Patterns in adaptive developmental biology and symbioses of small-sized deep-sea chemosymbiotic mussels (Bathymodiolinae)

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Université Pierre et Marie Curie

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ED227

UMR7208 BOREA Equipe : Adaptations aux milieux extrêmes

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PhD in Marine Biology

Supervised by Sébastien Duperron, Marina Cunha, Sylvie Gaudron

To be defended 24th September 2014

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Aspects adaptatifs de la biologie du développement et des symbioses chimiosynthétiques chez les petites moules Bathymodiolinae de l'Océan profond

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Thèse de Biologie Marine

Dirigée par Sébastien Duperron, Marina Cunha, Sylvie Gaudron

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To my wife: best friend… and closest confidante

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ABSTRACTS

Patterns in adaptive developmental biology and symbioses of small-sized deep-sea chemosymbiotic mussels (Bathymodiolinae)

An array of deep-sea habitats are characterised by thermo- and/or biogenic production of chemically reduced compounds. Benthic communities thrive at these 'reducing habitats', due to trophic links to free-living and symbiotic chemosynthetic bacteria which derive energy from electron donors (sulphides, hydrocarbons) and acceptors (O_2), at reduction-oxidation boundaries. The bathymodiolin mytilids (*sensu lato*) are a keystone taxon in these habitats; all-but-one species host gill-associated symbiotic Gammaproteobacteria as adults, but data are scarce on the remaining lifecycle, particularly at organic falls. To understand how mussels are adapted to these habitats, this research characterises the reproductive, developmental and nutritional lifecycle biology of two species, *Idas modiolaeformis* and *I. simpsoni*. Anatomical, histological and molecular analyses on post-larval-to-adult size spectra are complemented with live observations. Contrasting and converging aspects of their biology and symbioses are presented. In both species, aposymbiotic post-larvae confirm strict, larval heterotrophy. While still very small, environmental symbiont infection is extracellular and initially non-specific, becoming progressively isolated to latero-abfrontal gill surfaces in adults. Maturation is rapid, in parallel with a transition from heterotrophy to chemosymbiotic mixotrophy: symbioses, filter-feeding, and the retention of a complete gut are observed simultaneously. Interspecific and habitat differences are discussed in the context of both species' evident evolutionary success in adapting to ephemeral, chemically reduced habitats in the deep sea.

Aspects adaptatifs de la biologie du développement et des symbioses chimiosynthétiques chez les petites moules Bathymodiolinae de l'Océan profond

Plusieurs habitats de l'Océan profond sont caractérisés par la présence de composés chimiques réduits. Les communautés benthiques prospèrent dans ces «habitats réducteurs», en raison de liens trophiques avec des bactéries chimiosynthétiques, qui tirent l'énergie de donneurs (sulfures, hydrocarbures) et accepteurs (O_2) d'électrons. Les moules bathymodiolines sont un taxon clé dans ces habitats. À l'âge adulte, presque toutes possèdent des bactéries symbiotiques, mais les données sont rares concernant leur cycle de vie. Cette recherche explore la biologie de la reproduction, le développement et la nutrition dans le cycle de vie des espèces *Idas modiolaeformis* et *«I.» simpsoni*. Les analyses anatomiques, histologiques et moléculaires à divers stades du développement sont associées à des observations *in vivo*. Une comparaison est proposée entre ces espèces. Chez les deux, les post-larves n'ont pas de symbiotes, suggérant des larves strictement hétérotrophes. L'infection par les symbiotes est environnementale, extracellulaire et initialement non-spécifique, se restreignant aux surfaces latéro-abfrontales des filaments branchiaux chez les adultes. La maturation est rapide, de même que la transition de l'hétérotrophie à la mixotrophie chimiosymbiotique: les symbioses, l'alimentation par filtration, et la rétention d'un système digestif complet coïncident. Les différences entre espèces et habitats sont discutées dans le contexte de leur évidente réussite évolutive à s'adapter aux habitats réduits et éphémères de l'Océan profond.

ABSTRACTS

Padrões na biologia do desenvolvimento adaptativo e simbiose em pequenos mexilhões (Bathymodiolinae) quimiossimbioticos de profundidade

Vários habitats de profundidade são caracterizados pela produção termogénica e / ou biogênica de compostos quimicamente reduzidos. Nestes habitats-reduzidos, as comunidades bentônicas prosperam, devido a ligações tróficas a bactérias quimiossintéticos de vida livre ou simbióticas. Estas bacterias derivam a energia a partir de dadores de elétrons (sulfetos, hidrocarbonetos) e receptores (O_2), nos limites de oxidação-redução. Os mitilídeos Bathymodiolinae (sensu lato) são um táxon importante nestes habitats; todos, excepto uma espécie, tem simbiontes Gammaproteobacteria associados aos adultos, mas os dados para o restante ciclo de vida são escassos, particularmente em acumulações de matéria orgânica. Para entender como os mexilhões estão adaptados a estes habitats, esta tese caracteriza a biologia reprodutiva, do desenvolvimento e nutricional do ciclo de vida de duas espécies: *Idas modiolaeformis* e *I. simpsoni*. Análises anatómicas, histológicas e moleculares de diversos tamanhos de pos-larva a adulto foram complementadas com observações ao vivo. Aspetos contrastantes e convergentes da biologia e simbiose são apresentados. Em ambas as espécies, a ausência de simbiontes em pós-larvas confirmam a heterotrofia larval estrita. Quando juvenil, a infecção pelo simbionte ambiental é extracelular, e inicialmente não-específica, tornando-se progressivamente mais isolada na superficie “abfrontal” dos filamentos das brânquias dos adultos. A maturação é rápida, em paralelo com uma transição de heterotrofia para mixotrofia quimiossintética: simbiose, com alimentação por filtração demonstrada, e com um intestino completo e em pleno funcionamento. Diferenças interespecíficas e entre habitats, são discutidas para ambas as espécies no contexto do seu evidente sucesso evolutivo na adaptação aos habitats efêmeros, quimicamente reduzidos no fundo do mar.

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ABBREVIATIONS

±	"either side of"
≈	approximately equal to
≡	equivalent to
µm	micrometre
mm	millimetre
‰	parts per thousand
σ	standard deviation
♀	female
♂	male
	hermaphrodite

I.	*Idas*
A.	*Adipicola*
B.	*Bathymodiolus*
Be.	*Benthomodiolus*
V.	*Vulcanidas*
C.	*Calyptogena*
R.	*Riftia*
T.	*Tamu*
d.f.	*De facto*
s.l.	*Sensu lato*
s.s.	*Sensu stricto*

AUV	Autonomous Underwater Vehicle
ChEss	Biogeography of Deep-Water Chemosynthetic Ecosystems
CoML	Census of Marine Life
EPR	East Pacific Rise
FISH	Fluorescence In situ Hybridisation
HE	Haematoxylin and Eosin-Y
JdF	Juan de Fuca Ridge
LD	Lacaze-Duthiers Canyon
MAR	Mid-Atlantic Ridge
mm	millimetre
MV	Mud volcano
NDSF	Nile Deep-sea Fan
PAR	Pacific-Antarctic Ridge
POM	Particulate Organic Matter
REDOX	Reduction-Oxidation
ROV	Remotely Operated Vehicle
SEPR	South-eastern Pacific Rise
TEM	Transmission Electron Microscopy

Chapter 1 General Introduction

1-1 History of deep-sea exploration

1-1.1. Overview

Deep-sea research still remains in its infancy, despite notable technological advances in the last hundred years and a considerable expansion of our scientific understanding (German et al. 2011). Accordingly, empirical observations and discoveries of deep-sea habitats have generally been as a result of technological advances in deep-water surveying (Koslow 2007; Ramirez-Llodra et al. 2010). During an intense period of exploration between the mid-19th century and early-20th century, advances in sea navigation, sounding equipment for measuring depth and dredging apparatus saw several independent discoveries of marine life at depths famously argued to be devoid of life by Edward Forbes previously (Koslow 2007). The first global oceanographic expedition aboard the HMS *Challenger*, was from 1872–1876 during which physical and chemical characteristics of the oceans were documented, alongside hundreds of newly described species (Murray et al. 1891). The sounding equipment, though primitive, also permitted the discovery of continental shelf breaks and continental slopes, and highlighted variability in the topography of the northern Atlantic (1853, Koslow 2007; Ramirez-Llodra et al. 2010). The first evidence for submarine ridges, trenches and seamounts were collected soon after using both the Thomson and Sigsbee sounding machines (Koslow 2007), just prior to the beginning of the 20th century. Both the Juan de Fuca ridge and Gorringe ridge were identified during this period. With the advent of the acoustic sounder in 1914 (patented by Reginald Fessenden, Koslow 2007), mapping of ocean depths became possible. From World War I (1914–1919) to the present day, oceanic acoustic research has seen intensive international military investment with notable developments such as radio acoustic ranging, sonar and radar that paved the way for present-day global positioning systems (GPS) and sounding equipment with greater coverage, reliability and precision. The use of sensitive magnetometers in the mid-20th century provided critical evidence for plate-tectonic theory in the form of crustal magnetism striping, and therefore faulting, in the crust (Vine and Matthews 1963). Remote sensing has emerged as a critical tool in advancing our understanding of large-scale hydrodynamics (Kerr and Ostrovsky 2003). Advances in deep-sea exploratory equipment have been staggering, as global enterprises invested heavily into research and development in telecommunications, offshore drilling (and piping) for oil and gas and an impending rise in deep-sea mineral extraction (Van Dover 2011a). The consequences for deep-sea habitat conservation are thus bitter-sweet, as advances in underwater high-pressure technology provide the means to further understand, and exploit, deep-sea environments simultaneously.

1-1.2. Key advances and the current state of the art

Critical advances in deep-sea exploration can be broadly separated into several categories. These have been in navigation, depth-sounding approaches, means of profiling environmental and chemical water characteristics, underwater exploratory innovations and habitat and faunal sampling techniques. Note that

for the purposes of this manuscript, unless stated otherwise, 'deep' environments are those > 200 m, beyond the continental break, and in which sunlight is at such low intensities as to be of little *direct* ecological significance (material and nutrients of shallow-water origin, which may be photosynthetically derived, are considered indirect).

1-1.2.1 Navigation and positioning

Advances in navigation techniques within the last century have been from the use of hand-drawn maps, compasses, sextants and callipers (and of course, a cloudless sky) with >100-km margins of error, to the application of global positioning systems (GPS), informed by a network of 32 mid-orbiting satellites in space, ground-based control stations and antennae, with an accuracy of 10 metres or much less with land-referenced Differential GPS (Parkinson and Enge 1996). GPS-positioning has been incorporated into nearly all deep-sea measuring devices since, permitting the geo-referencing of any data to coincidental, potentially explanatory, environmental data. This is of particular importance when data is to be integrated into geographic information systems (GIS), a conceptual quantitative visual-mapping tool developed initially for use in multidisciplinary terrestrial geographic studies (Hatcher and Maher 2000).

1-1.2.2 Sounding to assess depth

In one of the first attempts to estimate depths in the 18th century, the French mathematician and astronomer Pierre-Simon Laplace argued (rather accurately) that the Atlantic ocean ought to be around 4000m deep on average, based on observed tidal patterns in the southern Atlantic (Gillispie 2000). However, attempts to actually assess water depths definitively would be made almost a hundred years later, employing basic 'sounding' apparatus (Koslow 2007). In stark contrast, scientists now employ a wide array of remote-sensing approaches including high-resolution bathymetric track data, Shuttle Radar Topography Mission data (Becker et al. 2009) and gravitational anomalies in the sea-surface geoid, identifiable when examining high resolution gravity field data (satellite radar altimetry data, Smith and Sandwell 1997). These measurements are then interpolated in suitable GIS software in order to create continuous high-resolution bathymetric data (e.g. Figure 1.1C) for the world's oceans: a powerful tool for predicting the locations of currently undocumented topographic features and thus potentially associated fauna (e.g. Marks and Smith 2006; Kim and Wessel 2011).

1-1.2.3 Environmental profiling

In terms of environmental data, the first attempts to map global temperatures and salinities both at the surface and at depth were during the Challenger Expedition (Dittmar 1880; Thomson et al. 1888). Approximately 300 profiles were recorded using pressure-protected thermometers and stopper bottles (for salinity) attached to sounding lines, subsequently demonstrating for the first time the relatively constant salinity of offshore marine waters, the presence of oceanic thermoclines, and inadvertently providing a baseline record for future global-warming studies (e.g. Roemmich et al. 2012). These samples required

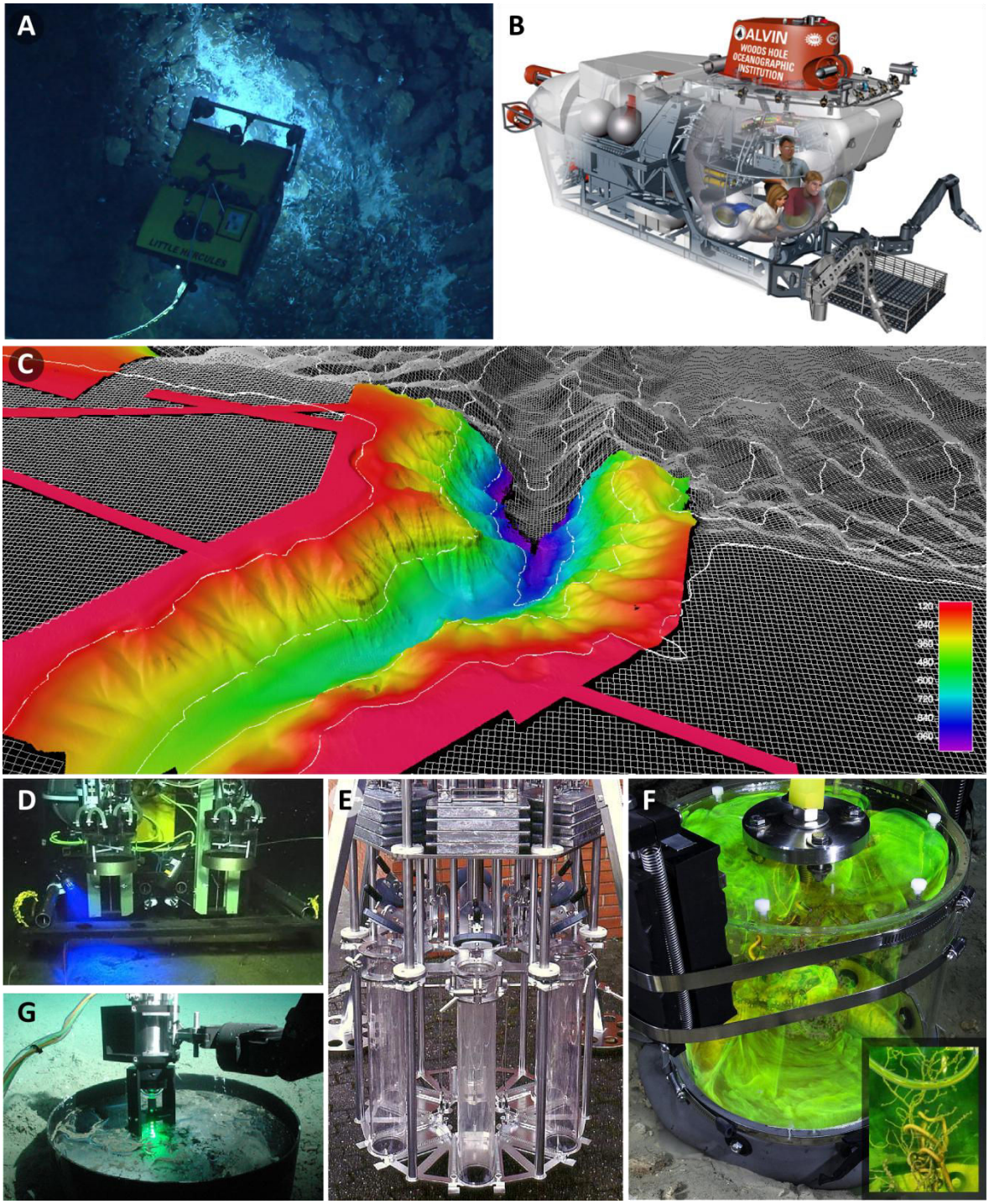

Figure 1.1 Various equipment used in deep-sea research

A) Little Hercules ROV exploring the Mid-Cayman Rise. Image courtesy of NOAA Okeanos Explorer Program, MCR Expedition 2011. **B)** Alvin submersible: plan for the 2013 refurbishment. Illustration by E. Paul Oberlander, for WHOI. **C)** 3-D multibeam sonar image of Norfolk Canyon, eastern United States, looking down the axis toward the canyon mouth (Steve Ross: Deepwater Mid-Atlantic Canyons cruise 2011). **D)** A benthic rover a mobile physiology lab assessing carbon supply and demand at the seafloor, being tested at the cabled observatory MARS. Image from MBARI website. **E)** Multicorer with 6 polycarbonate coring tubes. KC Denmark research equipment. **F)** Calcein fluorescence-labelling chamber for marking organisms after which growth can be tracked. Inset is view of coral inside. 2014 Nautilus Exploration Program. **G)** A Raman spectrometer, which uses laser excitation and measurement of molecular vibration, specific to the chemical bonds and symmetry of molecules. Image from MBARI website

lengthy post-expedition analyses however (Koslow 2007). In contrast, today such measurements can be made in real time using conductivity-temperature-depth (CTD) profilers, while current-velocity profiles are constructed from acoustic Doppler current profiler data (ADCP), both typically being mounted on a submerged platform (Ramirez-Llodra et al. 2010). Additional data on turbidity and light intensity within the euphotic zone (0–200 m, Gage and Tyler 1991) can be measured using dedicated instruments. Remote sensing in the deep-sea can provide broad scale environmental data at the surface (e.g. temperature and chlorophyll anomalies, Kerr and Ostrovsky 2003), identifying zones of upwelling and the presence of large-scale eddies potentially important for long-distance larval dispersal (Pineda et al. 2007). Some of this equipment is also used in the deep sea, and where appropriate, can be installed on remotely operated vehicles (ROVs, Figure 1.1A), on landers, or at cabled observatories. The latter often has a dedicated array of technology at its disposal (e.g the MARS benthic rover Figure 1.1D). The exploration of deep-water environments where the water chemistry is of particular interest (e.g. chemically reduced end-member fluid emission in vent fields) has fuelled further innovations in measuring devices for assessing water chemistries *in situ* and at great depths. Many compounds which would formerly have needed to be analysed at the surface in a laboratory, can now be recorded *in situ* using highly sensitive potentiometric sensors (Le Bris et al. 2008) or pressure-housed spectrometers (e.g. the Raman spectrometer, Brewer et al. 2004; Zhang et al. 2010; Figure 1.1G), removing the effects of alterations in water chemistry during water sample recovery.

1-1.2.4 Underwater exploration

Accessing the deep-sea directly was simply impossible prior to the advent of submersible exploratory equipment in the 20th century (Koslow 2007; Ramirez-Llodra et al. 2010). This was demonstrated to be possible through the pioneering work of Edward Beebe (1934, 923 metres**,** tethered bathyscaph), the French research submersible F.N.R.S. (1954) and the Bathyscaph Trieste submersible (1960, maiden Mariana Trench dives, Koslow 2007; Figure 1.2A), while the advent of SCUBA-diving by Jacques Cousteau in 1943 popularised underwater exploration. Today, deep-sea exploration is either by AUV (autonomous underwater vehicles), ROV, and to a lesser extent by research submersibles that remain in operation, with the pioneering submersible Alvin (WHOI) having been refitted for the 8th time (Figure 1.2C; newest design in Figure 1.1B). The most recently constructed submersible to be used for scientific exploration is the DEEP Challenger submersible (Figure 1.2B). However, exploration of the deepest parts of the ocean remains challenging exemplified by the probable implosion of the ROV “Nereus” during exploration of the Kermadec trench (WHOI Media-Relations Office 2014). ROVs today can be mounted with all the equipment necessary to document a deep-sea habitat in its entirety, using the systems already described. With geo-referenced data, this can then be coupled with remote sensing data to provide a complete picture of the environment in question.

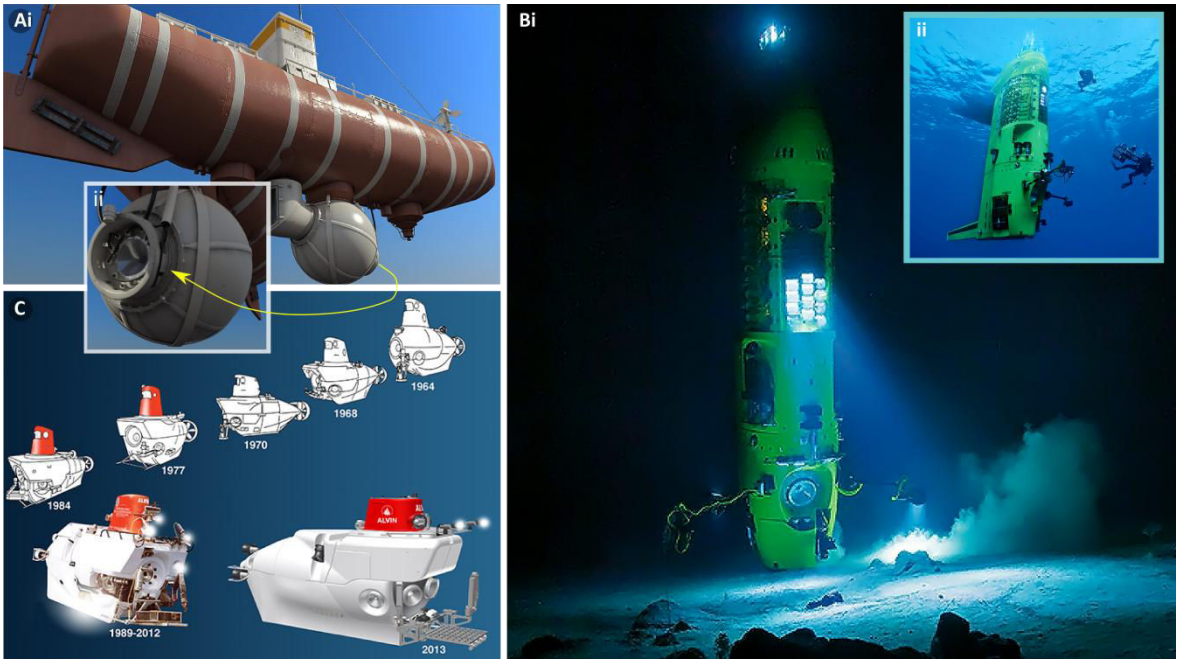

Figure 1.2 The evolution of manned submersible technology
A) The Bathyscaph Trieste [i], made the first voyages to the Marianas Trench. View of pilot sphere from front [ii]. B) The CHALLENGER DEEP submersible having just reached a predetermined depth (during mid-depth test dive). All bodywork except the pilot sphere is made from advanced syntactic foam to reduce operating weight. Scuba divers in support during pre-dive checks [ii]. C) The various incarnations of Alvin, the figurehead submersible for the Woods Hole Oceanographic Institute, whose debut dive was in 1964.

1-1.2.5 Biological sampling

While all these developments in technology have placed deep-sea research at the cutting edge of marine sciences, methods for sampling the benthos and pelagos remains limiting in deep-sea research. Deep-sea benthic sampling is still relatively cumbersome, patchy and extremely expensive as it is necessarily vessel-based and evermore ROV-orchestrated. Although deep-sea trawling/dredging is best avoided being highly destructive, it's use is still prevalent, particularly for deep-sea science in developing countries or when no access to an ROV is available (e.g. Samadi et al. 2010). Cores and grabs have been optimised with the use of remotely-guided operation. Additional means of sampling are stationary deployments such as nets, traps (Beaulieu et al. 2009) and nascent substrata for colonisation (e.g. the European CHEMECO project, Gaudron et al. 2010; INDEEP's Serpent Project e.g. Higgs et al. 2014), alongside direct sampling using corers, suction dredges and articulated robotic arm. Deep-sea research has historically been hindered by poor sampling resolution with limited comparability across studies, as sampling approaches are often not uniform. The maintenance of international collaborations, with sustained active communications between experts, remains of prime importance. The whole field begins to benefit from the up-scaling of time-series multiple-site sampling where habitat surveillance is routinely performed, such as in cabled observatories. Innovative techniques that can be performed by ROV are now being developed for the deep sea (e.g. burrow casting, Seike et al 2012, or calcein staining for growth measurements, Nedoncelle et al. 2013; see also Figure 1.1).

1-1.2.6 Live maintenance of deep-sea organisms

Pressurised conditions present several problems, not least the requirement for pressure-resistant equipment with which to carry out scientific work, but also the singular most difficult aspect of the study organism's environment to sustain in the laboratory (Marsh et al. 2001). Even with the use of hypobaric chambers, reproductive and larval biology studies remain highly challenging, though possible (e.g. Young and Tyler 1993; Young et al. 1996; Eckelbarger et al. 2001; Marsh et al. 2001; Pradillon et al. 2005). An alternative, for species with a less constrained depth range, is to bring them to the surface and maintain them alive for reproductive experiments at atmospheric pressure (Mortensen 1921; Young and Cameron 1989; Young et al. 1989; Colaço et al. 2006; Dixon et al. 2006; Arellano and Young 2009; Arellano and Young 2011). Such examples include slope-based communities which can also be the sites of reducing conditions (e.g. species from cold seeps) or reducing habitats which can occur over large depth-ranges (e.g. sunken wood). Keeping these animals alive long enough for larval studies to be performed in the laboratory either requires fully equipped aquaria on the research vessel, or more typically, facilities at least equipped to transport organisms to land-based aquaria.

1-1.2.7 Perspectives in deep-sea

Deep-sea sciences have gained momentum over the last half-century with technical advances and relative reductions in cost. Exploration efforts increased with the discovery of highly productive chemosynthetic communities thriving in the immediate vicinity of hydrothermal vents in 1977 at the Galapagos Rift, a spur of the East Pacific Rise (Corliss and Ballard 1977; Lonsdale 1977; Corliss et al. 1979). If a research vessel is fully-equipped, it is now possible to collect sufficient data in order to document those environmental parameters and biogeochemical characteristics which define different deep-sea habitats, as well as interactions between habitats (e.g. fluxes, migration and the formation of environment boundaries which may promote allopatric speciation). The bathymetry, hydrodynamics and water or substrate chemistry may be documented using remotely-operated vehicles, gliders or autonomous underwater vehicles (AUV), coupled with multibeam arrays, ADCPs, CTD profilers and chemical picosensors with perhaps in the future, *in-situ*-mounted equipment for inductively-coupled plasma mass-spectrometry (ICP-MS) or gas chromatography (GC). Biological data can be obtained through geo-referenced video-surveying, the use of temporally-sequential multicorers (e.g. Figure 1.1E), continuous plankton recorders, permanent deep-sea monitoring stations, manipulative experimental installations. The use of naturally occurring (or experimentally-derived) chemical tracers to directly assess growth (e.g. Tada et al. 2010; Nedoncelle et al. 2013), transport, connectivity and nutrition *in-situ* in deep-sea benthic species, has already proved informative in shallow-water environments (Becker et al. 2005; Becker et al. 2007; Cowen and Sponaugle 2009). However, these techniques require considerable investment in terms of resources as well as overcoming the logistical difficulties of the environment in question (Van Dover 2011b). The sheer expanse of deep-sea environments also presents a daunting prospect for marine scientists. If an ephemeral habitat is under investigation, much time and expenses must be spent in order to simply locate the habitat (Rogers

et al. 2012). By definition, these habitats are also the most challenging habitats to sample temporally (and for studies examining temporal variations in recruitment for example), though they may also be the most informative (e.g. Mullineaux et al. 2010). In habitats that occur over large spatial scales (such as abyssal plains, oceanic trenches, and continental slopes), researchers may be tempted (inappropriately) to interpret findings in isolated studies and make wide-sweeping conclusions about larger regions (e.g. Thorson 1950). Understanding such habitats is essential however, if the more "atypical" dynamics of some deep-sea habitats are to be placed in an appropriate biological context.

Cost-effective innovations for the future to address the historical imbalance might include remote-release sampling methods, combined with permanent high-resolution remote monitoring stations (e.g. NSF-Ocean Observatories Initiative, U.S.A; NEPTUNE, Canada; DELOS project, Scotland; the European Seas Observatory NETwork or ESONET; DONET, Japan) and periodic geo-referenced video surveys using autonomous underwater vehicles (AUV).

1-2 Deep-sea benthic habitats

1-2.1. Habitat diversity and productivity in the deep-sea

Despite historical opinion to the contrary, it appears that the deep-sea is not a "desert abyss". This is all the more impressive considering approximately 75% of extant species are thought to have evolved following the Cretaceous–Paleogene (K–Pg) mass extinction event (Jacobs and Lindberg 1998; Schulte et al. 2010), possibly subsequent to a currently hypothesised reinvasion by species from shallow-water environments. Even in the abyssal plains, traditionally thought to be highly conservative, stable environments (and thus not sufficiently dynamic to peak scientific interest), growing evidence suggests that environmental and organismal processes act to maintain highly levels of biodiversity (with low species densities) through macro- and mesoscale habitat disturbance (e.g. formation of massive burrow complexes by deep-sea bivalves, Seike et al. 2012; mound formation through bioturbation, Kukert and Smith 1992). In habitats where a persistent REDOX boundary occurs (reducing habitats), symbiotic adaptations have evolved in deep-sea metazoans to harness the energy available from reduced electron-acceptor compounds (e.g. H_2S and $S_2O_3^{2-}$, and CH_4) by hosting bacteria and archaea capable of either chemoautotrophy or -heterotrophy, using inorganic minerals (chemolithotrophy) or organically derived reduced compounds (chemoorganotrophy), reviewed in Vrijenhoek (2010). This results in highly productive, specialised communities in the absence of sunlight, though with relatively poor species diversity (Grassle 1989).

Between the discovery of habitats that can support highly productive communities in the absence of light (e.g. hydrothermal vent fields, hydrocarbon 'cold' seeps and organic falls) and the associated media interest in such 'alien' habitats, a renewed interest in the deep-sea has emerged in recent decades. With technical advances in the field continually emerging, discovery rates of new habitat types in the deep sea

have consequently accelerated exponentially over the last hundred years (Figure 1.3). Such interest is justified; the deep-sea is arguably the largest biome on Earth. Of the planet's 510 million km^2 surface, 71% is coastal and ocean seafloor where approximately 90% is classified as deep – i.e. beyond continental shelf breaks (Ramirez-Llodra et al. 2010; Van Dover 2011b). Global interactions between plate-tectonics and hydrological, meteorological, biogeochemical, and geological processes combine to sculpt ocean-floors and, together with organic fluxes to the seabed, determine habitat characteristics. The level of habitat diversity on a global scale in the deep sea is astounding, but the horizontal coverage of various formations and habitats is extremely asymmetric, according to the most current data available from bathymetric topography (Ramirez-Llodra et al. 2010, the values cited in this section henceforth are from this review, unless stated otherwise).

