (e.g. Yebra et al., 2004). The expression of biomass as protein content has many advantages, such as: (1) relative ease and low cost of analysis; (2) being directly associated with structural weight and organism condition; (3) reduction in error (because the proportion of N and C in protein is

generally constant, a conversion from protein estimated production to these units with a conversion factor is likely to generate negligible errors when compared to other conversion factors used in production assessments such as volume or length to weight equations, except during periods of significant lipid accumulation); and (4) both protein content and biochemical indices can be assessed from the same sample homogenate. For this reason, protein content is likely to be the most appropriate biomass estimator for biochemical indices.

1.3 Growth Rate

The direct estimation of growth rate, the second term of the production calculation, is usually more time consuming to measure than biomass, since the latter can be estimated directly after sampling (e.g. dry weight), whereas the direct estimation of growth requires repeated sampling and/or incubations (see Section 1.3.1). Since growth is a dynamic process intrinsically linked with environmental conditions, there is an interaction at both the organism and population level. Production of a population is derived from both somatic and generative (reproductive) growth (Zaika, 1973). Somatic growth can be estimated in terms of structural and non-structural weight (see Section 1.2). This has important implications for production assessments due to the potential high variability in non-structural components of total weight (Hirst and McKinnon, 2001). If production is assessed using total weight, negative growth can be measured, for instance, during starvation periods (Shaw et al., 2010) or just after moulting (e.g. Shreeve and Ward, 1998). A good example of the theoretical interplay between structural and non-structural components in growth and egg production was illustrated with the individual-based model for the copepod *Calanus finmarchicus* by Carlotti and Hirche (1997).

Production can be estimated by following population cohort development, provided unique cohorts can be defined (see Winberg, 1971). However, most zooplankton populations (including copepods) are characterized by continuous reproduction, which impedes clear cohort definition (Kimmerer, 1987). Copepod growth (g) is normally exponential and can be calculated using the following equation (as per the instantaneous growth method):

$$g = \left(\frac{1}{D}\right) \times \ln\left(\frac{m_{\max}}{m_{\min}}\right), \tag{2}$$

where m_{min} and m_{max} are the minimum and maximum individual biomass within a size class, respectively, and D is the mean time an animal takes to grow from m_{min} to m_{max} (Downing and Rigler, 1984). The theory behind growth and production estimation for continuously reproducing populations has been described in detail by Downing and Rigler (1984) and Kimmerer (1987).

1.3.1 Traditional Growth Estimation Methods

Zooplankton production rates are key to our understanding of both biogeochemical fluxes and the transfer of energy to higher trophic levels. It is not surprising, therefore, that past decades have seen the development and application of several methods for measuring both *in situ* zooplankton biomass and growth rates (see Omori and Ikeda, 1984; Runge and Roff, 2000). Traditionally, growth and production rate measurements have focussed on copepods, the main component of marine zooplankton. These 'copepod-centric' methods are based on either direct or indirect procedures (Fig. 2). The direct

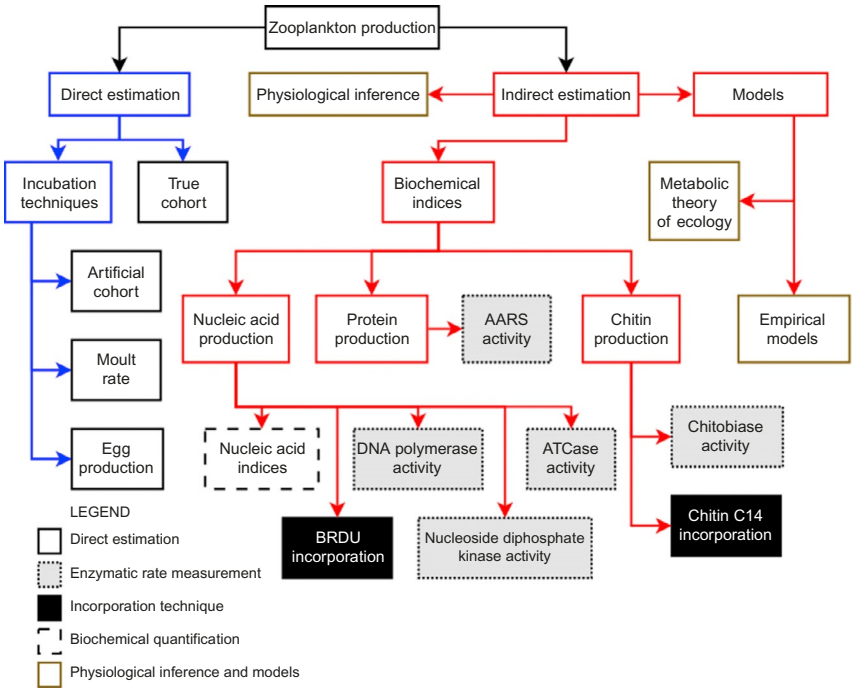

Fig. 2 Categorization diagram of zooplankton growth and production estimation methods.

estimates of growth assess changes in weight or reproductive output (see Downing and Rigler, 1984; Omori and Ikeda, 1984; Runge and Roff, 2000; Winberg, 1971).

One of the first methods used for estimating *in situ* growth rates was the natural cohort technique (e.g. Heinle, 1966). It does not require incubations, but requires: (1) relatively high frequency of sampling appropriate for the target population, and (2) populations with recognizable cohort structure (i.e. non-continuous reproduction). The problems with applying this approach to marine zooplankton include: (1) logistical difficulties following the same population over time in the marine environment (due to processes such as mixing and advection), (2) sampling with an appropriate frequency is difficult for remote locations (e.g. offshore and deep ocean) or hard to assess *a priori* for poorly known populations (Huntley and Lopez, 1992; Kimmerer et al., 2007), and (3) many zooplankton populations reproduce continuously, and therefore, natural cohorts are not identifiable.

These issues are overcome using incubation techniques, such as the egg production (Marshall and Orr, 1955), moulting rate (e.g. Burkill and Kendall, 1982) and artificial cohort (Kimmerer and McKinnon, 1987) methods, as they can be applied to continuously reproducing populations. Due to its simplicity, the egg production method for the estimation of *in situ* rates of copepod fecundity and production is the most widely used technique (Runge, 1984). The method is based on the assumption that juvenile growth is equivalent to adult female egg production (Berggreen et al., 1988). However, this is assumption is not always true (Hirst and McKinnon, 2001; Hopcroft and Roff, 1995, 1998; McKinnon, 1996; Peterson et al., 1991). Also, egg production rates (EPRs) can be underestimated in incubation experiments due to bottle effects and cannibalism of eggs.

Measuring moult rates is the most widely applied incubation technique for the direct *in situ* estimation of somatic growth in copepods (e.g. Burkill and Kendall, 1982; Hutchings et al., 1995; Peterson et al., 1991; Richardson and Verheye, 1998; Richardson et al., 2003). The assumptions underpinning this method are that: (1) growth is exponential during the incubation, (2) age within stage is uniform, and (3) stage duration can be applied to growth increments estimated from differences in the mean weight of consecutive stages. The 'modified moult rate' method (Hirst et al., 2005, 2014) clearly demonstrates that this last assumption is wrong and attempts to resolve a more precise growth rate estimate while still using stage duration and differences in mean weight-at-stage.

The artificial cohort method (Kimmerer and McKinnon, 1987) is another incubation technique used for growth estimation of continuously reproducing populations. This technique assumes exponential growth during the incubation, and that growth is not altered by the manipulation required by the technique (Kimmerer et al., 2007). The procedure for the artificial cohort method is simple and is probably the best technique applied to continuously reproducing populations. Its advantage over the moult rate technique is that it is less or not affected by variation of age within stage in the population. Consequently, this approach has been widely used to calibrate biochemical (indirect) indices of growth (see Section 2). Nevertheless, the main drawback of this and other incubation techniques is the difficulty maintaining incubation conditions comparable to those in the field, since: (1) handling of live organisms may induce stress or damage to them, (2) food provided may not reflect *in situ* diversity and concentrations, and (3) scaling is a major issue: constraining organisms and their prey in mesocosms or bottles may account for up to a 10^6 volume reduction with respect to field conditions, which influences the natural behaviour of organisms. Furthermore, both cohort-tracking and incubation-type methods suffer several disadvantages and limitations when they are applied to zooplankton *in situ* (Hirst and McKinnon, 2001; Hirst et al., 2005; Kimmerer et al., 2007), such as having very low temporal and spatial resolution due to laborious on-board incubations and time-consuming sample analysis, limiting their application to entire communities in the field.

Indirect methods for measuring growth and production rates consist of modelling and biochemical approaches (see Fig. 2). The use of generalized models of growth has the advantage that growth can be predicted from easily obtained *in situ* data, and no incubations are necessary. Empirical models estimate growth from relationships fitted with environmental variables such as temperature and chlorophyll *a*, and some characteristics of the zooplankton group studied, such as body size, stage of development, spawning type, sex and broad taxonomic grouping (e.g. Banse and Mosher, 1980; Hirst and Bunker, 2003; Hirst and Lampitt, 1998; Hirst and Sheader, 1997; Huntley and Lopez, 1992). In addition, the metabolic theory of ecology relates physiological processes such as growth or development to ambient temperature and individual weight (Brown et al., 2004; Gillooly et al., 2001, 2002). Moreover, the so-called physiological method (Ikeda and Motoda, 1978; Winberg, 1956) is based on the measurement of metabolism (T). This approach assumes an empirical relationship between respiration or excretion and growth (G). In this sense, ingestion (I) is the addition of assimilation

(A) and egestion (E), while the former is also the addition of the metabolic expenditure (e.g. respiration) and growth. Thus, $I = A + E$ and $A = T + G$. Also, T and G are related through the net growth efficiency (K_2): $K_2 = G/A = G/T + G$ and therefore, $G = (K_2/1 - K_2) \cdot T$. Similarly, Ikeda and Motoda (1978) proposed to estimate production using values of assimilation and gross growth efficiency ($K_1 = G/I$) gathered from the scientific literature. Thus, $G = K_1 \cdot T/A - K_1$. They assumed a 30% for K_1 and 70% for A, being $G = 0.75 \cdot T$ and $I = 2.5 \cdot T$. However, net and gross growth efficiencies as well as assimilation efficiency display a high range of variability (see Banse, 1979; Le Borgne, 1982; Omori and Ikeda, 1984; Vidal, 1980; Welch, 1968).

All these models are based on specific assumptions, such as: (1) some physiological rates and factors are assumed constant, and (2) growth is not dependent on food availability for the Huntley–Lopez (1992) model. We note that models are best applied to the species/groups used in their assembly and cannot be generalized. Hence, both physiological and empirical models may induce uncertainties when estimating growth rate (Hirst and Lampitt, 1998; Kobari et al., 2003; Lin et al., 2013; Runge and Roff, 2000).

As alternatives to these traditional methodologies, other techniques using biochemical measurements have been developed for the estimation of weight-specific growth rates (see Fig. 2), such as those using radio-labelled compounds, nucleic acids and enzyme activities as proxies for growth. In this review, we summarize the advances in biochemical indices for measuring weight-specific growth rates developed since the publication of the ICES (International Council for the Exploration of the Sea) Zooplankton Methodology Manual (Harris et al., 2000). We also describe the general concepts behind the most widely used methodologies and review the advantages and limitations of their *in situ* application to zooplankton communities.

2. BIOCHEMICAL METHODS FOR ZOOPLANKTON PRODUCTION

Several biochemical techniques have been developed to overcome the limitations of traditional methodologies (Fig. 2), with different levels of success. For example, incorporation techniques have had limited development in zooplankton studies, despite being widely adopted for phytoplankton (^{14}C, Steemann Nielsen, 1952; ^{13}C, Hama et al., 1983) and prokaryote production assessments (^{3}H thymidine, Fuhrman and Azam, 1982). Only two incorporation techniques (Fig. 2) have been investigated as potential indices

of zooplankton growth: the estimation of chitin production by the incorporation of ^{14}C NAG into the chitinous exoskeleton of planktonic crustaceans (Roff et al., 1994) and the incorporation of bromodeoxyuridine (BrdU) into the DNA (Gómez et al., 2001). To date no advances in these techniques have been made as the only application of these methods has been in the laboratory experiments describing them.

In addition to incorporation techniques, diverse alternative biochemical approaches have been proposed as proxies for growth and include methods based on nucleic acid quantification (e.g. Båmstedt and Skjoldahl, 1980; Dagg and Littlepage, 1972; Ota and Landry, 1984; Wagner et al., 2001) and enzyme activities involved in synthesis pathways (e.g. Alayse-Danet, 1980; Berges et al., 1990; Oosterhuis et al., 2000; Sapienza and Mague, 1979; Sastri and Roff, 2000; Yebra and Hernández-León, 2004). A major advantage of these methods over traditional approaches is that they do not require incubation of organisms, thus overcoming the main drawback of some direct measurement techniques. In addition, biochemical methods normally rely on simple procedures and quick measurements with high precision and repeatability, which can yield high temporal and spatial resolution data. These advantages overcome the limitations of the traditional methodologies for estimating zooplankton biomass and growth rate. Indeed, information on the application of biochemical approaches to whole zooplankton communities *in situ* is increasing. However, we have little comprehensive knowledge on the suitability of these biochemical methodologies applied to natural populations. In the following sections, we discuss the advances in biochemical methods currently in use for the assessment of zooplankton production, which include nucleic acid indices and enzymatic methods (Fig. 2).

2.1 Nucleic Acid Indices

Protein synthesis is a complex process with multiple steps involving the assembly of proteins by ribosomes (i.e. translation) along with the generation of messenger RNA (i.e. transcription), aminoacylation of transfer RNA, co-translational transport and post-translational modification. The cellular contents of RNA (messenger RNA, transfer RNA and ribosomal RNA) relative to those of DNA or protein may vary with cellular activity or protein synthesis. Thus, the total content, concentration or ratio of nucleic acids for an individual can be theoretically representative of somatic growth. These physiological functions are common to the entire zooplankton community.

Hence, nucleic acid-based indices have been applied in various species as proxies of organismal overall condition, reproductive activity and somatic growth.

The existence of a potential relationship between nucleic acid content and growth or reproduction compelled the development of this biochemical index in zooplankton (Table 1). Initial studies focused mostly on the search for a functional relationship between growth rate and RNA content in planktonic crustaceans (Båmstedt and Skjoldahl, 1980, 1976; Dagg and Littlepage, 1972; Skjoldal and Båmstedt, 1976; Sutcliffe, 1965). The RNA:DNA ratio as an index was introduced some years later (Ota and Landry, 1984), and research about the relationship between nucleic acids and egg production started during the mid-1990s (Nakata et al., 1994). The index gained popularity with the improvement of experimental protocols and laboratory facilities, along with the development of sensitive nucleic acid dyes. These improvements allowed the assessment of individual content of nucleic acids in zooplankton organisms, increased the sensitivity in quantification and in the number of samples per analysis, and reduced the overall cost of the analysis. In addition, nucleic acid indices became widely applied for the assessment of nutritional condition of various marine taxa during this period, especially in fish larvae (e.g. Buckley, 1984; Chícharo et al., 1998; Clemmesen, 1994; Vidal et al., 2006), contributing to the increase in the application of nucleic acid indices in zooplankton. This index was used not only as proxies for growth or reproduction but also as a proxy of various other factors, such as gonad maturation (e.g. Biegala et al., 1999; Nakata et al., 1994), the relationship between nutritional condition and food quantity or quality (e.g. Becker et al., 2005; Holmborn et al., 2009; Wagner et al., 1998) and dormancy (e.g. Kobari et al., 2013).

The use of nucleic acid indices for zooplankton production assessment was reviewed by Runge and Roff (2000). Since then, few reviews on nucleic acids in zooplankton have been published, and all of them with particular interest in stoichiometry (e.g. Elser et al., 2003; Hessen et al., 2007; Vrede et al., 2002). Here we present an update, focusing on the production-related application of nucleic acid indices.