Descending down the continental slopes, depths range from around 150 m (with a few exceptions) at the shelf break to depths of around 3500m as the slope gradient flattens out at the continental rise. The continental slopes and rises together account for about 10% of the deep-sea ocean floor. In terms of surface-area however, the vast soft-sediment seascapes that extend beyond these margins, the abyssal plains, dominate the deep-sea (76%). Studies during the last half-century (reviewed in Gage 2003) have revealed previously hidden levels of dynamism in the ecology of abyssal plains. Localised macro- and mesoscale processes, both organic and environmental (Ramirez-Llodra et al. 2010), act to maintain high levels of diversity in these soft-sediment environments, but with typically low resource-limited productivity (Ramirez-Llodra et al. 2010). These community characteristics are assumed to apply for the majority of the abyssal soft-sediment deep-sea, forming what scientists studying chemically reduced systems refer to as the "background" fauna or communities. Though predominantly flat and featureless, these expanses are punctuated by other deep-sea structures. The most extensive are the mountain ridges along tectonic boundaries, being almost equivalent to continental margins in their coverage (9%). The formation of nascent oceanic crust occurs at mid-ocean spreading centres, flanked either side by semi-contiguous volcanic mountain ranges that extend along their entire lengths (depending on the rate of spread). Relatively isolated submarine mountains (seamounts) make up around another 2.6% (though estimates range up to 19.6%, or 67.4 x 10^6 km^2 of ocean floor >500m depth, for a predicted ca. 201,000 seamounts from 100–6700 m in height, Hillier and Watts 2007). The oceanic trenches normally located at sites of oceanic subduction, represent ≈2% of the horizontal coverage, though considerably more in vertical relief with depths of nearly 11 km in the Marianas Trench (10908 m, DEEPSEA CHELLENGER, 2013). Reducing environments and the habitats therein currently represent incidental features by comparison, with hydrothermal vents on spreading ridges, back-arc basins and volcanic seamounts, hydrocarbon seeps, which include methane seeps, gas hydrates, asphalt flows and oil seeps, cold-water coral reefs, whale falls and oxygen minimum zones (OMZ) representing <1% of the ocean floor, based on current knowledge.

Although Ramirez-Llodra et al. (2000) include whale-fall data in their estimates of global habitat coverage in the deep-sea, they do not account for sunken vegetative organic matter. This may because estimates of coverage by sunken wood and other debris in the deep sea are not currently feasible; it is not even clear whether sunken wood in the deep sea is more or less abundant than in coastal systems, as data on the volume and fate of wood exported to the open oceans have never been quantitatively assessed (though qualitative records suggest that they could be considerable, Maser et al. 1988). The influx of sunken wood to deep environments may be in the form of numerous but small fragments of submerged woody debris deposited in and exported down continental slopes (e.g. via submarine canyons, Josselyn et al. 1983), right up to whole trees weighing several tonnes that can take many months to become waterlogged and sink to the bottom (Maser et al. 1988; Maser and Sedell 1994). The distribution of vegetative debris already submerged on the continental shelf is likely to be restricted to continental margins, while the largest pieces of floating wood could sink practically anywhere on the ocean floor. In the presence of suitable offshore currents, the rate at which wood becomes waterlogged and sinks likely dictates its final settlement site, with 'intermediate' sizes of wood operating somewhere along this spectrum of transport potential. The distribution of wood in the deep-sea is probably asymmetric, favouring stretches of the continental margin that are strewn with canyons, and subject to fluvial discharge from rivers with flood plains in heavily forested areas, particularly if the region is subject to storms.

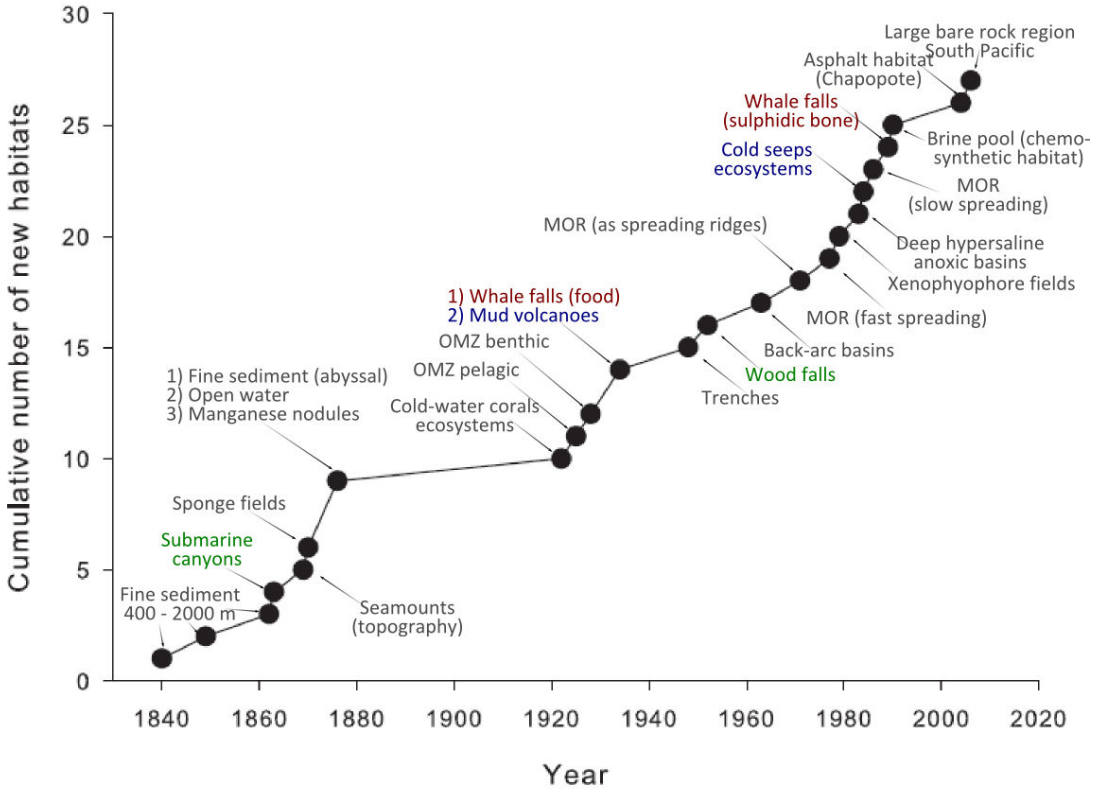

Figure 1.3 Rates of habitat discovery in the deep sea
Depicted is a plot of key moments in the last 160 years at which various aspects of novel habitats have been recognised by the scientific community. Highlighted, are those habitats most relevant to the current research presented in this thesis. Adapted from Ramirez-Llodra et al. 2010, with annotation from data therein.

1-3 Challenges for deep-sea metazoans: deeply difficult or just right?

1-3.1. General considerations

1-3.1.1 A life under pressure

The maximum known depth for any vertebrate, is currently 7.7 km for a hadal liparid fish (Jamieson et al. 2010). Invertebrates however appear less constrained by pressures at depth (except decapods <7 km, Jamieson et al. 2010) in the absence of large gaseous cavities (e.g. lungs or swim bladders) and being released from hypoosmotic constraints associated with the protein-stabilising molecule trimethylamine, present in hadal fish (Yancey et al. 2014). One of the adaptations proposed for deep-sea species is of a cellular nature. Phospholipid-membrane proteins, Na^+/K^+-ATPases, appear to be more resilient to increases in pressure and decreases in temperature in deep-sea species than in their shallow-water counterparts, owing to greater cell membrane fluidity at a given depth (Cossins and Macdonald 1989; Hazel and Williams 1990). Other adaptations to elevated pressures include chemically stabilised structuring in enzymes, adaptive functioning in nervous and musculoskeletal systems, differing osmolytes, and the loss of unsustainable carbonate skeletal structures, where applicable (Somero 1992). Some of these adaptations are even orchestrated by pressure-resistant operons within DNA (Li et al. 1998). Put simply, for species that have evolved in deep environments, such pressures are a habitual component of their lives. It's also worth noting that changes in pressure with depth are actually most abrupt at the surface; a transition from surface waters to 100-m depth results in a ten-fold increase in ambient pressure, whereas a transition of 5 km from 5000–10000 m, only equates to a doubling in pressure.

1-3.1.2 The absence of light and photosynthesis

In the absence of light, prey and predators alike have developed capacities for bioluminescence, employed complex and ingenious visual signalling (reproduction), distraction (predator evasion) and attraction (visualising and luring prey) in the deep sea, where a predator-prey arms race is ongoing (Herring 1984). The absence of light from the deep sea was also once thought to be the single-most limiting attribute of this environment's productivity for all organisms that lived below 200m depth. Even at the start of the 1970's, eminent scientists were still of the opinion that the deep sea was decoupled spatially and temporally from primary production in surface waters (Sanders and Hessler 1969). However, evidence of some deep-sea organisms timing their spawning events so as to take advantage of spring blooms in surface waters (e.g. Tyler 1988; Arellano and Young 2009) suggests that this is not the case, and of course, chemosynthetic primary production which does not rely directly upon the sunlight's energy (but does indirectly in the historical production of O_2), is now known to occur in multiple guises throughout the world's oceans (Dubilier et al. 2008).

1-3.1.3 Vertical flux of food supply

In non-reducing deep habitats, benthic assemblages are thought to be dependent wholly on the vertical flux of organic material from proximal surface waters. This comprises of organic aggregates – some derived

from plankton blooms – slowly sinking through the water column (collectively called 'marine snow' Turner 2002, i.e. macrozooplankton, fish faecal pellets and sinking phytoplankton). The vertical flux thus varies in quality, on account of inherent variability in contributing sources and due to nutrient recycling and repacking during descent (Turner 2002). Since the composition of vertical organic flux is derived from primary productivity in the euphotic zone, it is worth considering how this can vary from coastal systems to the open oceans and with increasing depth, since the underlying soft-sediments will mirror the productivity of proximal surface waters, but an increase in the depth of descent reduces the volume of material that can reach the seafloor (Levin et al. 2001).

The productivity of surface waters will depend upon seasonal levels and latitudinal gradients of irradiance and the availability of nutrients; rising irradiance drives photosynthesis while the nutrient supply sustains algal growth, resulting in a surge in planktonic densities (a bloom). Net surface primary production varies 11-fold world-wide (excluding highly variable polar regions, Turner 2002), from around 40–50 g C m^{-2} yr^{-1} at oligotrophic gyres, Jenkins 1982 versus 300 – 600 g C m^{-2} yr^{-1} in regions of upwelling, Suess 1980; Turner 2002 and references therein), with general proportionality between surface primary productivity and net downward organic carbon flux for the first 2000 m at least (Suess 1980; Lampitt and Antia 1997). The arrival of particulates to the seabed is known to be seasonal even in deep environments (Gooday et al. 1990; Lampitt and Antia 1997; Turner 2002), evidenced by seasonality in soft-sediment community oxygen consumption rates (e.g. Smith and Baldwin 1984) and in cyclical reproductive patterns of some responding species (summarised in Gooday et al. 1990). Bacteria and protozoan flagellates and foraminifera rapidly settle upon and proliferate in phytodetritus when it settles, while deposit feeding organisms appear to consume it directly (reviewed in Gooday et al. 1990). Larger organic falls also transport food to the seafloor, but with habitat-specific responses by benthic organisms (discussed in section 1-5.3). The nature of arriving taxa appears to depend on the form of the organic material (Gooday et al. 1990).

1-4 Deep-sea conservation: research and challenges

1-4.1. The paucity of baseline data

Although the recent advent and rapid development of equipment capable of sampling the ocean's depths has enabled the exploration of habitats under pressure, technical and habitat-specific biases persist in our understanding of deep-sea biology and ecology. While global patterns in bathymetry, marine hydrography and geochemical processes are being resolved, to date, many deep-sea community-based studies are systematically hindered by low sample sizes and a lack of replicate sampling both temporally and spatially. The exceptions are large-scale sampling campaigns undertaken on the world's continental slopes to asses biodiversity in the deep sea (Sanders and Hessler 1969; Hessler and Jumars 1974; Grassle and Maciolek 1992; Snelgrove and Smith 2002). Due to the operational costs of performing detailed ecological studies in deep-sea environments, ecologically significant biological interactions are poorly defined (other than

commensal and symbiotic associations), even in chemosynthetic environments (Glover et al. 2010; Ramirez-Llodra et al. 2010). Genetic connectivity (e.g. Bors et al. 2012), biogeography (Bachraty et al. 2009; Olu et al. 2010; Rogers et al. 2012) and levels of endemism (Howell et al. 2010) have formed the focus of recent studies. Within single communities, data is fragmented concerning interactions within and between cohabitant species and their environment. This fundamentally limits our ability to assess the sensitivity of communities to environmental disturbances both in terms of physiological tolerance and resilience through inter-population connectivity. Basic data concerning the physiological flexibility and the life-histories of deep-sea organisms are scarce (Tyler and Young 1999). Whether observed ecological patterns in shallow-water habitats are even applicable to the deep-sea remains to be seen. Behavioural data on deep-sea organisms is extremely limited. Evolutionary alterations to the mytilid bauplan are generally inferred from shallow-water species and preserved-tissue analyses only. On an organismal level, a basic understanding of the developmental, reproductive and nutritional biology of select model species throughout its lifecycle could provide insights into the origin and maintenance of high biodiversity in deep-sea assemblages.

1-4.1. Identified challenges of deep-sea conservation

1-4.1.1 An impact assessment of the deep-sea

In the first assessment of its kind for the deep sea, Ramirez-Llodra et al. (2011) collated the documented reports of anthropogenic impacts on deep-sea environments. The themes (Figure 1.4) were “disposal” (human-origin waste and litter), “climate” (ocean acidification and climate change, exacerbated by anthropogenic activities) and “exploitation” (the impacts of deep-sea resource prospecting and exploitation). These were considered for 12 deep-sea habitats: mid-ocean ridges (MOR), sedimentary slope (SLO, background fauna), canyons (CA), seamounts (SM), cold-water coral reefs including associated species (CO), active hydrothermal vents (VENT), cold seeps (SEEP), oxygen minimum zones (OMZ), abyssal plains (AP), manganese nodule provinces (MnAP), trenches (HT), and bathypelagic water column (BP; see Ramirez-Llodra et al. 2011 for more detailed habitat descriptions). Based on their collective judgement each of the impacts was assessed in detail as far as data would allow.

Mean “disposal” impact for all habitats was highest in the past, on account of a recent worldwide ban on offshore waste disposal. Mean impact indices for “exploitation” increased for those habitats in which current or predicted commercially valuable resources occur (particularly mineral extraction and fish stocks). It was in mean “climate” impacts however that the greatest increases were predicted reflecting the hitherto-underestimated impact that climate change, and in particular, ocean acidification is expected to have upon the deep sea in the foreseeable future (Smith et al. 2008).

1-4.1.2 Areas of focus for conservation

Ramirez-Llodra et al. (2011) identified four deep-sea habitats that are at greatest risk in the near future. These were upper-slope sedimentary benthos (accumulative effects of climate change-related shifts in nutrient input, ocean acidification and spreading hypoxia; global fishing effort), cold-water corals (for

identical reasons), seamount communities (fishing effort, fishing gear damage to the benthos, climate change-driven changes in circulation and increased stratification), and finally the canyon-based benthos (innovative fishing activity, increases in refuse, chemical pollution and nutrient loading, climate change-driven changes in circulation and increased stratification).

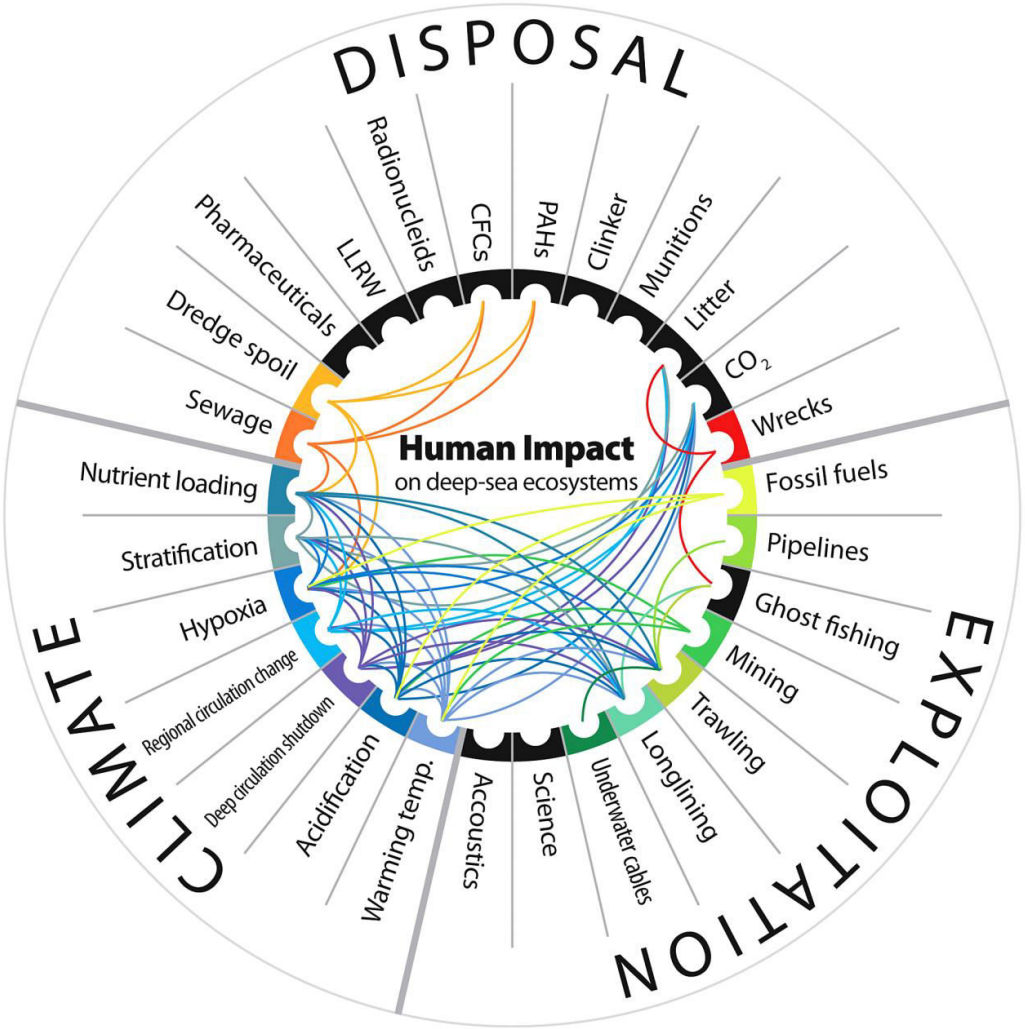

Figure 1.4 Impacts in the deep sea
Schematic of all impacts on the deep sea considered in Ramirez-Llodra et al. (2011). Each impact was initially scored from 0–5 based on the collective expertise of 23 deep-sea researchers (0 = *no impact*, 5 = "*Major anthropogenic impact including death of all life at the point of impact. Likely to have subsequent regional effects*", with a spectrum of impacts in between). A matrix was then compiled to identify the presence or absence of antagonistic interactions between impacts. The cross-links displayed are the results of this analysis. CFCs = Chlorofluorocarbons; PAHs = Polycyclic Aromatic Hydrocarbons. From Ramirez-Llodra et al. (2011)

In acknowledgement of their current vulnerability, measures have been taken to design and install Marine Protected Areas (MPAs) and areas closed to bottom fisheries, both within EEZs and to a lesser extent, international waters (Ramirez-Llodra et al. 2011; Figure 1.5). MPAs for the four high-risk habitats previously listed include the Northeast Atlantic Fisheries Commission Regulatory Area for upper-slope sedimentary benthic communities, numerous MPAs which restrict or prohibit bottom fishing on seamounts

communities (e.g. Formigas-Dollabarat Bank, Azores, Portugal; Sitka Pinnacles Marine Reserve, Alaska; Bowie seamount, Canada; Tasmanian seamounts, Australia and; fisheries closures over 19 seamounts spread throughout the New Zealand, Pitcher et al. 2007), no-fishing zones in cold-water coral reef habitats (e.g. Bering Sea–Aleutian Islands ecosystem, Ruckelshaus et al. 2008, assessed globally with generally positive results, Selig and Bruno 2010), and MPAs prohibiting the disturbance, damage, destruction or removal of any living marine organism from submarine canyons (The Gully, Canada; Soquel Canyon State Marine Conservation Area, Monteray Bay, US, pelagic fin-fishing accepted).

Future human activities could place additional habitats at threat through the extraction of lucrative mineral resources such as polymetallic sulphide deposits at hydrothermal vents, cobalt-laden ferromanganese concretions on seamounts, and hydrocarbon reserves (e.g. methane, gas hydrates, oil) buried at hydrocarbon seeps (Ramirez-Llodra et al. 2011; Van Dover 2011a). This was the argument behind the establishment of the International Seabed Authority (ISA): an intergovernmental body which seeks to orchestrate and control all mineral-related commercial activities expected to take place beyond the limits of national jurisdiction, i.e. in the High Seas. The ISA itself was a product of UNCLOS (United Nations Convention on the Law of the Sea) which delineates the entitlements and responsibilities of ramified nations concerning global oceanic ecosystem services, by creating guidelines for enterprise, conservation, and the ethical, sustainable management of natural marine resources.

What troubles researchers presently, is the rampant pace at which commercial interests in the deep sea moves forward with plans to make full use of deep-sea ecosystem services. As the forerunners, with the means to explore the deep sea extensively, they outpace scientists, management authorities and legislators whose aim it is to control and regulate the exploitation of deep-sea services. Not included in the assessment performed by Ramirez-Llodra et al. (2011) is the range of large-sized organic falls that reach the deep-ocean floor. Organic falls are highly ephemeral with somewhat less predictable distributions than other reducing habitats. Their conservation status is therefore poorly defined. However, larger organic falls may provide intermediary settlement sites for long range multigenerational dispersal in species that bridge these, and other reducing habitats. In addition, wood accumulations in submarine canyons appear to be the preferred habitat context for deep-sea wood-boring bivalves *Xylophaga* spp. (Romano et al. 2013; Fagervold et al. 2014), which frequently occur at staggering densities, in conjunction with an array of other opportunistic organisms, chemoautotrophic free-living material mats and chemosymbiotic bivalves from the genus *Idas* (e.g. Gaudron et al. 2010; Bienhold et al. 2013; Cunha et al. 2013; Thubaut et al. 2013a). They represent production hotspots at the juncture between coastal- and deep-sea- dominated systems, both in terms of hydro-geography and their ecology.

Human activities are placing mounting ecological pressure on deep sea habitats, where the severity of these impacts will likely worsen with time. If humankind is to exploit the deep seabed now and increasingly in the future, as seems inevitable (Ramirez-Llodra et al. 2011; Van Dover 2011a; b), then the

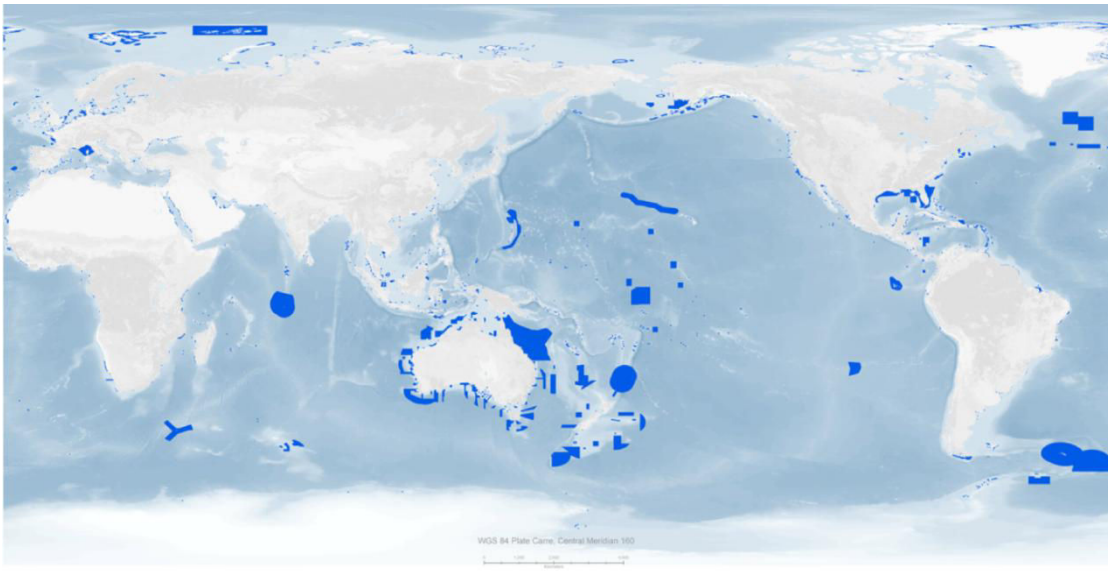

Figure 1.5 Map of Marine Protected Areas generated by UNEP World Conservation Monitoring Centre
This map is the most recent official map of MPAs available, generated by UNEP World Conservation Monitoring Centre (UNEP WCMC) using data from the World Database on Protected Areas (WDPA)

potentially destructive nature of commercial deep-sea exploration and resource extraction deserves a better notion of what we have to lose. Such threats to a domain for which so much remains unknown or poorly studied, have created the impetus to gather foundational data that can reliably inform decisions regarding conservation. The sustainable and ethical use of deep-sea resources can only be realised through ongoing exploration of poorly surveyed regions of the oceans, routinely assessing biodiversity and ecosystem function in known habitats and initiating measures to optimise conservation efforts (German et al. 2011; Ramirez-Llodra et al. 2011).

One critical line of research lies in describing deep-sea invertebrate lifecycles in relation to the physical, chemical and biological processes that impact upon their development, as this will help us understand and predict which factors will control spatial and temporal variability in populations (i.e. distribution patterns, composition, stability, migration, resource partitioning and larval recruitment). This goal can be achieved in part through the design, validation and application of population dynamics models informed by crucial lifecycle metrics from the community (Eckman 1996). This requires a full account of the developmental biology of, at least, a selection of 'keystone' species in an ecosystem (Le Pennec and Beninger 2000) to constraint the model. Larvae and their ecological characteristics are important in the establishment and maintenance of many marine communities and associations (Levinton 2001), while the settled and established benthic community utilise a multitude of developmental strategies. Understanding deep-sea larval-to-adult biology is thus critical in describing how communities temporally and spatially persist.

1-5 Reducing Habitat characteristics

1-5.1. An ecosystem map of benthic deep-sea habitats

Visualising the heterogeneity and distribution of deep-sea habitats requires a global perspective. These habitats frequently arise from coastal hydrodynamic processes and thermogenic and biogenic processes (hydrothermal vents and hydrocarbon seeps, discussed in section 1-5.1, p. 16). Consequently, such habitats are inextricably linked to regions where these processes sculpt the local environment (Figure 1.6, Figure 1.7). Hydrothermal vents may be found at mid ocean ridges, back arc basins and on isolated volcanic seamounts. Hydrocarbon seeps are found close to continental margins (Figure 1.6, Figure 1.7) though gas hydrates are more widespread (Figure 1.7). Organic fall locations are probably related partly to their origin: concentrated along nektonic megafaunal migratory routes and breeding / feeding grounds (e.g. for whales, Figure 1.7) and regions of elevated wood and macroalgal offshore transport. Historic sighting and catch data for whales suggest that certain regions of the ocean are likely to see disproportionately high numbers of natural whale deaths. As part of the Census for Marine Life such data has been compiled for the most frequently sighted whale species (Smith et al. 2012; redrawn in Figure 1.7B). Available whale-fall data appears to agree with whale sightings and catch data (Figure 1.7B). By layering global remote sensing data of the present-day distribution of forests worldwide and the main (i.e. navigable) global network of rivers, their catchment areas and their associated mean monthly discharge, it is possible to identify, semi-objectively, regions of the planet that are most likely see the greatest proportion of coastal efflux of woody material (Figure 1.7C). Together these maps suggest that open abyssal expanses are dominated by soft sediments, but punctuated with volcanic and geothermal features, which include hydrothermal vents. Coastal systems are far more complex, where habitat heterogeneity is high and dependent on both hydrodynamics and depth. These are discussed in detail.

1-5.2. Hydrocarbon seeps and hydrothermal vents

Since the processes that form hydrocarbon seeps are linked intrinsically to the offshore export of coastal detritus, they are distributed predominantly along continental margins, in areas with a history of organics-rich sedimentary deposition. At passive margins, deep layers of sediment have formed due to the transport of terrigenous and marine material to the slope and deep-sea ocean basins, delivered by turbidity flows, sedimentary slumps and offshore detrital deposition (Boetius and Wenzhofer 2013). Thus continental slopes and oceanic basins that are coupled with passive continental margins are frequently subject to high degrees of organic-enriched sediment loading. When sedimentary deposition continues in this way over

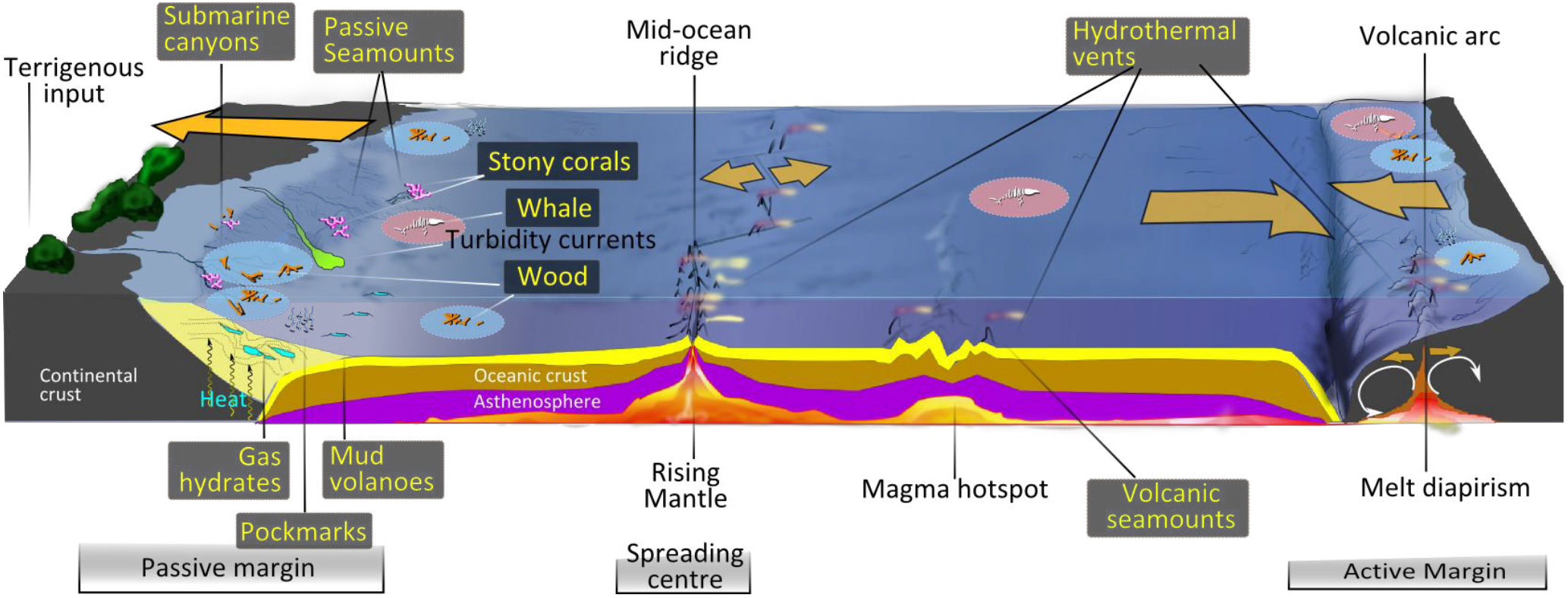

Figure 1.6 Distribution of deep-sea habitats within an ocean bordered by a passive and an active margin

The schematic displayed attempts to summarise the principal processes and resulting habitats which are known in deep-sea environments. Not marked but occupying the vast majority of the deep sea, are soft-sediment environments. See text for description of each habitat. Habitats which relate directly to this research are highlighted in blue (wood falls) and pink (whale falls). Conceptual schematic compiled by S.R. Laming.

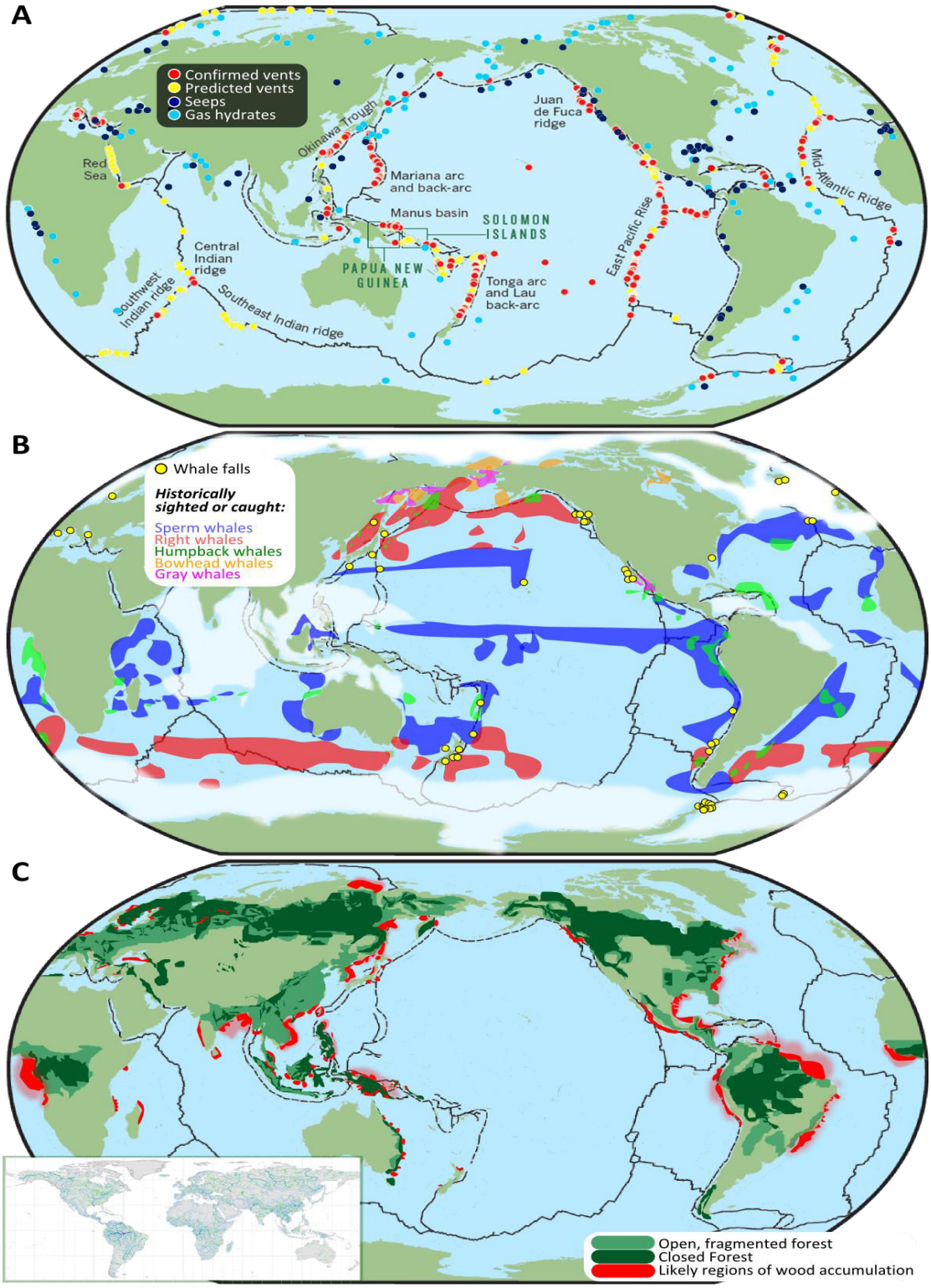

Figure 1.7 Data and predictions for hydrothermal vents, hydrocarbon seeps, gas hydrates, and organic falls
A) Map compiled from vent, seep and hydrate distribution data from InterRidge Vents and ChEss Databases and the MARUM website. **B)** Reproduction of data on caught and sighted whale species since records began from Smith et al. 2012, overlaid with whale fall locations from the ChEss Database. C) Objective predictions (by S.R. Laming) for wood falls accumulations based on the locations of forested areas on land (2009 global forest/non-forest map from the Japan Aerospace Exploration Company) and river distribution/discharge data (navigable rivers from the CIA World DataBank II/ Major river discharge data from polylines of 687 rivers, classified by discharge from the Global River Discharge Centre, inset map).

geologically significant periods of time, the buried organic matter undergoes chemical maturation during lithification (Figure 1.8: "POC burial"), caused by an elevation in geothermal temperatures and the considerable compressive forces exerted by millions of tonnes of sediment and water (Didyk et al. 1978). These processes alter interstitial pore-waters chemically, forming hydrocarbon-laden fluids, which readily percolate towards seabed surfaces when destabilised (Figure 1.8). These emissions manifest as "cold" hydrocarbon seeps, frequently with methane as a principal component (Sibuet and Olu 1998), while processes such as the anaerobic oxidation of methane along with sulphate-reduction can generate local enrichments of sulphide (Duperron 2010). When gas-hydrates occur – natural gases such as methane dissolved in seawater, previously subjected to pressures over ≈5000 kPa, (Maini and Bishnoi 1981) – their destabilisation can provide high-flux seepage (Mienert et al. 1998). In other instances the flows can be viscous or even semi-solid, as in oil seeps (e.g. Sassen et al. 1993), asphalt volcanism (e.g. MacDonald et al. 2004), mud volcanoes (e.g. Vogt et al. 1997) and brine pools (Mapelli et al. 2012). Hydrocarbon seepage can occur at active margins such as sedimented vent/seep sites on the Juan de Fuca Ridge, or at the unusual CO_2-vent systems of the Mediterranean, however this is not so common.

These sites of hydrocarbon seepage are an example of reducing environments which are all characterised by the release of chemically reduced fluids which interact with oxygenated seawater at the reduction-oxidation (REDOX) interface. These reducing compounds can support free-living and symbiotic chemosynthetic bacteria, which derive their energy from the metabolic oxidation of reduced compounds. Typically, they employ hydrogen, or sulphidic compounds as electron donors and gain their carbon from CO_2 (i.e. chemoautotrophic primary production) though several other electron donors have also been implicated (Anantharaman et al. 2013). Sulphides at hydrocarbon seeps can be generated in large amounts at sites where the anaerobic oxidation of methane occurs (Boetius et al. 2000; Treude et al. 2003). Some bacteria can also derive their energy from the metabolic oxidation of methane (methanotrophs, methane supplies a portion of their carbon also). The result is a myriad of bacterial biofilms and mats comprised of chemoautotrophic and methanotrophic bacteria, as well as bacterivorous primary consumers, specialist secondary consumers and a number of chemosymbiotic metazoan species that are seep- or reducing-habitat specialists. At times, biofilms are so ubiquitous and dense that they act as a biofilter, significantly lowering the concentrations of reducing compounds exiting the mats as free-fluid production (e.g. up to 40% reduction, Niemann et al. 2006). Those chemosymbiotic organisms which can survive and prosper at seeps include Bathymodiolinae mussels and Pliocardiinae clams (Figure 1.9) forming dense beds, and narrow, elongate *Lamellibrachia* siboglinid tubeworms, aggregating in bushes (Le Pennec et al. 1990). In addition to this core community, opportunistic "background" fauna occur. A high diversity of gastropod species also appears to reside at mud volcanoes in the eastern Atlantic, in the Gulf of Cadiz (Génio et al. 2013).

The seepage of chemically reduced fluids capable of supporting specialised communities is not restricted to hydrocarbon cold seeps. In fact, the acknowledgement that cold seeps were a habitat type in their own right (Paull et al. 1984) came almost a decade after another chemically reduced habitat – the hydrothermal vent – was described for the first time (Corliss and Ballard 1977; Lonsdale 1977). By September 2010, over 150 confirmed deep-sea vent sites and over 130 cold seep sites had already been studied with respect to the fauna communities associated with them (Baker 2010; German et al. 2010; Van Dover 2011b), illustrated in Figure 1.7. Approximately 70 additional unconfirmed vent-site locations have been postulated, extrapolated from water-chemistry anomalies recorded during oceanic surveys (Van Dover 2011b; Figure 1.7), as well as just less than 80 known methane hydrate sites, though not all in marine environments.

The geothermal processes that form vent fluids are quite well established for basalt-hosted hydrothermal activity (Tivey et al. 1995; Figure 1.10). Initially, seawater containing Mg^{2+} and SO_4^{2-} ions among others (both notable by their removal or reduction in vent end-member fluids), must permeate faulted and fissured igneous crust often rich in metal ores and minerals. If the crust is subject to proximal volcanism, infiltrating water can be chemically altered by a number of ion exchanges with the rock as it penetrates the deeper "recharge" zone (Tivey 2007). By the time the deepest "HT reaction" zone is reached (Figure 1.10) under elevated temperatures and pressures, evolved fluids are notably stripped of Mg^{2+}, while the liberation of H^+, Na^+, K^+, Ca^{2+} ions has increased fluid acidity and caused the precipitation of $CaSO_4$ anhydrite. Water and Fe-bearing minerals reacting together create conditions favouring the reduction of SO_4^{2-} to a mixture of H^+, HS^- and H_2S. Thus, reactions occurring in the recharge zone result in an evolved fluid that is somewhat acidic, anoxic, rich in alkalis and Mg-poor compared with entering seawater. The reaction zone sees the solubilisation of sulphur and metals (e.g., Cu, Fe, Mn, Zn, Li, Pb) from the crust, usually a period of phase separation into brines and vapour (the critical point between both being 407 °C at 298 bar, Tivey 2007) and varying degrees of magmatic volatiles degassing into the fluid (CO_2, CH_4, H_2). As a consequence, 'end-member' fluids are buoyant and ascend rapidly to the crust-sea interface via the path of least resistance (Figure 1.10). On contact with seawater and with a fall in temperature and pressure, some minerals are precipitated to form chimneys and deposits (e.g. CuFeS, FeS_2, ZnS, Tivey et al. 1995). End-member fluids from on-axis peridotite-hosted (ultramafic) hydrothermal activity differs by being marginally less acidic and H_2 and CH_4-enriched (by serpentisation, except 'Rainbow' MAR, which is H_2 and CH_4-enriched but more acidic and Fe-saturated). The single known off-axis ultramafic vent site exhibits atypically alkaline end-member fluids ('Lost City', off-MAR, H_2 and CH_4-enriched, pH 9-10, Kelley et al. 2001; Kelley et al. 2005). Andesite-hosted hydrothermal activity at back-arc basins in contrast are enriched in Ca, Mn, Si, and Fe and even more acidic (due to magmatic off-gassing of SO_4), relative to basalt-hosting.

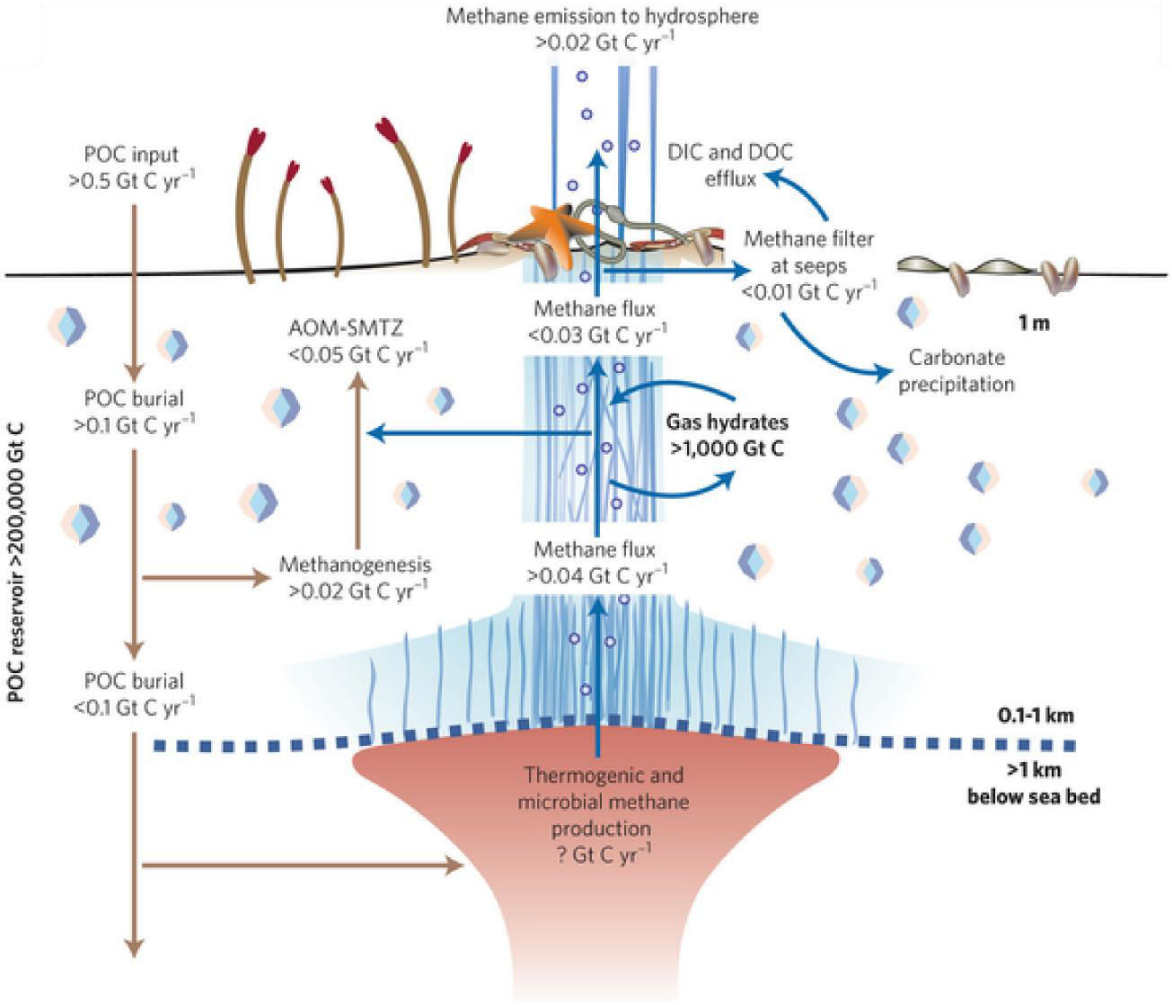

Figure 1.8 Methane production in a hydrocarbon seep environment
The < and > signs indicate if fluxes are likely to be smaller or larger in reality, respectively, based on the underlying uncertainties. Figures are derived from literature reviewed by Boetius and Wenzhofer 2013. Brown arrows indicate fluxes in diffusion driven systems, blue in advective (seep) systems. POC *particulate organic matter*, AOM *anaerobic oxidation of methane in the sulphate–methane transition zone*. From Boetius and Wenzhofer 2013

Vents are most numerous within the valleys and upon the flanks of spreading mid-ocean ridges, but also occur on subducting back-arc basins, on volcanic arcs and on active (volcanic) seamounts (Figure 1.6–1.5). The chemosynthetic basis for primary production is analogous to that of hydrocarbon seepage, driven generally by free-living and chemoautotrophic symbionts (though not always exclusively, e.g. Duperron et al. 2006a). Vent communities vary depending on the end-member fluid composition (e.g. across different vent fields), the ratio of mixing of these fluids with ambient oxygenated seawater within a single field, and on the geographic setting. Based on the current data available, a complex global biogeography for vent communities is known to exist (discussed in section 1-5.4, Van Dover et al. 2002; Bachraty et al. 2009; Rogers et al. 2012). Generally however, mid-ocean spreading ridges in the Atlantic and Pacific are both characterised by the presence of chemosymbiotic mytilids and large vesicomyid clams, forming extensive aggregations around diffuse vent flows. In addition on the Mid-Atlantic ridge species of hydrothermal vent shrimp, move around in search of suitable crevices with diffuse flow, and together these

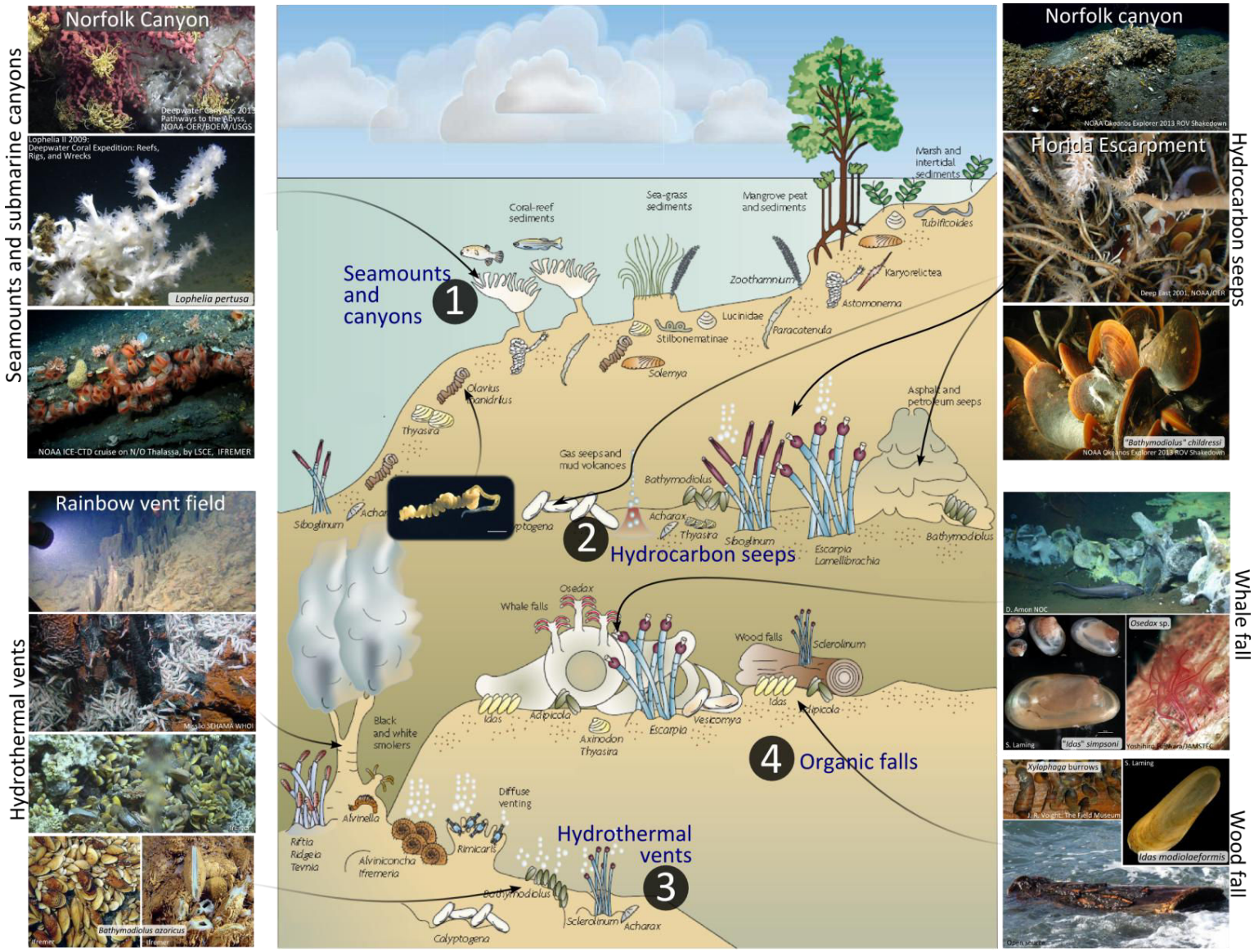

Figure 1.9 Chemosymbioses in different marine habitats

Chemosynthetic symbioses occur in a multitude of habitats. Aside from shallow-water sediments, these habitats include continental slope sediments, submarine canyons and seamounts, mainly as reduced sediments but also due to the accumulation of organic matter, which enriches localised patches of sediment [1]; hydrocarbon seeps [2], nektonic megafaunal carcasses and wood falls [3], and hydrothermal vents [4]. Some host groups are restricted to one habitat (such as Osedax on megafaunal bones), whereas others are pervasive in several different environments (such as thyasirid clams, which are found in shallow-water sea-grass sediments, deep sea cold seeps, whale falls and hydrothermal vents), while the bathymodiolins, occur in the three deepest habitats, at vents, seeps and organic falls. Animals are not to scale. Adapted from Dubilier et al. 2008. Photo acknowledgements are cited on photos when known (see electronic version, with high resolution).

three taxa dominate macrofaunal community compositions (Figure 1.9). In the Pacific, such shrimp do not occur, however in their place are perhaps the most extraordinary chemosymbiotic organisms to occur in the marine environment, the vestimentiferan (Siboglinidae) tubeworm *Riftia pachyptila*. These worms display the fastest growth rates in any marine metazoan (Lutz et al. 1994). Smaller but equally impressive polychaetes from the Alvinellidae, tolerate some of the hottest temperatures of any multicellular marine organism, spiking at 80 °C maximums, but typically around 40 – 45 °C.

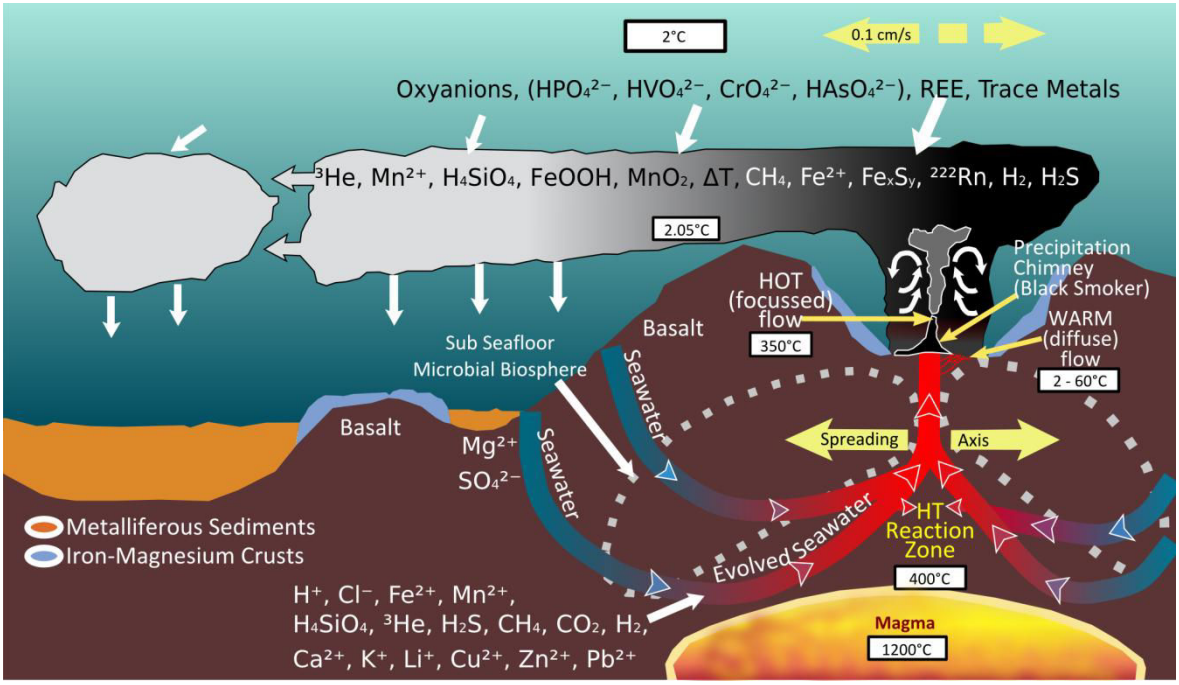

Figure 1.10 The biogeochemistry of hydrothermal-vent fluid formation
Above is a schematic of the hydrothermal circulation in a basalt-hosted mid-oceanic ridge vent system. Seawater infiltrates the semi-porous igneous rock, is heated and compressed. The chemistry of the evolved fluid is determined by the minerals available but also the temperature reached at the HT reaction zone. For details see main text. Diagram is adapted from an open domain figure.

1-5.3. Organic falls: megafauna and plant-derived material

Since the discovery of communities at hydrothermal vent and hydrocarbon seeps, additional habitats have been established as productivity 'hotspots' in the deep-sea, including communities that settle and thrive on, in or around large megafaunal carcasses at various stages of their decomposition and accumulations of sunken vegetation on the deep-sea floor (Van Dover et al. 2002). Unlike vents and seeps, organic falls are not linked inextricably to biogeochemical or geothermal processes, but rather to the relative productivity of overlying eutrophic waters as feeding grounds (e.g. in the case of baleen whales) or the localised import of coastal and terrestrial vegetative material to the deep sea (e.g. wood and kelp). By far the best understood of these are whale-falls (Baco and Smith 2003; Smith 2006). When averaged over the entire deep-sea floor's surface area (3.6×10^8 km^2), whale falls worldwide probably only represent ≈0.1% of the background particulate organic carbon (POC) flux to the deep-sea even under the most oligotrophic central gyre waters (Baco and Smith 2003, details and assumptions therein). However the reality at the habitat scale is that whale falls provide massive influxes of labile organic matter to localised areas of the seabed (typically

around 50 m^2, Baco and Smith 2003). These can be in the region of 2000 years worth of background POC flux at abyssal depths in a single pulse (Baco and Smith 2003). What's more, if conservative predictions for whale-fall frequencies based on natural surface aggregations are accurate (e.g. nearest neighbour distance of <16 km for gray whales within their known North Pacific range, Baco and Smith 2003) then whale-fall aggregations may provide conduits for multi-generational dispersal in the endemic fauna (Levin 2007).

Like vents, these habitats are ephemeral and ultimately become sulphidic (e.g. Treude et al. 2009). In the case of megafaunal nektonic carcasses, four stages of community succession have been recognised during decomposition, of which the first three are expedited by biological processes (Bennett et al. 1994). Depending on the carcass size, some of the stages may be truncated (Baco and Smith 2003; Smith 2006). Initially however, the carcass must first settle on the seafloor: given that sinking rates for naturally deceased megafauna are thought to be rapid, it is argued that relatively little tissue removal occurs during the carcass' descent (explained in detail in Smith 2006). Once the carcass arrives and settles on the sea floor, the first stage is effectively a feeding frenzy where bacteria and mobile necrophagous scavengers feed at high densities and breakdown or remove the bulk of soft tissues ("mobile scavenger", or "necrophage stage", Bennett et al. 1994).

Particles of soft-tissue and faecal matter that sink to the seabed during scavenger feeding, organically enrich the underlying sediments up to 3 metres from the carcass, hosting microbial mats and within 4 months, densities of heterotrophic and chemosymbiotic macrofauna an order magnitude higher than in background communities (*enrichment-opportunist stage*, Bennett et al. 1994; Baco and Smith 2003; Bernardino et al. 2012). Many of the taxa present during this stage are documented at other organic-enriched habitats and may be considered as opportunistic, but a select number appear to be specialist to carcass-falls (Smith 2006), though not necessarily whales only (e.g. bone-eating siboglinid polychaete, *Osedax* spp., Jones et al. 2008; Rouse et al. 2011). The end of this stage is marked by the depletion of all organic tissue, other than those internal to the skeleton, and the disappearance of the opportunist community indicative of these sediment enrichment conditions.

Internal lipid-rich bone marrow within the skeleton probably begins to undergo sulphate-reductive anaerobic degradation soon after external tissue putrefaction begins. Once bones are exposed, the resultant efflux of sulphides (Treude et al. 2009) can fuel the metabolism of chemoautotrophic bacteria, both free-living (e.g. microbial mats) and as symbionts in specialist chemosymbiotic metazoans (e.g. the siboglinid polychaete *Osedax* spp.; the mussels, *Idas washingtonius*, Baco and Smith 2003; and *Adipicola pelagica*, Fujiwara et al. 2007; Figure 1.9). This stage has been described as the *sulphidic stage* (Bennett et al. 1994), and has been documented to last for at least two years in 4 whale skeletons on the California slope (Bennett et al. 1994; Smith et al. 2002). The proportion of natural whale falls found at this stage of degradation, in comparison to the initial two stages described by Bennett et al. (1994), suggests that the sulphidic stage may in fact be the most extended of the biodegradation phases (e.g. Smith et al. 2002; Baco

and Smith 2003; Glover et al. 2005; Smith 2006; Treude et al. 2009; Tyler et al. 2009; Bernardino et al. 2012; Amon et al. 2013). Radiochemical methods based on $^{210}Pb/^{226}Ra$ disequilibria have identified sulphidic-community colonised skeletons up to 85 years old (Schuller et al. 2004). Exceptions may be in juvenile whale falls (Smith and Baco 2003), and whale falls at shallower depths (e.g. Fujiwara et al. 2007), which can deviate from the described timings and patterns of succession. In practice, evidence suggests that no clear temporal delineation exists between any of the biodegradation stages; in particular, characteristics of the enrichment-opportunist stage and the sulphidic stage are often observed simultaneously (Lundsten et al. 2010).

With the depletion of internal lipids, all organic tissues within and upon the skeleton are consumed, after which only hollow bones remain. Frequently, a proportion of these bones are elevated from the seafloor. Consequently they could provide a hard substrate for filter-feeding organisms adapted to low particulate flux to the deep-sea floor for as long as the bones persist (coined the final *reef stage*, Bennett et al. 1994).

Like whale falls, wood and macroalgae settling on the sea floor deliver organic material in bulk to sediments, ultimately enriching them via similar avenues (vegetative fragments and faecal matter, Wolff 1979; Bernardino et al. 2010; Bienhold et al. 2013) and initiating reducing conditions both within the wood (Yücel et al. 2013) and to a limited extent in the sediments (Bernardino et al. 2010). However, estimating the supply of vegetative material that enters deep-sea marine environments is complicated by – and likely dependent upon – conditions during their long journeys from source locations. Environmental transitions through which these materials must pass are more diverse for terrestrial wood than coastal macroalgae: the two principal types of plant debris to reach deep-sea environments (Bernardino et al. 2010). Macroalgae are highly labile and are consumed and degraded quickly in coastal and deep sea systems (Bernardino et al. 2010). Being most prevalent in the intertidal rocky shore, they are predominantly exported to the deep-sea floor by tidal currents and wave-driven hydrodynamics (e.g. down-slope flushing) and are most abundant during severe storm events, particularly when higher densities of kelp grazers and encrusting epibionts compromise kelp-blade integrity (Josselyn et al. 1983; Vetter 1994; Vetter and Dayton 1998; Krumhansl and Scheibling 2012). It is only during storms and down-slope flushing that transport rates will be sufficiently rapid to deliver largely un-degraded material to the deep sea floor providing high quality nutrient input to the deep-sea benthos (Bernardino et al. 2010; Krumhansl and Scheibling 2012). Based on the limited data available Bernardino et al. 2010, 'modest' carbon and nitrogen enrichment in sediments impacted by kelp parcels actually reflect rapid rates of utilisation by specialised infauna, which outstrip rates of carbon and nitrogen loading in the sediment (Bernardino et al. 2010). By comparison, the arrival of wood to the deep-sea floor does not result in immediate carbon-enrichment of sediments; the initiation of deep-sea sediment enrichment by refractory woody substrata is markedly delayed (>1.8 years) compared with labile macroalgal enrichment (Bernardino et al. 2010). However, levels of enrichment at 3 years far

exceeded rates at which they could be utilised, resulting in massive carbon enrichment 3–6 times higher than background sediments. The differences in C/N ratios between the two types of plant material reflect the relatively nitrogen-rich composition of kelp versus the extremely nitrogen-poor composition of wood.