2.1.1 Quantification of Nucleic Acids in Zooplankton

Most of the studies investigating the relationship between growth or reproduction and nucleic acid content quantified nucleic acids using specific dyes following the enzymatic degradation of either RNA or DNA. The only exception was Johannsson et al. (2008), who extracted the nucleic acids from

Table 1 Studies Applying Nucleic Acids (DNA and RNA) and Protein (PRO) Analysis to Zooplankton Growth and Production

Zooplankton Taxon	Type or Region	Proxies				Objectives	Reference
		DNA	RNA	RNA: DNA	RNA: PRO		
Culture							
Benthic amphipods	Marine		O			First application to growth estimation	Sutcliffe (1965)
Artemia	Brackish		O			Growth	Dagg and Littlepage (1972)
Copepods	Marine	O	O	O		Temperature effect on growth	Ota and Landry (1984)
Cladocerans	Freshwater				O	Growth	McKee and Knowles (1987)
Copepods	Marine		O	O		Temperature effect on egg production	Saiz et al. (1998)
Copepods	Marine			O		Egg production	Biegala et al. (1999)
Copepods	Marine			O		Egg production	Bersano (2000)
Copepods	Marine			O	O	Temperature and food quality effects on growth	Wagner et al. (2001)
Cladocerans	Freshwater	O	O			Development and moult	Gorokhova and Kyle (2002)
Cladocerans	Freshwater			O		Effect of food quality on growth and nucleic acids	Vrede et al. (2002)
Copepods	Marine			O		Egg production	Durbin et al. (2003)
Cladocerans, mixed zooplankton	Freshwater		O			Growth	Elser et al. (2003)

Copepods	Marine	O	O	O		Egg production	Gorokhova (2003)
Copepods	Brackish			O		Effect of food quantity on growth	Hansen et al. (2003)
Copepods	Marine			O		Effect of food quality on growth	Becker et al. (2005)
Cladocerans	Freshwater		O			Growth	Acharya et al. (2006a,b)
Cladocerans	Freshwater		O			Growth	Kyle et al. (2006)
Copepods	Marine			O		Nutritional condition	Speekmann et al. (2007)
Rotifers	Brackish			O		Growth	Hessen et al. (2007)
Mysids	Marine		O	O		Growth	Johannsson et al. (2008)
Copepods	Marine		O	O	O	Effect of food quantity on growth	Holmborn et al. (2009)
Copepods	Marine			O		Effect of food quality/quantity on growth	Gusmão and McKinnon (2009)
Cladocerans	Freshwater			O		Temperature effect on growth	Van Geest et al. (2010)
Copepods	Marine		O	O		Effect of temperature and food quality/quantity on growth	Gusmão and McKinnon (2011)
Copepods	Marine			O		Effect of starvation and temperature on growth	Yebra et al. (2011)
Rotifers	Brackish				O	Growth	Wojewodzic et al. (2011)
Copepods	Marine		O		O	Growth	He et al. (2015)

Continued

Table 1 Studies Applying Nucleic Acids (DNA and RNA) and Protein (PRO) Analysis to Zooplankton Growth and Production—cont'd

Zooplankton Taxon	Type or Region	Proxies				Objectives	Reference
		DNA	RNA	RNA: DNA	RNA: PRO		
Field							
Copepods	Canadian coast		O			Growth	Dagg and Littlepage (1972)
Copepods	Norwegian coast		O			Growth	Båmstedt and Skjoldal (1976)
Euphausiids	Norwegian coast		O			Growth	Skjoldal and Båmstedt (1976)
Mixed zooplankton	Norwegian coast		O			Growth	Båmstedt and Skjoldahl (1980)
Copepods	Kuroshio			O		First application to egg production	Nakata et al. (1994)
Copepods	Gulf of Maine			O		Egg production	Durbin et al. (2003)
Copepods	Baltic Sea	O	O	O		Growth	Gorokhova (2003)
Euphausiids	Southern Ocean	O	O	O		Growth	Shin et al. (2003)
Copepods	Baltic Sea		O			Egg production	Holmborn and Gorokhova (2008)
Copepods	Baltic Sea		O			Nutritional condition	Hook et al. (2008)

Copepods	Canadian coast		O	Effect of food quality on growth	Pommier et al. (2010)
Cladocerans	Lake Erie	O		Growth	Goto et al. (2012)
Copepods	Canadian coast		O	Effect of food quality on growth	Pommier et al. (2012)
Copepods	North Atlantic		O	Nutritional condition	Hauss et al. (2013)
Copepods	North Pacific		O	Dormancy	Kobari et al. (2013)
Cladocerans, Copepods	Spanish lakes	O	O	Effect of food quality on growth	Bullejos et al. (2014a,b)
Copepods, Rotifers	Baltic Sea		O	Effect of food quality on growth	Gorokhova et al. (2014)
Copepods	Baltic Sea	O		Growth	Hogfors et al. (2014)

mysids before quantifying RNA and DNA individually. There are several variations in protocols for the quantification of nucleic acids in zooplankton using the fluorochromes RiboGreen (Gorokhova and Kyle, 2002), ethidium bromide (Wagner et al., 1998) and SYBR Green (Berdalet et al., 2005a,b), but all follow similar steps. All available protocols require the homogenization of the sample in a small volume of extraction buffer as a first step, then reading the fluorescence of the mixture in cuvettes or microplate readers following the sequential degradation of RNA with the addition of RNase. The RNA and DNA contents are inferred from the difference in fluorescence before and after the addition of nucleases. The analytical procedures for nucleic acid quantification are very simple and show some advantages in comparison to other biochemical indices.

2.1.2 Application of Nucleic Acid Indices to Zooplankton

Since Runge and Roff (2000) reviewed the biochemical methods applied for zooplankton production, there has been a marked increase in studies investigating the relationship between nucleic acid content and growth or reproductive output in zooplankton (Table 1). In addition, there is considerable variation in the choice of nucleic acid index reported in the scientific literature.

Despite this, very few studies compared the efficiency of different nucleic acid indices as predictors of either growth or reproduction in zooplankton. Those studies suggest that the RNA:DNA ratio appears to be the best index of egg production in copepods. For instance, Saiz et al. (1998) reported that the RNA:DNA ratio is a slightly better predictor of egg production in the copepod *Paracartia grani* than individual RNA content. Gusmão and McKinnon (2011) found that both RNA:DNA and the total individual RNA content are better predictors of egg production than the relative concentration of RNA (per individual carbon or nitrogen content) in the copepod *Acartia sinjiensis*. On the other hand, nucleic acid concentration appeared to be more appropriate as an index of somatic growth. For example, Gorokhova (2003) recommended the use of RNA concentration as an index of growth rate instead of RNA:DNA ratio for *Acartia* spp. In fact, RNA concentration is the most common index in studies investigating the relationship between nucleic acid indices and growth rates in crustacean zooplankton, while the RNA:DNA ratio is applied for copepod reproductive output experiments (Table 2). However, any generalization should be interpreted with caution as there is limited evidence supporting either type of index.

Table 2 Models Relating Growth and Reproduction Rates as a Function of Nucleic Acid Indices in Zooplankton Species

Species	Empirical Relationship	r^2	Type	Source
Acartia bifilosa	$sep_W = 0.0043 \times [RNA_C] - 0.125$	0.92***	Field and Exp	Gorokhova (2003)
Acartia bifilosa	$sep_W = 0.027 \times [DNA_C] - 0.471$	0.42*	Field and Exp	Gorokhova (2003)
Acartia bifilosa	$sep_W = 0.116 \times (RNA{:}DNA) - 0.152$	0.75**	Field and Exp	Gorokhova (2003)
Acartia bifilosa	$efd = 14.74 + 106.17 \times [RNA_{ind}] + 43.79 \times PL + 0.24T$	0.49***	Field	Holmborn and Gorokhova (2008)
Acartia sinjiensis	$efd = 1.078 \times ([RNA_{ind}] \times T) - 7.204$	0.64***	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$efd = 0.561 \times (RNA{:}DNA \times T) - 8.683$	0.70***	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$efd = 0.410 \times ([RNA_N] \times T) - 6.756$	0.41*	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$sep_C = 0.013 \times ([RNA_{ind}] \times T) - 0.08$	0.63**	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$sep_C = 0.007 \times (RNA{:}DNA \times T) - 0.1$	0.70***	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$sep_N = 0.013 \times ([RNA_{ind}] \times T) - 0.081$	0.63**	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$sep_N = 0.007 \times (RNA{:}DNA \times T) - 0.1$	0.70**	Exp	Gusmão and McKinnon (2011)
Acartia sinjiensis	$sep_N = 0.005 \times ([RNA_N] \times T) - 0.079$	0.42*	Exp	Gusmão and McKinnon (2011)
Acartia tonsa	$efd = 2.8 \times (RNA{:}DNA) - 8.2$	0.86**	Exp	Bersano (2000)
Acartia tonsa	$efd = 26.23 \times [RNA_{ind}] - 0.114$	0.20***	Field	Hogfors et al. (2014)[a]
Calanus finmarchicus	$g_{\%PROT} = 1.29 \times (RNA{:}DNAr) - 28.39$	0.73*	Exp	Wagner et al. (2001)
Calanus finmarchicus	$g_{\%C} = 1.27 \times (RNA{:}DNAr) - 32.39$	0.66*	Exp	Wagner et al. (2001)
Calanus finmarchicus	$g_{protein} = 1.407 \times RNA{:}PRO - 0.074$	0.66*	Exp	Wagner et al. (2001)

Continued

Table 2 Models Relating Growth and Reproduction Rates as a Function of Nucleic Acid Indices in Zooplankton Species—cont'd

Species	Empirical Relationship	r^2	Type	Source
Calanus finmarchicus	$g_N = 0.956 \times \text{RNA:PRO} + 0.110 \times (\text{RNA:PRO} \times T) - 0.111$	0.86***	Exp	Wagner et al. (2001)
Calanus finmarchicus	$g_C = 1.208 \times \text{RNA:PRO} + 0.131 \times (\text{RNA:PRO} \times T) - 0.155$	0.86***	Exp	Wagner et al. (2001)
Calanus finmarchicus	$g = 1.549 \times \text{RNA:PRO} + 0.145 \times (\text{RNA:PRO} \times T) - 0.189$	0.9***	Exp	Wagner et al. (2001)
Calanus finmarchicus	$\text{efd} = 0.820 \times (\text{RNA:DNA}) - 32.264$	0.85	Exp	Durbin et al. (2003)
Calanus finmarchicus	$\text{efd} = 0.889 \times (\text{RNA:DNA}) - 29.128$	0.70	Exp	Durbin et al. (2003)
Calanus pacificus	$g = 0.0850 + 0.0315 \times (\text{RNA:DNA})$	0.32	Field	Ota and Landry (1984)
Calanus pacificus	$g = 0.0903 + 0.0407 \times (\text{RNA:DNA})$	0.54	Field	Ota and Landry (1984)
Calanus pacificus	$g = 0.0795 + 0.0306 \times (\text{RNA:DNA})$	0.01	Field	Ota and Landry (1984)
Mixodiaptomus laciniatus	$\text{RNA\%} = 2.32 + 5.73\, g$	0.84*	Exp	Bullejos et al. (2014a)
Paracartia grani	$\text{efd} = 38.7 \times [\text{RNA}_{\text{ind}}] - 11.8$	0.88***	Exp	Saiz et al. (1998)
Paracartia grani	$\text{efd} = 126 \times [\text{RNA}_{\text{ind}}] - 40.8$	0.93***	Exp	Saiz et al. (1998)
Paracartia grani	$\text{efd} = 1.76 \times (\text{RNA:DNA}) - 19.4$	0.92***	Exp	Saiz et al. (1998)
Paracartia grani	$\text{efd} = 3.41 \times (\text{RNA:DNA}) - 29.6$	0.96***	Exp	Saiz et al. (1998)
Paracartia grani	$\text{efd} = -25.7 - 1.67 \times (\text{RNA:DNA}) + 0.21(T \times (\text{RNA:DNA}))$	0.96***	Exp	Saiz et al. (1998)

Paracalanus sp.	efd = 1.57 × (RNA:DNA) − 5.52	0.84***	Field	Nakata et al. (1994)
Pseudodiaptomus annandalei	$g_{\text{nauplii to copepodites}} = 0.493 \times$ RNA:PRO + 0.051	0.52**	Exp	He et al. (2015)
Pseudodiaptomus annandalei	$g_{\text{copepodites}} = 0.304 \times \text{RNA:PRO} + 0.046$	0.81**	Exp	He et al. (2015)
Pseudodiaptomus annandalei	$g_{\text{copepodites}} = 0.225 \times [\text{RNA}_{\text{DW}\mu\text{g}}] + 0.031$	0.86**	Exp	He et al. (2015)
Oithona davisae	$\text{RNA:DNA}_{\text{nauplii}} = 39.03 \times g - 2.36$	0.84***	Exp	Yebra et al. (2011)
Oithona davisae	$\text{RNA:DNA}_{\text{copepodites}} = 41.15 \times g - 4.18$	0.90***	Exp	Yebra et al. (2011)
Euchaeta norvegica	$g_{\%} = -0.545 + 0.224 \times [\text{RNA}_{\text{DW}}]$	0.90	Field	Båmstedt and Skjoldal (1976)
Euphausia superba	$\text{RNA:DNA} = 0.2483 \times g(\%) + 1.1563$	0.29***	Field	Shin et al. (2003)
Euphausia superba	$[\text{RNA}_{\text{WW}}] = 0.3317 \times g(\%) + 1.0398$	0.47***	Field	Shin et al. (2003)
Euphausia superba	$[\text{DNA}_{\text{WW}}] = 0.0392 \times g(\%) + 0.9534$	0.14**	Field	Shin et al. (2003)
Meganyctiphanes norvegica	$g_{\%} = -0.761 + 1.526 \times \log[\text{RNA}_{\text{DW}}]$	?	Field	Skjoldal and Båmstedt (1976)
Mysis relicta	$g_{\%\text{wt}} = 1.607 + 0.029 \times [\text{RNA}_{\text{ind}}] - 0.352 \times [\text{RNA}_{\text{WWF}}] - 0.102 \times \text{Wwi} + 0.008$ RNA: DNA $\times T$	0.59***	Exp	Johannsson et al. (2008)
Bosmina spp.	$g = 0.11 \times \text{RNA\%} + 0.03$	0.67***	Exp	Acharya et al. (2006a)

Continued

Table 2 Models Relating Growth and Reproduction Rates as a Function of Nucleic Acid Indices in Zooplankton Species—cont'd

Species	Empirical Relationship	r^2	Type	Source
Daphnia galeata	$g = 0.03 \times (\text{RNA:DNA}) - 0.19$	0.94***	Exp	Vrede et al. (2002)
Daphnia galeata	$\text{RNA\%} = 1.74 + 4.53 \times g$	0.65*	Exp	Elser et al. (2003)
Daphnia lumholtzi	$\text{RNA\%} = 2.252 + 9.819 \times g$	0.48**	Exp	Acharya et al. (2006b)
Daphnia magna	$\text{RNA:PRO}_{\text{mg}} = 114 + 0.546 \times g_{\%\text{DW}}$	0.68	Exp	McKee and Knowles (1987)
Daphnia magna	$\text{RNA\%} = 0.98 + 5.30 \times g$	0.65*	Exp	Kyle et al. (2006)
Daphnia pulex	$\text{RNA\%} = -1.70 + 8.47 \times g$	0.89***	Exp	Kyle et al. (2006)
Daphnia pulicaria	$\text{RNA\%} = 1.92 + 8.69 \times g$	0.91***	Exp	Elser et al. (2003)
Daphnia pulicaria	$\text{RNA\%} = 2.12 + 7.96 \times g$	0.91*	Exp	Kyle et al. (2006)
Daphnia pulicaria	$\text{RNA\%} = 2.63 + 6.16 \times g$	0.81**	Exp	Kyle et al. (2006)
Artemia salina	$g_{\%} = -2.33 + 0.35 \times [\text{RNA}_{\text{DW}}]$	?	Exp	Dagg and Littlepage (1972)
Orchestia platensis	$g_{\%} = -5.041 + 21.667 \times \log[\text{RNA}_{\text{DW}}]$	?	Exp	Sutcliffe (1965)[b]
Brachionus calyciflorus	$\text{RNA:N} = 2.23 \times g + 0.13$	0.98	Exp	Hessen et al. (2007)
Brachionus calyciflorus	$g_{\text{H}} = 0.37 \times \text{RNA:PRO} - 0.017$	0.75	Exp	Wojewodzic et al. (2011)
Various (freshwater)	$\text{RNA\%} = -0.55 + 15.9 \times g$	0.99**	Exp	Elser et al. (2003)
Various (marine)	$k \times 10^3 = 0.795 \times [\text{RNA}_{\text{DW}}]$	?	Field	Båmstedt and Skjoldahl (1980)

Growth Variables	Nucleic Acid Indices	Reproduction Variables	Other Variables
k = coefficient of daily exponential growth	DNA_{WW} = μg DNA mg wet weight^{-1}	efd = eggs Fem^{-1} d^{-1}	PL = prosome length (mm)
$g = d^{-1}$	DNA_C = μg DNA mg C^{-1}	sep_W = weight-specific egg production rate (d^{-1})	Wwi = initial wet weight (mg ind^{-1})
$g_H = h^{-1}$	RNA_{DW} = μg RNA mg dry weight^{-1}	sep_C = carbon-specific egg production rate (d^{-1})	T = temperature (°C)
$g_{\%}$ = % tissue growth d^{-1}	$RNA_{DW\mu g}$ = μg RNA μg dry weight^{-1}	sep_N = nitrogen-specific egg production rate (d^{-1})	
$g_{\%PROT}$ = % protein growth d^{-1}	RNA_{ind} = μg RNA ind^{-1}		
$g_{\%C}$ = % carbon growth d^{-1}	RNA_{WW} = μg RNA mg wet weight^{-1}		
$g_{\%wt}$ = % wet weight growth d^{-1}	RNA_{WWF} = μg RNA mg final wet weight ind^{-1}		
$g_{\%DW}$ = % dry weight growth d^{-1}	RNA% = % of dry mass		
$g_{protein} = d^{-1}$ (protein)	RNA_N = μg RNA μg N^{-1}		
$g_N = d^{-1}$ (nitrogen)	RNA_C = μg RNA mg C^{-1}		
$g_C = d^{-1}$ (carbon)	RNA:PRO = μg RNA μg protein^{-1}		
	$RNA:PRO_{mg}$ = μg RNA mg protein^{-1}		
	RNA:N = RNA to nitrogen ratio		
	RNA:DNA = RNA to DNA ratio		
	RNA:DNAr = relative RNA:DNA		

[a]Regression calculated from all data from supporting information.
[b]Equation defined for *Orchestia platensis* (a benthic organism) but used successfully to estimate the growth rate of *Artemia salina*.
Exp, laboratory experiments; *Field*, field assessments.
$^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

Regardless of the index used, the nucleic acid content often shows a linear relationship with growth or egg production in zooplankton species, which makes nucleic acid indices attractive in zooplankton research. However, the universal applicability of such empirical relationships is unlikely for diverse zooplankton species, as these equations may be affected by several factors (see Section 2.1.4.2).