The physical breakdown of wood is a consequence of intensive burrowing activity by deep-sea wood-boring bivalves (e.g. *Xylophaga* spp., Pholadidae) and to a lesser extent through grazing by other groups including the limpet genus *Pectinodonta*. Both these taxa host cellulolytic symbiotic bacteria (Distel and Roberts 1997; Zbinden et al. 2010) that aid in the assimilation of cellulose as a carbon source and in some cases contribute to nitrogen fixation. This also facilitates burrowing activity, which greatly accelerates wood degradation in the deep sea (Voight 2009). Fragments of wood and vast volumes of faecal matter then settle on the surrounding sediment, enriching the underlying seabed locally (<0.5m from wood, Bernardino et al. 2010). Simultaneously, anaerobic sulphate-reducing heterotrophic bacteria deriving their carbon from the wood's core produce sulphides as a by-product (Treude et al. 2009; Khelaifia et al. 2011; Bienhold et al. 2013; Yücel et al. 2013). These sulphides sustain chemoautotrophs occurring both free-living in microbial films and mats and as symbiotic bacteria within hosts that belong to similar higher taxonomical ranks to those at seeps or whale falls (depending on the species: e.g. small bathymodiolin mussel *Idas modiolaeformis*, Duperron et al. 2008b; siboglinid polychaetes, Dando et al. 1992).

In the past, organic falls have been proposed as potential evolutionary 'stepping stones' for the ancestral invasion and eventual adaptation of bivalve species to other reducing habitats, such as vents and seeps (Smith et al. 1989; Distel et al. 2000). The most up-to-date extant phylogenies of wood-fall species (Jones et al. 2006; Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b) appear to support this hypothesis for Bathymodiolinae (*s.l.*) living on wood. More controversial however, is the notion that organic falls may be used as conduits for dispersal in contemporary vent and seep species across consecutive generations. Experiments employing coincident reducing habitats which display similarities in fluid emissions generated by different chemical processes, have identified little overlap in between-habitat communities (e.g. whale-fall community in comparison with nearby seeps and vents, Japan, Fujiwara et al. 2007; organic falls in relation to mud volcano [MV] seeps, Gulf of Cadiz, Cunha et al. 2013).

1-5.4. Biogeography of deep-water ecosystems

Several projects within the CoML initiative (e.g. ChEss, COMARGE, MAR-ECO, and CenSeam) set out to define the biogeographic distribution of deep-water chemosynthetic and hotspot provinces worldwide, while gaining a better understanding of the processes maintaining these ecosystems. The biogeography of at least one of the deep-sea habitats concerned, hydrothermal vents has been investigated in some detail, however disagreement persists over differing interpretations. Based on a biogeographic model using phylogenetic data available at the time, Bachraty *et al*. in 2009 suggested that at least six major hydrothermal provinces in the world's oceans exist (in agreement with Van Dover et al. 2002), with seven

paths for dispersal wherein the Northern East Pacific Rise (NEPR) was identified as a likely dispersion centre. However in this study, the authors acknowledge some potential bias in relation to sampling effort. In addition, flaws in the choice of statistical approach have been inferred in a subsequent study casting doubt upon the number of provinces proposed, which are postulated to be far more numerous than originally believed; 11 with additional data from vent-field communities discovered in the Southern Ocean (Rogers et al. 2012; Figure 1.11).

In contrast, any trends that may exist in the faunal biogeography of seeps along the Atlantic Equatorial Belt (AEB) were overwhelmingly dominated by depth-dependent clustering (Bray-Curtis and Hellinger similarities), particularly in specialist seep fauna (Olu et al. 2010). Similar depth-dependent trends have been identified in cold seeps of the Gulf of Mexico (Cordes et al. 2007). Relatively giant species of the Bathymodiolinae are conspicuously absent from the Mediterranean Sea, where small-sized members of this subfamily and siboglinid *Lamellibrachia* sp. are the dominant chemosymbiotic species found on carbonate concretions at seeps, with additional sediment-based Vesicomyidae, Lucinidae and Thyasiridae bivalves.

Identifying biogeographic patterns for organic falls is confounded by the infrequency with which they have been discovered. Whale-fall assemblages may have more restricted biogeographic ranges, but the degree to which this trend is biased towards inherently low sampling effort, as opposed to constrained gene flow in spatially and temporally fragmented habitats, remains unclear (McClain and Hardy 2010). Similarly, for wood, the majority of studies to date have been on experimentally deployed wood substrata, and about three-quarters of the limited number of natural wood studies appear to originate from a single sampling region around the Pacific Islands (e.g. Palacios et al. 2006; Pailleret et al. 2007a; Lorion et al. 2010;

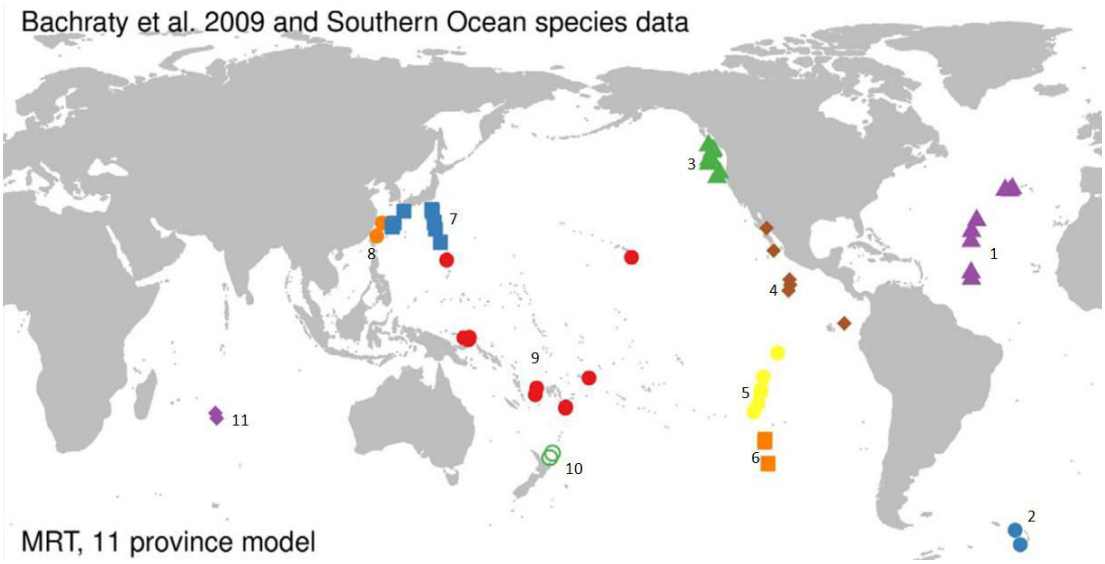

Figure 1.11 Eleven-provinces biogeography for vent species
Based on Vent provinces are resolved comprising the Mid-Atlantic Ridge, the ESR, the northern, central, and southern East Pacific Rise, a further province located south of the Easter Microplate, four provincesin the western Pacific, and a further Indian Ocean province. From Rogers et al. 2012

Samadi et al. 2010; Zbinden et al. 2010), making biogeographic assertions difficult. That said, several reducing-habitat genera appear to be widely distributed among deep-sea habitats, including siboglinid polychaetes and chemosymbiotic bivalves (Van Dover 2000; Cordes et al. 2007). These patterns of large deep-sea species ranges appear to hold true for vents, seep, seamounts, and soft-sediments also (Bisol et al. 1984). Global biogeographic patterns of genetic heterogeneity across bathymetric gradients of a few kilometres only, are recurrent on slopes, at seeps and vents. In view of the fact that deep-sea chemosynthetic habitats continue to be discovered (Domack et al. 2005; Rogers et al. 2012), the resolution of biogeographic complexity can only increase in the coming years.

1-6 Adaptations to reducing environments: A benthic lifecycle perspective

1-6.1. Environmental constraints

In addition to the constraints of the deep sea, specific characteristics of reducing habitats make them challenging places to live. First, chemosynthetic environments are typically ephemeral over short time-scales, normally decades (Tunnicliffe et al. 1997; Smith et al. 2002; Snelgrove and Smith 2002; Smith and Baco 2003; Smith 2006; Gaudron et al. 2010; Mullineaux et al. 2010; Gaudron et al. 2012 except perhaps seeps, von Cosel and Olu 1998). This may be caused by geological instability (e.g. fast-spreading vent-ridges or changes in fluid supply at back-arc basins due to continental subduction) or it may relate to the finite nature of the organic supply of reduced compounds (lipids in whale-bone, sulphidic core of sunken, decomposing wood, Metaxas and Kelly 2010). Second, these habitats are not continuous (occasionally separated by up to 1000 km, Mullineaux et al. 2010) interspersed among a seemingly unsuitable background soft-sediment habitat (e.g. Figure 1.6; Figure 1.7). Third, endemicity (or at the very least strict habitat specificity) is a common feature, which can place species adapted to these habitats at high risk of local extinction. Finally, the dominant species that successfully colonise chemosynthetic environments rely on larval dispersal to populate new habitat, being generally sessile or of limited mobility (Paull et al. 1984; Olu et al. 1996; Metaxas 2001; Ramirez-Llodra et al. 2010),

Therefore, in order to exist and persist in the more ephemeral, patchy, chemically reduced habitat ‘islands’, benthic organisms in these habitats must have initially had (or subsequently evolved) advantageous physiological, behavioural, and life-history traits. Physiological and behavioural adaptations would permit the identification and colonisation of sparse suitable, habitat, with mechanisms in place to tolerate and even harness the reducing conditions of said habitat indirectly. Life-history traits which would be well adapted to overcome geographic isolation and ephemeral timescales, are probably those that favour dispersal phases capable of reaching the nearest suitable habitats, and rapid rates growth, maturation and reproduction respectively. Given such life history traits are energetically demanding, a reliable supply of nutrition would be required. Thus biological communities that thrive in reducing habitats are faced with specific obstacles above and beyond those of soft-sediment environments that dominate the deep-sea in surface area (Figure 1.12).

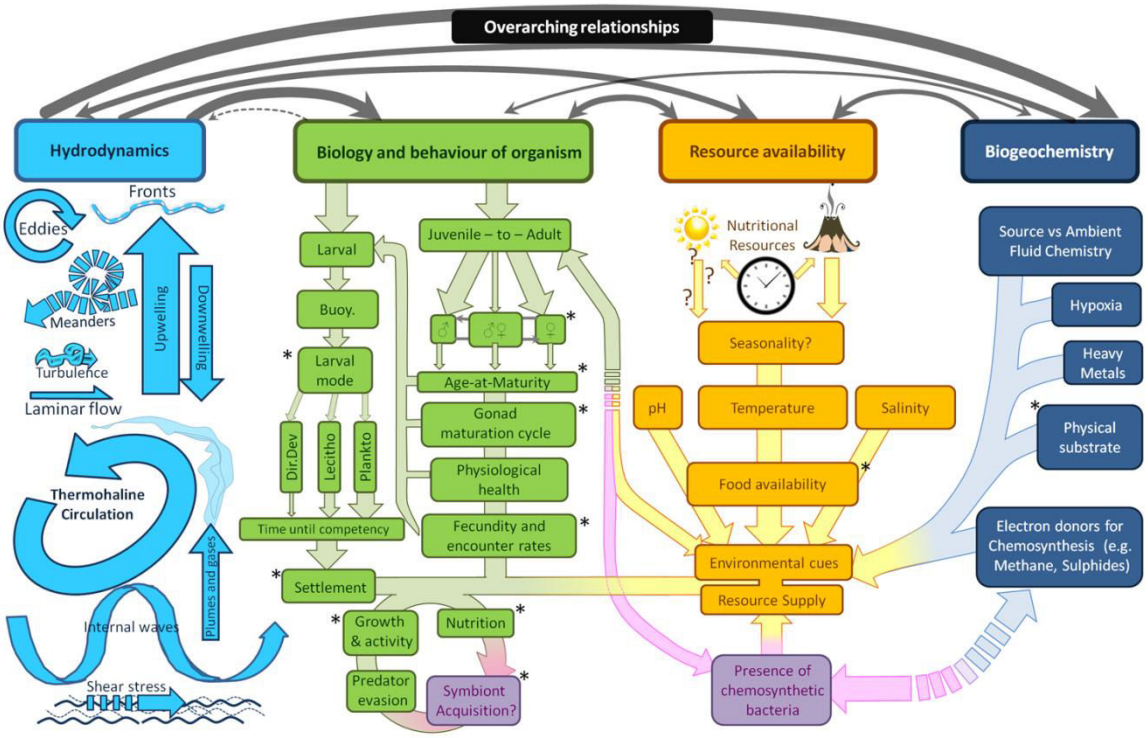

Figure 1.12 Flow-diagram depicting those principal themes and factors in the marine environment that affect the lifecycle of a chemosymbiotic invertebrate species

The diagram displays factors within each of 4 themes that influence the lifecycle of a generic deep-sea invertebrate with chemoautotrophic symbionts. Interactions between components of themes are indicated. The diagram, though not exhaustive, helps to convey the complexities that face marine organisms (and scientists studying their lifecycles). The highlighted components (*) are those areas into which this PhD investigates directly or for which it provides additional insight indirectly. Arrow widths indicate relative perceived importance/impact of relationships. Bi-directional coloured links are highlighted with broken transitions. Compiled by S.R. Laming

1-6.2. Biology, behaviour and critical junctures in the benthic lifecycle

Benthic invertebrates found in deep-sea reducing habitats usually exhibit complex life cycles that include a planktonic larval phase, a demersal or benthic juvenile phase, and a benthic adult phase (Figure 1.12, see also Figure 1.13). Critical junctures in development occur throughout the benthic invertebrate's lifecycle including metamorphoses, a period of small-sized developmental, reproductive activities, and transitions between the pelagic and benthic environment (Figure 1.13). Such junctures carry high levels of mortality (e.g. export of larvae off-habitat, developmental abnormalities during complex ontogenetic changes, and post-settlement mortality) and represent some of the most vulnerable phases in the lifecycle (Morgan 2001). On top of this are multiple stressors typical of benthic and pelagic environments alike (Figure 1.13, core of schematic). At reducing habitats, juveniles are prone to predation on account of their small size, while competition for space and access to reducing fluids is probably fierce in juveniles and adults.

1-6.3. Adult reproduction

Reproductive traits of adult deep-sea benthic invertebrates are well-documented in comparison with larval characteristics, but represent a small fraction of what is known for shallow-water species. Most reproductive processes identified in deep-sea metazoans appear to be phylogenetically conservative, even

in ‘extreme’ vent and seep habitats (Tyler and Young 1999). Species in the deep-sea have been identified as being gonochoristic; including many annelids, most gastropods, nearly all bivalves and crustacea and the majority of echinoderms and octacorals (Young 2003, Figure 1.12). Exceptions exist of course, such as protandry in gastropods (e.g. *Haliella stenostoma*, Warén 1983), bivalves (e.g. *Idas* spp., Tyler et al. 2009; Gaudron et al. 2012) and pandalid shrimp (Young 2003) and simultaneous hermaphroditism in holothurians (Tyler et al. 1992). The advantages of being either gonochoristic or hermaphroditic in the deep-sea are unclear, other than constraints upon phylogeny, but probably depend upon the evolutionary history of a species distribution and densities.

The move from benthic to pelagic occurs subsequently to spawning or hatching (Figure 1.13). By this stage, gonads have ripened and gametes are mature in the chemosymbiotic adult, prior to the release of gametes (in broadcast spawners). The timing and frequency of spawning in reproductive adults in the deep-sea are known to strongly influence the direction of transport and therefore dispersal with consequences for marine connectivity (reviewed in Carson et al. 2010), be it seasonal (e.g. bathyal echinoids, Young et al. 1992, or continuous (e.g. polychaetes in reducing habitats, Rouse et al. 2009; scleractinian coral, Waller et al. 2002). The timing will dictate the water mass into which the gametes or larvae begin their journey, whilst a higher frequency (number of cohorts per unit time) will mitigate the impact of incidents of mass-mortality during a single spawning event. For example, high rates of fecundity and continuous spawning in the genus *Osedax* and several bathymodiolins are thought to support their capacity to populate nascent habitats on the seafloor (Rouse et al. 2009; Mullineaux et al. 2010). In fact, semi-continuous reproduction is a common strategy in deep-sea fauna (reviewed in Young 2003), particularly in polychaetes but with examples within the molluscs (Olabarria and Ramirez-Llodra 2004; Villanueva 1992). Seasonal spawning has been documented in bathyal environments (e.g. Young et al. 1992) and particularly in the Bathymodiolinae (Van Dover et al. 2002; Van Dover et al. 2003; Colaço et al. 2006; Dixon et al. 2006; Tyler et al. 2007; but not *B. thermophilus* Tyler and Young 1999), and even alternating spawning strategies, correlated with habitat depth (Mercier and Hamel 2008). Behaviour in deep-sea broadcast-spawners has generally been attributed to constraints relating to sperm concentration and thus lowering encounter and fertilisation rates in gametes with increasing diffusion (Levitan and Petersen 1995; Powell et al. 2001). Other hypotheses cite a combination of extended gamete longevity, coupled with the increased viscosity and capacity for retention at the benthic boundary layer (e.g. echinoids, Yund and Meidel 2003). Documented examples of adaptive behaviour compensating for these problems include pairing in echinoderms (e.g. Tyler et al. 1992; Young et al. 1992; Mercier and Hamel 2008) or relocation to a bathyal habitat for spawning in mesopelagic cephalopods (e.g. Seibel et al. 2000). Timing adaptations may include taking advantage of environmental cues (e.g. snow crabs, Starr et al. 1994) or the presence of mating partners in close proximity (e.g. Tyler et al. 1992). What’s more, spawning and reproductive biology can be depth-dependent (and presumably resource-specific) in starfish *Henrica lisa* (Mercier and Hamel 2008).

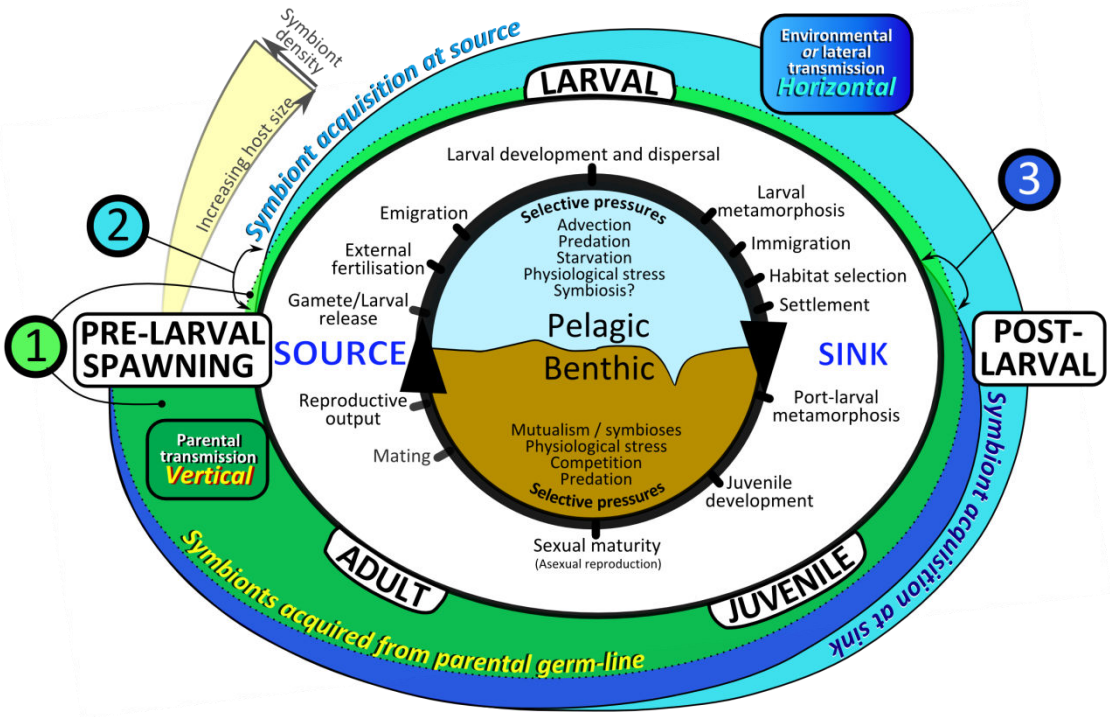

Figure 1.13 Critical junctures in the lifecycle of chemosymbiotic organism
Centre: Selective pressures which impact on physiological health and mortality, for both pelagic and benthic lifecycle phases. Surrounding white region: key junctures in the benthic and pelagic periods of the lifecycle (pelagic phases may be demersal in select species). Emigration is away from the source habitat and immigration is towards the sink, however during self-recruitment, sink and source habitats are the same geographic location. The peripheral coloured graphic relates to symbiont densities (width of band = density) in the tissue of the host, where zero density indicates the aposymbiotic state (absence of symbionts in a typically symbiotic species). In vertical transmission (green), symbionts are passed down the germ-line in gametes from parent to offspring (①). Thus the organism is never strictly aposymbiotic. In horizontal transmission (two shades of blue), symbionts are either acquired laterally, through parental non-germ-line, conspecific or interspecific host transmission, or environmentally, as free-living bacteria. This is likely to occur at source or at sink habitats (②and ③ respectively), where environmental conditions favour the presence of symbiotic bacterial candidates. Where acquisition is at the source (②), only a very brief aposymbiotic period exists prior to acquisition. In contrast, acquisition at the sink habitat (③) enforces a longer aposymbiotic period during larval development and dispersal. Intermediate acquisition during larval dispersal is thought to be unlikely due to a lack of symbiont supply in the pelagos. In demersal larvae, this assumption may not apply. Adapted from the lifecycle of benthic organisms in Morgan 2001.

Invertebrates achieve fertilisation in various ways from releasing gametes at high densities (broadcast spawning) with pelagic embryos (Porifera, echinoderms, bivalves and corals (Levinton 2001), to internal fertilisation via copulation, with viviparity (e.g. some crustaceans, polychaetes, and ascidians (Levinton 2001), or external egg masses/capsules (some anthozoans, nemerteans, polychaetes, and gastropods (Levinton 2001). Most species' mode of fertilisation will reside within this spectrum. The method of fertilisation and the dominant larval mode employed typically co-evolve, and the costs and benefits of both stages in development are intertwined (Morgan 2001). Fertilisation success in some situations will be limited by access to gametes of the opposing sex (Levitan 1995; Levitan and Petersen 1995), but in others, the reverse is true with fertilisation success curtailed by incidents of polyspermy (Franke et al. 2002). After hatching, a period of larval transport takes places, the duration of which depends on the mode of development and physical oceanographic conditions.

1-6.4. The larval phase

Following successful fertilisation, the development of larvae is typically in the water column (Figure 1.13, except in larval-brooding species) where several modes of development are known to exist (Figure 1.12). The most common are planktotrophic larvae (represented by the widest arrow in Figure 1.12), which can actively feed and are thus assumed to be capable of remaining in the plankton for longer than non-feeding larvae that rely on a maternally-derived yolk reserve (lecithotrophy); both have far greater larval durations than brooded larvae (few seconds to hours). Note that, though planktotrophy permits higher fecundity in adults due to minimal energy investment per gamete, it is also believed to result in higher rates of mortality when compared with yolk-nourished larvae (Strathmann 1985). Logically, the larval mode will reflect cost to benefit trade-offs between the number of larvae that can be produced and the actual success of dispersal in species-specific situations. These developmental modes are often ranked in terms of a hierarchy of increasing larval duration as follows (assuming identical conditions during transport): brooding (direct-development) << lecithotrophy < planktotrophy (which includes extreme teloplanic[1] examples, Scheltema 1971) and is either pelagic or demersal. However, when environmental conditions differ between similar larval modes, the afore-stated hierarchy in duration regularly breaks down (e.g. Mercier et al. 2013). Temperature increases are known to have a universal impact upon all modes of development increasing rates of development presumably through increased metabolism (Reitzel et al. 2004). Consequently, even though a lack of feeding in lecithotrophy places an upper limit upon energy resources, many extreme cold-water species' lecithotrophic larvae display prolonged, retarded larval development due, it's believed, to metabolic suppression (Young et al. 1997). In addition, at least in the echinoderms, a synergistic influence of phylogeny and climate has greater influence on larval dispersal than larval mode (Mercier et al. 2013). Given a large enough yolk reserve, lecithotrophy may even be competitively comparable to planktotrophy (Marshall and Keough 2003) and potentially the more reliable strategy, if local planktonic nutritional resources are unpredictably patchy (Pearse et al. 1991). Extreme examples of unexpectedly far-reaching dispersal have been documented in larvae undergoing direct development (brooding), where otherwise-inexplicably broad adult distributions are maintained by "rafting" on flotsam over great distances (Johannesson 1988; Helmuth et al. 1994; Foighil et al. 1999). Two additional considerations must be the hydrodynamics that determine the transport environment for larvae, and the active mediation of transport by larvae themselves.

1-6.4.1 Physical oceanography: Hydrodynamics and its impact on dispersal

Hydrodynamic processes that are known to affect benthic invertebrates (Figure 1.12) operate on a vast temporal and spatial scale. Large-scale but slow-moving hydrodynamic processes, in particular thermohaline (Figure 1.12), or "conveyer belt" circulation results from temperature- and salinity-driven

[1] A larval duration which is extensive enough to serve as a means for long range pelagic dispersal (~100's km or more) and which originates from continental shelf benthic species

density differences in oceanic water masses, due to wind-driven Eckman transport, solar heating and evaporation. These large-scale movements transport energy (kinetic and heat) and substances in suspension and dissolution around the globe, with residency times in the order of 1000s of years. Mesoscale processes such as the formation and movement of meanders, upwelling and downwelling currents, mesoscale eddies, frontal boundaries and complex coastal operate on regional km-wide scales. Smaller-scale interactions then act from the kilometre-wide scale to the molecular level, such as the conditions that favour either laminar or turbulent flow (the basis for frontal formation) and dictate how fluids in the deep oceans interact with abiotic and biotic processes (e.g. the initiation of fluid-driven lift in grains of sediment). Together these hydrodynamic processes can result in very low and high-energy environments, with far-reaching ecological consequences from zones of permanent hypoxia on some continental margins to the complex energetic environment of submarine canyons and upwelling zones respectively. In the context of reducing habitats, hydrodynamics govern the delivery of sunken wood the deep sea and provide the oxygenated ambient water present at all anoxic-oxic interfaces, critical to chemosynthetic life. Propagule dispersal, typically associated with the earliest life history stage (as gametes, fertilised eggs, larvae), is assumed to be the principal means by which the distribution of benthic species is maintained in chemosynthetic deep-sea habitats (Mullineaux et al. 2010). For these organisms including those from deep-sea reducing habitats, hydrodynamics provide a vector for planktonic propagule transport at scales of dispersal that would otherwise be unachievable.

Examples of current-aided larval dispersal from coastal (mainly) and deep-sea environments include the transport of larvae entrained in and conveyed by mesoscale eddies (Lobel and Robinson 1986; Limouzy-Paris et al. 1997; Siegel et al. 2003; Aiken et al. 2007; Paris et al. 2007; Christie et al. 2010); complex but predictable circulations generated by tides, such as salt intrusions in estuarine systems (Shen et al. 1999; Shanks et al. 2002; Hsieh et al. 2010; Narvaez et al. 2012); net transport during long-shore drift (Richards et al. 1995; Siegel et al. 2003); the action of internal waves (Wing 1998; Epifanio and Garvine 2001); peripheral larval transport during the formation of oceanic gyres (Lee et al. 1992); upwelling zones (Shanks et al. 2000) and the particular case of geothermal-based plume circulation (e.g. Kim et al. 1994; but Marsh et al. 2001; Bailly-Bechet et al. 2008). Hyper-evaporative processes such as deep-water cascading may represent an additional means of dispersal (Canals et al. 2006; Canals et al. 2009). With exception to geothermal systems (and even then occasionally; Marsh et al. 2001), most of these phenomena can be partially or wholly attributed to wind- or tide-driven currents, interacting with topography where relevant.

Conversely, interactions between larvae and counter-dispersive features can assert sub-maximal dispersal range limits (i.e. oceanographic or topographic barriers) such as in coastal boundary layers (Lee et al. 1992; Gaylord and Gaines 2000) or transverse faults in ridge valleys for vent fauna (McGillicuddy et al. 2010), or they can induce widespread mortality-by-export into unsuitable environments (e.g. Van Den Avyle and Maynard 1994).

1-6.4.2 Larval mediation during transport

Meroplankters have historically been thought of as almost entirely passive: insufficiently motile to overcome currents which drive local and regional water circulation; the physical environment that will largely dictate the direction and speed of larval transport from source to sink. However, growing evidence suggests that while this is true for horizontal water mass speeds, it may not apply to vertical migration between two horizontal water masses. If these act in opposing directions, it is possible to preferentially navigate between favourable currents. This is well-documented for larval marine fish (reviewed in Neilson and Perry 1990; with examples more recently in Cowen et al. 2000; Kingsford et al. 2002; Gerlach et al. 2007) and often enables high levels of retention at natal sites for endemic species (Jones et al. 2005; Almany et al. 2007; Gerlach et al. 2007). Tendencies towards natal larval retention have also been postulated for hydrothermal vent species (e.g. vent tubeworm *Riftia pachyptila,* Shank and Halanych 2007). Incidents of vertical migration have also been documented to a lesser extent in marine invertebrates (e.g. in response to light and food availability, Starr et al. 1994; Gallager et al. 1996; Manuel et al. 1996; or diel-migration in reverse to counteract offshore-export in an upwelling zone, Poulin et al. 2002. In fact, examples from upwelling zones are documented so frequently as to suggest that this is the norm, employing various documented or assumed migrations (Shanks et al. 2000; Kingsford et al. 2002; Shanks and Brink 2005; Marta-Almeida et al. 2006; Morgan et al. 2009a; Morgan et al. 2009b). Larvae will even perform migrations that place them at higher risk of predation, if it can result in finding suitable habitat and prevent exportation to unsuitable environments (e.g. inverse vertical migrations during daylight hours in estuaries (Morgan and Anastasia 2008).

Thus, although far-reaching dispersal necessitates a long larval duration in order to accommodate extensive larval transport, extensive duration will not necessarily equate to a far-reaching dispersal (see Figure 1.14). This is because larval transport is the accumulative distance covered along the path of motion during the entire planktonic existence prior to settlement in the form of a translocation between two points (Figure 1.14; blue path in A and B), and is a direct function of planktonic duration (Pineda et al. 2007). It is subject both to larval behavioural mediation and physical mechanistic processes, where the spatiotemporal scales of variability are vast (Shanks 2009). In contrast, the distance *dispersed* is effectively a measure of linear *spread* from the source (Pineda et al. 2007). The concept of the pre-competent duration *not* correlating with the dispersal distance on account of these phenomena is an increasingly pervasive one in marine science; larval durations of less than a day predict dispersal capacity well, whereas the true dispersal distance realised by larvae with extended larval durations, frequently falls well-short of predictions (Shanks 2009).

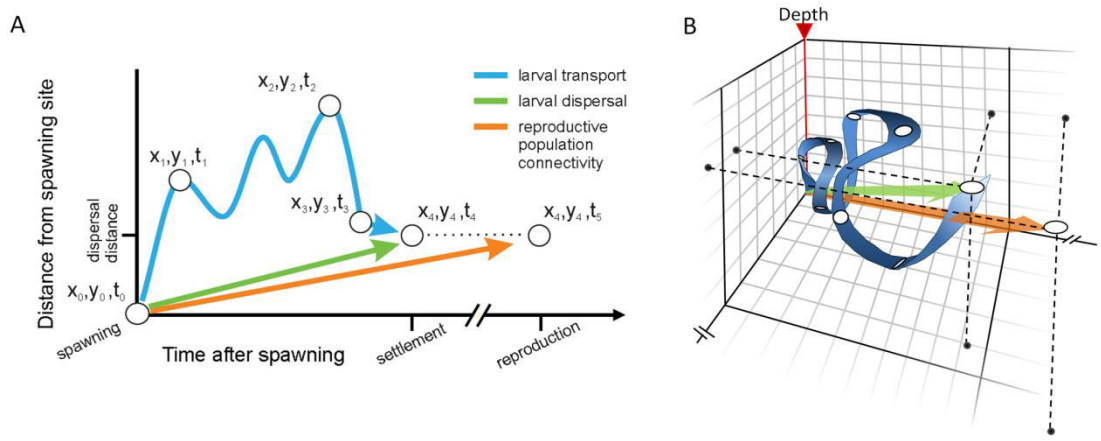

Figure 1.14 Theoretical relationships between the spatial and temporal aspects of larval transport, dispersal, and (reproductive) population connectivity for a sedentary species
Survivorship is not represented for the sake of clarity. In both figures the sum of larval transport distances is assumed to be greater than the ultimate (vectoral) dispersal distance. White circles are locations in space with coordinates x-y at times t in figure A, and x-y-z at times t in figure B (a 3D interpretation of the theory behind A, accounting for depth), though the white circles are not intended to be directly comparable across the figures. All locations are pelagic except x0,y0 and x4,y4 (in A), which are benthic. N.B. between settlement and sexual maturity there is only a passage of time. Figure A taken from Pineda et al Pineda et al.., 2007, figure B was drawn by S.R. Laming.