2.1.3 Advances in Nucleic Acid Indices Application in the Field

The use of sensitive fluorescent dyes has improved the limit of detection and the precision of nucleic acid analysis (see Section 2.1.4.1). These improvements have increased the accuracy of measurements of nucleic acids even on small zooplankton species and their larvae, which also contribute to the production of populations and communities (e.g. Hopcroft and Roff, 1995, 1998). For example, Yebra et al. (2011) studied changes in RNA:DNA ratios of cyclopoid nauplii (*Oithona davisae*) for several days after hatching, and Gorokhova and Kyle (2002) determined the differences of nucleic acid contents of *Daphnia pulex* juvenile instars.

Improved (sensitive, user-friendly) experimental instruments, such as microplate readers, have allowed simplified procedures, quick measurements and enhanced cost–benefit ratios. Improvements have allowed simultaneous measurements of many samples and/or replicates, providing for high spatial and temporal resolution of field measurements. For instance, Ikeda et al. (2007) showed RNA:DNA ratios for more than 80 copepod species from epipelagic to abyssopelagic depths in oceanic waters of the North Pacific Ocean. Currently, empirical equations relating nucleic acid indices to growth or egg production are available for various species of crustaceans and rotifers (Table 2). In particular, empirical models were described for eight of these species in the field. However, to date, there is no universal equation or factor applicable to multiple species or communities as described later (see Section 2.1.4.2). Nevertheless, the identification of empirical equations for additional species (especially for species other than crustaceans) would increase opportunities for applying nucleic acid indices to zooplankton production rate estimates in the field.

2.1.4 Advantages and Limitations of In Situ Application of Nucleic Acid Indices

2.1.4.1 Advantages

2.1.4.1.1 Simple and Rapid Procedures Initially, nucleic acid concentrations were determined with multiple steps of enzyme treatments (Bentle et al., 1981) and the number of samples analysed was limited.

Wagner et al. (1998) proposed a simple procedure for quick and routinely applicable measurements, allowing processing of several samples at once. Furthermore, the fluorescent dye ethidium bromide is mutagenic and more sensitive non-mutagenic dyes such as RiboGreen and SYBR Green have been more widely used in recent years (Berdalet et al., 2005a,b; Gorokhova and Kyle, 2002), allowing for the measurement of individual nucleic acid contents even in small zooplankton species. The use of nucleic acid specific dyes has improved the detection limit and allowed the use of small sample volumes in the procedure, thus reducing the overall cost of the analysis per sample and the processing speed in the laboratory.

2.1.4.1.2 Broadly Applicable Across Taxonomic Groups Protein synthesis is a universal physiological function in animals and the process is quite conservative among different taxa. Therefore, nucleic acid indices can be theoretically used for any zooplankton taxa. Indeed, nucleic acid indices have been applied to rotifers, cladocerans, copepods, euphausiids, amphipods and mysids (see Table 1). However, there is no information on gelatinous zooplankton groups such as pelagic tunicates, hydrozoans or ctenophores, which have low protein content.

2.1.4.1.3 Requires no Incubation Traditional methodologies require incubations of animals, whereas no incubation is necessary for indices reliant on nucleic acid estimation. Once zooplankton samples are collected, they are transferred to RNA*later*© solution (Gorokhova, 2005) or frozen in liquid nitrogen on board and subsequently transferred to a deep freezer for long-term storage prior to analysis. However, there is one exception. The estimation of the relative RNA:DNA ratio (Wagner et al., 2001) requires the incubation of animals under saturated food condition in order to evaluate the relative change in RNA:DNA. This procedure has not been widely incorporated into zooplankton research and might be not applicable for animals accumulating lipids or experiencing food-limiting conditions (Wagner et al., 2001).

2.1.4.2 Limitations

2.1.4.2.1 Uncertainty of the Theoretical Assumptions The application of nucleic acid content as a proxy for growth or reproduction relies on two main assumptions: (1) synthesized protein represents weight-specific growth, and (2) DNA content is relatively constant and represents the cell number. Evidence suggests that both assumptions do not always hold for zooplankton. The cellular nucleic acid machinery synthesizes proteins, which

is a main component of body mass. For estimating zooplankton growth, therefore, it is assumed that the synthesized protein will be accumulated into tissues such as muscles, hence reflecting an increase in body mass. Many laboratory experiments demonstrated that a gain in weight is generally followed by a similar trend in nucleic acid content in zooplankton (e.g. Vrede et al., 2002; Wagner et al., 2001; Yebra et al., 2011). However, a large variability is usually observed in the relationship between weight and nucleic acids, which may be a result of three factors.

First, several types of proteins produced by the cellular nucleic acid machinery may not necessarily be accumulated in tissues. For example, enzymes are generally short-lived (hence not contributing significantly to increase in weight) and are used in various metabolic processes related to the maintenance of the cell. In addition, cells continuously produce proteins that are incorporated into tissues, but may be used for repairing them, so no weight gain would be observed (see also Section 2.4). The proteins used for the maintenance of the cell function are often observed in diapausing copepods as a low nucleic acid machinery in the cell, represented by a low nucleic acid index, with no observable gain in weight.

Second, weight (or C or N content) gain may be associated with the accumulation of biochemical compounds other than proteins, such as lipids and carbohydrates. These compounds are not directly associated with the nucleic acid machinery, so nucleic acid indices are unlikely to be associated with the production of these compounds, such as lipids and chitin, which can contribute significantly to weight variation in zooplankton organisms. Moreover, the proportion of water content to body mass is high in gelatinous zooplankton (e.g. Postel et al., 2000), suggesting the low contribution of protein synthesis to weight-specific growth in these organisms.

Third, weight-specific growth estimated using nucleic acids relies upon protein synthesis derived from the substrates of ingested and assimilated materials. However, not only externally derived but also internally derived materials seem to be available as substrates for protein synthesis. For crustacean zooplankton, *Artemia* spp., proteolytic activities increase with yolk degradation in newly hatched nauplii and genital structures develop in late life stages (Slegers, 1991). Laboratory experiments showed that the nucleic acid ratio (i.e. RNA:DNA) was high for newly hatched nauplii and late life stages of *Artemia salina*, due to yolk consumption and gonad maturation (Kobari et al., 2016). Therefore, protein synthesis from internally derived materials (e.g. yolk, stored lipids or somatic protein turnover) would be a potential source of error when using nucleic acid indices (see also Section 2.4) to infer weight-specific growth.

The second assumption concerns the constancy of DNA content per cell, for which there is also contradicting evidence. For instance, it has been observed a significant decline in the total DNA content of *Acartia* copepods under starvation (Speekmann et al., 2007) or exposed to low food concentrations (Gusmão and McKinnon, 2011) in comparison to those exposed to saturating food conditions. There is also evidence that DNA content or concentration may be correlated with growth rates (Gorokhova, 2003; Gusmão and McKinnon, 2011), which may be an additional source of error when using nucleic acid indices in zooplankton. Little is known on the variation of DNA content among zooplankton taxa, but two processes are believed to explain such variation: (1) endopolyploidy and expansion of DNA, and (2) chromatin diminution (see Gorokhova, 2003). However, the variation of DNA content seemed to follow a similar trend to RNA content (e.g. Gusmão and McKinnon, 2011; Speekmann et al., 2007), which lead Wagner et al. (2001) to suggest that, despite DNA content apparently not representing the cell number, the RNA:DNA ratio can still be used as an index of growth or reproduction.

2.1.4.2.2 No Universal Equation or Factor If nucleic acid indices are to be used for estimating zooplankton production in mixed populations, then a universal equation or a factor is required. The search for such universal equation was one of the main objectives of the early zooplankton nucleic acid research. Empirical equations developed for one organism were used to predict growth rates in others (e.g. Dagg and Littlepage, 1972; Sutcliffe, 1965). The absence of a universal equation or a factor is likely a consequence of three factors. First, production-related data are species specific and limited to a few taxa. Most of the nucleic acid data gathered so far are for planktonic crustaceans. Consequently, any generalization made from these equations may be affected by specific developmental or reproduction characteristic of these groups, such as the development in copepods and the parthenogenetic reproduction in cladocerans. In fact, among crustaceans, cladocerans show much higher RNA:DNA ratios (e.g. Gorokhova and Kyle, 2002) than copepods (e.g. Gorokhova, 2003). Even for copepods, RNA:DNA ratios fluctuate considerably among species (Ikeda et al., 2007), and the relationships between nucleic acid indices and growth are species and sometimes stage specific (e.g. Yebra et al., 2011). Second, there are no standardized indices of nucleic acid content or growth and reproduction. There is large variability in the way both nucleic acid indices and growth or reproduction are represented (see Tables 1 and 2), and the

conversion from one nucleic acid index to another cannot be done, making the direct comparison among studies difficult. Finally, potential covariates are often not accounted for in the relationship between nucleic acid content and productivity indices in zooplankton. A major consequence of the lack of a universal equation or a factor is that nucleic acid indices are unlikely applicable to community-based growth estimates, such as those provided by enzymatic indices (e.g. AARS (aminoacyl-tRNA synthetases) and chitobiase).

2.1.4.2.3 Factors Affecting the Relationship Between Nucleic Acid Content and Growth Several factors are known to influence the nucleic acid content of zooplankton organisms, such as temperature, food quantity and quality, and body size or weight, but few studies have investigated the effect of these factors on the relationship between nucleic acid indices and growth or reproductive rates of zooplankton. Among these factors, only the temperature and body size or weight are presented as covariates in empirical relationships between nucleic acid content and growth-related indices in the literature. Nucleic acid indices often show a strong negative correlation with temperature (Gusmão and McKinnon, 2011; Ota and Landry, 1984; Wagner et al., 2001), which is believed to be linked to a higher RNA activity at higher temperatures (Buckley et al., 2008; Wagner et al., 2001). However, the effect of temperature on the empirical relationship remains unclear. Some studies found that the incorporation of temperature as an interactive term with nucleic acid indices improves the predictability of empirical relationships (e.g. Gusmão and McKinnon, 2011; Saiz et al., 1998), while other studies have not (e.g. Gorokhova, 2003). Body size has been incorporated as an independent factor, concomitantly with temperature, in models predicting the growth of mysids (Johannsson et al., 2008) and egg production in copepods (Holmborn and Gorokhova, 2008). Food quantity and quality are the most frequently manipulated factors in nucleic acid experiments with zooplankton, but neither factor is included in empirical relationships in the literature. This is likely because most empirical relationships are based on linear models, and the effect of food quantity and quality appears to be complex, following non-linear relationships. For instance, the effect of food quantity on the nucleic acid content appears to follow a non-linear (sigmoid) function up to a saturation level (Gusmão and McKinnon, 2009), and food quality appears to affect nucleic acid content in an unimodal way (Bullejos et al., 2014b). Since growth is affected by both food quantity and quality, understanding how these factors correlate with nucleic acid content is likely to lead to better predictive models in zooplankton.

2.2 Enzymatic Methods

In contrast to the ratio method, the enzymatic approach to measure growth rates gained momentum during the 1980s based on the parallelism between growth and enzymatic activity as both are measured as rate processes. Sapienza and Mague (1979) were the first to propose such a relationship comparing growth rates and the activity of the enzyme DNA polymerase (Table 3). Following previous attempts to relate metabolic and enzyme activities (e.g. respiration and succinate dehydrogenase, Curl and Sandberg 1961) identified a strong relationship between growth and DNA polymerase in *Artemia salina*, but unfortunately the method was no longer used. Perhaps, the need for radioactive thymidine triphosphate to run the assay precluded its use in oceanography. Almost coincident in time with the publication of the previous method, the French laboratories proposed the activity of

Table 3 Summary of Enzymes Applied to Assess Zooplankton Production Rates

Enzyme	Acronym	IUBMB Nomenclature	Associated Process	Calibration Against Growth Rate
DNA polymerase		EC 2.7.7.7	DNA replication	Sapienza and Mague (1979)
Aspartate transcarbamylase	ATC	EC 2.1.3.2	Cell proliferation	Alayse-Danet (1980), Hernández-León et al. (1995)
Nucleoside diphosphate kinase	NDPK	EC 2.7.4.6	Nucleoside synthesis	—
Glucose-6-phosphate dehydrogenase	G6DPH	EC 1.1.1.49	Nucleic acid synthesis	—
Electron transport system enzymes	ETS	EC 1.6.5.3	Respiration	Ikeda and Motoda (1978)[a]
Chitobiase	CBA	EC 3.2.1.52	Moult	Oosterhuis et al. (2000)
Aminoacyl-tRNA synthetases	AARS	EC 6.1.1	Protein synthesis	Yebra and Hernández-León (2004), Yebra et al. (2005, 2006b, 2011), Herrera et al. (2012)

[a]Respiration rate (not ETS activity) was related to growth rate.
IUBMB, International Union of Biochemistry and Molecular Biology.

aspartate transcarbamylase (ATC) as a suitable index of growth. This enzyme controls the first specific step to *de novo* synthesis of pyrimidine bases, which are used in the cell to build RNA and DNA. Bergeron and Buestel (1979) and Bergeron and Alayse-Danet (1981) found good relationships between growth rates, and the gonad and mantle of molluscs. Later, Bergeron (1982) also found good relationships between growth and ATC activity in fishes, although the regression coefficients varied among species. However, the first attempt to study this relationship in planktonic crustaceans (*A. salina*) failed (Alayse-Danet, 1980). Although significant relationships were observed, the poor correlation ($r^2 = 0.39$) gave no confidence for growth prediction.

Since ATC controls the synthesis of pyrimidine bases, it is expected that its activity should reflect cell multiplication as observed by Koueta and Boucaud-Camou (1992). ATC activity does not measure growth *sensu stricto*, but cell proliferation could be assimilated to growth in some organisms such as molluscs and fishes. In crustaceans, however, moulting is a process in which cell multiplication and growth are not coupled. Crustaceans increase in weight before moulting by increasing the amount of water in their cells, they cast the exoskeleton (negative growth) and finally they increase the protein content of cells (positive growth) (see Section 2.3). Thus, maximum of ATC activity is expected just before moulting. This feature was evidenced by Hernández-León et al. (1995), who observed a peak of ATC activity coinciding with the moulting period.

In any case, the relationship between enzymatic activity and metabolic rates is not straightforward. As observed for other proxies of respiration and ammonia excretion such as the electron transfer system (ETS) and glutamate dehydrogenase (GDH) activities, the ATC technique was also affected by methodological constrains related to the proper enzymatic assay. Enzymes are normally measured at V_{max} in the laboratory, and this is achieved at substrate saturation. However, cells in nature are normally affected by substrate levels, as related to the organism feeding history. Hernández-León and Gómez (1996) found different relationships between ETS activity and respiration rates during bloom and no-bloom conditions in subtropical waters. They interpreted this difference to indicate that the organisms were well fed during the period of increased primary production and, therefore, experienced increased substrate concentration in the cell. This different relationship was also evidenced by Hernández-León and Torres (1997) in GDH activity as observed from the parallel regressions between ammonia excretion and GDH at different levels of metabolized nitrogen (measured as the ratio between oxygen consumed and ammonia

excreted). Those results were quite similar to the different relationships observed by Hernández-León et al. (1995) between ATC activity and growth rates.

Other enzymes were also assayed during these decades in the race to find a proxy for growth (see Table 3). Berges et al. (1990) assayed the activity of the nucleoside diphosphate kinase (NDPK), an enzyme involved in biosynthesis pathways from di- to triphosphate nucleosides (cystidine, guanosine, uridine and thymidine). Although they measured the increase in biomass of individuals of *A. salina*, they only provided the scaling factor between this biomass and the enzymatic activity. They suggested its suitability as an index of growth because of the decrease in activity as organisms reached the adult size. Later, Berges and Ballantyne (1991) also assayed NDPK and glucose-6-phosphate dehydrogenase. They also studied the scaling exponent between biomass and enzymes, also suggesting that their variability may relate to differences in growth rates. However, no calibration was shown in any of both studies. Growth rates and NDPK were also measured in the marine diatom *Thalassiosira pseudonana* (Berges and Harrison, 1993), observing a statistically significant regression. However, the poor correlation found also precluded its use for predictive purposes.