Given the importance that larval motility appears to play in mitigating unfavourable physical transport processes in shallow-water systems, a better understanding of larval motility and behaviour in the deep sea is of fundamental importance. Categorising motility remains challenging however. Larval-tracking in the deep-sea is not tractable, so larval studies must be performed *ex situ* with the associated difficulties of recreating pressurised conditions. In addition the requirements for larval pilotage and settlement remain untested, in oceanographic current regimes which are poorly described. (Metaxas 2001). These could include micro-turbulence at physical and biological discontinuities in the water column, behaviour at the benthic boundary layer during demersal transport and all settlement behaviour; maintaining location (retention) or changing direction (mediated transport) in laminar flow and transport in internal waves, eddies and meanders. The difficulties are compounded by the likelihood that larvae might also respond to other directional stimuli such as electro-magnetic energy (intensity and frequency e.g. phototaxis) or display capacities for chemotaxis (particulates and solutes), thermotaxis, galvanotaxis, magnetotaxis or engage in spatial orientation (gravitaxis and linear acceleration) and respond to the kinematic viscosity of seawater. One of the most widely acknowledged applications of motility in shallow-water species is in vertical migration through the water column. The degree to which deep-sea invertebrate larvae mediate their transport with swimming behaviour is unclear, with limited information available concerning actual larval trajectories (e.g. Arellano et al. 2014). The high densities of larvae found in coastal current regimes which have permitted reliable interpretations of mediated transport, are not reflected in the open ocean where larvae of deep-sea species are rarely collected in large numbers. This is particularly true for reducing habitats. However it is known that, with the exception of siboglinids and vesicomyids, many of the organisms that dominant vent and seep habitats including bathymodiolin mussels, alvinocaridid shrimps and vent crabs, have either indirectly or directly, have been shown to employ long-lived planktotrophic

larvae. The demonstrated ability to colonise nascent sites following natural disturbances by organisms adapted to these habitats (e.g. Shank et al. 1998; Mullineaux et al. 2010), where supply is known to be from 'neighbouring' distant sites, provides evidence that the larvae of deep-sea chemosymbiotic invertebrates are capable of long-range dispersal, requiring long planktonic duration times. Other than apparently long larval durations (Scheltema 1971; Arellano and Young 2009), the means by which long-ranging dispersal is achieved, specifically with regard to mediated behaviour, remains unknown. In the deep-sea only limited examples exist that successfully identify a larval trait as having acted to influence dispersal: the possible effects of positive buoyancy in *Riftia pachyptila* in vent-plumes (Marsh et al. 2001) and negative buoyancy in another siboglinid genus, *Osedax* sp., found on sunken carcasses (Rouse et al. 2009).

The apparent level of connectivity over long distances maintained by some species from reduced habitats (e.g. Olu et al. 2010, Rogers et al. 2012), is even more extraordinary when one considers the effect that extended larval transport could have on densities of surviving larvae in the deep sea, by the time they are competent to settle (Figure 1.15). It is evident that from the moment of spawning, larval transport is subject to a plethora of forcing variables (Figure 1.12–15) which dictate dispersal capacity, successful settlement on a suitable substrate and survival through to recruitment (reproductive maturity).

1-6.5. Metamorphosis and post-larval to juvenile development

If a larva has survived the period of dispersal (the lucky 1%, Cowen and Sponaugle 2009, Figure 1.15), and assuming it has sufficient energy reserves, it becomes 'competent', i.e. physiological ready to settle and metamorphose. Once competent, larvae must detect proximal colonisable habitat. Settlement behaviour studies into shallow-water invertebrates have recorded various environmental cues which may predominantly influence habitat selection, including but not restricted to irradiance, salinity, currents, micro-topography and texture (hard substrata), grain size and organic matter (soft sediments), food availability (biofilms, chemosynthetic resources) and adult conspecifics. Substrate selection has been identified at the benthic boundary layer (e.g. Pawlik and Butman 1993; Krug and Zimmer 2004), while other responses to cues include active sinking in turbulent flow to facilitate retention (Fuchs et al. 2004; Fuchs et al. 2007) and settlement and metamorphosis in response to conspecific cues (e.g. Tamburri et al. 1996). The delaying of the onset of metamorphosis subsequent to reaching competence has also been reported (e.g. Bishop et al. 2006). Presumably however, in deep sea reducing habitats, larvae must precisely identify chemical traces which indicate suitable substrate, while 'weighing up' competitive and facilitative intra- and interspecific interactions and environmental habitability (Levin 2006), since settling in high-output fluid emissions may be sub-lethal due to fluid toxicity. Examples of chemotaxis towards reduced fluids as cues for larval settlement are limited to chemical traces of conspecific or interspecific exudates (e.g. *Bathynerita naticoidea* veligers cueing to *Bathymodiolus childressi*, Dattagupta et al. 2007). Chemotaxis towards sulphides though inferred in adult shrimp (Renninger et al. 1995) has not been identified in the larvae of deep-sea reducing habitats. Evidence for cueing to chemical plumes does exist in opportunistic polychaete

Capitella sp. larvae (sulphides, Cuomo 1985). Cueing to physical parameters in deep-sea invertebrates is also documented (e.g. thermotaxis in *Bythograea thermydron* megalopae, Epifanio et al. 1999). Analogous parasite-host scenarios provide examples of the level of cueing precision that is possible, where *Heterosaccus dollfusi* naupliar 'settlement' cues are highly localised (the body of the brachyuran crab *Charybdis longicollis*) and involve complex mediated swimming responses (Pasternak et al. 2004). For chemosymbiotic species which engage in environmental acquisition of symbionts, the presence of free-living chemoautotrophic bacteria rather than reduced compounds may be of fundamental importance and a powerful cue for settlement.

Once cues have been identified, larvae are assumed to sink rapidly towards the identified signature and begin trying to locate the habitat while minimising predator encounters, by actively mediating their position in the environment. If contact is made, then proximal, directional environmental cues are thought to further dictate microhabitat selection. Assuming that a suitable microhabitat has been identified, remaining energy reserves within each larva must be sufficient in order to complete metamorphosis and sustain the newly formed post-larva until it can independently acquire energy for growth. Once larvae have successfully metamorphosed into juveniles, metamorphosed individuals face multiple sources of post-settlement mortality (Figure 1.15) through predation, competition for resources (i.e. starvation, smothering

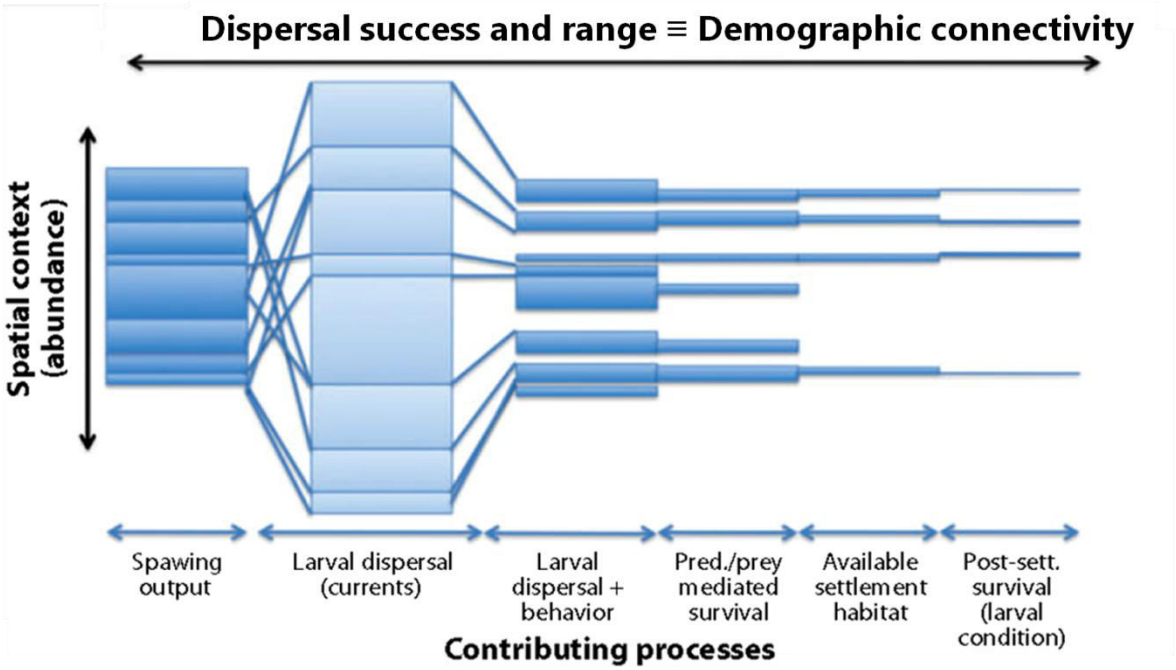

Figure 1.15 The combined processes contributing to larval dispersal and survival and thus demographic connectivity
The spawning output (abundance) for each of the populations (represented by a single bands on the left) is spatially variable due to variability in the adult reproductive capacity of each. Advection and diffusion causes mixing and dilution of larvae: crossing lines and increases larval distribution spatially (expanded size, lighter colour). Larval behaviour (e.g., vertical migration), acts to mitigate diffusion through biophysical interactions; spatial extents decrease and densities increase. Spatially dependent and therefore variable larval loss (mortality: predator/prey interactions, availability of settlement habitat, and post-settlement survivorship with carryover effects) result in ongoing depletion of survivors (progressively smaller bands). Evidently the source (left) and sink locations (right) have markedly differing spatial distributions and densities providing a picture of the scale of successful dispersal and demographic patterns (from Cowen and Sponaugle 2009).

and exclusion), immuno-incompetence with death from bacterial, viral and parasitic disease, environmental pressures such as toxic fluid exposure or sediment loading and anthropogenic disturbance (Levinton 2001). Thus, recruitment in to the adult population will reflect the net contributions of larval supply, successful settlement and metamorphosis, and rates of post-settlement mortality. Recruitment rates for a given species population (when not due to larval retention) are a major determinant of between-population dynamics and when combined with birth and death rates, will dictate population connectivity within a metapopulation (Cowen and Sponaugle 2009). This universal process ultimately governs global biogeography.

1-6.6. Resource availability and environmental cues

The availability of resources and the concept of environmental cues are not necessarily independent (Figure 1.12). In coastal systems it is understood that successfully 'cueing' to resource availability, be it nutritional or habitat-based, is fundamental to effective dispersal and in mitigating mass-mortality (Levin 2006). Habitat availability certainly impacts on dispersal success, since patchiness of habitat will reduce substrate encounter rates for sessile species. The availability of suitable nutrition is evidently pivotal in the survival of planktotrophic larvae, particularly in environments where available nutrition are limiting (Starr et al. 1994). In chemosynthetic-based deep-sea environments where scarcity in habitat and food are linked intrinsically, the relative importance of photosynthetically derived organic material arriving seasonally (e.g. Tyler et al. 2007) and nutrition derived directly from symbioses with chemoautotrophic bacteria (e.g. Childress et al. 1991; Robinson and Cavanaugh 1995; Marsh et al. 2001; Duperron et al. 2008a; Duperron 2010; Vrijenhoek 2010; Duperron et al. 2011) remains unclear for many reducing habitats. It is likely that at least in some adult metazoans, changes in the availability or quality of either of these nutritional resources (or both) may instigate the onset of gametogenesis in preparation for spawning (Colaço et al. 2006; Gaudron et al. 2012), the initiation of spawning itself (Fujiwara et al. 1998) or a change in the dominant means of energy acquisition in adults (Tyler et al. 2007). Given the ephemeral nature of vent, seep (to a lesser degree) and organic-fall habitats, it is believed that some species must have evolved a means to detect certain anomalies during larval dispersal, cueing for substrate selection and settlement (discussed previously). The alternative is that larvae are ubiquitous and omnipresent which, while feasible given the high productivity of these ecosystems (Olu et al. 1996; McCollom 2000), would incur heavy energy investments reproductively. Given these considerations, the availability of suitable habitat or nutritional resources (or potentially both) in the vicinity of source populations could affect larval transport pathways, assuming that larvae can actively respond to these directional environmental cues and, alter their behaviour accordingly in order to take advantage of those currents orientated favourably. Finally, mediated selection of bacteria during infection and the ensuing symbiont acquisition (in the case environmental transmission, as in Figure 1.12) is suspected, given the prevalence for exclusively symbiotic, and frequently monophyletic, associations in many chemosymbiotic species (Dubilier et al. 2008; Bright and Bulgheresi 2010)

1-7 Chemosynthetic bacteria as symbionts in marine systems

1-7.1. Symbioses: definitions and application

Symbiosis and its definition have evolved conceptually over time, as new data comes to light describing symbiotic function both extrinsically within the context of the surrounding environment, and intrinsically between the host (usually ascribed to the larger or more complex organism in terms of anatomy) and the symbiont. A common interpretation of symbiosis is being of mutual 'benefit' to host and symbiont. However symbioses are often asymmetric, where benefit is difficult to demonstrate, as partner(s) can be constrained physiologically-speaking (as in some vertically transmitted bacterial symbionts). Examples are available throughout nature, frequently when a symbiont organism must derive its nutrient supply by means of host intervention (e.g. symbiotic bacteria in some insects have a limited ability to synthesise nucleotides and amino acids, Zientz et al. 2004).

Symbioses are not completely mutualistic and between-partner conflict is a widespread feature, though it is generally managed and contained (Douglas 2008; Figure 1.16). Reciprocated mutualisms may in fact be host-enforced in cases where one partner stops investing in the symbiotic exchange of resources but continues to receive the benefit of it. In other words the symbiosis is no longer co-operative. This viewpoint presumes some asymmetry in the association, with one partner exacting control over the other(s), enforcing 'good behaviour' in its partner(s). The asymmetry presents a means by which the enforcing partner might provide incentives for cooperation, while punishing cheats. This process is described well in Douglas (2008), from which Figure 1.16 is derived, and takes examples founded on known plant physiology or known animal behaviour. The concept is quite intuitive: take a two-partner symbiosis where each gains some advantage from the other, the 'benefit' outweighing the energetic cost in both cases (Figure 1.16b). The risk to each partner is that the opposing partner can "cheat" the system by not committing any energetic investment, while benefiting from its partner's supply: this can result in further net energy-gain for the cheat, while the partner that continues to co-operate incurs a net loss through unreciprocated investment (Figure 1.16c). Recent modelling studies investigating symbiotic conflict (see Douglas 2008) have found that the initially co-operative partner can 'punish' the cheat by imposing sanctions on supply (Figure 1.16d), which forces the supplicant partner to reinitiate investment, so resetting conditions to those depicted in Figure 1.16b. Repeated or persistent interactions of this sort are thought to temper conflict in reciprocal interactions (Douglas 2008), and create a steady state. Examples of such situations may be drawn from the 450-million year-old symbioses between most land plants and arbuscular mycorrhizal fungi, where positive feedback is through an exchange of surplus 'luxury' resources between partners, regulated through sanctions by one or both partners (Kiers et al. 2003; Kiers and Heijden 2006).

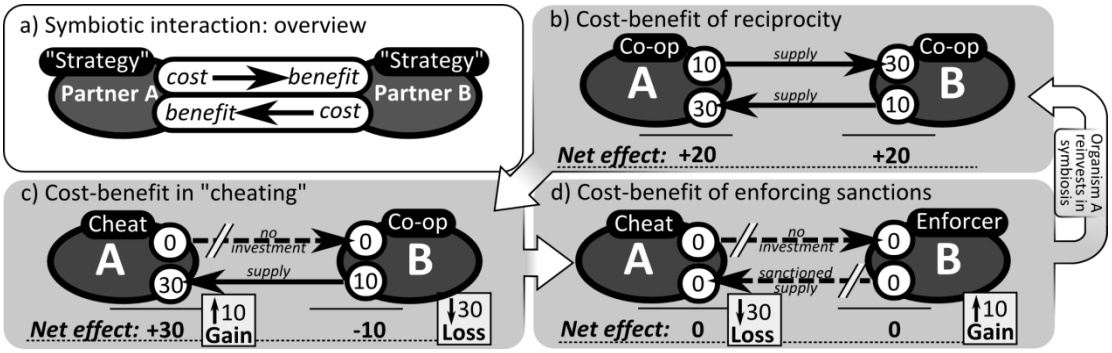

Figure 1.16 The cost of reciprocal benefit between two organisms (A and B).
a) The cost of reciprocal benefit: each organism provides a service at a cost of 10 arbitrary units, which has a value of 30 units to the recipient, yielding a net benefit of 20 units for each organism. (b) The 'temptation' to cheat: organism A does not provide the service and obtains increased net benefit (30 units) to the detriment of organism B, which derives a net cost of 10 units. Note that for the 'cheating' organism A, the benefit of being ineffective as a partner in a symbiosis is a gain of 10 additional arbitrary units. Adapted from Douglas (2008).

The concept of 'mutual benefit' is extremely difficult to test empirically[2] since experiments would first have to identify an unequivocal advantage to both partners; and then importantly, that their integration into a single, collective organism is a 'superior' arrangement than if the two partners lived independently. In theory, this might be achieved by applying with-, versus without-association experimental treatments and employing an index of 'benefit' (e.g. changes in rates of growth, reduced mortality, etc), but in practice, symbioses are often so physiologically entangled that this is impossible. Therefore, many scientists have explicitly abandoned the traditional view that symbioses are mutually beneficial relationships, in favour of symbioses representing vehicles by which many organisms have garnered complex metabolic capabilities (Douglas 1994), providing augmented fitness for the host as a minimum. For the purposes of this research, concepts from (Douglas 1994) and (Zook 1998) may be combined to form a loose definition. Thus, symbiosis is considered herein to be the association of one (or more) organism(s) to another – host – organism for all or part of the host's lifecycle, granting the host novel metabolic capabilities and providing such fitness advantage that the symbiosis persists in this way throughout the course of natural selection.

1-7.2. Evolutionary transition hypotheses

Intracellular endosymbiosis, wherein the internalised organism resides non-destructively within its host's tissue beyond the barrier of epithelia, represents the most integrated of interspecific organismal interactions (Petersen et al. 2012). In this sense, bacterial symbioses have been pivotal in eukaryote evolution and diversity, since mitochondria and chloroplasts are thought to have arisen from endosymbiotic inclusion of prokaryotic cells (something similar to an α-proteobacteria, Gray et al. 2001 and α-proteo-cyanobacteria transitional organism respectively, Moreira et al. 2000; Yoon et al. 2005; Figure 1.17). The exact mechanisms involved in Endosymbiotic Theory are still a matter of considerable ongoing debate (e.g.

[2] Unfortunately, neither bacteria nor their hosts have developed the ability (or inclination) to fill out scientific questionnaires.

Moran and Wernegreen 2000; Rivera and Lake 2004; Wächtershäuser 2006; Poole and Penny 2007). Genomic studies which have sought to resolve the Tree of Life have employed novel genomic analyses, revealing that one possible origin of the eukaryotic genome may be through the ancient ancestral fusion of two diverse prokaryotic genomes, linking prokaryotes and eukaryotes and the deepest levels of the tree of life, which in this context is more akin to a "ring of life" (Rivera and Lake 2004). Thioautotrophic bacteria in marine invertebrates (e.g. Kuwahara et al. 2007) display certain traits that have been proposed as the beginnings of a new eukarya lineage, the thiotrophic eukaryotes (Vetter 1991; Lallier 2006). However, organelles as we understand them currently display bidirectional gene exchange with their hypothetical hosts, and are present in many types of tissue. Thiotrophic bacteria have not yet been shown to display either of these characteristics. Thus, currently it is difficult to imagine a situation in which this will arise in multicellular organism, though it does not rule out the possibility of such a novel eukarya in single-celled organisms. The obligatory, vertically (maternally) transmitted symbionts of Vesicomyid clams display some of the most reduced genomes (1,022,154 bp with 31.6% guanine + cytosine content, the smallest reported genome in autotrophic bacteria at the time of publication, Kuwahara et al. 2007). This is coupled with a loss of both essential genes (e.g., *ftsZ* for cytokinesis) for functioning and genes that are superfluous for an intercellular existence. The genomes of other chemosymbiotic deep-sea marine invertebrates symbionts (horizontally and vertically transmitted) are currently being sequenced in order to understand the broader context of endosymbiosis from both a physiological and evolutionary perspective (Lallier 2006).

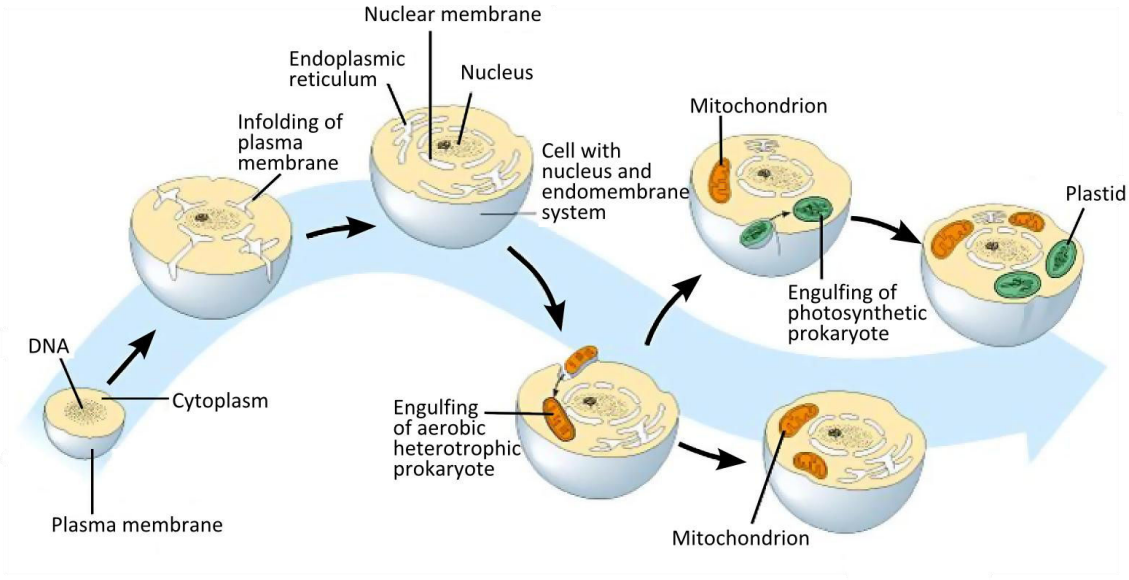

Figure 1.17 Endosymbiotic theory
The diagram depicts a hypothesised evolution of multicellular Eukaryotes, where the origins of plastids and mitochondria in contemporary eukaryotes were through incidences of endosymbiosis of prokaryotes/archea. Source: Benjamin Cummings, Pearson education

1-7.3. Known interactions: free-living bacteria, to obligate symbionts

1-7.3.1 Overview

One of the indicative features of reducing habitats is the prevalence of metazoans such as siboglinid polychaetes and several bivalve families, which have evolved intrinsic and complex symbiotic relationships with chemosynthetic bacteria, most notably sulphur- or methane-oxidisers (Childress et al. 1991; Dufour 2005; DeChaine and Cavanaugh 2006; Nussbaumer et al. 2006; Gros et al. 2007; Duperron et al. 2008a; Duperron et al. 2008b; Thornhill et al. 2008; Lorion et al. 2009; Bright and Lallier 2010; Bates et al. 2011; Duperron et al. 2011; Oliver et al. 2011; Rodrigues and Duperron 2011; Rodrigues et al. 2011; Stewart et al. 2011; Levin et al. 2012) though examples of methylotrophic-type bacteria (Duperron et al. 2008a) and hydrogen-oxidisers (Petersen et al. 2011) are also documented. The energy from these reduced compounds permits the synthesis of ATP through oxidative phosphorylation, used in cellular metabolism (DeChaine and Cavanaugh 2006). Courtesy of the interaction, bacteria are believed to provide a sizeable fraction of their hosts' nutritional requirements through the sustainable production of organic carbon otherwise inaccessible to metazoans (e.g. Conway et al. 1989; Duperron et al. 2011). Bacteria are thought to benefit from physical shelter and increased access to oxygen and reduced compounds, delivered collectively by the host (Duperron 2010). This removes the oxidative constraints normally placed upon aerobic free-living chemosynthetic bacteria when metabolising reduced compounds typically produced in anoxic environments. However, for those symbionts who live part of their life-cycle freely outside the host, metazoan symbiosis is evidently just one of several suitable niches available to them in the deep-sea environment. Consequently, for these host-symbiont associations, the interaction is assumed to be obligatory for the host, but requiring some level of mediation in order to secure their symbionts. Symbioses may also mitigate reduced-fluid toxicity, imparting a greater tolerance in the host, and so extending the ecological niche for the host (and possibly the bacteria). The formation of symbioses between deep-sea metazoans and chemosynthetic bacteria thus allows the metazoan hosts to tolerate (initially) and then ultimately thrive in reducing habitats which would have been uninhabitable otherwise, where contributions from photosynthetically derived organic material can be low (Van Dover et al. 2002).

Within most deep-sea chemosymbiotic metazoa, details regarding the contribution of chemosynthetic bacterial metabolism to the host's energy demands during development are unknown. In fact, even the means by which this association is initiated (i.e. maternal or environmental symbiont transmission) and integrated at all stages of the host's development (i.e. timing of bacterial symbiont acquisition and patterns of spread) remain elusive for many if not most, deep-sea chemosymbiotic species (Dubilier et al. 2008).

1-7.3.2 Vertical transmission of symbionts (via the germ-line)

The acquisition of symbionts by offspring through vertical transmission is via one or both of the parental organisms (Bright and Bulgheresi 2010; Bright and Lallier 2010; Figure 1.13). Consequently, bacteria are

present throughout the dispersal of larvae (Figure 1.13; Figure 1.18B, red bacterium). In the deep sea, the largest group known to employ vertical transmission are the contemporary vesicomyid clams. Symbiont and host alike depend upon their symbioses; the consequences for either party if the symbiosis was somehow terminated, is assuredly death. Vertical transmission in the vesicomyid subfamily Pliocardiinae, occurs during gametogenesis (i.e. maternally, e.g. Endow and Ohta 1990; Cary and Giovannoni 1993), with evidence of resulting host-symbiont co-speciation (Peek et al. 1998a; Peek et al. 1998b; Hurtado et al. 2003). This form of transmission creates evolutionary bottlenecks in the bacterial genome, with far-reaching consequences for their survival. These include the increased frequency of deleterious (nearly neutral) substitutions (Peek et al. 1998b) right up to a genome-wide reduction through deletions in the thioautotrophic symbiont of the vesicomyid bivalve *Calyptogena okutanii*, causing the loss of genes that code for proteins essential for their survival (Kuwahara et al. 2007). These proteins, which remain necessary even as a symbiont, are believed to be provided by the host directly. Likewise, *C. okutanii* is constrained nutritionally, having neither the means to filter feed (extreme hypertrophy of gills that accommodate bacteria intracellularly, results in ciliated surfaces being dwarfed), nor a true digestive system. This extreme interaction is in fact quite rare, even vesicomyids have been shown to supplement maternal symbionts with chemosynthetic bacteria acquired from the environment (Kuwahara et al. 2007), and transmission may often be a combination of the two (Figure 1.18).

1-7.3.3 Horizontal transmission of symbionts

For the majority of chemosymbiotic associations currently documented to occur in reducing habitats in the deep sea, the mode of transmission is partially or entirely horizontal (e.g. as in the lecithotrophic larva of *Riftia pachyptila*, Nussbaumer et al. 2006). Horizontal transmission may be at some initial period during larval development (at the source habitat), while settling at the sink location, or following metamorphosis as a post-settlement larva (Bright and Bulgheresi 2010; Figure 1.13). Bacteria are likely acquired environmentally from free-living bacterial pool or laterally from spatially proximal hosts (Figure 1.13; Figure 1.18). For the former, the host-based symbiotic existence is not obligatory for the bacteria, but may be for the host (e.g. *Riftia pachyptila*, Nussbaumer et al. 2006) or perhaps facultative (evidence for filter feeding in *B. thermophilus* and *B. azoricus*, Page et al. 1990; Page et al. 1991; Dixon et al. 2006). Once infection has occurred, some level of mediation on the part of the host may occur, as bacterial densities are often actively constrained. Examples of free-living populations which are also symbiotic in the marine environment are known to occur in both coastal waters (Lee and Ruby 1992; Gros et al. 2003; Aida et al. 2008) and the deep-sea (Harmer et al. 2008). While known occurrences are limited, they may not be isolated, in a discipline which for now remains in its infancy. This is despite the obvious advances that might be gained from better understanding horizontal, and potentially environmental, acquisition (Bright and Bulgheresi 2010; Bright and Lallier 2010).