Thus, cumulative problems comparing enzymatic activities and physiological rates promptly arose. The fact that ATC activity measures cell multiplication, or NDPK the nucleoside synthesis, instead of protein building, the inherent issue related to measurement of enzymatic activities at V_{max}, the uncoupling between the physiological process and the enzymatic processes, and the low precision normally achieved to measure growth rates as it is not possible to work using single animals (errors in measuring biomass in tiny organisms) makes difficult to find the expected relationship with physiological rates.

Another way of estimating growth is by use of the physiological method, which is based on the empirical relationship between respiration and growth rates (see Section 1.3.1). Respiration could ostensibly be estimated from the measurement of the ETS activity (Packard, 1971). This method has been widely applied in biological oceanography, but has some inherent assumptions and shortcomings. For instance, the respiration to ETS ratio normally ranges between 0.5 and 1.0 depending on the oligo- or mesotrophic state of the system (Hernández-León and Gómez, 1996). McKinnon et al. (2015) compared growth estimated from AARS and ETS activities, the latter following Ikeda and Motoda (1978), and observed similar values of growth assessed on the basis of both of these enzymatic activities. However, the

use of ETS activity to infer growth implies a combination of the assumptions and limitations of two indirect methods (i.e. the physiological model and the R/ETS ratio).

Recently, the use of two additional enzymatic approaches has gained momentum. These are chitobiase activity, related to the moult process, and AARS activity, which catalyse the first step in protein synthesis.

2.3 Chitobiase Activity

Chitobiase is a chitinolytic enzyme participating in the moulting process of all arthropods. Variation in the activity of this enzyme has been investigated as either an index or a quantitative measure of crustacean zooplankton moulting, growth and biomass production rates (BPRs). Therefore, methodological techniques reliant on chitobiase activity are specific to actively moulting arthropods (typically crustaceans in marine systems). Accordingly, this technique encompasses the productivity of what is usually the biomass-dominant zooplankton group and dominant intermediary between the base of marine food webs and higher trophic levels. However, the productivity of non-crustaceans (i.e. gelatinous zooplankton) or non-moulting crustacean stages (i.e. adult copepods) is not measured with chitobiase. Thus, with respect to crustacean zooplankton productivity, the chitobiase assay has been applied to: (1) indexing moult rates in homogenates (Espie and Roff, 1995a,b), (2) indirect measurements of moulting rates of single and mixed populations in the laboratory and field (Sastri, 2001; Sastri and Dower, 2006; Sastri and Roff, 2000) and (3) indirect measurements of BPRs for single populations in the laboratory (Oosterhuis et al., 2000; Suchy et al., 2013) and for entire crustacean zooplankton communities in the field (Avila et al., 2012; Sastri and Dower, 2009; Sastri et al., 2012, 2013; Suchy et al., 2016a,b; see Table 4). The earlier work by Espie and Roff (1995a,b) was discussed in detail by Runge and Roff (2000) in the ICES Zooplankton Methodology Manual (Harris et al., 2000). Here, we briefly review some of the relevant background covered by Roff et al. (1994) and Runge and Roff (2000) and focus on the development and application of the assay since 2000.

2.3.1 Crustacean Moult Cycle and Chitin Metabolism

Following the rate of synthesis of mass by tracking a single structural component (e.g. protein) is the underlying principle common to most biochemical and radiochemical techniques for measuring or indexing the growth of

Table 4 Studies Applying the Chitobiase Method to Zooplankton

Zooplankton Taxon	Type or Region	Measurements	Objectives	Reference
Culture				
Cladocerans	Freshwater	Liberated enzyme activity	Enzyme characterization	Vrba and Machacek (1994)
Copepods	Marine	Liberated enzyme activity, degradation rate, enzyme characterization	Method development: biomass production rate	Oosterhuis et al. (2000)
Cladocerans	Freshwater	Liberated enzyme activity, degradation rate	Method development: developmental rates	Sastri and Roff (2000)
Copepods	Marine	Enzyme characterization	Characterize salinity responses	Avila et al. (2011)
Cladocerans	Freshwater	Liberated enzyme activity	Method development: toxicological assay	Duchet et al. (2011)
Copepods	Marine	Liberated enzyme activity, degradation rate	Productivity responses to diet mixture and toxicity	Suchy et al. (2013)
Culture/field				
Copepods	Strait of Georgia, Canada	Liberated enzyme activity, degradation rate, enzyme characterization	Field method validation: developmental rates	Sastri and Dower (2006)
Crustacean Zooplankton	Strait of Georgia, Canada	Liberated enzyme activity, degradation rate, enzyme characterization	First field estimates of biomass production rates	Sastri and Dower (2009)

Continued

Table 4 Studies Applying the Chitobiase Method to Zooplankton—cont'd

Zooplankton Taxon	Type or Region	Measurements	Objectives	Reference
Field				
Crustacean Zooplankton	Rivers/ Streams, Europe	Liberated enzyme activity	First field evaluation of benthic invertebrate productivity index and toxicological response	Hanson and Lagadic (2005)
Crustacean Zooplankton	Rivers, North America	Liberated enzyme activity	Toxicological response to pharmaceuticals	Richards et al. (2008)
Crustacean Zooplankton	Temperate-Arctic, Gulf of Alaska, Bering, Chukchi and Beaufort Seas	Liberated enzyme activity, degradation rate	Broad-scale characterization of production rates in high latitude waters	Sastri et al. (2012)
Crustacean Zooplankton	Subtropical Estuarine Lagoon, Brazil	Liberated enzyme activity, degradation rate	Seasonal patterns of production rates; comparison to empirical models	Avila et al. (2012)
Crustacean Zooplankton	Temperate Freshwater Lake	Liberated enzyme activity, degradation rate	Field evaluation of biomass and production rate estimates	Sastri et al. (2013)
Crustacean Zooplankton	Temperate Freshwater Lake	Liberated enzyme activity, degradation rate	Zooplankton productivity responses to variable thermal water column structure and diversity	Sastri et al. (2014)

Table 4 Studies Applying the Chitobiase Method to Zooplankton—cont'd

Zooplankton Taxon	Type or Region	Measurements	Objectives	Reference
Crustacean Zooplankton	Temperate Fjord; Canada	Liberated enzyme activity, degradation rate	Characterize temporal patterns of production rates and phytoplankton-zooplankton transfer efficiency	Suchy et al. (2016b)
Crustacean zooplankton	Subtropical Bay, Brazil	Liberated enzyme activity, degradation rate	Characterize temporal patterns of production rates	Suchy et al. (2016a)

planktonic organisms. One such structural component is the carbohydrate chitin, which represents the major constituent of the crustacean exoskeleton (Muzzarelli, 1977). All crustacean zooplankton must periodically shed their exoskeleton (moulting) as part of their development and often to accommodate changes in body size (mass) attributed to somatic growth.

The process of moulting is characterized by changes in the condition of the exoskeleton that were first described in detail by Drach (1939) and later depicted by Roff et al. (1994). In brief, immediately following moult, the exoskeleton consists of a single pre-ecdysial layer. An additional chitinous post-ecdysial layer is synthesized between the epidermis and pre-ecdysial layer during the inter-moult phase. This synthesis period accounts for approximately two-thirds of the duration of any given stage (Kramer et al., 1985; Roff et al., 1994; Stevenson and Hettick, 1980) and is followed by the apolytic phase which is characterized by an initial separation of the epidermis and post-ecdysial layer. Entrance into apolysis is initiated by the moulting hormone ecdysone (Muzzarelli, 1977). The space now separating the epidermis from the post-ecdysial layer is termed the apolytic space, and it is into this space that the moulting enzymes chitinase (poly-β;1,4(2-acetamido-2-deoxy)-D-glucoside gluconohydrolase, EC 3.2.1.14) and chitobiase (β-*N*-acetylglucosaminidase or β-*N*-acetylhexosaminidase, EC 3.2.1.52) are secreted from vesicles in the epidermis. Chitin in the post-ecdysial layer is composed of relatively long chains (polymers) of *N*-acetyl-glucosamine (NAG_n), which are susceptible to hydrolysis into shorter chain lengths (i.e. trimers (NAG_3)

and dimers (NAG_2)) by chitinase (Skinner and Kumari, 1992). Chitobiase then catalyses the hydrolysis of NAG trimers and dimers to monomeric NAG (Fig. 3). As apolysis proceeds, the monomeric NAG in the apolytic space is recycled toward the synthesis of a new pre-ecdysial layer (Gwinn and Stevenson, 1973). Buchholz (1989) found that the activity of both enzymes in euphausiid exoskeletons varied with the moult phase, with the highest activity occurring during apolysis and probably indicative of significant resorption of chitin from the post-ecdysial layer of the old exoskeleton. When the animal moults (ecdysis), the apolytic space and the enzymes present in it become continuous with the surrounding water. Synthesis of the post-ecdysial layer is *de novo* and does not utilize chitin from the old exoskeleton (Roff et al., 1994).

2.3.2 Chitin Metabolism and Weight-Specific Growth and Moulting Rates

The studies discussed earlier demonstrate that chitin metabolism in the crustacean exoskeleton is periodic and intimately linked to degradation and synthesis of the exoskeleton. These processes were quantified by Roff et al. (1994) who fed radio-labelled *Chlamydomonas* spp. to *Daphnia magna* and monitored the rate of uptake (and therefore chitin synthesis) of the radiolabel into the newly synthesized exoskeleton. They demonstrated that specific pathways of chitin metabolism varied directly with growth rates; however, the radiochemical method proved to be too tedious for routine field

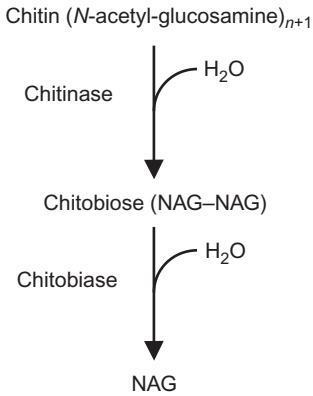

Fig. 3 A simplified illustration of the pathway for chitin degradation during the crustacean apolytic phase. *After Roff, J.C., Kroetsch, J.T., Clarke, A.J., 1994. A radiochemical method for secondary production in planktonic crustacean based on the rate of chitin synthesis. J. Plankton Res. 16, 961–976.*

application. Espie and Roff (1995a,b) then explored relationships between chitobiase activity in homogenates of *Daphnia* spp. They showed that chitobiase activity in homogenates of single stages of asynchronously developing animals varied with moulting rates. However, the optimized activity (V_{max}) also overestimated the flux of chitin from the old to new exoskeleton (see Runge and Roff, 2000). Espie and Roff (1995a) also found that chitobiase activity varied with body size, but that relationship was significantly different between species. They hypothesized that the overestimation of chitin flux by chitobiase activity in homogenates was because: (1) the optimized activity overestimates the actual *in vivo* rate (see Newsholme and Crabtree, 1986) of hydrolysis of oligomeric chitin, and/or (2) the small size of most planktonic crustacea prohibits physical separation of the integument from the digestive tract in which isomers of both chitinolytic enzymes are also active but non-varying over time (Buchholz, 1989). Thus, it was not possible to fully distinguish chitobiase activity actually associated with moulting in homogenates from the activity of additional chitobiase in the digestive tract and stored in vesicles present in the epidermis.

A novel approach to measuring chitobiase activity directly associated with moulting (i.e. without interference from digestive tract isomers) was introduced by Vrba and Machacek (1994), who were interested in variation of the cell-bound microbial chitobiase activity and wanted to address the extent to which planktonic crustacea contributed to the total chitobiase pool. Overall, Vrba and Machacek (1994) found that: (1) chitobiase activity in the environment immediately following ecdysis of *Daphnia pulicaria* was significantly higher than before ecdysis, (2) the activity of the enzyme positively varied with body size, and (3) the microbial (cell-bound) and dissolved crustacean chitobiase could be physically separated via filtration of water through 0.2 μm. Later, several freshwater studies including Vrba (2000) and Vrba et al. (2004) also identified measurable differences in both the kinetic properties and pH optima of the two chitobiase sources in the water column.

2.3.3 Variation of Liberated Chitobiase Activity With Body Size and Estimates of Developmental Rates

Since 2000, most of the literature concerned with chitobiase activity and crustacean zooplankton productivity has been concerned with the activity of the enzyme liberated into the water. Building on the measurements made by Vrba and Macachek (1994), both Oosterhuis et al. (2000) and Sastri and Roff (2000) considered the body size dependence of chitobiase activity in

the water following ecdysis. Measurements of chitobiase liberated into the water carried out by Sastri and Roff (2000) found a positive relationship between enzyme activity and body size and in contrast to work with homogenates (Espie and Roff, 1995b). The relationship between individual post-moult body size and liberated chitobiase activity was generalizable across three species of freshwater cladocera (*D. magna*, *D. pulex* and *Ceriodaphnia dubia*; Sastri and Roff, 2000). The differences between body size-dependent relationships of homogenate-specific and liberated chitobiase activity suggested that there may be species-specific differences in chitobiase concentration in the digestive tract and/or stored in the epidermis. However, chitobiase activity in the apolytic space varied as a function of body size across species and its activity *in situ* has the potential to be applied to entire assemblages. Unfortunately, the activity of chitobiase liberated into the water was still too high to serve as quantitative measure of the flux of chitin (as estimated by Roff et al., 1994) during the moulting process (Sastri, 2001). This overestimation suggests that even though chitobiase activity in the water can be attributed to the moulting event exclusively, the optimized activity of the enzyme still overestimates the *in vivo* rate (as per Newsholme and Crabtree, 1986) of hydrolysis of oligomeric chitin in the apolytic space. It may yet be possible that liberated chitobiase activity represents a direct measure of chitin flux. However, this relationship may only be evident with enzyme activity assayed under *in vivo* conditions with special attention paid to substrate concentration (see Section 2.4). Alternatively, since liberated chitobiase activity varies with body size when individuals moult, Sastri and Roff (2000) proposed that the turnover rate of the enzyme dissolved in the water represented a measure of moulting rate.

The approach taken by Sastri and Roff (2000) involved measurements of chitobiase decay rates and following liberated chitobiase activity (CBA_{NAT}) over time in laboratory cultures. The cultures were composed of asynchronously moulting single and mixed populations which were in steady state as was reflected in a near-constant CBA_{NAT} over time. Thus, the decay rates measured by Sastri and Roff (2000) did not require correction (steady-state conditions) and were used to calculate the average moulting rate (or its reciprocal, stage duration) of their culture as:

$$D = \sum (\mathrm{CBA}_i \times N_i)/(\Delta T_{\mathrm{DAY}}), \tag{3}$$

where D represents the stage duration of the mean-sized individual in the community and CBA_i is the activity of chitobiase liberated by an individual

of size i and N is the number of individuals in that size class. Thus, $\Sigma(\mathrm{CBA}_i \times N_i)$ is an expression of developing biomass in chitobiase activity units (nmol methylumbelliferone liberated $\mathrm{L}^{-1}\ \mathrm{h}^{-1}$). The term ΔT_{DAY} is the measured absolute change in chitobiase activity over 24 h. Hence, Eq. (3) represents the turnover time for the developing community biomass (i.e. the stage duration attributable to the mean-sized individual in the community). In the laboratory, Sastri and Roff (2000) found a good agreement between chitobiase-based estimates of stage duration and conventional estimates (absolute differences between 3.4 and 8 h).

The first marine field test of chitobiase-based stage duration estimates was carried out during the spring of 2004 in the Strait of Georgia, Canada, by Sastri and Dower (2006). They assessed the body size dependence of post-moult chitobiase activity for four copepod species: *Tigriopus californicus*, *Pseudocalanus* spp., *Metridia pacifica* and *Calanus pacificus*, and found that the positive relationship between body size and chitobiase activity could be generalized across species. This relationship was later expanded by Sastri and Dower (2009), to include mysid (*Neomysis* sp.) and decapod larvae (*Lopholithodes foraminatus*). Moulting rates (MR) and stage duration (1/MR) were calculated for the most abundant species/stage following the field approach of Peterson et al. (1991) using on-board incubations of zooplankton. Over the course of the sampling period, the chitobiase-based stage duration estimates (relying on Eq. 3) varied with, but underestimated (>2 times), the conventional measure of stage duration. Sastri and Dower (2006) suggested that the underestimation was linked to underestimates of biomass by their plankton nets. They also considered the potential of a stage duration estimate calculated solely on the basis of the turnover rate of the enzyme in the water column (T_{CBA}). This value is estimated via measurements of the rate of decay of chitobiase in several depth-specific water samples free of crustacean zooplankton (<45 μm size fraction), and does not demand an independent measure of biomass (i.e. contents of a plankton net). Thus,

$$T_{\mathrm{CBA}} = 1/-k, \tag{4}$$

where k represents the slope of the relationship between ln(CBA) and seawater incubation times (see Sastri et al., 2013). Overall, T_{CBA} values varied with conventional stage duration estimates and differed by only 5–15%. In contrast to the approach taken by Sastri and Roff (2000), a T_{CBA}-based stage duration estimate does not depend on an independent measure of biomass,

and therefore assumes that the specific turnover rate (via moulting) of the CBA pool equals the specific turnover rate (via moulting) of the biomass pool. Subsequently, CBA_{NAT} should: (1) vary with and not represent a fraction of (i.e. less than) $\Sigma\ CBA_i \times N_i$, and (2) not necessarily vary with T_{CBA} (Sastri, 2007). More recent field studies have found significant positive relationships between CBA_{NAT} and biomass ($\Sigma\ CBA_i \times N_i$), with CBA_{NAT} greater than the developing crustacean zooplankton sampled by plankton nets (Sastri and Dower, 2009; Sastri et al., 2012, 2013).

2.3.4 Liberated Chitobiase Activity and BPRs

Oosterhuis et al. (2000) took a novel approach and found that the difference in body mass between successive stages of the marine calanoid *Temora longicornis*, varied with liberated chitobiase activity subsequent to ecdysis. This relationship can be scaled up to estimate the sum of all somatic growth (biomass production) for an entire population of *T. longicornis*, since the relationship between growth increment and chitobiase activity was linear with a slope of ~1. In order to calculate the rate at which biomass (equivalent to chitobiase activity in the water) was being produced via growth, they measured the rate of production of the enzyme in their laboratory culture. By measuring the standing activity of the enzyme and its dissolved rate of production, they were able to estimate BPRs of their cultures that were in excellent agreement with direct measurements (Fig. 4). To calculate the rate of chitobiase production by moulting animals in the water, Oosterhuis et al. (2000) measured the rate of decay of the dissolved enzyme in water samples free of zooplankton. This approach was taken because, as a protein, the dissolved crustacean chitobiase is susceptible to degradation by cell-bound microbial proteases (Chróst, 1991; Hollibaugh and Azam, 1983). Thus, it is impossible to directly measure its rate of production by moulting animals. However, the rate of decay of chitobiase can be assumed to be balanced by its rate of production by moulting animals provided that the population or community is in steady state (i.e. CBA_{NAT} in the water column be in equilibrium over short periods of time; Sastri and Dower, 2006). Note, however, that this assumption of steady state was not met by Oosterhuis et al. (2000), because they followed the development of a synchronous single cohort (from naupliar through adult stage *T. longicornis*), and the size frequency distribution and biomass of the population were increasing over time. To account for the non-steady-state conditions, Oosterhuis et al. (2000) effectively corrected their decay rate measurements with knowledge of the change in CBA_{NAT} over time. This non-equilibrium

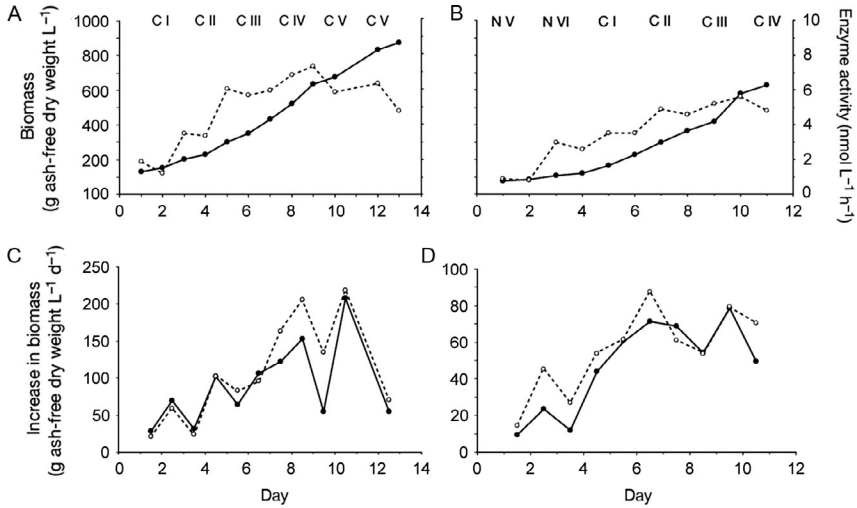

Fig. 4 (A, B) The results of two time-course experiments tracking both the activity of dissolved chitobiase (*unfilled symbols* and *dashed line*) and the biomass of *Temora longicornis* (*filled symbols* and *solid line*) in a culture vessel; (C, D) the temporal correspondence between biomass production rates calculated directly (*filled symbols* and *solid line*) and using the chitobiase method during two experiments tracking the synchronous development of a *Temora* sp. culture. *From Oosterhuis, S.S., Baars, M.A., Klein-Breteler, W.C.M., 2000. Release of the enzyme chitobiase by the copepod* Temora longicornis*: characteristics and potential tool for estimating crustacean biomass production in the sea. Mar. Ecol. Prog. Ser. 196, 195–206.*

calculation was also applied by Suchy et al. (2013), who tracked growth of laboratory cultures of the harpacticoid copepod *Tigriopus californicus*.

BPR measurements for the entire crustacean zooplankton community (as first proposed by Oosterhuis et al., 2000) are as simple as the radiochemical methods for estimating the community-level rates of production for phytoplankton and bacterioplankton communities (Fuhrman and Azam, 1982; Steemann Nielsen, 1952). Measurements of BPRs using this method rely entirely on collections and short-term incubations of seawater. This approach does not require separate collections or incubations of zooplankton. A BPR estimate is calculated as

$$\mathrm{BPR} = \Delta B / T_{\mathrm{CBA}}, \tag{5}$$

where ΔB (total community biomass produced due to somatic growth) is calculated from the relationship between the change in individual mass between successive stages and the individual post-moult chitobiase activity.

Sastri and Dower (2006) found that ΔB varied with CBA for three calanoid copepods (*Pseudocalanus* spp., *M. pacifica* and *C. pacificus*) as follows:

$$\log \Delta B = 0.864 \times \log(\mathrm{CBA}) - 1.78. \quad (6)$$

Another positive relationship describing variation of ΔB on the basis of CBA for common freshwater groups (cyclopoid copepods, calanoid copepods and cladocerans) was recently assembled by Sastri et al. (2013). Single species relationships relating variation of ΔB to CBA were also assembled by Oosterhuis et al. (2000) for *Temora longicornis* (ash-free dry weight) and by Avila et al. (2012) for *Acartia tonsa* (dry weight). The generalized relationship (Eq. 6) assembled by Sastri and Dower (2006) is useful in cases where no prior knowledge of the crustacean zooplankton community exists. However, when the community biomass is dominated by one or two dominant species (e.g. Avila et al., 2012), the use of a species-specific relationships is more appropriate. Thus, the use of a ΔB–CBA relationship allows for the calculation of the total biomass produced (i.e. production) by all developing (moulting) animals via direct measurements of $\mathrm{CBA_{NAT}}$, at the depth or depths sampled. The rate component (T^{-1}) of the BPR estimate requires an estimate of the rate of chitobiase production (via decay rate measurements) in the water column.

2.3.5 Assumptions and Potential Sources of Error

Because chitobiase is constantly decaying in the water column, the rate of chitobiase production by moulting animals in the field cannot be easily measured by following $\mathrm{CBA_{NAT}}$ over time. However, assuming that the rate at which the enzyme is being produced by moulting animals equals the rate of decay *in situ*, we can estimate CBA production as the negative rate at which the enzyme decays in water samples free of zooplankton. In the field, this assumption is reasonable over short periods of time (24 h or less), since perfectly synchronous populations are unlikely and communities are typically composed of multiple populations that are not developing in the same manner. For example, Sastri and Roff (2000) followed cultures of asynchronously developing populations and found $\mathrm{CBA_{NAT}}$ to remain approximately constant over time. However, it is possible to establish synchronous populations in the laboratory, and in these instances, an assumption of a balance between chitobiase production and decay can be violated since the size frequency distribution of the population is constantly changing with time. To account for this non-steady-state condition, Oosterhuis et al. (2000), and more recently Suchy et al. (2013), calculated the production rate of the enzyme as the

sum of the change in CBA_{NAT} between two time steps and the decay rate. In the field, however, the assumption of steady state must be made since the design of most sampling programs makes logistically difficult to track a parcel of water over time.

Chitobiase-based estimates of BPR are entirely dependent on enzyme assays carried out on relatively short-term incubations of seawater. Hence, they do not explicitly depend on knowledge of the contents of conventional plankton net hauls. However, knowledge of species and stage compositions, biomass and size-frequency distributions provides useful information necessary for understanding factors contributing to variation of measured rates. Thus, if an objective of applying chitobiase-based methods is to understand how the crustacean zooplankton community influences spatio-temporal patterns of BPR in the field, care should be taken to collect seawater samples spanning a depth range corresponding to that of plankton net hauls. Moreover, the depth range of both should account for the depth range traversed by high-amplitude migrators (i.e. *Metridia* spp.). It may otherwise be difficult to relate the contents of net casts to chitobiase activity when animals are moulting in surface waters but present at depths below the vertical range of nets hauls when samples are collected.

2.3.6 Method Validation and Measurement Calibration

The chitobiase-based stage duration estimates measured by Sastri and Dower (2006) represent the only field comparisons to conventional methods. In that study, Sastri and Dower (2009) found that incubation-based developmental rate estimates compared favourably to chitobiase-based moulting rates. However, the only validation for chitobiase-based estimates of BPR comes from the laboratory-based studies of a single synchronous population (*Temora longicornis*) that were conducted by Oosterhuis et al. (2000) (see Fig. 4). A comprehensive *in situ* validation of chitobiase-based BPR estimates would demand calibration with conventional estimates of both weight-specific growth rate (e.g. natural or artificial cohort method) and biomass for entire communities. Thus, a community-level estimate of BPR would demand growth rates estimated across the entire crustacean zooplankton community size spectrum, as well as the multiple types of sampling gear required to accurately represent community biomass. Moreover, a true validation should make such comparisons over a variety of conditions known to influence variation of productivity (i.e. temperature, food concentration and biomass). Continued detailed work with laboratory cultures growing under a variety of conditions may also provide validation tools.

2.3.7 Application to the Natural Environment

Measurements of liberated chitobiase in the field began with freshwater studies (e.g. Vrba et al., 1997). This, and similar studies, found that seasonal patterns of crustacean zooplankton biomass varied with the activity of the low-affinity (i.e. relatively high K_m values) isomer of chitobiase in water samples passed through 0.2 μm. There have since been a number of freshwater studies (in streams and ponds) investigating variation of native chitobiase activity as a component of toxicological response (Conley et al., 2009; Hanson and Lagadic, 2005; Richards et al., 2008). The first field estimates of chitobiase decay rates were reported for the Strait of Georgia (Sastri and Dower, 2006) as a field validation of laboratory-based estimates of stage duration (Sastri and Roff, 2000). Instantaneous estimates of biomass production rates for Strait of Georgia crustacean zooplankton communities developing in the spring and early summer were measured by Sastri and Dower (2009). Cross-shelf measurements were also carried out in 2005 on the west coast of Vancouver Island, and along the oceanographic Line P (extending from coastal waters to high-nutrient low-chlorophyll offshore waters) in the Gulf of Alaska, by Sastri (2007) and Suchy (2014), respectively. Sastri et al. (2012) also measured BPR across a broad range of temperatures, food concentrations and zooplankton community biomasses along a transect extending from southern Vancouver Island through the Gulf of Alaska, along the Bering Sea shelf break, through the Bering Strait, southern Chukchi Sea and western Beaufort Sea. The range of conditions measured along this transect was suitably broad that the authors were able to attribute 67% of the variation in measured BPR to variation of phytoplankton biomass and temperature. Also, Avila et al. (2012) characterized and compared seasonal variation of crustacean zooplankton production rates to empirical models in a subtropical estuary in southern Brazil. More recent field studies (see Table 4) have begun to utilize chitobiase-based BPR estimates to explore temporal patterns of phytoplankton to zooplankton transfer efficiency (Suchy et al., 2016b), and the impact of water column mixing on interactions between zooplankton community composition and productivity (Sastri et al., 2014).

2.3.8 Advantages and Limitations of Chitobiase-Based Estimates of Productivity

2.3.8.1 Advantages

2.3.8.1.1 Community-Level Estimate Chitobiase-based development and BPR measurements all yield community-level estimates. This is an advantage from the perspective of foodweb and biogeochemical studies concerned

with mass and energy transfer. Thus, calculations of lower trophic level transfer efficiency (i.e. ratio of production rates for adjacent trophic levels; Lindeman, 1942) are routinely possible since both phytoplankton and bacterial production rate measurements are similarly non-specific. We note that it is theoretically possible to make productivity estimates for all populations in a given crustacean zooplankton community using traditional methods (see Section 1.3.1). However, this would not be practical, because of the prohibitive number of incubations required and time spent at the microscope.

2.3.8.1.2 Broadly Applicable Across Environmental Conditions The chitobiase assay is apparently applicable across a wide variety of environments and range of conditions (see Table 4). Meaningful productivity estimates have been measured in freshwater, estuarine and marine environments, with estimates from subtropical (Avila et al., 2012; Suchy et al., 2016a), temperate (Sastri and Dower, 2009; Suchy et al., 2016b) and polar waters (Sastri et al., 2012), and spanning water column temperatures from <0 to >20°C.

2.3.8.1.3 Routinely Applicable at High Spatio-temporal Resolution Since chitobiase-based estimates rely on relatively small seawater samples, measurements can be resolved both vertically and horizontally. For instance, variation in rates of production can be compared throughout the water column or measured at relatively high spatial resolution in order to follow physicochemical gradients (e.g. riverine to oceanic conditions), or target physical features such as fronts. Thereby, finely resolved estimates of surface production could be reasonably approached using the flow through systems often used on oceanographic cruises. Automated enzyme samplers (see Jaeger et al., 2009) specific to fluorogenic substrates (e.g. methylumbelliferone) have been developed, and could be applied for this purpose or on-board ocean observing systems such as moorings or autonomous profiling buoys.

2.3.8.1.4 Relatively Quick and Sensitive Analytical Procedure The chitobiase enzyme assay is a relatively simple and sensitive assay that can be carried out easily at sea with a small-footprint bench top fluorometer. The enzyme assay itself relies on the addition of a single fluorogenic substrate (see Hoppe, 1983, 1993) without the addition of cofactors. Since chitobiase-based rate measurements are based on relatively short (i.e. 12 h in temperate waters) incubations of seawater, production rate estimates can ostensibly be completed within hours of the last sampled station.

2.3.8.2 Limitations

2.3.8.2.1 Applicable Only to Crustacean Zooplankton The chitobiase method as described here yields indirect estimates of BPRs of crustacean zooplankton communities. The method captures production linked to changes in body mass accrued between moulting events (i.e. production rates can only be measured for animals that moult). This is of particular significance for adult copepods, which do not moult and still contribute to production in terms of gamete (egg and spermatozoa) production. Moreover, this method cannot be applied to estimate growth or production rates of important non-crustacean groups such as gelatinous plankton. Moreover, the contribution of non-planktonic crustaceans (late-stage decapods and cirripeds) to chitobiase activity will also contribute to production rate estimates. This would occur when water samples are collected close to the sea floor or in very shallow well-mixed environments.

2.3.8.2.2 Not Applicable to Single Populations in the Field Although also described as an advantage, applying the chitobiase assay in studies concerned with the production rates of single populations would not be suitable, unless the target species dominated the community biomass. This limitation is somewhat rectifiable since the decay rate of chitobiase can be scaled with body size in order to calculate body size-specific estimates of stage duration (Sastri and Dower, 2006). However, this approach does demand knowledge of the size frequency distribution of the community. It may also be possible to distinguish broadly among groups (class to order), by developing isomer-specific chitobiase antibodies in a manner analogous to that used by Peters et al. (1998) to distinguish between euphausiid digestive and moulting chitobiase activities.

2.3.8.2.3 Growth Increment Assumptions The existing relationships between growth increment (ΔB) and chitobiase activity (Avila et al., 2012; Oosterhuis et al., 2000; Sastri and Dower, 2009; Sastri et al., 2013) all rely on stage weights based on length–weight regressions. Thus, these estimates suffer a bias similar to those identified for the moult rate method (Hirst et al., 2005; Rey-Rassat et al., 2004). This issue is rectifiable with the assembly of growth increment–chitobiase relationships based on post-moult weights. Similarly, chitobiase-based estimates of ΔB assume that all groups have a generally positive relationship between body size and the increment of growth between moults. For copepods, this assumption is generally appropriate; however, for other groups such as certain developmental stages of euphausiids, somatic growth may be marginal or even negative between

moults. Thus, the application of existing ΔB–chitobiase activity relationships implies the risk of overestimating production when and where these developmental stages dominate crustacean zooplankton biomass.