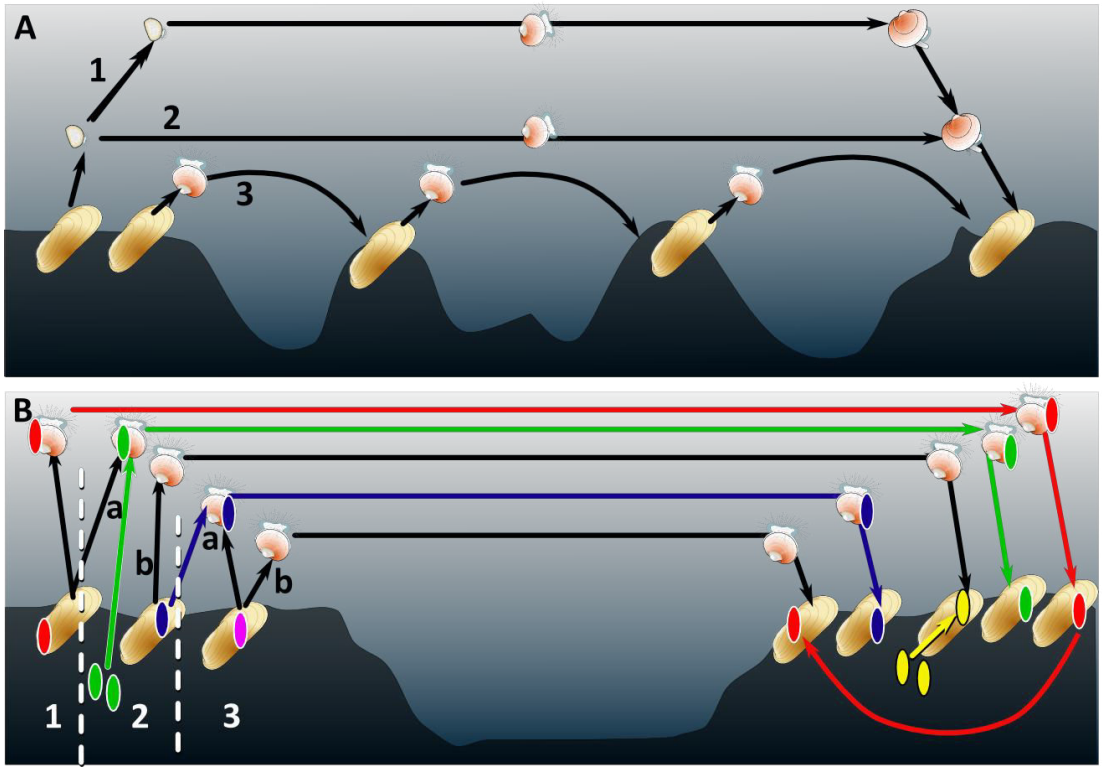

Figure 1.18 Dispersal, and links with symbiont transmission
Depicted, are the various proposed scenarios for transmission of symbionts, in the context of dispersal in bivalves. **A)** Displayed are the various dispersal strategies of bivalve molluscs. [1] Teleplanic, with (typically) planktotrophic larvae dispersing either in the euphotic zone [**1**] or in deeper water layer (**2**). Larvae can grow during dispersal and can settle far from the natal site. Shorter-distance dispersal (**3**) can be achieved by lecithotrophic larvae, which live on maternal reserves and settle closer to their natal site in theory. **B**) Bacterial symbionts can be maternally inherited (**1**), acquired from free-living populations of bacteria at site of origin (2a, green bacterium) or at site of settlement (**2b**, yellow bacterium), or laterally acquired from another host either at site of origin (3a, blue bacterium), or at site of settlement (**3b**, red bacterium). Situations **1**, **2a** and **3a** lead to host–symbiont co-dispersion, as emphasized by the colour arrows. Different transmission modes can co-exist within a given species. Redrawn from Duperron et al. 2013

1-7.3.4 Symbiont localisation and compartmentalisation

Intracellular endosymbioses are known to occur in many bivalves from reducing environments including the Vesicomyidae (e.g. Fujiwara et al. 2000; Hurtado et al. 2003; Szafranski et al. 2014), Lucinidae (e.g. Distel and Felbeck 1987), Thyasiridae (e.g. Brissac et al. 2011) with most documented in Mytilidae, e.g. *Bathymodiolus* sp. (though in the genera *Idas* and *Adipicola*, symbionts are generally to be extracellular[3], when present, Halary et al. 2011). In polychaetes, known symbioses are not only endosymbiotic but sometimes highly integrated physiologically (e.g. in siboglinids which lack a digestive tract, bacteria are housed in a dedicated organ, the trophosome: Vestimentifera, Nussbaumer et al. 2006; Frenulata and Monilifera, Lösekann et al. 2008). Siboglinid symbionts are acquired horizontally following settlement and metamorphosis (Nussbaumer et al. 2006). They enter the host by infecting the epidermis, and subsequently the adjacent muscle-layer and underlying mesodermal tissue, ultimately becoming enclosed in the vacuoles of mesodermal cells which eventually develop into a dedicated chemosynthetic organ, the trophosome

[3] Except Idas (s.s.) washingtonius

(Nussbaumer et al. 2006). Bacterial ectosymbioses (reviewed in Goffredi 2010) in chemosynthetic deep-sea habitats are known to occur in association with nematodes (Polz et al. 1994), the thermo-tolerant polychaetes *Alvinella pompejana* and *Paralvinella grasslei* (Chevaldonné et al. 2000), the highly productive Mid-Atlantic vent-shrimp *Rimicaris exoculata* (Zbinden et al. 2004), the recently described yeti crab (*Kiwa* spp.; e.g. Goffredi et al. 2008) and various examples of limpet (e.g. Bates et al. 2011).

1-8 Symbiotic bivalves

1-8.1. The ubiquity of bivalves in reducing environments

1-8.1.1 The bivalve bauplan

In terms of abundance, functional ecology, distribution and adaptation to varying habitats, bivalve molluscs represent a remarkably adaptable class in the animal kingdom, often representing dominant or important groups in most benthic ecosystems (Mikkelsen 2011). The evident success of Bivalvia in reducing habitats is believed to be attributable to several governing class-specific characteristics. The class is known to inhabit a wide variety of physical substrata with an extensive depth range worldwide (Barnes et al. 2001). Flexibility in habitat use across symbiotic bivalves owes its roots to the considerable plasticity of the bivalvian (and more generally molluscan) bauplan. This has given rise to wide variability in species body size, diverse shell morphologies and variation in their anatomy. Depending on the family and the selective pressures imposed by the surrounding environment, diverse life habits have been adopted both within and across families. The net result is that bivalves are prolific in habitats within reducing environments.

1-8.1.2 Body size

The range of sizes displayed in bivalves both generally, but particularly within the chemosymbiotic families is worthy of note; variability in maximum body sizes has allowed bivalves to make maximal use of resources in the context of bodily demand, under differing environmental conditions. At hydrothermal vents and methane seeps the abundant availability of electron donors for chemosynthesis-driven productivity has led to frequent cases of bivalve gigantism. Gigantism is particularly apparent in species in the Mytilidae (e.g. seeps, *Bathymodiolus boomerang* von Cosel and Olu 1998), Vesicomyidae (e.g. *Calyptogena* spp. Boss and Turner 1980) and the Solemyidae (deep-sea genus *Acharax*, shell lengths up to 200 mm (*A*. *bartschii*, Taylor and Glover 2010). The highly productive environment at vents (and some seeps) are thought to permit rapid growth rates (e.g. the mytilid, *B. thermophilus*, Nedoncelle et al. 2013; the vesicomyid *Calyptogena magnifica*, Lutz et al. 1988), while it has been suggested that the relative temporal stability of less productive hydrocarbon seeps which can persist for many decades to millennia, permits extended life spans at slower growth rates (e.g. *B. boomerang*, as suggested by von Cosel and Olu 1998). Gigantism seems to have been a relatively ubiquitous trait in extinct species from several of the above families (Kelly et al. 2000; Campbell 2006) and may in fact be more prevalent in extant soft-sediment taxa than current records suggest, as these habitats are severely underrepresented in the literature (e.g. the genus *Meganodontia*,

Bouchet and von Cosel 2004). At the other end of the size spectrum, a large number of the described Heterodonta (includes most of the burrowing species from the Thyasiridae, Lucinidae and Vesicomyidae) are in the range of a few to tens of millimetres in length. For example thyasirids display size ranges of 1–110 mm (upper limit is for *Conchocele*), but the majority are relatively tiny, at <10 mm (Taylor and Glover 2010); similar sizes are reported for most members of the vesicomyid subfamily Vesicomyinae, in contrast to the larger Pliocardiinae. The diversity of smaller-sized representatives from each of the families is likely to be highly underestimated particularly in poorly explored biogeographic regions, since these habitats are patchy and many of the families are infaunal.

A wide range of shell lengths also characterises the Bathymodiolinae (Mytilidae), which display shell lengths from less than 20 mm to around 50 mm in the smaller bathymodiolin species predominantly found on wood- and whale falls (*Idas*, *Adipicola*, *Benthomodiolus*, *Tamu*), 80–316 mm at vents (*B. marisindicus* and *Gigantidas gladius*, respectively), and from 80–360 mm at hydrocarbon seeps (*B. hirtus* and *B. boomerang*, respectively). Paedomorphism has been posed as one means by which this has occurred in the smaller 'bathymodioliform' mussels (Génio et al. 2012), though it could equally be matter of supply versus demand, where reducing environments that sustain the largest species within this (and other) symbiotic bivalve families are those where energy supply can meet the demands of rapid or extended growth. This is supported generally by the differences in size between bathymodiolin species from vents and seeps and those found at organic falls, being considerably smaller, though exceptions exist in *I. macdonaldi* at the Garden Banks seeps, Gulf of Mexico (Gustafson et al. 1998) and *Adipicola* sp. and *I. washingtonius* at vents on the Juan de Fuca ridge (McKiness et al. 2005; Southward 2008).

1-8.2. Distribution and phylogeny

1-8.2.1 Overview

Since the discovery of atypically massive bivalves (e.g. the genera *Bathymodiolus* and *Calyptogena*) at vents and seeps, bivalves engaging in chemosynthetic and heterotrophic bacterial symbioses have been found in a comprehensive array of reducing environments ranging from organically enriched sands and muds, mangroves, seagrass beds, accumulations of sunken vegetative debris, offshore sewage sites and nektonic megafaunal carcasses (Taylor and Glover 2010). In the deep sea, bivalve assemblages regularly figure as dominant macrofaunal components of reducing habitats (Duperron 2010).Understanding the lifecycle biology of model species from this group can provide insight into the different, habitat-specific processes that allow communities to persist (their suitability for lifecycle studies is considered in more detail as part of the following chapter on the rationale for the research undertaken during the PhD)

Bivalves engaging in chemosymbiotic associations have been reported or postulated for seven distinct families in the deep sea that vary markedly in their basic anatomy and life habits (Taylor and Glover 2010),

based on information from several reviews and extraneous incidental records (Tunnicliffe et al. 1998; Taylor and Glover 2010; Brissac et al. 2011; Roeselers and Newton 2012). These are the Solemyidae, Mytilidae, Lucinidae, Thyasiridae and Vesicomyidae, with more speculative accounts in the Nucinellidae (synonym = Manzanellidae) and Montacutidae, where the first five are by far the most dominant in reducing environments, when all habitats are considered. At present, these proven (or hypothesised) symbiotic bivalves are recorded across deep-sea reducing habitats as follows:

1) **Hydrothermal vents (all types**):
    a. the Mytilidae, genera Bathymodiolus, Gigantidas, Idas, Adipicola, Benthomodiolus, Vulcanidas[4];
    b. the Solemyidae, genera *Acarax, Solemya*;
    c. the Vesicomyidae, genera Abyssogena, Calyptogena, Vesicomya, Waisiuconcha;
    d. the Thyasiridae, genus *Thyasira*
    e. the Lucinidae, genus *Bathyaustriella*
2) **Hydrocarbon seeps (all types):**
    a. the Mytilidae, genera Bathymodiolus, Gigantidas, Idas, Tamu;
    b. the Solemyidae, genera *Acarax, Solemya*;
    c. the Thyasiridae, genera Adontorhina, Conchocele, Parethyasira, Spinaxinus, Thyasira, Axinulus, Maorithyas;
    d. the Vesicomyidae, genera Calyptogena, Laubiericoncha, Vesicomya, Isorropodon, Chrisinteconcha, Pliocardia, Elenaconcha;
    e. the Lucinidae, genera *Myrtea*, *Lucinoma*, *Mesolinga*[5]
3) **Wood- and whale-falls**
    a. the Mytilidae, genera Idas, Adipicola, Benthomodiolus;
    b. the Solemyidae, genus *Solemya*;
    c. the Thyasiridae; genus *Thyasira* (juveniles)
    d. the Vesicomyidae; genera *Archivesica*, genus unknown (juveniles)
    e. the Nucinellidae, genus *Nucinella*[6]
4) **Deep-sea soft sediments with REDOX layers and oxygen-minimum zones**[7]
    a. the Lucinidae, genus Lucinoma, Meganodontia[8]
    b. the Nucinellidae, genus *Nucinella*
    c. the Vesicomyidae, *Isorropodon*
5) **Other**[9]
    a. the Montacutidae, genus Syssitomya

[4] N.B. deeper end of depth range is barely deep at ≈500m for both *Vulcanidas* and *Gigantidas*
[5] Putatively assigned to this genus based on shell morphology (Okutani and Hashimoto 1997).
[6] This species was collected in a JAMSTEC cruise, but it is uncertain whether it is actually associated with a chemosynthetic environment (Okutani and Iwasaki 2003). The genus is generally known from normal deep-sea environments.
[7] NOT EXHAUSTIVE, Likely to be several other families, given the lack of sampling effort in both environments, which frequently overlap
[8] Valves only, thus chemosymbiosis is inferred only
[9] Oxygenated deep-sea soft sediments, but as a commensal on the deep-sea echinoid *Pourtalesia* sp. (possibly utilising commensal organic waste products)

1-8.2.2 The Protobranchia: Solemyidae

The ancient family Solemyidae (Protobranchia, with putative Paleozoic representatives, Taviani et al. 2011, and references therein) have an extensive bathymetric range to 6,000 m depth (Fujikura et al. 2002) and include two extant genera *Solemya* (from shallow shelf to bathyal depths) and *Acharax* (bathyal), distributed world-wide often at low densities, except for the most extreme Polar Regions where they are absent (Taviani et al. 2011). The two genera are clearly distinct genetically and to a lesser extent morphologically (mainly due hinge internalisation in *Solemya*, and its smaller size) with evidence of an ancient divergence in their evolutionary history (Neulinger et al. 2006).

1-8.2.3 The Heterodonta: Lucinidae, Thyasiridae and Vesicomyidae

The family Lucinidae also occurs ubiquitously throughout the world's oceans, being particularly prevalent in tropical and temperate seagrass beds (Taylor and Glover 2000, and references therein). Having been formerly classified along with Thyasiridae, up-to-date molecular evidence has revealed that common taxonomic features to both families are convergent; the two families are only distantly related (Taylor and Glover 2000). *Lucinoma* currently represents the prevalent genus of this family in the deep sea (Cary et al. 1989; Okutani and Hashimoto 1997; Salas and Woodside 2002; Olu-Le Roy et al. 2004; Holmes et al. 2005; Holmes and Oliver 2006; von Cosel 2006; von Cosel and Bouchet 2008; Cordes et al. 2009).

Thyasirids are found from coastal intertidal habitats to hadal depths and from both the poles to the equatorial latitudes in sediments often with hydrocarbons-enriched, of varying grain size including mud, silt and sand (Dufour 2005; Taylor and Glover 2010). Thyasirids can also be found at oxygen minimum zones (Oliver and Levin 2006), and in the vicinity of high-productivity reducing habitats including hydrocarbon seeps (Clarke 1989; Fujikura et al. 1999; Kamenev et al. 2001; Gebruk et al. 2003; Olu-Le Roy et al. 2004; Holmes et al. 2005; Rodrigues et al. 2008; Oliver et al. 2013), and hydrothermal vents (Oliver and Holmes 2006; Oliver and Holmes 2007). Extant thyasirids are divided into approximately 12 genera, based principally upon rather poorly defined morphometrics which have led to taxonomic difficulties, with frequent arbitrary placement of species in the genus *Thyasira* in particular (see Oliver et al. 2002). The family is thus in need of revision.

The Vesicomyidae consists of over 100 described species, which occur worldwide within an impressive depth-range of (100–9500m, latter limit is for *Vesicomya* (*s.l.*) *sergeevi*, currently the deepest recorded bivalve living in abundance at hadal depths, Krylova and Sahling 2010), although within-genus depth ranges vary, some being relatively narrow (see table 1 of Krylova and Sahling 2010). The depth ranges of large-bodied vesicomyid genera of the subfamily Pliocardiinae appear to be restricted to deeper habitats, in contrast to the small-bodied pliocardin genus *Isorropodon* and the subfamily Vesicomyinae (which includes the genus *Vesicomya* only) which are eurybathic and transoceanic (Krylova and Sahling 2010). Vesicomyinae and Pliocardiinae can be found worldwide and a little less than a third of known species have panthalassic-type distributions (from the genera *Vesicomya*, *Abyssogena*, *Laubiericoncha*, *Pliocardia* and

Phreagena, Krylova and Sahling 2010). That said, the deep-sea subfamily Vesicomyinae are mainly known from trenches and abyssal plains, whilst the Pliocardiinae appear to be highly specialised for sulphide-rich environments and are so far documented to occur principally on continental margins and along mid-ocean ridges (Krylova and Sahling 2010). The phylogenetics of this family has seen several revisions (Krylova and Sahling 2010; Krylova and Cosel 2011; Decker et al. 2012) where even today polyphyletic genera persist, alongside some taxonomic misidentifications (Krylova and Sahling 2010; Krylova and Cosel 2011; Decker et al. 2012), particularly the inaccurate classification of several 'small' vesicomyid clams as the genus *Vesicomya* (Krylova and Sahling 2010). Consequently, several species assigned at some time or other to the "Calyptogena" genus based on gross morphology, have been relocated to other existing or recently erected genera (e.g. *Abyssogena*, *Christineconcha*), based both on molecular analyses and more detailed morphoanatomies.

1-8.2.4 The Pteriomorpha: Mytilidae: Bathymodiolinae (*sensu lato*)

Current phylogeny and morphology-based taxonomy for the Bathymodiolinae (s.s.) are not yet concurrent and the status of this subfamily remains ambiguous. It's evident from a wealth of molecular work carried out on chemosynthetic mytilids that the Bathymodiolinae (s.s.) fails to account for several genera of smaller mussels which fall within this clade and for which symbiosis has been demonstrated in several members. These small-sized mussels have historically been placed in the subfamily Modiolinae (having been described prior to the last century in some cases) and are usually found on organic falls, though some species also occur at seeps (e.g. Gustafson et al. 1998; Olu-Le Roy et al. 2004; Rodrigues et al. 2010; Ritt et al. 2012; Rodrigues et al. 2013) and in rare instances, vents (e.g. McKiness et al. 2005). Current understanding of the systematics of these two subfamilies (Jones et al. 2006; Kyuno et al. 2009; Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b), undoubtedly warrants the inclusion of all known chemosymbiotic Mytilidae into a single subfamily (be it the Bathymodiolinae [*s.l.*] or Modiolinae [*s.l.*]), including the additional genera *Idas*, *Adipicola* (formerly *Teru*/*Myrina*), *Benthomodiolus*, *Tamu* and *Vulcanidas*). The subfamily name Bathymodiolinae (*sensu lato*), though incorrect, is used to collectively discuss these genera from here on in.

Within the subfamily Bathymodiolinae (*s.l.*), 55 species are currently described in the World Register for Marine Species (Bouchet 2014c; a; b; Bouchet and Gofas 2014; Sartori 2014; Table 1.1). However, only a fraction of these species have undergone molecular analysis, particularly lacking in the smaller mussel genera *Idas* and *Adipicola* for which species descriptions are sometimes restricted to shell characteristics. That said, the multigene Bayesian trees of Lorion et al. (2013) and Thubaut et al. (2013b) generally appear to corroborate one another, thus bringing a great deal of new information to the phylogeny.

Of the 55 species in Bathymodiolinae (*s.l.*), three are *Benthomodiolus* spp., presently known from the Pacific only (but, with possible addition of two more species from Mid Valley, Juan de Fuca Ridge and an unspecified locality in the South Atlantic, Thubaut et al. 2013b, Figure 1.19; Table 1.1). The next two most

deep-branching clades of the Bathymodiolinae are represented by monospeciate genera. *Vulcanidas isolatus*, occurs on relatively shallow-water hydrothermal vents (140 – 504m) at the Kermadec Ridge (von Cosel and Marshall 2010). Additional undescribed elongate congenerics may exist (Thubaut et al. 2013b, Figure 1.19). The second genus, *Tamu fisheri*, occurs on relatively shallow sulphidic hydrocarbon seeps on the Louisiana slope, Gulf of Mexico (Gustafson et al. 1998; Table 1.1).

The remaining 'modiolin' small-mussel genera, *Adipicola* and *Idas,* predominantly live on sunken wood or whale skeletons (Table 1.1). They are represented by 9 and 16 described species respectively (Bouchet 2014c; Bouchet and Gofas 2014; for *Idas* distribution, Figure 1.20). Both genera remain contentious, as each has a long history of existence and both are evidently polyphyletic (Lorion et al. 2013; Thubaut et al. 2013b), where nucleotide sequences are not available for either holotype (*A. pelagica* and *I. argenteus* respectively). The genus *Idas* (*s.l.*) remains poorly resolved phylogenetically; many species were described based upon malacological characteristics alone, sometimes by default, being the only material available. Examples are: the type species *I.* (*d.f.*) *argenteus, I.* (*s.l.*) *lamellosus, I*. (*s.l.*) *coppingeri*, *I.* (*s.l.*) *dalli*, *I.* (*s.l.*) *simpsoni, I.* (*s.l.*) *indicus,* and *I.* (*s.s.*) *modiolaeformis*. Two species, (*I.* [*s.l.*] *simpsoni* and *I.* [*s.s.*] *modiolaeformis*) have recently been sequenced for fragments of mitochondrial and nuclear DNA from paratypes that were confirmed taxonomically by Thubaut et al. (2013b), and by both Olu-Le Roy et al. (2004) and Lorion et al. (2012), respectively. These two species, along with the three other species that have been sequenced, *I.* (*s.s.*) *macdonaldi*, *I.* (*s.s.*) *washingtonius* and *I.* (*s.l.*) *japonicus*, frequently feature in phylogenetic analyses, wherein marked levels of divergence warrant their classification in at least two distinct genera (e.g. Thubaut et al. 2013b). An additional species, *A.* (*s.l.*) *iwaotakii*, was cited as *I.* (*s.s.*) *iwaotakii* in a study describing this species general biology (Thubaut et al. 2013a) with phylogenetic analysis that suggests it does indeed belong in the clade within which most *Idas* (*s.l.*) spp. appear to occur (Thubaut et al. 2013a; Thubaut et al. 2013b). However, this species' official classification remains within the genus *Adipicola* (perhaps incorrectly). Remaining morphospecies were described recently from the Mediterranean include: *I. ghisottii*, *I. cylindricus*, *I. cristiani*, *I. emmae*, *I. filippoi*, and *I. jaclinae*, whose morphoanatomies though quite distinct, bear some resemblance to type specimens of both *I. modiolaeformis* and *I. simpsoni* (particularly in the cases of *I. cylindricus* and *I. ghisottii*, see Pelorce and Poutiers 2009; Giusti et al. 2012). Several of these species are assigned to the genus somewhat arbitrarily, by the authors own admission. An additional collection of COI-sequenced *Idas*-like Evolutionary Significant Units (ESUs) for which morphoanatomies have not been formally described, are known to colonise various types of shallow wooden substrata in the South Pacific (10 distinct clades, 20+ ESUs, Lorion et al. 2010).

The Bathymodiolinae (*s.s*) as it currently stands (i.e. excluding 'modiolin' sister clades) is composed of the relatively giant genera *Gigantidas* and *Bathymodiolus*: 25 species in total (Table 1.1)). Twenty-three are *Bathymodiolus* (*s.l.*) species, of which 10 cluster in the *Bathymodiolus* (s.s.) clade (Thubaut et al. 2013b, holotype *B. thermophilus*), including a recent description of *B*. *antarcticus,* (synonymous with *B*. aff.

thermophilus, Johnson et al. 2013). The majority of the *Bathymodiolus* (*s.l.*) reside in the West Pacific (Figure 1.21) – the most diverse region in terms of the Bathymodiolinae (13 sp. [10]). Endemism is high in the West Pacific (Figure 1.21) for example, *B.* (*s.l.*) *taiwanensis* is only known from shallow vent sites near Kueishan Island. A species from of the genus *Gigantidas*, *G. horikoshii* also occurs in this region at the Kaikata seamount, southwest of the Ogasawara (Bonin) Islands. The North Fiji Basin thus hosts *B.* (*s.s.*)

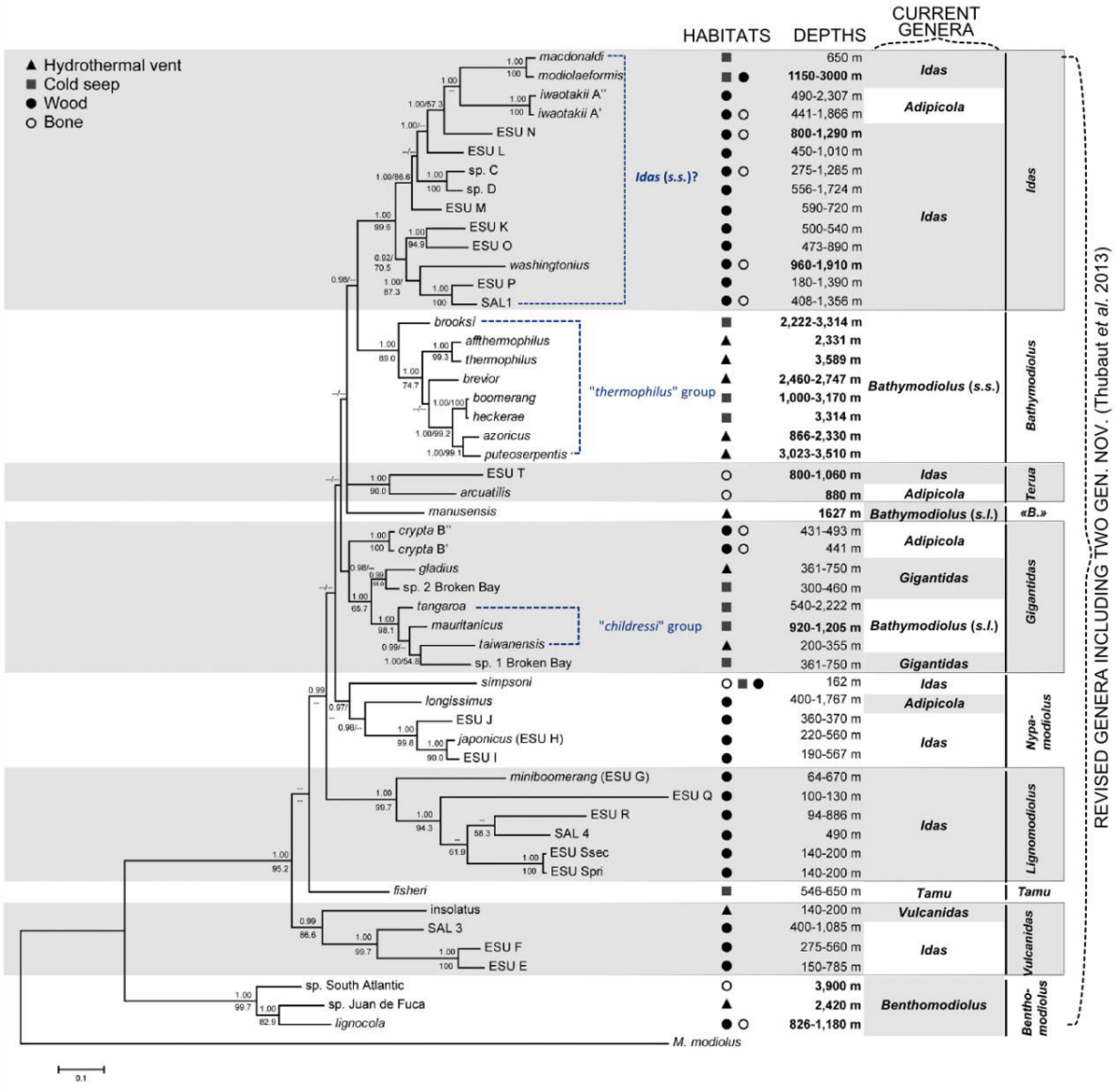

Figure 1.19 Multigene tree for Bathymodiolinae
Adapted from Thubaut et al. 2013b with genera as they currently stand included for comparison

[10] Some authors argue against this level of speciation: observed morphological variation among the '*brevior*' mytilid-complex may be due to adaptive radiation, but equally it could be a product of developmental plasticity in shell growth patterns (Vrijenhoek 2009).

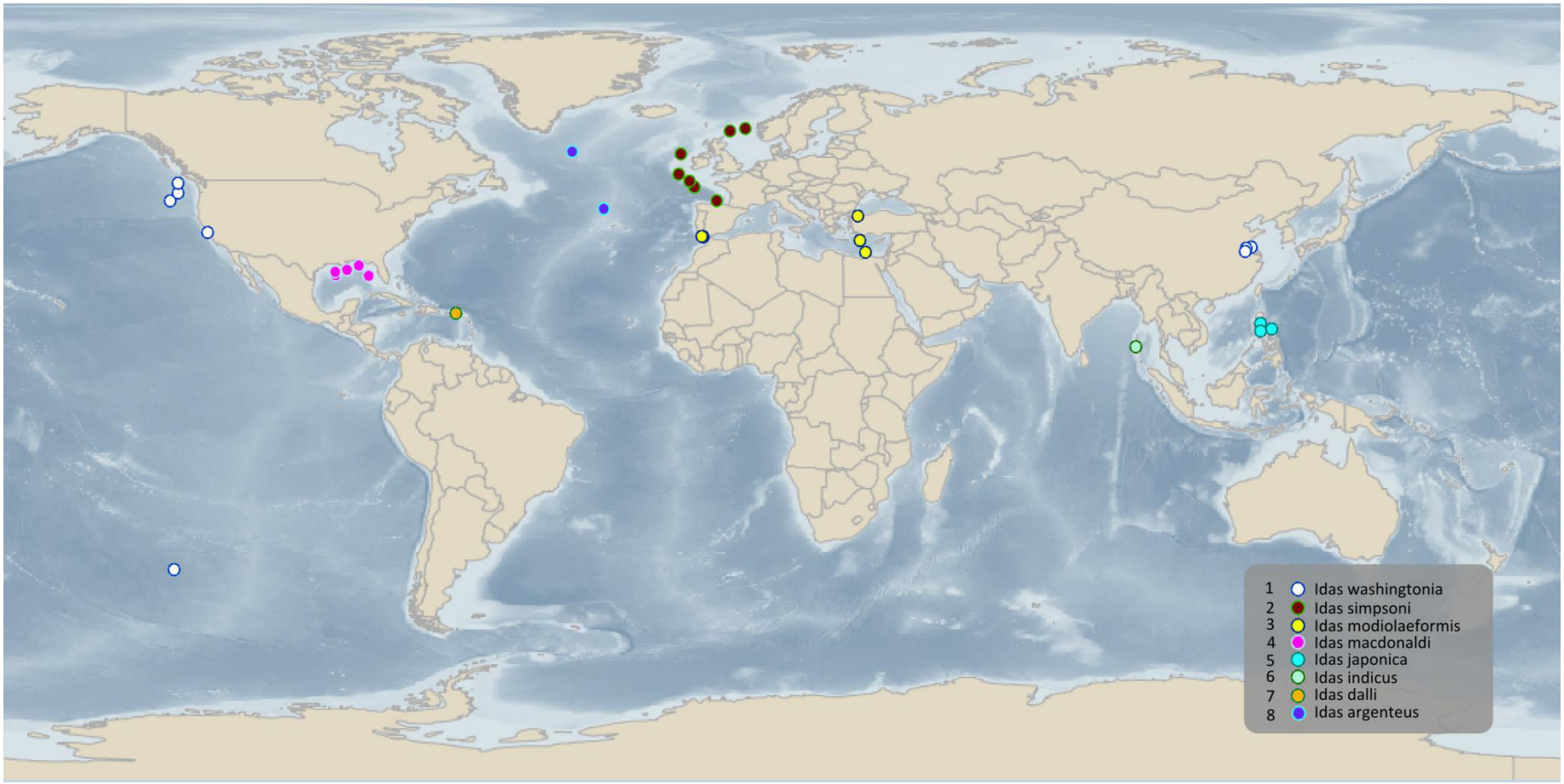

Figure 1.20 Global distribution of members of the Bathymodiolinae: genus *Idas*
Data from OBIS principally, created by S.R. Laming