2.4 AARS Activity

2.4.1 Protein Synthesis Rate and AARS Activity

Somatic growth is defined as increase in biomass, mostly protein content of organisms. Protein synthesis is a complex process, which involves interaction of nucleic acids (RNA) and proteins (enzymes) (see Section 2.1). For this reason, several indices based on RNA and enzyme activities related to synthesis processes were developed to assess either somatic growth or female reproduction rates (Fig. 2). These indices are based on the relationships between protein and nucleic acid metabolic pathways involved in the process of growth. In the case of the protein metabolism, the first step in protein synthesis, prior to translation, is the amino acid activation and the aminoacylation of tRNA, that is, the union of amino acids to the tRNA (Schimmel and Söll, 1979) (Fig. 5). This step is catalysed by a group of enzymes called aminoacyl-tRNA synthetases (EC 6.1.1; AARS) (see Table 3). There is a specific AARS for each amino acid to be activated. Aminoacyl-tRNA synthetases activity is related to protein synthesis rates

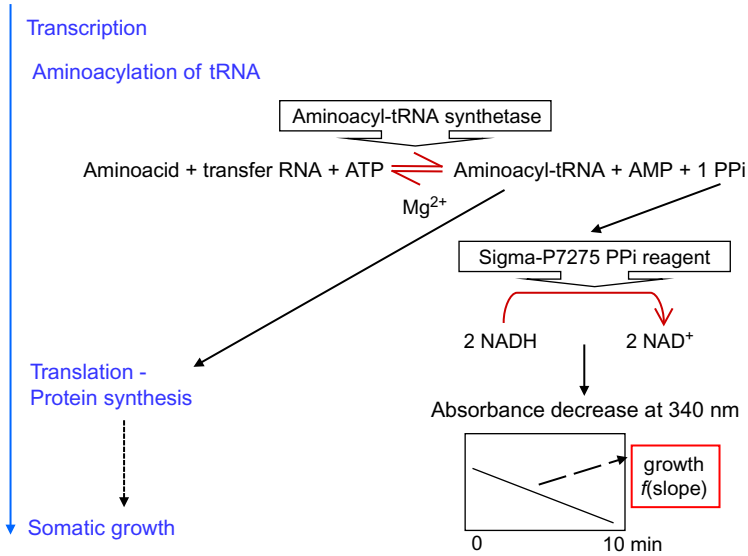

Fig. 5 Simplified protein synthesis pathway detailing the step catalysed by the AARS enzymes (*top*) and the AARS spectrophotometrical assay (*right*) by Chang et al. (1984).

(Johnson et al., 1977; Neidhart et al., 1975; Parker et al., 1974), and protein synthesis can be inhibited by disrupting the function of the AARS enzymes (Larrinoa and Heredia, 1991; SirDeshpande and Toogood, 1995). Therefore, AARS activity represents a limiting step in the protein synthesis pathway. The activity of these enzymes has been widely studied using labelled amino acids (mainly leucine), to assay their incorporation into tRNA (e.g. Anderson and Neidhart, 1972; Del Rio and Heredia, 1983; Deutscher, 1967; Eigner and Loftfield, 1974; Garlick et al., 1980; Houlihan et al., 1986; Nielsen and Haschemeyer, 1976; among others). These studies found a direct relationship between AARS activity and protein synthesis. Therefore, as protein synthesis is related to somatic growth, AARS activity should also be related to growth. In this sense, Parker et al. (1974) showed that the rate of protein synthesis in bacteria was correlated with an increase in AARS activity. Using ^{14}C, they found a positive relationship between growth rate and AARS activity. Also, Neidhart et al. (1975) and Johnson et al. (1977) found that high AARS activities coincided with high growth rates in yeast. The activity of AARS has also been applied to the study of protein synthesis and growth rates in cephalopods (Houlihan et al., 1990) and fishes (Bolliet et al., 2000; Carter et al., 1993; Clemmesen, 1994; Foster et al., 1992; Sveier et al., 2000). Given the consistent demonstrations of its relationship with growth among broad taxonomic groups, AARS activity represents a good candidate as a proxy of zooplankton growth.

In addition to radio-labelling amino acids, there are two other non-radiochemical methods to measure AARS activity. Both of these approaches directly measure the activity of different AARS based on the release of pyrophosphate (PPi) during aminoacylation of tRNA. In order to measure PPi release, Upson et al. (1996) developed a very sensitive method. However, it is not suitable for assays of cell homogenates (Lloyd et al., 1995) and thus cannot be used to assess AARS activity on zooplankton. On the other hand, Chang et al. (1984) developed a spectrophotometrical continuous assay for AARS activity, by assessing PPi release as the oxidation of NADH by the PPi (O'Brien, 1976). The method of Chang et al. (1984) is very simple and is applied by using a spectrophotometer and a commercially available non-radioactive PPi-reagent kit manufactured by Sigma-Aldrich (Fig. 5). Note, however, that the enzyme assay described by Chang et al. (1984) measures the activity of isolated enzymes with the addition of specific substrates and registers the maximal potential activity (V_{max}) of the enzymes.

Enzyme activities are routinely measured under optimal conditions, which include substrate saturation. Nonetheless, these conditions rarely

reflect *in vivo* reaction rates for cells in nature (e.g. Båmstedt, 2000; Newsholme and Crabtree, 1986; Packard et al., 1996). In order to better reflect the *in vivo* cell condition, enzyme activities should be assayed based on the endogenous substrates. To overcome this issue, Yebra and Hernández-León (2004) applied the Chang et al. (1984) method to zooplankton homogenates without the addition of substrates. In this way, AARS activity was assayed without adding amino acids in order to measure the capability to synthesize proteins that individuals had in the field at the moment of capture. The AARS activity measured with this protocol would then reflect their previous food and development history. The AARS method (Yebra and Hernández-León, 2004) was first applied to the study of growth of the freshwater cladoceran *Daphnia magna* in the laboratory. Since then, AARS activity has been successfully calibrated as index of growth for marine calanoid and cyclopoid copepods (Herrera et al., 2012; Yebra et al., 2005, 2006b, 2011), euphausiid larvae (Guerra, 2006) and fish larvae (Herrera, 2014) in the laboratory and/or in the field (Table 5).

These advances have allowed the application of this proxy in oceanographic studies for the assessment of zooplankton growth and production rates at basin scale (see Table 6).

2.4.2 Potential Sources of Mismatch Between Growth and Protein Synthesis Rates

2.4.2.1 Moult

The first assessment of crustacean growth using the AARS method showed that peaks of enzyme activity coincided with or were in advance of peaks of protein growth, although in some cases a delay of 1 day was observed in the growth response (Yebra and Hernández-León, 2004). Those experiments were run with the cladoceran *D. magna* and moulting of the cultures was isochronal. The moult processes were recorded as dramatic decreases in both growth rates and enzyme activities (see fig. 3 in Yebra and Hernández-León, 2004). However, daily protein content increases and AARS activities were sometimes uncoupled. This uncoupling may be explained by the delay between the start of protein synthesis and its accumulation as body mass. Decreases in AARS activity were also followed by growth rates reduction, but not immediately. This suggests that the pool of tRNA already aminoacylated could continue the process of protein synthesis through the ribosome, even though AARS activity had diminished. The numerous steps between tRNA aminoacylation and protein accumulation as body mass as well as the protein turnover in the cells were suggested to be the main

Table 5 Models Relating Growth Rates and Indirect Growth Indices With Aminoacyl-tRNA Synthetases (AARS) Activity in Zooplankton Species

Species	Empirical Relationship	r^2	Type	Reference
Calanus finmarchicus	$g = -0.09 + 0.009 \times \text{spAARS}$	0.55***	Field	Yebra et al. (2006b)
Calanus finmarchicus	$g = -0.046 + 0.008 \times \text{spAARS}$	0.52***	Field	Yebra et al. (2006b)
Calanus helgolandicus	$g = 0.08 + 0.002 \times \text{spAARS}$	0.55*	Exp	Yebra et al. (2005)
Calanus helgolandicus	$g_{\text{WIf}} = 0.452 + 0.022 \times \text{spAARS}$	0.17*	Field	Yebra et al. (2005)
Calanus helgolandicus	$g_{\text{WIm}} = 0.435 + 0.021 \times \text{spAARS}$	0.05 ns	Field	Yebra et al. (2005)
Calanus helgolandicus	$g_{\text{WIf+m}} = 0.446 + 0.022 \times \text{spAARS}$	0.10*	Field	Yebra et al. (2005)
Calanus helgolandicus	$g_{\text{HB}} = 0.125 + 0.001 \times \text{spAARS}$	0.13*	Field	Yebra et al. (2005)
Calanus helgolandicus	$\text{spEPR}_{\text{RS}} = 0.051 + 0.018 \times \text{spAARS}$	0.40***	Field	Yebra et al. (2005)
Calanus helgolandicus	$\text{spEPR}_{\text{NRS}} = 0.051 + 0.003 \times \text{spAARS}$	0.07 ns	Field	Yebra et al. (2005)
Paracartia grani	$g = 0.13 + 0.007 \times \text{spAARS}$	0.95***	Exp	Herrera et al. (2012)
Paracartia grani	$g = 0.25 + 31.65 \times \text{AARSind}$	0.83***	Exp	Herrera et al. (2012)
Paracartia grani	$g = -0.59 + 204.46 \times \text{AARSind}$	0.95***	Exp	Herrera et al. (2012)
Paracartia grani	$g = 1.49 - 0.025 \times \text{spAARS}$	0.96***	Exp	Herrera et al. (2012)
Paracartia grani	$g = -0.14 + 0.002 \times \text{spAARS}$	0.46 ns	Exp	Herrera et al. (2012)
Paracartia grani	$g = -0.03 + 0.008 \times \text{spAARS}$	0.15 ns	Exp	Herrera et al. (2012)
Paracartia grani	$g = -0.57 + 0.038 \times \text{spAARS}$	0.80**	Exp	Herrera et al. (2012)

Oithona davisae	$spAARS_{nauplii} = 23.93 \times e^{5.96g}$	0.53*	Exp	Yebra et al. (2011)
Oithona davisae	$spAARS_{copepodite} = 20.03 \times e^{6.22g}$	0.80*	Exp	Yebra et al. (2011)
Oithona davisae	$spAARS_{nauplii+copepodite} = 24.35 \times e^{5.51g}$	0.70*	Exp	Yebra et al. (2011)
Oithona davisae	$(RNA:DNA)_{nauplii} = 5.83 + 0.0414 \times spAARS_{nauplii}$	0.33*	Exp	Yebra et al. (2011)
Oithona davisae	$(RNA:DNA)_{copepodite} = 7.26 + 0.0716 \times spAARS_{copepodite}$	0.75*	Exp	Yebra et al. (2011)
Euphausia superba	$g^* = 0.0002 + 0.007 \times spAARS$	0.93***	Field	Calculated from Guerra (2006)
Daphnia magna	$g_{DW} = 0.568 + 0.691 \times spAARS$	0.45*	Exp	Yebra and Hernández-León (2004)
Daphnia magna	$g_{PR} = -0.191 + 1.103 \times spAARS$	0.69*	Exp	Yebra and Hernández-León (2004)
Clupea harengus larvae	$g = -0.033 + 0.001 \times spAARS$	0.42*	Exp	Herrera (2014)
Various	$g = -0.012 + 0.004 \times spAARS$	0.74***	Field + Exp	S. Hernández-León (unpublished data)

Continued

Table 5 Models Relating Growth Rates and Indirect Growth Indices With Aminoacyl-tRNA Synthetases (AARS) Activity in Zooplankton Species—cont'd

Species	Empirical Relationship	r^2	Type	Reference
Various (marine)	$g_H = 0.009 + 0.636 \times G_{AARS}$	0.19*	Field	Garijo and Hernández-León (2015)
Various (marine)	$g_{HB} = 0.004 + 0.324 \times G_{AARS}$	0.07*	Field	Garijo and Hernández-León (2015)
Various (marine)	$g_Z = 0.006 + 0.636 \times G_{AARS}$	0.08*	Field	Garijo and Hernández-León (2015)
Various (marine)	$g_{GH} = 0.013 + 0.946 \times G_{AARS}$	0.19*	Field	Garijo and Hernández-León (2015)

Exp, laboratory experiments; *Field*, field assessments.
Variables: *AARSind*, nmPPi ind^{-1} h^{-1}; *DW*, dry weight; *g*, d^{-1}; *g**, mg protein ind^{-1} d^{-1}; G_{AARS}, d^{-1}, calculated from spAARS with equation from S. Hernández-León, unpublished data; *GH*, Garijo and Hernández-León (2015) equation; *H*, Hirst et al. (2003) equation; *HB*, Hirst and Bunker (2003) equation; *NRS*, non-reproductive season; *PR*, protein; *RS*, reproductive season; *spAARS*, nmPPi mg $prot^{-1}$ h^{-1}; *spEPR*, d^{-1}; *WI*, weight increment method; *Z*, Zhou et al. (2010) equation.
*ns: $p > 0.5$, *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Table 6 Studies Applying the AARS Method to Zooplankton

Zooplankton Taxon		Objectives	Reference
Culture			
Cladocerans	Freshwater	Method development and calibration: growth rate	Yebra and Hernández-León (2004)
Euphausiids	Marine	Assessment of biochemical proxies: krill larvae growth rate	Guerra (2006)
Copepods	Marine	Assessment of biochemical proxies: female growth and metabolism	Holmborn et al. (2009)
Copepods	Marine	Method development and calibration: cyclopoid nauplii growth rate	Yebra et al. (2011)
Copepods	Marine	Method calibration: calanoid nauplii growth rate	Herrera et al. (2012)
Fish larvae	Marine	Method calibration: fish larvae growth rate	Herrera (2014)
Copepods	Marine	Effect of oxygen minimum zone on female metabolism	P. Ruz (unpublished data)
Culture/Field			
Copepods	English Channel	Field method validation: growth rates; comparison to empirical models	Yebra et al. (2005)
Copepods	North Atlantic	Field method validation: growth rates and dormancy	Yebra et al. (2006b)
Copepods	Norway fjord mesocosm	Effect of toxic phytoplankton bloom on nauplii growth rate	Koski et al. (2012)
Various	Marine	Modelling of global equation for zooplankton growth rate	S. Hernández-León (unpublished data)

Continued

Table 6 Studies Applying the AARS Method to Zooplankton—cont'd

Zooplankton Taxon		Objectives	Reference
Field			
Copepods	Canary Islands	Effect of eddies and upwelling filaments on zooplankton metabolism	Yebra et al. (2004)
Zooplankton	Irminger Sea, North Atlantic	Broad-scale characterization of zooplankton community production rates	Yebra et al. (2006a)
Zooplankton	Bransfield Strait, Antarctica	Broad-scale characterization of zooplankton community production rates	Yebra et al. (2009a)
Zooplankton, Copepods	Labrador Sea, North Atlantic	Effect of mesoscale hydrography on production rates	Yebra et al. (2009b)
Euphausiids	Mexican pacific	Oxygen minimum zone effect on migrants metabolism	Herrera (2014)
Zooplankton	Canary Islands	Time series of zooplankton metabolism	Herrera (2014)
Zooplankton	Canary Islands	Comparison to empirical models; specific growth and community production	Garijo and Hernández-León (2015)
Zooplankton	Australian Indian and Pacific	Broad-scale characterization of zooplankton community production rates	McKinnon et al. (2015)
Zooplankton	Alboran Sea, Mediterranean Sea	Broad-scale characterization of zooplankton community production rates	L. Yebra (unpublished data)

reasons for the observed time lags (Yebra and Hernández-León, 2004). The uncoupling was solved when growth rates and average specific AARS activities were compared over longer than 24-h periods. Then, the dramatic biomass daily changes due to moulting were smoothed and a more accurate growth rate of the organisms was calculated. Following this approach, significant positive correlations between a culture growth rate and its averaged biomass-specific AARS activity have been determined for various crustacean species (see Table 5).

The way in which we obtain biomass data to calculate growth affects the rates obtained. Hence, the calculation of growth can influence the calibration and the observed relationships between growth and protein synthesis rates or AARS activity. For example, weight changes are traditionally inferred from length–weight relationships that are obtained under food-saturating conditions. This may lead to gross errors on the assessment of growth when the organisms studied are not growing under saturating levels of food (see Section 1). Also, the application of length–weight relationships does not account for biomass losses within a crustacean developmental stage, as their exoskeleton length does not vary between moults. The use of direct biomass measurements (e.g. dry weight, protein or carbon content) to assess growth variation would minimize the errors induced by the assumption of fixed length–weight relationships in crustaceans. In addition, the biomass unit chosen to determine growth might also induce uncertainties in the validation of enzymatic methods related to somatic growth. In the case of protein synthesis, variability is reduced by measuring growth and AARS activity on a protein basis (see Yebra and Hernández-León, 2004). As the activity of these enzymes is a measure of the rate of amino acid incorporation into protein, protein-specific AARS activity should be a suitable index of protein-specific growth. The relationship between somatic growth and the AARS activity in terms of dry weight also gave a significant but poorer correlation (Yebra and Hernández-León, 2004). This was probably due to the variability in the percentage of dry weight corresponding to proteins in each organism.

2.4.2.2 Severe Starvation

Food limitation affects both growth and protein synthesis rates. Also, starvation produces a dramatic reduction of RNA:DNA levels and somatic growth rates (see Section 2.1). However, increases on specific AARS activities have been reported in cases of severe starvation (0–11 μg C L^{-1}). These low growth-high activities were observed in nauplii of calanoid

Paracartia grani (Herrera et al., 2012) and cyclopoid *Oithona davisae* (Yebra et al., 2011) copepods. Initially a methodological interference was suggested. Nonfeeding organisms may use their lipid reserves as fuel to survive. The β-oxidation of fatty acids could produce PPi during the AARS assay, which might interfere with the enzyme assay as AARS activity is measured as the PPi release rate in the homogenate sample. However, in gilt-head bream, *Sparus aurata*, larvae, the low growth-high enzyme activity periods were unrelated to the consumption of lipid reserves by the larvae, demonstrating that the lipid catabolism did not affect the AARS assay (S. Hernández-León, unpublished data). In all these cases, the increase in protein-specific enzyme activity corresponded neither to a higher growth rate nor to an artefact, but to the coupling of a high protein turnover and a decrease in individual biomass. That is, starved organisms use their own proteins as fuel, and with no food intake and enhanced protein turnover, they lose protein weight. Consequently, in starved organisms (or with food intake insufficient for growth), proteins are degraded faster than they are synthesized (Hawkins, 1985), producing a mismatch between AARS activity and growth rates (similar to that observed for nucleic acids). Previous studies on the effects of feeding frequency on overall growth and protein metabolism in the European lobster, *Homarus gammarus*, showed both elevated protein synthesis and protein degradation rates. This resulted in high turnover and reduced growth (Mente et al., 2001). Thus, the degradation of proteins under severe starvation conditions may affect the relationship between specific AARS activity and somatic growth rates, precluding the application of this method as index of growth for organisms surviving on the degradation of their own protein bulk (e.g. starvation experiments). Nevertheless, the artificially induced mismatch observed in the laboratory between specific AARS activity and somatic growth rates due to prolonged starvation is unlikely to occur in the field, considering that typical particulate organic carbon values observed in coastal (40–5000 μg C L^{-1}) and oceanic waters (14–1166 μg C L^{-1}) are above the levels of experimental starvation (Duforet-Gaurier et al., 2010; Gardner et al., 2006; Huntley and Boyd, 1984).

2.4.3 Validation of the AARS Method Through Calibration With the Direct Measurement of Growth

As with any proxy, prior to its application to cultures or natural populations, the AARS method needs to be calibrated with the rate we aim to approach, in this case somatic growth. Hence, the first calibration of the AARS activity as index of growth rate for zooplankton (Yebra and Hernández-León, 2004)

followed three essential steps: (1) the methodological test of the method; (2) the comparison of enzyme activity in animals of similar age and individual biomass growing at different rates; and (3) the direct comparison of growth rates with enzyme activity.

The first step, set-up of the method, included the study of: (1) the optimum pH and temperature of the enzyme reaction, (2) the linearity of the enzyme-catalysed reaction without addition of substrates, (3) the linearity of the enzymatic activity vs the concentration of the analytical sample, and (4) the biomass concentration range required to perform the assay. Once the method was optimized, the second step required observations of the possible variations in AARS activity on organisms of the same cohort, growing at different rates. This was a prerequisite to demonstrating the suitability of the method, as the true test of an index of growth is that it should vary among animals of the same size when they are growing at different rates (Runge and Roff, 2000). These variations in growth rate may be easily achieved by culturing the organisms at different temperatures and/or food concentrations. As a third step, the different levels of AARS activity had to be compared with their corresponding growth rates. If the different AARS values in organisms of similar body weight are significantly related to the growth rates of the same individuals, then the AARS method could be used as a proxy of growth rate of the species studied. To accomplish this last step of the calibration, the assessment of direct somatic growth rates was needed. Direct growth rates could be measured as variations in biomass or in length. The variations in biomass over time were then used to calculate biomass-specific growth rates (d^{-1}) following the approach of Kimmerer and McKinnon (1987), in which the slope of the regression of the mean log-transformed biomass over time is the instantaneous growth rate.

Given that AARS catalyse the first step of the protein synthesis process, the correlation observed between AARS activity and protein-specific growth was higher than with dry weight-specific growth (Yebra and Hernández-León, 2004). This might also have been due to the fact that protein content was measured from the same homogenate (i.e. same individuals) as the AARS activity, whereas when calculating growth from dry weight or carbon content, the enzyme activity and the biomass were determined from different aliquots of the same culture. Also, it must be noted that growth rates might also be negative. Decreases in biomass are not detectable when assessing growth as variations in length of crustaceans, because their exoskeleton does not shrink when losing weight. In addition, most of the length–weight relationships used to transform length to biomass units are obtained

under optimum temperature and feeding conditions. The use of such conversions in organisms growing below these optimum conditions (for example, during the calibration experiments) might lead to errors in the calculation of growth and would increase the uncertainty of the calibration of the AARS method as index of growth rate. Hence, to increase the accuracy of the calibration and to reduce the number of organisms required for the experiments of such calibration, we recommend assessment of growth rate as protein-specific growth (see Section 1.2.1). Nevertheless, calibrations of the AARS method against direct growth rates were successfully performed using the different options for growth rate calculation mentioned earlier (e.g. Herrera et al., 2012; Yebra and Hernández-León, 2004; Yebra et al., 2006b).

2.4.4 Application to Natural Communities

The first application of the AARS activity as a proxy for copepod growth in the field was in the subtropical waters of the Canary Islands (Yebra et al., 2004). The aim was to study the effects of upwelling filaments and island-induced eddies on the growth of two copepod species abundant in the region. The use of indirect indices allowed assessing how hydrography (mesoscale structures) affected the distribution and metabolic rates of copepods, including growth, with both vertical and horizontal mesoscale resolution. Later, subsequent to calibration in the laboratory, the AARS method was applied to monitor the growth of *Calanus helgolandicus* late developmental stages in the English Channel (Yebra et al., 2005). Also, AARS activity was validated as an index of *Calanus finmarchicus* somatic growth over a wide range of geographical areas, seasons and feeding regimes (Yebra et al., 2006b). Given the encouraging results obtained in the assessment of *C. finmarchicus* growth, from both laboratory cultures and at-sea cruise experiments, the AARS method was applied to study natural zooplankton communities in the North Atlantic Ocean. In the Irminger Sea, the AARS method showed that summer epipelagic zooplankton production distribution matched the major physical zones described in the area and that this distribution was mainly driven by food availability rather than temperature (Yebra et al., 2006a). Further west, in the Labrador Sea, this biochemical proxy allowed the effect of mesoscale hydrography on zooplankton production during both spring and winter (when it is not possible to assess female EPR), and from surface to 1000 m depth, to be studied for the first time (Yebra et al., 2009b). In the same way, the AARS method allowed the broad-scale characterization of zooplankton production in Antarctic, Mediterranean, Indian and Pacific waters (Table 6). Recently, the AARS method has been applied to study the growth of euphausiids

migrating within the minimum oxygen zone off Mexico (Herrera, 2014). These examples show how the application of the AARS method simplifies large-scale monitoring of zooplankton in the field, not only at the surface but also in deep waters.

2.4.5 Activation Energy of the Enzymes

As mentioned earlier, one way of obtaining different growth rates to proceed with the calibration of the AARS method is by growing organisms at different temperatures. In order to transform the AARS activity assayed in the laboratory to the *in situ* AARS activity at the experimental or field temperature, it is necessary to know the activation energy (E_a) of the enzymes AARS. The E_a of an enzyme could be calculated by dividing a sample in several aliquots and running the enzymatic assay at different temperatures covering the range of field/experimental values where the organisms were grown or captured. Then the E_a (kcal mol^{-1}) is obtained as

$$E_a = -R \times S, \tag{7}$$

where S is the slope of regression line between the AARS activity and the corresponding temperature (K) (e.g. fig. 1 in Yebra et al., 2005) and R is the gas constant (1.987 cal°K^{-1} mol^{-1}). Then, the AARS activity can be corrected for the *in situ* temperature applying this E_a to the Arrhenius equation:

$$\mathrm{AARS}_{\mathrm{situ}} = \mathrm{AARS}_{\mathrm{incubation}} \times \exp\left[E_a \times \left(T_{\mathrm{incubation}} - T_{\mathrm{situ}}\right)^{-1} \times R^{-1}\right], \tag{8}$$

where $\mathrm{AARS}_{\mathrm{incubation}}$ is the enzyme activity at the $T_{\mathrm{incubation}}$ during the enzymatic assay and $\mathrm{AARS}_{\mathrm{situ}}$ is the enzyme activity at T_{situ} (experimental or field temperature in K).

The E_a of AARS enzymes is rather similar in calanoids and mixed zooplankton in temperate waters. However, it is slightly higher in polar euphausiids and mixed zooplankton of upwelled subtropical waters (Table 7). Further research on the variability of E_a should be conducted prior to the application of a given E_a in different areas.

2.4.6 Use of the AARS Method as a Proxy for Other Metabolic Processes

The positive relationships between AARS activity and somatic growth of marine zooplankton permitted its use as tool to study the changes in the relationship between somatic growth and the metabolism of proteins under different stressing factors, environmental variables, ontogenetic processes, etc. In this sense, the AARS method was tested as an index of dormancy and female EPR with different results:

Table 7 Aminoacyl-tRNA Synthetases (AARS) Activation Energies (E_a, kcal mol^{-1}) Obtained in Planktonic Organisms

Species	Site	Latitude	Temperature	E_a (r^2)	Reference
Calanus helgolandicus	L4 station, English Channel	50°15′N	2–40	8.6 (0.85)	Yebra et al. (2005)
Euphausia superba	Lazarev Sea, Antarctica	64–70°S	5–40	10.5 (0.93)	Guerra (2006)
Mixed zooplankton (200–2000 μm)	Almería Bay, Alboran Sea	36°48′N	8–30	8.4 (0.73)	L. Yebra (unpublished data)
Mixed zooplankton (200–2000 μm)	Two Rocks, West Australia	31°50′S	5–37	9.5 (0.64)	L. Yebra (unpublished data)

Temperature (°C) indicates the temperature range tested for the biochemical determination of E_a.

In the case of dormancy, although there is no growth in overwintering organisms, protein synthesis can still be measured. This is because the net protein growth rate equals the synthesis rate minus degradation rate. Hence, AARS activity is expected even when growth is negative, owing to the continued protein turnover in the cells. Yebra et al. (2006b) studied the levels of enzyme activity in overwintering *C. finmarchicus* copepodites to assess the possibility of determining the state of dormancy through the analysis of AARS activity. They found significant AARS activity when growth was negative, and also significant reductions in AARS activity from active copepodite V (CVs) to those just starting diapause (predormant) ($p < 0.02$) and also between predormant and overwintering CVs ($p < 0.001$). They observed that a single correlation between AARS activity and growth rates was applicable to both active and diapausing organisms. However, AARS activity could not be established as index of dormancy *per se* given that very low values of AARS activity could also be indicative of negative growth rates related to starvation or unfavourable field conditions on active (i.e. non-diapausing) organisms.

Adult female EPR is used to assess copepod production in most field studies (Hirst et al., 2003) by assuming that reproductive growth is equivalent to the copepodites somatic growth (McLaren and Leonard, 1995; Poulet et al., 1995; Sekiguchi et al., 1980). However, this assumption is not always correct as both processes respond differently to environmental factors (Calbet et al., 2000; Hirst and Bunker, 2003; Hirst and McKinnon, 2001; McKinnon, 1996). In addition, EPR is usually lower than

somatic growth rates of juveniles when food is limiting (Hopcroft and Roff, 1998; Rey-Rassat et al., 2002, 2004). These differences are probably due to the fact that metabolism of larvae is directed to somatic growth, whereas metabolism of adult females is focussed on egg production, and both pathways (somatic and reproductive growth) are different. For example, Yebra et al. (2005) found that the relationship between *C. helgolandicus* females specific AARS activity and specific EPR in the field was different during the reproductive and non-reproductive seasons. Biegala et al. (1999) also observed a seasonal mismatch between this species female EPR and ATC activity, the enzyme that catalyses the synthesis of pyrimidine nucleotides (fundamental units of RNA and DNA). In a later laboratory study, Holmborn et al. (2009) also found no significant relationship between *Acartia bifilosa* adult EPR and AARS activity. However, they observed a good relationship between EPR and a combination of AARS activity and RNA:DNA ratio. For these reasons, despite the AARS method has been validated as an index of somatic growth for adult copepods (Yebra et al., 2005, 2006b), due to the marked differences between somatic and reproductive metabolic pathways, it does not seem suitable as index of adult female EPR in the field.

Recently, the advances in the assessment of growth on small nauplii with this method (Herrera et al., 2012; Yebra et al., 2011) led to the use of the AARS activity as index of protein metabolism in *C. finmarchicus* prefeeding nauplii during a mesocosm experiment (Koski et al., 2012).

Apart from the mentioned calibrations, the AARS method has been compared to theoretical models of growth (Garijo and Hernández-León, 2015; Yebra et al., 2005). These comparisons, despite they do not constitute true calibrations, might be useful to ascertain the degree of accuracy of methods or models that had not been adequately calibrated against the direct measurement of growth.

2.4.7 Advantages and Limitations of the AARS Method

2.4.7.1 Advantages

2.4.7.1.1 Simple, Quick and Non-radioactive Assay The AARS index has the advantages of an enzymatic assay (sensitivity, precision and repeatability) and it has a biomass-specific basis. Besides, this method solves some of the disadvantages of previous enzymatic assays as it is non-radioactive and it is a simple assay that minimizes manipulation of organisms. Also, the assay can be performed during cruises or samples can be frozen and analysed in the laboratory.

2.4.7.1.2 *In Situ* Approach to Growth Rate Assessment Despite the non-addition of substrates, the enzyme-catalysed reaction is not substrate-limited during the length of the assays. This allows assessing the enzymes activity without addition of substrates, which reflects the ability to synthesize proteins that individuals had in the field and depends exclusively on their endogenous amino acids resulting from the history of the organism. In this way, the AARS method provides an *in situ* approach to the assessment of growth rates in contrast to the traditional *in vitro* maximum potential activity (Yebra and Hernández-León, 2004).

2.4.7.1.3 Broadly Applicable Across Taxonomic Groups Conventional methods used in measuring copepod growth are generally most suitable for measuring rapid rates of growth. When growth is low or negative, when stage durations are long or when moulting rate is very low, results from these methods can be inaccurate (Rey-Rassat et al., 2004; Yebra et al., 2005). Their inability to provide accurate results in such circumstances depends not only upon the general technique applied (e.g. artificial cohort, modified moult rate method, see Section 1 for further details) but also upon the specific methods within these two (e.g. direct weight of animals or application of length–weight equations). The use of the AARS method can overcome these disadvantages. The method does not require incubations, it does not require moult to occur and it can be used to study growth both across and within developmental stages. Thus, AARS activity can be measured in non-moulting organisms such as adult and dormant crustaceans as well as in non-crustacean zooplankton. Hence, future studies are encouraged to include taxa such as protozoans, gelatinous plankton, pelagic tunicates and molluscs. Besides, although there is no growth in overwintering organisms, AARS activity can still measure their protein synthesis rates.

2.4.7.1.4 Compatible With Other Biochemical Measurements The AARS method can be combined with other biochemical analyses (e.g. RNA: DNA, ETS activity, gut fluorescence, protein content, etc.). Such combined protocols allow for the optimization of sampling and the assessment of multiple variables from the same organisms in order to achieve a more comprehensive assessment of the organisms/population physiological status (e.g. Holmborn et al., 2009; Koski et al., 2012; Yebra et al., 2004, 2009a, 2011).

2.4.7.2 Limitations

2.4.7.2.1 Requires Calibration The limitations of the method reside, as with all indices, in the need for calibration with the real rate to be approached (somatic growth in this case) before its application in the field or the laboratory. Also, protein synthesis rate is not a measure of growth itself. The protein turnover rate (recycling rate) must be taken into account, as there could be low growth-high enzyme activity scenarios (see earlier). Hence, AARS activity is not a measurement of growth rate but a correlative.

2.4.7.2.2 Different Sources of PPi There might be a potential uncoupling between growth rates and specific AARS activity measured from cell homogenates because there are other enzymes in the cytosol able to produce PPi. Despite this, the use of plankton homogenates instead of purified extracts or radioactive compounds simplifies the enzyme assays, while providing significant relationships between growth of a zooplankton population and its AARS activity.

2.4.7.2.3 Activation Energy of the Enzymes As the biochemical assays are run in the laboratory under controlled temperature conditions, it is needed to know the activation energy of the enzymes in order to calculate the *in situ* AARS activity at field temperature.