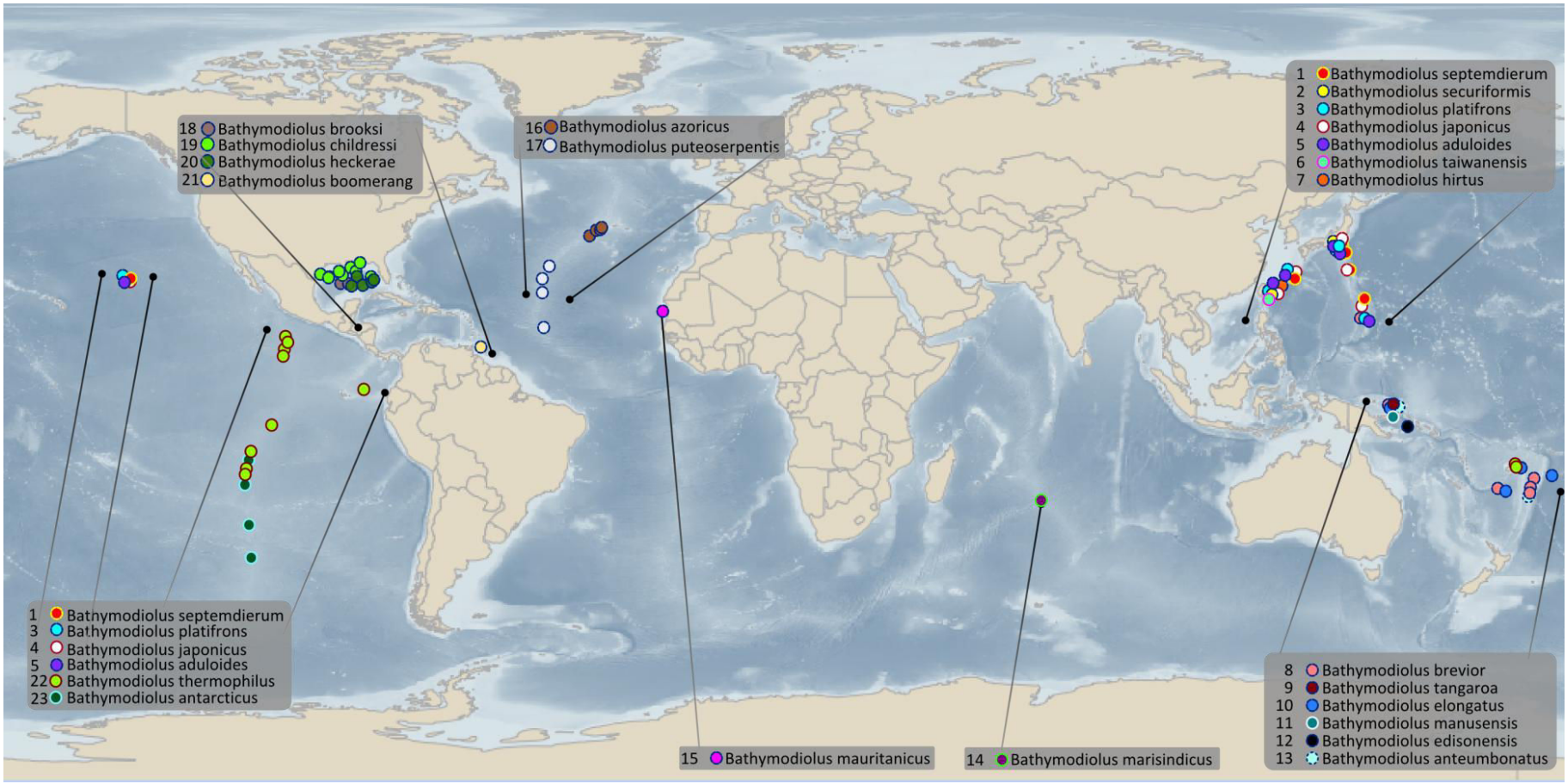

Figure 1.21 Global distribution of members of the Bathymodiolinae: genus *Bathymodiolus*
Data from OBIS principally, created by S.R. Laming

Table 1.1 Depth and geographic distributions of the Bathymodiolinae (*s.l.*)

Species	Authority	Locality	Depths (m)	
Adipicola				
A. arcuatilis	(Dell, 1995)	NE of Chatham Islands, NZ, South Pacific	880	B
A. crypta	(Dall, Bartsch & Rehder, 1938)	Multiple locations on Ryukyu Ridge,	225–229	B
A. dubia	(Prashad, 1932)	unknown	?	?
A. iwaotakii	(Habe, 1958)	Japan, Philippines, Vanuatu, N. California	490 - 1745	Wo
A. longissima	(Thiele & Jaeckel, 1931)	Solomon islands, Philippines SW Pacific	?	Wo
A. osseocola	(Dell, 1987)	N of Chatham Islands, NZ	?	B
		Near Nugget Point, South Island, NZ	?	B
A. pacifica	(Dall, Bartsch & Rehder, 1938)	Off Noma Cape, Kagoshima	225–501	B
A. pelagica	(Forbes in Woward, 1854)		unknown	
A. projecta	(Verco, 1908)		unknown	
Bathymodiolus				
B. aduloides	(Hashimoto & Okutani, 1994)	Off Kikaijima Island	1 451	S
B. anarcticus	(S. B. Johnson & Vrijenhoek, 2013)	32S East Pacific Rise	2 331	V
B. anteumbonatus	(Cosel & Janssen, 2008)	Methane seeps near Edison Seamount, off New Ireland	?	
B. azoricus	(Cosel & Comtet, 1999)	Lucky Strike, Mid-Atlantic Ridge	?	V
		Menez Gwen, Mid-Atlantic Ridge	866–2330	V
B. boomerang	(Cosel & Olu, 1998)			
B. brevior	(Cosel, Métivier & Hashimoto, 1994)	Mussel Valley, North Fiji Basin	?	V
		Mariana Trough	3589	V
		White Lady, North Fiji Basin	?	V
B. brooksi	(Gustafson, Turner, Lutz & Vrijenhoek, 1998)	Alamiños Canyon	2222	S
		West Florida Escarpment	3314	S
B. childressi	(Gustafson, Turner, Lutz & Vrijenhoek, 1998)	Gulf of Mexico	1859	S
		Alamiños Canyon	540–2222	S
B. edisonensis	(Cosel & Janssen, 2008)	Methane seeps near Edison Seamount	?	
B. elongatus	(Cosel, Métivier & Hashimoto, 1994)		?	
B. heckerae	(Turner, Gustafson, Lutz & Vrijenhoek, 1998)	Blake Ridge	2 155	S
		West Florida Escarpment	3 314	S
B. hirtus	(Okutani, Fujikura & Sasaki, 2004)	Kuroshima Knoll, Off Yaeyama Islands	637	S
B. japonicus	(Hashimoto & Okutani, 1994)	Off Hatsushima, Sagami Bay	1170–1180	S
B. manusensis	(Hashimoto & Furuta, 2007)	PACKMANUS Field E, Manus Basin	1627–1629	V
B. marisindicus (= B. brevior?)	(Hashimoto, 2001)	Kairei Field, Southern Central Indian Ridge	2443–2454	V
		Kairei Field, Southern Central Indian Ridge	2415–2460	V
B. mauritanicus	(Cosel, 2002)	West Africa	1000–1267	S
B. platifrons	(Hashimoto & Okutani, 1994)	Off Hatsushima, Sagami Bay	1029–1180	S
		Off Hatsushima, Sagami Bay	?	S
		Off Hatsushima, Sagami Bay	1029	S
		North Iheya Ridge, Mid-Okinawa Trough	1028	V
		Hatoma Knoll, Okinawa Trough	1523	V
		Dai-yon Yonaguni Knoll, southern Okinawa Trough	1336	V
B. puteoserpentis	(Cosel, Métivier & Hashimoto, 1994)	Snake Pit, Mid-Atlantic Ridge	3023–3510	V
		Snake Pit, Mid-Atlantic Ridge	3023–3510	V
B. securiformis	(Okutani, Fujikura & Sasaki, 2004)	Kuroshima Knoll, Off Yaeyama Islands	641	S

B. septemdierum	(Hashimoto & Okutani, 1994)	Myojin Knoll, Izu-Ogasawara Island-arc	1288–1290	V
		Myojin Knoll, Izu-Ogasawara Island-arc	1346	V
		Suiyo Seamount, Izu-Ogasawara Island-arc	1373–1382	V
B. taiwanensis	(Cosel, 2008)	Kueishan Island, Okinawa Arc	200–355	V
B. tangaroa	(Cosel & Marshall, 2003)	Off Turnagain Cape, New Zealand	920–1205	S
B. tangaroa tuerkayi sub sp.	(von Cosel and Janssen 2008	Near to Edison seamount, New Ireland Fore-Arc Basin	1600	V
B. thermophilus	(Kenk & B. R. Wilson, 1985)	9N East Pacific Rise	2524	V
		9N East Pacific Rise	2460–2747	V
		7S East Pacific Rise	2460–2747	V
Benthomodiolus				
Be. abyssicola	(Knudsen, 1970)	Trans-Pacific	1500–3670	
Be. geikotsucola	(Okutani & Miyazaki, 2007)	Torishima Seamoun	4 051	B
Be. lignicola	(Dell, 1987)	Chatham Ris	826–1174	B-Wo
Gigantidas				
G. gladius	(Cosel & Marshall, 2003)	Rumble III	300–460	V
G. horikoshii	(Hashimoto & Yamane, 2005)	Kaikata Seamount	486	V
Idas				
I. argenteus	(Jeffreys, 1876)	North Atlantic	150–3600	Wo
I. coppingeri	(E. A. Smith, 1885)	Indonesia	2000–2800	Wo
I. cristiani	(Giusti, Mietto & Sbrana, 2012)	Mediterranean Sea	500–700	?
I. cylindricus	(Pelorce & Poutiers, 2009)	Mediterranean Sea	?	Wo
I. dalli	(E. A. Smith, 1885)	Culebra Island, West Indies	713	?
I. emmae	(Giusti, Mietto & Sbrana, 2012)	Mediterranean Sea	500–700	?
I. filippoi	(Giusti, Mietto & Sbrana, 2012)	Mediterranean Sea	500–700	?
I. ghisottii	(Warén & Carrozza, 1990)	Mediterranean Sea	?	W
I. indicus	(E. A. Smith, 1904)	Andaman Islands	338	-
I. jaclinae	(Giusti, Mietto & Sbrana, 2012)	Mediterranean Sea	500–700	?
I. japonicus	(Habe, 1976)	Japan, Philippines	220–560	Wo-W
I. lamellosus	(Verrill, 1882)	North Atlantic	613–944	?
I. macdonaldi	(Gustafson, Turner, Lutz & Vrijenhoek, 1998)	Garden Banks	650	S
I. modiolaeformis	(Sturany, 1896)	Meditteranean Sea and East Atlantic	350–3015	S-Wo
I. simpsoni	(J. T. Marshall, 1900)	NE Atlantic, Mediterranean Sea, Marmara Sea	150–1000	B-Wo-S
I. washingtonius	(Bernard, 1978)	NZ (Chatham Isl.),Japan (e.g. Torishima Seamount),USA (Washington, Cape Flattery, California), Panama (Gulf)	500–4037	B-Wo-V
Tamu				
T. fisheri	(Gustafson, Turner, Lutz & Vrijenhoek, 1998)	Garden Banks	546–650	S
Vulcanidas				
V.insolatus	(Cosel & B. A. Marshall, 2010)	Macauley Cone	140–504	V

brevior, *B.* (*s.l.*) *anteumbonatus*, *B.* (*s.l.*) *elongatus* and the most westerly record for *B.* (*s.s.*) *thermophilus* (based on OBIS data), though a peer-reviewed account of this last record could not be identified (in fact, obviously erroneous data occurred quite frequently on OBIS during distribution searches). In contrast the East Pacific plays host to only two species: *B.* (*s.s.*) *thermophilus* and *B.* (*s.s.*) *antarcticus*. Hybridisation between these two species has been identified (Johnson et al. 2013). The type species for *Gigantidas*, *G. gladius* forms dense beds near vent sites on Rumble III seamount, northwest of New Zealand's North Island, in the South-West Pacific.

In the Atlantic, a total of seven species occur; three in the West Atlantic at hydrocarbon seeps in the Gulf of Mexico (*B*. (*s.l.*) *childressi*, *B*. (*s.l.*) *heckerae , and B*. (*s.l.*) *brooksi*), two at hydrothermal vent fields in the Mid-Atlantic Ridge (*B. azoricus, B. puteoserpentis*) and two which are trans-Atlantic seep species (*B. mauritanicus, B. boomerang*[11]). Similar to *B. thermophilus* and *B. antarcticus* in the Pacific, a hybridisation zone exists between *B. azoricus* and *B. puteoserpentis* from 37°N and 14°N on the Mid-Atlantic Ridge, (O'Mullan et al. 2001). Curiously, shell morphometry and variability in mitochondrial sequences indicate that the *azoricus-puteoserpentis* complex is more divergent than the *thermophilus-antarcticus* complex, but the opposite is the case when considering nuclear genes (Johnson et al. 2013). An additional species, *B*. (*s.l.*) *marisindicus* occurs at vents in the Indian Ocean (e.g. Kairei field, 2460m): both this species and *B.* (*s.l.*) *aduloides*, warrant placement in their own genus (Lorion et al. 2013; Thubaut et al. 2013b), as do the second lineage of *Bathymodiolus* (*s.l.*) spp. that fall within the "*childressi* group" including *B*. (*s.l.*) *childressi*, *B*. (*s.l.*) *tangaroa*, *B*. (*s.l.*) *securiformis*, *B*. (*s.l.*) *japonicas*, *B*. (*s.l.*) *mauritanicus*, *B*. (*s.l.*) *hirtus* and *B*. (*s.l.*) *platifrons* (Kyuno et al. 2009; Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b).

1-8.3. Habitats and life habits: driven by symbiosis

The habitats in which chemosymbiotic bivalves live tend to reflect adaptations that have permitted maximal reducing-habitat exploitation. These have defined chemosymbiotic bivalvian modes of existence or 'life habits' (Stanley 1970), i.e. behavioral and physiological adaptations employed in relation to feeding, interactions with their habitat substratum and gaining access to reduced compounds.

1-8.3.1 Characteristics of the physical substratum

Chemically-reduced pore-waters in the deep-sea are most common in sedimentary substrata, occurring in interstitial sub-surface sediments below the REDOX boundary layer, in organically enriched fine silt and mud including those near whale- and wood-falls, and in zones of soft-bottom hydrocarbon seepage (e.g. mud volcanoes). For this reason the greatest diversity of bivalve genera are found at these habitats. In relatively rare instances, substrata can also be solid, associated with seepage of percolating reduced fluids, such as in venting chimneys and diffuse flows from fissured igneous rocks at hydrothermal vents, on the solid surfaces of decomposing nektonic megafaunal skeletons and wood, and on authigenic carbonate crusts or asphalt concretions formed at some hydrocarbon seeps. Bivalve assemblages that occur on solid substrata, subjected to reducing conditions, are dominated by the chemosymbiotic Mytilidae (with a few exceptions from the Pliocardiinae), which are well adapted to these habitats on account of being byssate (a common neotenous post-larval feature of substratum attachment by byssus threads retained in adults of Mytilidae, Morton 1992).

[11] *B.* (*s.l.*) *mauritanicus* is considered by many to be synonymous with *B.* (*s.l.*) *childressi* and similarly, *B.* (*s.l.*) *boomerang with B*. (*s.l.*) *heckerae*: they are sister species at their most distant

1-8.3.2 Feeding modes and reducing fluid access

Feeding modes appear to provide the means to exclusively target reduced compounds for nourishing chemosymbiosis on which the host depends, while in the chemosymbiotic Mytilidae, diet may be supplemented significantly by heterotrophy (e.g. particle assimilation in *B. azoricus and B. thermophilus*, Page et al. 1990; Page et al. 1991). Thus feeding modes depend upon the species location in the substratum and in relation to the REDOX boundary in particular, with contributory roles from hydrodynamics and sea surface processes in some instances (e.g. vertical flux of organic material).

The Solemyidae are infaunal and make U- or Y shaped burrows that permit simultaneous access to aerated seawater at the sediment boundary layer, and interstitial pore-waters in reducing sediments (Stanley 1970; Reid 1980; Fisher 1990; Stewart et al. 2011). Despite weak filter feeding capabilities (Krueger et al. 1992), symbiotic nutrition is presumed to be obligatory and the principal source of nutrition (Duperron et al. 2013), evidenced by the radical reduction (e.g. *Solemya velum*), or complete loss of a digestive system, with only basic labial palps remaining, as in *S. pervernicosa* (formerly *S. reidi*).

The most diverse of the chemosymbiotic bivalve families[12], Lucinidae, are also infaunal, found in a multitude of soft sediment habitats with high plant-derived organic enrichment, with some additional species from hydrocarbon seeps and vents (Taylor and Glover 2010). Lucinidae can often burrow deeply into sulphidic, anoxic sediments using their foot (e.g. Cary et al. 1989) and typically live in proximity to the REDOX boundary layer. While orientated vertically in the sediment with the hinge-line uppermost, aeration and sulphide acquisition are both performed by the foot, the former by maintaining a mucous-reinforced inhalant tunnel linked to the surface, the latter by probing down into anoxic reducing layers (Taylor and Glover 2000).

Second only to the Lucinidae, Thyasiridae are the most diverse, most varied in their morphology and most extensive in their habitat range (Taylor and Glover 2010). The Thyasiridae are infaunal, burrowing into suboxic-to-anoxic sediments (including those in oxygen minimum zones), in which the interstitial porewater is enriched with organic matter, hydrocarbons, or reducing fluids. Means of sulphide acquisition is presumed to be in line with that of other Heterodonta, although evidence suggests that many thyasirids do not rely on chemosymbiotic bacteria at all (summarised below). This family is poorly studied in the deep sea by comparison. Species which engage in symbiosis generally burrow deeper than non-symbiotic species and use their super-extensile foot, which can extend 30 times the length of the shell, to search for and extract sulphides from deeper reducing sediment patches, consequently creating a network of tunnels (the number being dependent on sulphide levels, Dufour and Felbeck 2003). The extraordinary length of the extended foot is made possible through having evolved dorso-ventrally elongate shells, which can

[12] This may be influenced by sampling bias given their pervasive presence in shallow-water environments

accommodate a longer foot than those in the antero-posteriorly extended asymbiotic thyasirids (Dufour and Felbeck 2003).

Unlike other Heterodonta, the Vesicomyidae display two separate life habits. Most are partially or almost entirely infaunal (all Vesicomyinae, and Pliocardiinae associated with non-vent sedimentary habitats) inhabiting the abyssal plains in association with subsurface REDOX boundaries or pockets of hydrocarbon seepage that underlie surface sediments, from which their posterior half protrudes (Krylova and Sahling 2010. Pliocardiinae inhabiting vents however, orientate themselves along seeping fissures in igneous rocks. The Vesicomyidae frequently contribute to community composition in hydrocarbon seeps at continental margins (Sibuet and Olu 1998), at mid-ocean hydrothermal vents (Taylor and Glover 2010), and occur both on and in proximity to organic falls (e.g. whale bones and adjacent sediment, Bennett et al. 1994; Smith and Baco 2003). The sheer abundance and frequency of the Pliocardiinae discovered at multiple sites along continental margins are thought to be indicative of more common occurrences of reducing environments on both active and passive margins than previously realised (Krylova and Sahling 2010). Access to sulphides and oxygen are via two separate avenues. Sulphides are absorbed by the foot (Childress and Mickel 1982), extending into reduced sediments or seeping fissures (as in the case of vents), transported to the gills by the blood (Arp et al. 1984; Goffredi and Barry 2002) while oxygen uptake is via the gills directly. In pliocardiins, sulphide may be transported by zinc-based sulphide-binding molecules, sulphide oxidation maintaining sub-saturation levels (Childress et al. 1993), and so protecting host tissues from poisonous respiratory inhibition. Additional oxidation of unbound sulphides to thiosulphate in blood serum may occur also (Childress et al. 1993). Sulphide absorption from water drawn in through the gills may present a secondary means of supply, as the gills of certain vesicomyid species are arranged in tubular bacteriocyte-lined channels (Fiala-Médioni and Le Pennec 1988; Krylova and Sahling 2010).

Collectively, the genera of the mytilid subfamily Bathymodiolinae have evolved to take advantage of a multitude of reducing habitats. In contrast to the other chemosymbiotic bivalve families discussed so far,

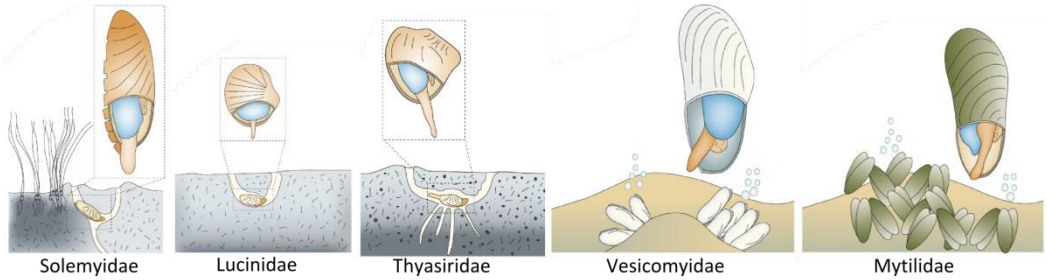

Figure 1.22 Habitat use in chemosymbiotic bivalves
Pictured are the five main bivalves discussed in this introduction. In the Solemyidae, Lucinidae and Thyasiridae anoxic sulphide rich sediments (darker regions are mined with a vermiform foot). In most Vesicomyidae, this remains the case, however the unusual Pliocardiinae reside on fissures of on the surface of sediments subject to diffuse flows loaded with reducing compounds. Mytilidae that are chemosymbiotic are almost always epibenthic (see text for exceptions), taking advantage of free fluids being released at vents, seeps, and organic falls. Blue internal organs are one of the organism's two gills. Brown internal organs are foot (extended) and viscera (internal). Images from Dubilier et al. 2008

the members of this byssate mytilid subfamily are generally not infaunal, living instead as epibenthos attached to hard substrata which can include authigenic carbonates and asphalt concretions at hydrocarbon seeps, igneous formations in the vicinity of hydrothermal vents, the outer walls of large polycheate tubes (Annelida: Siboglinidae, with accounts both at vents and seeps) or on the shells of other organisms including conspecifics (Duperron 2010). Since the upflux of subsurface reduced interstitial fluids to the sediment boundary layer in the vast majority of deep-sea habitats is effectively nil (except during large scale disturbance), the chemosymbiotic Mytilidae, which exclusively harbour symbionts in their gills and are not known to mine for sulphides with their foot, must rely on being bathed in reducing fluids, in the absence of direct access to anoxic sediments. To date, two exceptions to this life habit are found in *Bathymodiolus boomerang* and *Gigantidas horikoshi* (Hashimoto and Horikoshi 1989; Hashimoto et al. 1995; Desbruyères and Segonzac 1997; von Cosel and Olu 1998), who bury themselves by up to two-thirds of their shell length in sediments subject to sulphide and methane seepage (though it's thought that the former still attaches by byssus threads to subsurface solid-mud accretions, von Cosel and Olu 1998). Even these two partially infaunal species, appear to be restricted to seeping sediments however. Consequently, combined with the almost universal requirement for byssate attachment, it is only in association with active reduced-fluid seepage at vents and seeps that the relatively giant chemosymbiotic mussels occur. Remaining smaller-sized chemosymbiotic Mytilidae, whose nutritional demands per individual are presumably much reduced, are capable of colonising decomposing organic substrates (particularly nektonic megafaunal carcasses and accumulations of sunken wood) where reduced fluid production is less dramatic but where hard substrate for attachment is available, although select species colonise seeps and vents (Gustafson et al. 1998; Olu-Le Roy et al. 2004; Rodrigues et al. 2010; Ritt et al. 2012).

1-8.3.3 Chemosymbioses in bivalves

Chemosymbiosis has been identified in all species of Lucinidae, Solemyidae and Vesicomyidae studied to date, suggesting it has an obligatory role in the biology of these families (Taylor and Glover 2010), though weak filter-feeding capacity is retained in *Solemya velum* (Krueger et al. 1992). In the Thyasiridae, while many species engage in symbioses, including the deepest known chemosymbiotic species, *Maorithyas hadalis* (Fujiwara et al. 2001), others do not (Dufour 2005). In Mytilidae, chemosymbiosis is confined to members of the subfamily Bathymodiolinae while all other mytilids are believed to be asymbiotic, though the two remaining deep-sea mytilid genera have not been examined for symbioses in detail (i.e. *Amygdalum* and *Dacrydium*, Salas and Gofas 1997; Oliver 2001). Symbioses almost invariably involve chemoautotrophic (typically sulphur-oxidising) bacteria and often, in the case of the chemosymbiotic Mytilidae, additional methane-oxidising methanotrophs (Dubilier et al. 2008). Rare instances of other bacterial phylotypes are detailed subsequently, pertaining to mytilids specifically. Most symbioses in chemosymbiotic bivalves involve Gammaproteobacteria, though rare instances of possible Epsilonproteobacteria, Spirocheates, Bacteroidetes subdivisions have also been suggested, though their

functional significance remains speculative (e.g. Duperron et al. 2006b; Rodrigues et al. 2010; Brissac et al. 2011).

In the Solemyidae, endosymbiotic coccoid-to-rod-shaped Gammaproteobacterial phylotypes predominate and cluster cladistically with those of lucinid and thyasirid bivalves (Taylor and Glover 2010), excluding *Acharax*, whose symbionts are phylogenetically distinct (Imhoff et al. 2003). Strong host mediation over bacterial division is suspected, as bacteria undergoing cell division are rarely witnessed (Duperron et al. 2013), crammed into bacteriocytes occupying the abfrontal regions of the individual filaments of the large posterior gills (Taylor and Glover 2010). Symbiont transmission is thought to be vertical, at least in the genus *Solemya* (symbiont DNA found in ovaries, oocytes and larvae of two species Cary 1994; Krueger et al. 1996).

Lucinidae gills are usually thickened and optically opaque, representing about one-third of the animal's biomass, and regions of coincident gill filaments are frequently fused to create channels, densely populated by bacteriocytes (Distel and Felbeck 1987). Much like the Solemyidae, host species are thought to exert considerable physiological restraint upon their symbiont densities; curiously, bacteria appear unable to escape host tissues once acquired (Brissac et al. 2009) and while genomic replication can take place, bacteria do not actively divide within hosts cells (Caro et al. 2007). Lucinid symbionts cluster in the Gammaproteobacteria, in a clade that also includes *Solemya* and vestimentiferan tubeworm symbionts (Cavanaugh et al. 2006; Dubilier et al. 2008). Based on tropical shallow-water research, symbiont transmission is believed to be horizontal following settlement and metamorphosis (Gros et al. 1997; Gros et al. 1999), acquired in some instances from a pool of free-living bacteria present in the sink environment (e.g. Gros et al. 2003), where ongoing acquisition may occur as adults (Le Pennec et al. 1988).

In stark contrast to the other families discussed, a notable number of the Thyasiridae examined for bacterial symbionts, have been shown to be asymbiotic (Dufour 2005), with varying densities of and nutritional dependencies upon symbiosis in those that are symbiotic (Dando 1993). The family is thus considered to be at an earlier stage of evolution in the context of symbiosis (Dufour 2005). Across the family, for species in which symbiosis has been examined to any degree, a spectrum of gill characteristics appears to exist that roughly place into three types. They pass from a typical homorhabdic filibranch gill with inter-filamental fusion dorsoventrally (along 70% of filament length) and only a few (if any) bacteria nestled between the microvilli that are located abfrontally, through a state where bacteria are numerous but filaments are thin and unmodified abfrontally, up to a state where gill filaments are abfrontally thickened and the greater non-ciliated epithelial surface area houses distinct bacteriocyte zones adorned with microvilli, among which extracellular bacteria are dispersed. In one species (*Maorithyas hadalis*) where bacteria are intracellular however, internalised bacteria-filled vacuoles are often located apically such that they remain partially open to external water (Fujiwara et al. 2001). This fourth state is considered intermediate between extracellular and intracellular symbiosis where only bacteria in vacuoles close to the

apical end of bacteriocytes are in contact with seawater (Endow and Ohta 1990). Dual, spatially partitioned symbioses occur in this species between a thioautotrophic bacteria and a second unknown phylotype, distantly related to the Thiomicrospira and Hydrogenovibrio (Fujiwara et al. 2001). In thyasirids, symbiont acquisition is preceded by the abfrontal development of filaments, which expands colonisable non-ciliated surfaces (Dufour 2005). Transmission though not rigorously examined, is thus suspected to be horizontal.

Studies into symbioses in the Vesicomyidae have focussed on those of the subfamily Pliocardiinae (Taylor and Glover 2010; Duperron et al. 2013), for which sulphur-oxidising symbionts have been identified without exception in species examined in detail, suggesting that symbiosis is a pan-pliocardiin characteristic. One record of gill bacteria, not yet confirmed to be chemoautotrophic, has been published for the species "*Vesicomya*" sergeevi, however current taxonomic opinion places this species in a sister family, the Kelliellidae (i.e. *Kelliella sergeevi*[13]). The principal features that characterise the Pliocardiinae are medium-to-large white shells (though they are smaller in *Isorropodon*), well-developed siphons, labial palps that are reduced to simple plicated ridges around the mouth, a basic gut and a small stomach (Morton 1986; Fiala-Médioni and Le Pennec 1988; Krylova and Cosel 2011) and sub-filamental tissue in the gills (Krylova and Sahling 2010) which are thickened. The Vesicomyinae by contrast possess a fully retained gut, relatively short siphons and an absence of sub-filamental tissue in the gills. In adult Pliocardiinae, domed, microvilli-covered gill epithelial bacteriocytes – packed with intracellular sulphur-oxidising autotrophic Gammaproteobacteria – are aligned in layers within the abfrontal region of the engorged gill filaments, separated by a central blood lacuna and interspersed with intercalary cells (Fiala-Médioni and Le Pennec 1988; Taylor and Glover 2010). These pliocardiin symbionts, which form a compact clade with highly homogeneous 16S rRNA sequences, are related to those within mytilid and poriferan hosts, and various free-living bacteria (Duperron et al. 2012).

Current evidence available indicates that when examined, symbiont transmission is vertical with maternal origins, where primary oocytes are known to be already infected with symbiotic phylotypes in *Phreagena* (*Calyptogena*) *soyoae* (Endow and Ohta 1990). Further putative evidence for vertical transmission is in apparent host-symbiont co-speciation based on mirrored phylogenies (Peek et al. 1998a; Peek et al. 1998b), and in reported genome reduction in the symbiotic bacteria, in comparison to free-living relatives (Kuwahara et al. 2007), indicative of vertical transmission and the absence of a free-living mode (e.g. as in some insects, Gil et al. 2004). In addition to the vertical transmission of symbionts, rare incidents of both intraspecific variability in symbiont lineages (e.g. *Calyptogena valdiviae*, Duperron et al. 2013) and non-parental lateral transmission are documented in species occurring in proximity to one another (Stewart et al. 2008; Decker et al. 2013); these phenomena may be linked.