3. SUMMARY

The usefulness of current biochemical indices in relation to classical methods and models is numerous, and their specific advantages and limitations are summarized in Fig. 6. The most compelling advantage of biochemical methods is that no incubations of animals are required, overcoming the artefacts induced by handling of the organisms, artificial feeding conditions and scaling issues, among others. Thus, they facilitate growth rate estimation in the field at the time of collection, independent of any interventions. In addition, they allow for both relatively rapid basin-scale wide and vertically resolved sampling, which permits to estimate the growth rate in the field with a resolution similar to other environmental variables (e.g. chlorophyll). Furthermore, enzyme activities in particular are measured as rates; thus, they are dimensionally correct as indices of growth (note, however, that nucleic acid ratios do not reflect rate process since ratios are dimensionless). Also, by standardization with protein content, rates can be easily translated into nitrogen units (suitable for basin-scale production models). Furthermore, given

Advantages:

- No incubations required
- No radioactive compounds used
- Easy and economic to measure
- Assays can be run under controlled conditions
- High precision, good repeatability
- High temporal/spatial resolution
- Combination of indices possible

Limitations:

- Rates must be standardized with biomass
- Rates must be corrected for the *in situ* field T
- *In vitro* rate may not reflect an *in vivo* process
- Indices must be calibrated against direct growth rate before its application in the field or laboratory
- Some indices are stage/species/taxa specific

Fig. 6 Summary of advantages and limitations of biochemical indices in relation to classical methods used to assess zooplankton growth rates.

the universality of biochemical pathways, the use of biochemical indices would facilitate filling gaps in knowledge through its application to the assessment of growth rates of often neglected taxa like microzooplankton (e.g. protozoan, copepod nauplii, meroplankton larvae) and non-crustacean zooplankton (e.g. gelatinous plankton, fish larvae).

There are several biochemical methods in use. The choice of method depends on: (1) the goals (field coverage, depth, toxicology, etc.); (2) the time and facilities available (either in the laboratory or on a cruise); and (3) the calibration needed. It is of utmost importance that any index is calibrated against the direct rate to assess prior to its application (see Tables 2, 3 and 5). Users must be aware that calibration is not the same as comparison, and if a method has not been calibrated, they will need to test it before its use. Also, once a method is calibrated, any modifications of the biochemical assay must be tested to ensure that the usefulness of the index is maintained (e.g. change of homogenising buffer, pH, reagents proportion, etc.). It is also essential to know the target species, taxa or population and the adequacy of the index to be applied to the assessment of its production

Table 8 Suitability of Various Methods for Application to Zooplankton Production Assessment in the Field

Application	Methods						
	NC	AC	MR	EP	NA	CB	AARS
Organism/population properties							
Crustacean	Y	Y	Y	Y	Y	Y	Y
Noncrustacean	Y	Y	N	Y	Y	N	Y
Mixed species	N	N	N	N	N	Y	Y
Body size small	Y	Y	Y	Y	Y	Y	Y
large	Y	Y	Y	Y	Y	Y	Y
Moulting larvae/adult	Y	Y	Y	N/Y	Y	Y	Y
Nonmoulting adult	N	N	N	Y	Y	N	Y
Stage duration long	Y	Y	Y	—	Y	Y	Y
short	Y	Y	Y	—	Y	Y	Y
Environment properties							
Water mass exchange low	Y	Y	Y	Y	Y	Y	Y
high	N	Y	Y	Y	Y	Y	Y
Coastal	Y	Y	Y	Y	Y	Y	Y
Offshore	N	Y	Y	Y	Y	Y	Y
Polar–subpolar	Y	Y	Y	Y	Y	Y	Y
Temperate	Y	Y	Y	Y	Y	Y	Y
Tropical–subtropical	Y	Y	Y	Y	Y	Y	Y
Surface	Y	Y	Y	Y	Y	Y	Y
Deep waters	N	N	N	N	Y	Y	Y
Data resolution							
Incubations needed	Y	Y	Y	Y	N	Y	N
Temporal	L	L	L	L	H	H	H
Spatial	L	L	L	L	H	H	H

AARS, AARS activity; *AC*, artificial cohort; *CB*, chitobiase activity; *EP*, egg production rate; *H*, high; *L*, low; *MR*, moult rate; *N*, no; *NA*, nucleic acid ratio; *NC*, natural cohort; *Y*, yes; —, no concern.

(see summary in Table 8). For example, the RNA:DNA index has proven to be very useful for estimating the growth of a specific target species, but not for mixed taxa samples. On the contrary, CBA is ideal to assess production of entire crustacean zooplankton assemblages, but is not applicable to non-moulting taxa/populations. With respect to AARS activity, its relationship with growth has been demonstrated across a wide variety of taxa, from bacteria to fish. Hence, it is theoretically applicable to the assessment of production for entire zooplankton communities, although for some taxa (e.g. gelatinous species and pelagic tunicates and molluscs) such relationships remain to be tested.

Ideally, a general equation relating a given index (i.e. enzyme activity) with the growth rate of the whole zooplankton community components would lead to the global adoption of such index. To date, no general biochemical-based equation exists with which to assess zooplankton production. Nucleic acid indices are species-specific, so generalization is not feasible, whereas CBA can be widely applied across species and genera within arthropoda dominated communities. Recently, by compiling existing calibrations relating AARS activity and growth rates of crustaceans and fish, a general equation has been proposed (S. Hernández-León, unpublished data), which has been successfully applied to field samples (see Table 6). Despite these advances, we are still missing many taxa such as protozoans, pelagic tunicates or gelatinous species, which may represent an important proportion of community production. There is a need for further studies on these groups in order to fully understand what these indices are telling us when applied to whole zooplankton samples.

Currently, scientists from around the globe are collaborating to pursue a global assessment of zooplankton production (Kobari and Yebra, 2016). The implementation of biochemical indices may represent a way forward to achieve goals similar to past assessments of global primary production (through the adoption of the ^{14}C technique as standard). In this sense, the next steps needed include: (1) calibration and intercomparison of available indices of zooplankton production (including empirical models), and (2) standardization of historical data for incorporation into predictive production models. Numerous time series on zooplankton standing stock and environmental parameters are available. Hence, such a global assessment of zooplankton production may be feasible with the incorporation of calibrated biochemical approaches in the near future.

Furthermore, training in biochemical techniques is crucial if we are to foster their application in future production studies, especially on understudied taxa that may be of great importance as components of the zooplankton communities in a changing ocean. For this purpose, the Appendix presents protocols for the most commonly used methods discussed in this review. The application of standardized biochemical indices to the assessment of zooplankton production in the field will provide information of high temporal and spatial resolution to ecologists and modellers, and also to stakeholders addressing issues such as marine resources management and environmental quality assessment, all of which may be of particular importance in the context of continued climate change.

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APPENDIX. BIOCHEMICAL PROTOCOLS

A.1 Nucleic Acid Ratio Method

A.1.1 Reagents

In the nucleic acid ratio method, the following chemicals are needed for the biochemical assay:

- Tris(hydroxymethyl) aminomethane
- Ethylenediaminetetraacetic acid (EDTA-2Na)
- Sarcosine
- Deoxyribonucleic acid
- Ribonucleic acid
- RNAse (and DNAse in some protocols)
- Ethidium bromide, SYBR® Green I or RiboGreen™

10 mM Tris–EDTA buffer is made with dissolution of Tris aminomethane and ethylenediaminetetraacetic acid in Milli-Q water. 10 mM Tris–EDTA buffer is used as a diluted solution for the other chemicals. Extraction buffer is made dissolving 5.05 g of Sarcosine in 500 mL of 10 mM Tris–EDTA buffer. Fluorescence dyes should be selected upon the amount of nucleic acids in the samples. Due to the mutagenicity and low sensitivity of ethidium bromide, SYBR® Green I and RiboGreen™ are recently recommended.

A.1.2 Sample Preparation and Preservation

Either in the laboratory or on board research vessels, individual or groups of a target species and stages are identified, sorted and quickly frozen in microvials with liquid nitrogen (−196°C). The quickly frozen samples can be stored in a deep freezer at −80°C for later biochemical assay.

A.1.3 Biochemical Assay

There are several protocols available. Here we present the method described by Wagner et al. (2001) as an example.

The frozen samples are vortexed with zirconia beads for 20 min after addition of extraction buffer (pH 7.5) and then vortexed again for 40 min after dilution with Tris–EDTA buffer. The sample homogenate is centrifuged for 10 min at 15,000 × *g*. Aliquots of the supernatant are used as samples for nucleic acid assay. The replicates of the extracted samples, dilution series of DNA and RNA standards, and controls (i.e. extraction and Tris–EDTA buffers) are added to each well of black solid flat-bottom microplates and incubated for 10 min after the addition of the fluorescence dye to each well. The fluorescence is measured using a microplate reader (first scan) with the excitation and emission filters which are specified for the fluorescence dyes. After the addition of RNAse to the wells (i.e. digestion of RNA), the microplate is incubated for 30 min and the fluorescence is then measured with microplate reader again (second scan).

A.1.4 Calculations

DNA and RNA standard curves are determined with the difference in fluorescence of the dilution series of DNA and RNA standards and controls between first and second scans. DNA and RNA contents of the extracted samples are calculated from the difference in fluorescence between first and second scans and the standard curves. Nucleic acids ratio (RNA: DNA) can be computed from RNA to DNA contents of the extracted samples. Because no universal equation exists, instantaneous growth rate (d^{-1}) is estimated from the nucleic acid ratio using the species-specific calibrated equations, e.g., Ota and Landry (1984), Wagner et al. (2001), Vrede et al. (2002) or Yebra et al. (2011) in Table 2. Zooplankton production rates can be then estimated by multiplying instantaneous growth rate by the corresponding biomass.

A.2 Chitobiase Method

A.2.1 Materials and Reagents

The method relies on short-term (12- to 24-h duration) incubations of seawater and a simple enzyme assay on subsamples of incubated seawater (see Sastri et al., 2012). Incubations and enzyme assays can be carried out at sea. Incubations require: (1) acid-washed Nalgene bottles for each sampled depth; (2) sterile syringes and 0.2 μm syringe filters; and (3) disposable glass test tube for storing subsamples. The enzyme assay reagents are: (1) methylumbelliferyl-β-D-*N*-acetyl glucosaminide (MBF-NAG); (2) citrate–phosphate buffer (pH 6.0); and (3) 2 N NaOH, 0.4 M EDTA solution.

A.2.2 Sample Collection, Incubation and Short-Term Preservation

Seawater samples are collected from multiple depths spanning the depth range sampled by a corresponding plankton net haul (or depth range of interest). On deck, depth-specific seawater is screened through a 40-μm mesh into a 500-mL Nalgene bottle. The mesh is used to exclude any moulting animals from the incubation (i.e. no further contribution of chitobiase). This process is repeated for each sampled depth. A subsample from each incubation bottle is drawn with a sterile syringe and passed through 0.2-μm syringe filter. This subsampling represents the native activity of the chitobiase (CBA_{NAT}) and should be processed as soon after sample collection as possible. In most cases, incubation bottles are amended with a 0.2-mm filtered homogenate of 20–30 copepods, in order to improve the sensitivity of measuring enzyme decay (see Sastri and Dower, 2006). Subsampling is carried out again following the addition of homogenate to each incubation bottle and at each predetermined time-step. Subsamples are retained in disposable glass test tubes and stored in a refrigerator (4°C) until enzyme assay. Incubation bottles are typically maintained in a bath fed by surface seawater and the temperature is recorded at each subsampling interval. Incubation subsamples have been stored in the fridge for up to 2 weeks without significant loss of chitobiase activity (Suchy, 2014). Samples should be left to warm to room temperature in the dark for 6–12 h before measuring dissolved chitobiase activity.

A.2.3 Chitobiase Assay

The assay for chitobiase activity in seawater presented here follows methods described by Sastri and Dower (2006, 2009); however a different approach is followed by Oosterhuis et al. (2000). The sample and reagent volumes described here can be modified to accommodate the cuvette or well-plate volumes used by the operator. Briefly, 2.0 mL aliquots from each time-step-specific subsample are incubated with 1.0 mL of pH 6.0, 0.15 M citrate–phosphate buffer at 25°C. Reactions are initiated with the addition of 0.5 mL 0.1 mmol MBF-NAG (final reaction volume concentration). Reactions are allowed to proceed for 60 min and quenched with the addition of 0.5 mL NaOH–EDTA solution. Note that the final pH of reaction volume (following the addition of NaOH–EDTA) should fall between 10 and 10.5. This pH will vary with both the volume of NaOH–EDTA solution and salinity range and can be tested before the assays either in the lab before field work or in the field if necessary. Both blanks and time step-specific assays are typically run in triplicate. The enzyme assay measures (as fluorescence) the

rate at which chitobiase hydrolyses the semisynthetic substrate, MBF-NAG. Net fluorescence is calculated as the difference between fluorescence in samples quenched at 60 min and blanks (samples quenched at 0 min, i.e., reaction not allowed to proceed). Net fluorescence is converted to units of methylumbelliferone liberated h^{-1} L^{-1} using a standard dilution curve of methylumbelliferone.

A.2.4 Calculations

BPRs are calculated as per the description in the main text and as described by Sastri et al. (2013). Briefly, depth-specific decay rates of chitobiase activity (T_{CBA}) are calculated as the reciprocal of the slope ($-k$) of incubation time step vs ln-transformed chitobiase activity. CBA_{NAT} values are converted to a corresponding biomass production value (ΔB) using the global relationship presented by Sastri and Dower (2009). Note that this relationship is general and can be substituted for relationships more specific to the assemblage or populations in question. BPR (mg C m^{-3} d^{-1}) is simply calculated as: $\Delta B/T_{CBA}$. Note that differences between incubation and *in situ* temperature may occur. This will often be the case when several depth-specific samples are incubated at sea surface temperature. Where discrepancies exist, a Q_{10} of 3.15 should be applied to T_{CBA} to correct enzyme turnover rates for temperature (see Sastri and Dower, 2006).

A.3 AARS Method

A.3.1 Reagents

The AARS method is very simple and only PPi reagent (P7275 from Sigma), Tris–HCl buffer (20 mM, pH 7.8) and Milli-Q water are needed to perform the enzyme assay.

A.3.2 Sample Preparation and Preservation

Either in the laboratory or on board a research vessel, groups of individuals of a target species can be identified, picked and quickly frozen inside microvials in liquid nitrogen at −196°C. Also, the whole sample from a zooplankton haul can be fractionated (or not) into different size categories (e.g. 100–200, 200–500, 500–1000 and >1000 μm) and immediately frozen in liquid nitrogen (−196°C) for later AARS analysis in the laboratory.

A.3.3 Biochemical Assay

The AARS assay described here follows the method of Yebra and Hernández-León (2004) modified by Yebra et al. (2011):

(1) Frozen samples are homogenized with Tris–HCl buffer (20 mM, pH 7.8) in an ice bath (0°C).
(2) Homogenates are centrifuged for 10 min at 5000 rpm at 0°C.
(3) Aliquots of the supernatant from each sample are used to assay AARS activity and protein content in order to standardize AARS to biomass-specific AARS activity.
(4) AARS activity assay: 250 μL of supernatant are added to a cuvette containing 200 μL of pyrophosphate reagent (PPi reagent, P7275 from Sigma) and 300 μL of Milli-Q water (volume of reaction mixture, $V_{rm} = 750$ μL). For microplate assay, all volumes are divided by five ($V_{rm} = 150$ μL).
(5) Enzyme activity is monitored spectrophotometrically at 340 nm for 10 min at 25°C. Note that spectrophotometer cuvettes or microplates need to be of PMMA or other material allowing UV readings.
(6) Protein content assay: 100 μL of supernatant is used to assess protein concentration following, for example, the Lowry et al. (1951) method adapted for microassay by Rutter (1967). Use bovine serum albumin (BSA) as a standard.

A.3.4 Calculations

(1) AARS activity in a sample is calculated with the equation given by Herrera (2014):

$$\text{AARS activity}\left(\text{nmol PPi} \cdot \text{h}^{-1}\right) = \left(\frac{\frac{\text{dAbs}}{\text{min}} \cdot 10^3 \cdot 60 \cdot V_{rm}}{V_s \cdot 6.22 \cdot 2 \cdot 1}\right) \cdot V_{hom}$$

where dAbs min^{-1} is the rate of decay in absorbance per minute, 10^3 is the conversion of μmol to nmol, 60 is the conversion from minutes to hours, V_{rm} is the volume of the reaction mixture (mL), V_s is the volume of the sample in the assay cuvette (mL), 6.22 is the millimolar absorptivity (L mmol^{-1} cm^{-1}) of NADH at 340 nm, 2 is the number of moles of β-NADH oxidized per mole of PPi consumed, 1 is the path length correction (cm) for the cuvette width or microwell depth (needs to be calculated according to the plate used) and V_{hom} is the total volume of sample homogenate (mL).

(2) AARS activities are corrected for the *in situ* temperature by applying the activation energy of the enzymes (e.g. 8.57 kcal mol^{-1}, Yebra et al., 2005 in Table 7) to the Arrhenius equation.

(3) Specific AARS activity (nmPPi mg protein^{-1} h^{-1}) is calculated dividing AARS activity of the sample by the protein content of each corresponding sample.
(4) Zooplankton growth rate (d^{-1}) is calculated from specific AARS activity with a calibrated equation, e.g. references in Table 4.
(5) Total zooplankton community production rates (mgC m^{-3} d^{-1}) can then be calculated by multiplying the growth rate by the corresponding integrated biomass (protein m^{-3}).

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