[13] Huber, M. (2014). *Kelliella sergeevi* (Filatova, 1971). Accessed through: World Register of Marine Species at http://www.marinespecies.org/aphia.php?p=taxdetails&id=505370 on 2014-07-02

As the most documented taxa to date, more is known about symbioses in the Bathymodiolinae then perhaps any other bivalve group employing symbionts for their nutrition (Duperron 2010). Part of their evident success is through the flexibility of their symbiotic associations. One notable exception may be *Idas argenteus* a deep-water, wood-associated mussel which has been described as a larviphage, ingesting larvae of the small wood-boring bivalve *Xyloteredo* (Ockelmann and Dinesen 2011), with no obvious bacterial symbionts in its gills. Otherwise, bacterial symbioses are at their most diversified within the bivalve family Bathymodiolinae based on symbiosis studies to date. These assemblages, like the monophylotypic symbioses of other bivalves discussed, almost invariably contain thiotrophic Gammaproteobacteria. However, the Bathymodiolinae (*s.l.*) are noted in particular for their incorporation of methanotrophic Gammaproteobacteria that employ methane monooxygenase in order to harness methane that both drives metabolic processes (by oxidation) and provides a source of carbon (Cavanaugh et al. 2006). These are normally part of dual symbioses, with occasional instances of exclusively methanotrophic associations (*B*. (*s.l.*) *childressi*, Childress et al. 1986; Duperron et al. 2007; *Bathymodiolus* (*s.l.*) *platifrons*, Barry et al. 2002; *Bathymodiolus* (*s.l.*) *japonicus*). These dual symbioses are known to occur with regularity in species found in the Gulf of Mexico, the Atlantic Ocean and the Mediterranean Sea (Fisher et al. 1993; Distel et al. 1995; Fiala-Médioni et al. 2002; Duperron et al. 2005; Duperron et al. 2006a; Rodrigues et al. 2013), but are conspicuously absent so far in well-documented symbioses in mussels found within the Pacific Ocean, and other oceanic regions. Multiple symbioses have been documented in select species (Duperron et al. 2007; Duperron et al. 2008a). The maximum number of symbiont lineages in a single species is currently six, in the host species *Idas modiolaeformis*, collected from authigenic carbonate crusts subject to methane seepage within the central pockmarks zone of the Nile Deep-Sea Fan, in the eastern Mediterranean (Duperron et al. 2008a). Recent studies have also identified alternative electron donors that may be used by symbiotic Gammaproteobacteria such as hydrogen in thiotrophs (based on presence of a hydrogenase encoding gene in *B. puteoserpentis*, Petersen et al. 2011), methylotrophs, an additional Gammaproteobacteria "G" (Duperron et al. 2007; Duperron et al. 2008a) and even the oxidation of aromatic compounds by a novel symbiont in *Bathymodiolus heckerae* (Raggi et al. 2013). *Bathymodiolus* (*s.s.*) *heckerae* as a species demonstrates the adaptability that multiple symbioses can afford a host, having different symbionts assemblages depending on its location. The species is known to harbour two distinct sulphur-oxidisers, a methanotroph and then either methylotroph-related symbiont in the northern Gulf of Mexico, or hydrocarbon-degrading bacteria in the southern Gulf of Mexico (Fisher et al. 1993; Distel et al. 1995; Duperron et al. 2005; DeChaine and Cavanaugh 2006; Duperron et al. 2007; Raggi et al. 2013).

The resulting advantages of genetically – and perhaps more importantly – nutritionally and metabolically diverse symbiotic assemblages are two-fold. The first, is that such assemblages bring an unrivalled level of nutritional flexibility with regards to the variety of reduced compounds present at reducing habitats, in environments where relative proportions fluctuate temporally (Dubilier et al. 2008). This permits the Bathymodiolinae (*s.l.*) to colonise habitats which are dominated by methane production,

such as periodite-hosted vents and many methane seeps (Tivey 2007). In addition, the relative dominance of co-occurring sulphur or methane oxidisers are known to reflect (Fiala-Médioni et al. 2002; Le Bris and Duperron 2010), and respond to (Halary et al. 2008; Riou et al. 2010b) changes in relative sulphide and methane concentrations within their environment. Astoundingly, bacterial – or host-mediated – responses to alterations in the proportional availability of reducing compounds are within the scale of hours (S. Duperron, unpublished data). The second, perhaps less evident advantage is that inter-bacterial competition is reduced (except perhaps, for vacuolar space), since the segregation of reduced-fluids across nutritionally independent bacterial populations with differing metabolic pathways allow resources to be partitioned (Dubilier et al. 2008). In some instances it is thought that such dual (or multiple) symbioses may even be synergistic, such as methanotrophs generating inorganic carbon by-products, which can be used by neighbouring thiotrophs (Dubilier et al. 2008). The level of symbiont integration in host organisation within the Bathymodiolinae varies across, and within genera. Bacteria although always documented to be endosymbiotic, may be either arranged extracellularly among microvilli that differentiate from the apical membranes of bacteriocytes (with one account of bacteria being partially housed open superficial pseudopodium-like structures, Fujiwara et al. 2010), or arranged intracellularly in the vacuoles of bacteriocytes that from part of the gill epithelium in close association with a central blood lacuna. In both cases putative lysosomes are often present, with bacteria restricted to the non-ciliated lateral-to-abfrontal regions of gill filaments in adult specimens (Duperron 2010; Duperron et al. 2013). In the small-sized mussels which are often able to colonise several types of reducing habitat, extracellular bacteria are documented for *Tamu fisheri* (Nelson and Fisher 1995) while both intra- and extracellular endosymbioses have been described in the genera *Idas* (*s.l.*) and *Adipicola* (*s.l.*); the former genus (*s.l*) is discussed in more detail in Chapter 2. No conclusive data is available for the genus *Benthomodiolus*. In the larger bathymodiolins (e.g. *Gigantidas*, *Bathymodiolus*), bacteria have been exclusively intracellular for those examined in detail, where several species of *Bathymodiolus* (*s.l.*) may in fact belong in the genus *Gigantidas* (s.s.), according to recent assertions by Thubaut et al. 2013b, though at the time of writing these had yet to be published in a formal nomenclature revision. As the phylogeny currently stands, localisation of thiotrophic symbionts in the genus *Gigantidas* has yet to be assessed.

Patterns of co-speciation seen in some other chemosymbiotic bivalves and their symbionts (e.g. the vesicomyids) are not reflected in the Bathymodiolinae, where transmission is suspected to be predominantly horizontal i.e. acquired from the environment or from adult host organisms laterally at any point in the lifecycle after the initiation of dispersal (i.e. post-spawning). Evidence for horizontal transmission have been posed for *Idas modiolaeformis* from the NDSF, based on the absence of bacteria in gonadal tissues in adults where densities of methanotrophic symbionts in gills were high (Gaudron et al. 2012), visualised using specific oligonucleotide probes and Fluorescence *in situ* hybridisation (FISH). Studies that have examined the ultrastructure of gill filaments in *Bathymodiolus* (*s.l.*) spp. have identified that intracellular bacteria are proximal to the bacteriocytes apical microvilli, where plasma membrane

invaginations are visible at various stages of bacteria isolation within endocytotic vacuoles (Le Pennec et al. 1988; Won et al. 2003b). This suggests that bacteria are internalised directly from the environment.

1-8.3.4 Acquisition of horizontally transmitted symbionts

Whilst there is growing molecular evidence that transmission in mussels is horizontal (Won et al. 2003b; Won et al. 2008; Fontanez and Cavanaugh 2014), limited data exists about the biological processes that govern infection, particularly in terms of host-symbiont recognition and dynamics of cellular communication, the incorporation of symbionts, and ultimately their assimilation. Apparent symbiont specificity in or on host tissues suggests mediation of bacterial distribution or resource partitioning. The timing of symbiont acquisition and the degree to which hosts depend upon symbionts at various stages of development are not well defined. Horizontal transmission invariably raises the question of timing of symbiont acquisition, given the nutritional consequences for being aposymbiotic at some point in the lifecycle of a chemosymbiotic species. Settling siboglinid tubeworms and bathymodiolin mussels that acquire locally adapted microorganisms might profit from the greater flexibility needed to take advantage of a wider range of reducing environments, when compared with vertically transmitted symbionts in Vesicomyidae hosts, occurring much less frequently in Indian and Atlantic vent sites (Won et al. 2003b). As bacteria are most likely to be present at either the source (spawning) or the sink (settlement) habitats being reducing environments, it is during these periods that acquisition is suspected to take place (Figure 1.13). However, information regarding the acquisition of symbionts is scarce for the deep sea generally. Data available for the Bathymodiolinae (*s.l.*) suggest that for *B*. (*s.s*.) *azoricus*, pediveligers are already symbiotic (Won et al. 2003b) where bacteria were almost certainly acquired horizontally, though perhaps with an un-quantified degree of 'leaky', parental transmission during post-embryonic development, based on the mixture of symbionts identified in host hybrid zones. In *B*. (*s.l*.) *childressi* methanotrophic symbionts appear to be already present in juveniles, but with less specific infection, occurring on other epithelial surfaces as well as the non-ciliated abfrontal and lateral regions of the gill filaments (Streams et al. 1997). This pattern of general-to-specific infection appears to occur in two other *Bathymodiolus* (*s.l.*), *B*. (s.s.) *azoricus* and *B*. (s.s.) *puteoserpentis*, confirmed by employing FISH upon host tissues using targeted oligonucleotide probes for the expected bacterial symbionts (Wentrup et al. 2013). Symbionts were present in all specimens, the smallest being juveniles of 4mm shell length, however, infection in the smallest individuals was specific in terms of phylotype but generalist in terms of tissue type. In adults, increased specificity resulted in the disappearance of non-gill epithelial symbionts. In *B.* (*s.s.*) *azoricus* at the Lucky Strike hydrothermal vents and the seep species *B.* (*s.l.*) *heckerae* at Blake Ridge cold seeps, patterns of symbiont infection appeared to vary independently of size (ranged down to post-larval specimens with no evidence of post-settlement growth, Salerno et al. 2005), instead reflecting variations in habitat fluid composition between the seep (predominance of methanotrophic symbionts) and vent environments (being chemoautotrophic bacteria dominated). Stable isotopes signatures supported chemosymbiotic life habits at all stages of benthic development.

1-8.4. Organic-fall mytilids

In the Bathymodiolinae, although knowledge on aspects of adaptive biology and physiology in the large Bathymodiolinae (s.s.) is growing, data are wholly deficient regarding the biology of small-sized genera that typically colonise decomposing wooden debris and megafaunal carcasses. This includes timing of symbiont acquisition in the context of other aspects of development, but also basic metrics of ontogeny, settlement, rates of growth, reproductive development and spawning habits, lifelong nutritional needs and anatomical or physiological adaptations (if any) to reducing habitats. Gaining a handle on these issues seems particularly pertinent considering some members of the small-sized Bathymodiolinae appear to branch basally within the subfamily (e.g. *Benthomodiolus*, *Vulcanidas*, *Lignomodiolus* and *Nypamodiolus*, Thubaut et al. 2013b), with obvious evolutionary implications. The suggestion that both *Idas* and *Adipicola* represent analogous genera to a *Bathymodiolus* ancestral state in terms of habitat use ("wooden steps" hypothesis, Distel et al. 2000), has since been tested more rigorously as sequence data amasses. For the most part, such a hypothesis remains plausible from a genetic point of view, with only a few examples which don't fit the proposed wood-whale-vent evolutionary progression, considered atypical habitat reversals (e.g. *I. macdonaldi*, *A*. [*s.l*]*. pacifica*). Their appropriateness as analogous species is sometimes called into question, based on the equivalent evolutionary position they adopt in most phylogenetic trees for the subfamily (Dubilier et al. 2008). Despite this, they present less-integrated, but more diverse symbioses and conversely, a greater flexibility in habitat suitability, which suggests that they represent less-specialised lineages. Developmental traits that permit the efficient colonisation of organic falls and their exploitation remain mysterious. What is more, the reproductive kinetics and ecology underpinning organic fall habitats are still extrapolated from either shallow water equivalents or "end-state" species of *Bathymodiolus*, restricted to highly specific vent habitats.

1-9 The hypothesised bathymodiolin lifecycle

1-9.1. What is known

Lorion et al. 2013 identified that bathymodiolin mussels (*s.l*.) at deep vents (particularly the *Bathymodiolus sensu stricto* clade) are end-state species with regard to habitat use. In other words they have reached an "evolutionary dead end". In light of this, it's seems counter-intuitive to back-cast biological assertions about the remainder of the subfamily from the described biology of these highly-derivative evolutionary states. Yet this remains the only course of action in the face of a 'data drought': much of the information gathered which follows is, by default, derived from these sorts of end-state species. Much about the bathymodiolin lifecycle remains inferred, no more so than in small-sized species. Both highly variable and notably conservative traits have been identified in several members of *Bathymodiolus* and *Idas*. Details identified for the subfamily (or at least inferred from the literature) are summarised below. A schematic is also

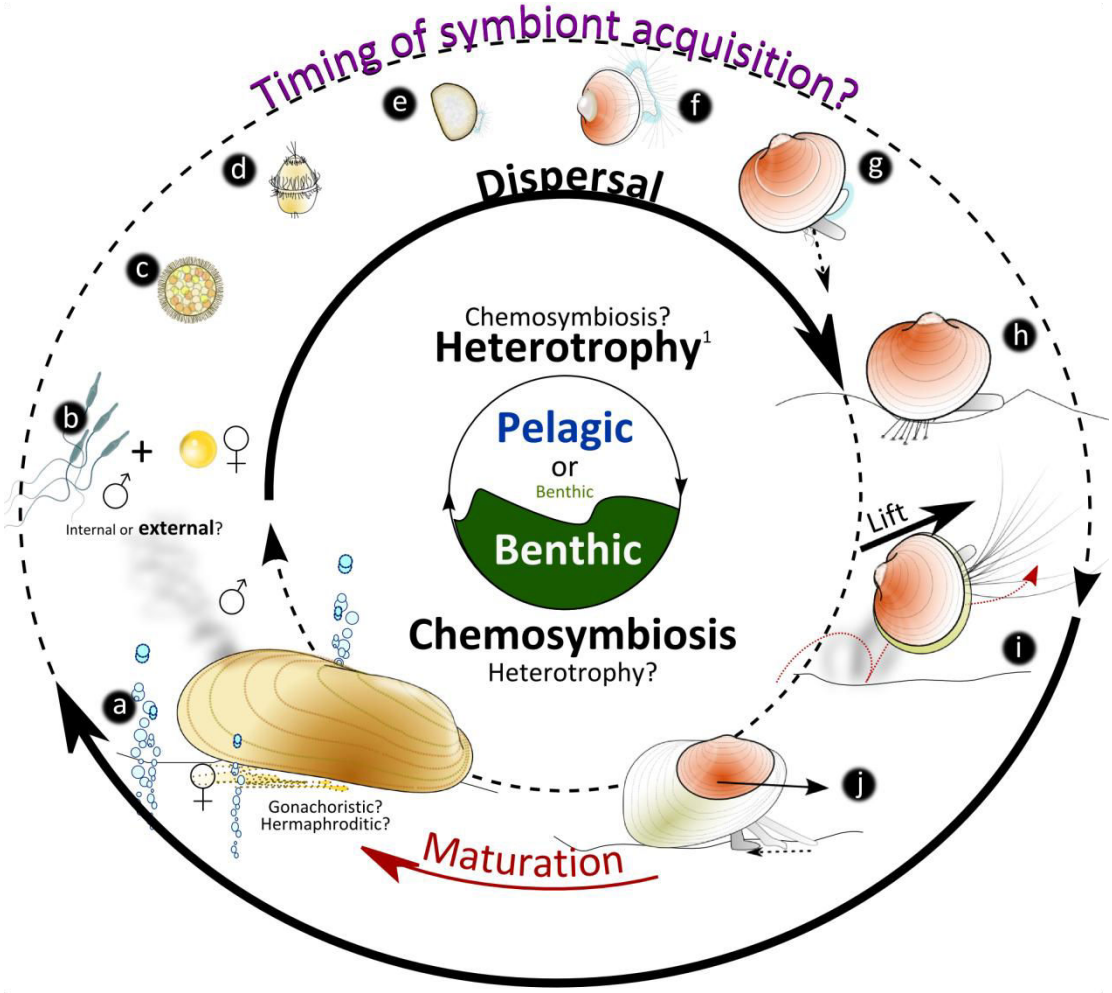

Figure 1.23 Hypothetical lifecycle of a Bathymodiolin
Cycle is inferred based on analogous species from shallow water and on limited data for Bathymodiolinae. a) Spawning. b) Fertilisation is probably external. c) Embryonic development as a morula. d) Gastrulation leads to formation of trochophore larva with two depressions: one is the gastropore, coupled with ciliary bands in a simple alimentary system; the other forms the origin of the shell field, at the gastrula's animal pole. e) The organic matrix of the shell field extends ventrally around the late trochophore from the dorsal hinge line, and ultimately becomes calcified, as it encloses the larva forming the straight-hinged "D" veliger stage. The prototroch develops to form the ciliated velum, for locomotion and filter-feeding. f) The development of the prodissoconch II (in red) in a veliger larva represents the period of greatest planktotrophic growth (1: Planktotrophic or lecithotrophic). g) Competent to settle: the velum regresses, as filter-feeding and locomotion are adopted by the developing gills and foot respectively. The pediveliger probably responds rapidly to a positive cue for suitable habitat. h) Initial attachment to substratum. i) Dissoconch development begins (yellow shell margin). In some shallow-water species, byssus drifting is a means for pediveligers/plantigrades to relocate using bottom currents (not yet documented in deep-sea mussel species). j) Some level of mobility is likely retained in developing juvenile mussels prior to maturation. Schematic created by S.R. Laming

presented in order to delineate the main junctures in the development of a generic bathymodiolin mussel (Figure 1.23). Some of these stages remain fundamentally hypothetical for deep-sea species.

1-9.2. Adult reproductive biology

1-9.2.1 Sex determination and ratios (Figure 1.23a)

One of the attributes which has been better documented in small-sized chemosymbiotic is their tendency towards protandric hermaphroditism, identified in all four species examined to date, of which three were *Idas* spp. (the seep and wood-based mussel *I. modiolaeformis*, Gaudron et al. 2012; the bone-colonising mussel *I. washingtonius,* Tyler et al. 2009; the wood-colonising, asymbiotic, larviphage *I. argenteus*,

Ockelmann and Dinesen 2011 and; the bone colonising mussel *A.* (*s.l.*) *pacifica*, Kinoshita et al. 2010). Sex determination appears to be one area within which the genera differ, since larger Bathymodiolus species are often gonochoristic, including *B. azoricus; B. childressi; B. mauritanicus* and *B. puteoserpentis* (Duperron et al. 2013). Reports can be conflicting however, as Comtet et al. 1999 suggest that *B. azoricus* might exhibit hermaphroditism, while in *B.* (*s.s.*) *thermophilus,* which was initially reported as protandric on the MAR (at 11°N to 13°N) as part of the species description (Kenk and Wilson 1985), has since been reported as gonochoristic based on specimens from the Galapagos rift and 13°N (Pennec and Beninger 1997). This study also identified *B. puteoserpentis* to be gonochoristic*,* while *B. elongatus* shows evidence of protandry (immature oocytes identified along the periphery of male acini). Thus for the smaller-sized species, at least from the genera *Idas* (s.s.) and *Adipicola* (s.l.), protandric hermaphroditism appears to be the norm, while in *Bathymodiolus* spp., both sequential hermaphroditism (apparently exclusively protandric) and gonochorism have been identified, where both processes of sex determination may exist within a single species (or an unidentified species complex), perhaps as a consequence of environmental drivers: the processes which determine the timing of sex-switching are not known, though they are thought to be epigenetic in origin (Tyler et al. 2009; Gaudron et al. 2012).

1-9.2.2 Spawning behaviour (Figure 1.23a)

The most thoroughly studied species in the Bathymodiolinae are *B*. (s.l.) *childressi*, a mixotrophic species (i.e. employing filter feeding and chemosymbiosis, Page et al. 1990) which harbours methanotrophs exclusively (Childress et al. 1986), residing at hydrocarbon seeps occurring on the Louisiana slope in the Gulf of Mexico, and *B*. (*s.s.*) *azoricus*, another mixotrophic (Riou et al. 2010a) species harbouring a dual thio-methanotrophic symbioses (Fiala-Médioni et al. 2002), on the MAR (e.g. Menez Gwen and Lucky Strike vent fields).

Gametogenesis, spawning behaviour, and aspects of larval development have been examined in *B*. (s.l.) *childressi* (Eckelbarger and Young 1999; Tyler et al. 2007; Arellano and Young 2009). The species is known to be gonochoristic, with gametogenic evidence to support a seasonal reproductive cycle (with spawning occurring sometime between December and March) and planktotrophic larvae that appear to be teloplanic (Arellano and Young 2009; Arellano et al. 2014). Gonads contained acini surrounded by inter-acinal tissue composed of adipogranular cells (Eckelbarger and Young 1999), whose nutritive function suggests non-continuous spawning (Le Pennec and Beninger 2000). A temporal sampling regime coupled with histological analysis established that, despite a continuous energy source in the form of methanotrophic chemosymbiosis, gametogenesis was seasonal at the Brine Pool (650 m depth, Louisiana slope). Gamete development between females and males was synchronous, with spawning beginning as early as November and continuing as far as February, with gametogenesis beginning in earnest again in March with evidence of oocyte growth and spermatozoan proliferation (Tyler et al. 2007). The shallower vent species B. (s.s.) azoricus on the MAR also displays seasonality in gamete production (Comtet et al. 1999), based on skewed

population structure (Comtet and Desbruyeres 1998), and analyses of seasonally collected samples from Menez Gwen (Colaço et al. 2006), which revealed not only that spawning was an annual event (at a slightly latter time to *B.* (*s.l.*) *childressi*, in January), but that mussels maintained this spawning regime in aquaria (a trend mirrored in spermatogenesis in this species, Kádár et al. 2006). In the bone-colonising species *Idas washingtonius*, gametogenesis also appears to be non-continuous, though gametogenic patterns were not so obviously synchronised across all individuals, such that a putative spawning period from March to April could be identified (Tyler et al. 2009). Spawning patterns remain unresolved for most other species on account of a lack of intra-annual temporal sampling, however there is strong evidence for discontinuous spermatogenesis in *B.* (*s.s.*) *puteoserpentis* and *B.* (*s.s.*) *elongatus* (Pennec and Beninger 1997) and a readiness to spawn in November in *Idas modiolaeformis* (only mature gametes were identifiable regardless of sex), from the central pockmarks methane-seepage region of the NDSF, eastern Mediterranean.

Periodicity in gametogenesis is unexpected in species where food supply is assumed to be continuous, particularly in light of the fact that many deep-sea species are continuous spawners (Tyler et al. 2007) with the exception of some species on continental slopes with planktotrophic larvae (Young 2003). However, some bathymodiolin species are known to be capable of mixotrophy, supplementing a chemosymbiotic diet with filter-feeding; this may explain their tendency towards seasonal spawning (Le Pennec and Beninger 2000; Dixon et al. 2006), since chemosymbiotic nutrition is likely to be nitrogen limited (Tyler et al. 2007). It's also possible that phylogenetic constraints act upon any capacity for alterations to gametogenic processes in the family Mytilidae (Eckelbarger and Young 1999), such that deep-sea mytilid spawning behaviour mirrors that of littoral species, considered by some to be analogous to the hypothesised ancestral origins of deep-sea mytilids.

That said caution is needed when extrapolating spawning behaviour and developmental trends from related species. In the genus *Mytilus*, astounding variability in reproductive traits exists even within a single habitat. For example, along the west coast of America in regions where *M. californianus* and *M. edulis* co-occur, their reproductive and morphological characteristics are surprisingly different (Suchanek 1981; Seed and Suchanek 1992). *M. californianus* is a bigger, more robust, but slower growing mussel, better placed to deter predators effectively and is a better competitor for spatial resources. It actually spawns continually with a considerably reduced reproductive output (Suchanek 1981). Comparatively, *M. edulis* is a typical "fugitive species" which rarely attains its maximum known size, maturing early and engaging in a single high-volume reproductive event per year (Suchanek 1981).

1-9.2.3 Fecundity and gamete characteristics (Figure 1.23b)

Reproductive and larval biology are well-documented for a number of commercially valuable species within fisheries (e.g. *Mytilus* spp., *Crassostrea gigas*, *Ostrea edulis*, *Cerastoderma edule*) providing useful analogues for relatively poorly studied deep-sea species, in terms of reproduction and larval biology. Of these families, the Mytilidae have in the form of the genus *Mytilus*, one of the more extensively studied

groups of marine benthic invertebrates, providing a useful analogue for deep-sea mytilid biology. Fertilization is external with mature oocytes around 60 – 90 µm in diameter, with optimal conditions being from 5–22 °C and at salinities of 15 - 40psu (Lutz and Kennish 1992). As in many molluscs, fecundity and reproductive effort increase with mounting age and size (though usually with an upper limit), as energy is invested mainly in growth as a young adult. Reproductive output tends to vary from year to year on account of temperature, resource availability and emersion.

In the deep-sea mytilids, limited data is available on fecundity and live-egg characteristics (as opposed to histological inferences), because of the invested effort required to keep deep-sea chemosymbiotic mytilids alive, both in terms of nutritional requirements and the physiological consequences for relocating these organisms to ambient conditions in the aquarium, which inevitably differ markedly from their local habitat. Fecundity appears to be 'high' in *Bathymodiolus* (*s.l.*) spp. (Tyler and Young 1999) although such qualitative assertions are not quantified in any published literature. In *I.* (*s.s.*) *washingtonius* fecundity is also described as being high (Tyler et al. 2009), however, once again no actual value per individual is provided and it's not clear whether this can be derived from data available in this paper. The asymbiotic species *I.* (*s.l.*) *argenteus*, during a wood-deployment experiment examining temporal trends in growth, one female at 5.26 mm shell length was identified to have around 3000 eggs at various stages of development (Dean 1993), suggesting quite high reproductive output for a relatively small organism.

In reality, 'high' fecundity is often inferred based on the bathymodiolin (and mytilid) predisposition for producing small oocytes. Egg diameters are cited in several *Bathymodiolus* (*s.l.*) spp. as ranging from 50 – 90 µm in the species *B.* (*s.s.*) *thermophilus*, *B.* (*s.s.*) *puteoserpentis*, *B.* (*s.s.*) *azoricus*, *B.* (*s.s.*) *elongatus* and *B.* (*s.l.*) *childressi* (Berg Jr 1985; Hessler et al. 1988; Pennec and Beninger 1997; Comtet and Desbruyeres 1998; Colaço et al. 2006; Arellano and Young 2009), while egg diameters are smaller again in *Idas* spp., being exclusively <50 µm in both *I. modiolaeformis* and *I. washingtonius* (Tyler et al. 2009; Gaudron et al. 2012). In both cases, these oocyte diameters are considered too small to contain the energetic yolk reserve necessary for lecithotrophy (Lutz et al. 1980), leading to the conclusion that larval development is likely to be planktotrophic in the *Bathymodiolus* (*s.l.*) spp. and *Idas* spp. examined.

1-9.3. Larval development

1-9.3.1 Fertilisation and early embryonic development (Figure 1.23b–c)

Following gamete release, fertilisation is presumed to be external (Figure 1.23b, Seed 1969) as in shallow water relatives; however no data confirming this one way or another is published. Cleavage is spiral in other Mytilidae, mirrored in those bathymodiolin species in which cleavage has been witnessed (e.g. Arellano and Young 2009). The rate of cell division and embryonic growth appears to be retarded in Bathymodiolinae (taking approximately twice as long) based on the limited data available (*B.* (*s.l.*) *childressi*, Arellano and

Young 2009), where it was only after 40 h that embryos had reached the hatched blastula stage (Figure 1.23c) in *B*. (*s.l*.) *childressi*.

1-9.3.2 Late embryonic and larval development

Subsequent to hatching, *B*. (*s.l*.) *childressi* (Arellano and Young 2009) took a period of about 7 days at 7–8 °C for embryos to pass through the relatively brief trochophore stage (Figure 1.23d), develop a velum and secrete a larval shell and, at 8 days, become straight-hinged, 'D'-shell veligers (Figure 1.23e). The trochophore was typical of mytilids: a multi-cellular embryo with a lateral perimeter of ciliary bands (the metatroch and prototroch). These cilia deliver food to the gastropore (a rudimentary mouth formed through gastrulation by invagination) and aid in locomotion (Bayne 1971). An opposing depression formed the site of shell field development: the origin of the larval shell. The shell field evaginated and extended ventrally from its dorsal origin around the embryo as it developed. This organic matrix folded to the right and left of the dorsal line, along which the two segments were connected by the thickened larval hinge-line (Arellano and Young 2009). The complete enclosure of the embryo by the shell field, once calcified, marked the formation of the two shell valves, and thus the D-shell veliger (Figure 1.23e). The prototroch was displaced by these valves ventrally, forming the velum. This ciliated circular membrane extended out from between the ventral shell margins, with an inner band of long cilia and a peripheral band of short cilia adorning the thickened margin of this "wing-like" structure. The former cilia aid in propulsion and both bands act as a basic conveyer mechanism for filter feeding (Bayne 1976). It was at this stage that larval development halted in the study by Arellano and Young (2009). However, typical mytilid development probably ensues at similarly retarded rates to those of early development (Bayne 1971). The larva typically increases considerably in size as a veliger, after which time a muscular larval foot begins to develop (Bayne 1971; Figure 1.23f). In Bathymodiolinae, veliger larvae probably use energy derived from filter-feeding (i.e. planktotrophy, Lutz et al. 1980). This last larval stage, the pediveliger, can then either continue to swim using its velum (which is eventually reabsorbed) or crawl using its developing foot, subsequent to initial settlement (larvae are thought to respond strongly to local settlement cues, descending rapidly, Figure 1.23g). While the veliger has relatively basic anatomical requirements as a pelagic dispersal phase, i.e. swimming apparatus, feeding apparatus and a shell for protection, the needs of the pediveliger phase are, by comparison, much more complex. It must select and colonise a suitable habitat and then make the physiologically demanding transition towards a benthic mode of life. This is reflected in the array of sensory and nervous anatomy already evident in pediveligers, and the presence of the main components of the gill-palp feeding assemblage (Figure 1.24). Thus by this stage, pediveligers already possess many juvenile characteristics including a muscular larval foot, photosensitive eye-spots, a fully developed mouth, oesophagus, stomach, intestine and anus.

Pediveligers select substrates on which to settle, and secrete byssal threads for attachment, undergoing post-larval metamorphosis to become settled plantigrades (Figure 1.23h). A process termed bysso-pelagic

drifting or casting (Figure 1.23i) is known to occur in some post-larval mytilids, where a network of byssus monofilaments form a 'sail' with which pediveligers can continue to disperse within the substratum-seawater boundary layer (Lane et al. 1985). These simple monofilaments are distinct both in their form and function, from the attachment threads of settled individuals (Lane et al. 1985). Bysso-pelagic drifting permits two phases of settlement following dispersal: the pediveligers first settle on filamentous substrates (adult conspecifics being notably absent) and then navigate to more 'permanent' substrata after a period of post-larval growth (up to 2 mm shell length in this species, Lane et al. 1985; Figure 1.23g–i, minus the filamentous substratum). Such behavioural features in deep-sea mytilid species settling within spatially constrained reducing habitats might prove pertinent since intraspecific larviphagi in adult bathymodiolins has been reported (unpublished observations in Comtet and Desbruyeres 1998), and is known to influence settlement mortality rates in shallow water mytilids when it occurs (Porri et al. 2008; *but* Tamburri et al. 2007). The propensity for this behaviour in deep-sea mytilids is however, unknown, though some juvenile traits, such as settlement on adult shells (e.g. *Bathymodiolus* (s.s.) spp. at Lucky Strike vent field, MAR) are mirrored in *M. edulis*.

In *Mytilus edulis*, planktotrophic larval development can pass through to the pediveliger stage (Figure 1.23g) in less than twenty days though mean time-to-competency is closer to 30 days (at 10 °C). A delay in metamorphosis can perhaps double this period, when substrate availability is poor (Bayne 1976). The development in *B*. (*s.l*.) *childressi* is also believed to be planktotrophic, though feeding behaviour was not witnessed during the aforementioned culturing experiment (Arellano and Young 2009). The frankly astounding larval pediveliger sizes identified to be those of *B*. (*s.l*.) *childressi* in Arellano et al. 2014, suggests a lengthy planktotrophic development for this species, corroborated by the considerable disparity between sizes at hatching (approximated by the size of the prodissoconch I <100 µm) and the size of the pediveliger shell (prodissoconch II, can be >600 µm).

Such differences in hatching and settling sizes (and consequently, larval tissue volumes) is a recurring theme in other members of the subfamily, where pediveliger size has been inferred from plantigrade sizes and prodissoconch II shell margins that persist in adults. The pacific hydrothermal vent mussel, *Bathymodiolus* (*s.s*.) *thermophilus*, the bivalve species characteristic of the EPR, has a larval prodissoconch I (PI) of between 95 and 110 µm in length with indeterminate granular sculpture and a prodissoconch II (PII) of 400–470 µm where incremental concentric growth can be identified (Lutz et al. 1980; Berg Jr 1985). These larval shell characteristics are indicative of planktotrophic development (Lutz et al. 1980). The minute hatching size (PI) prohibits the presence of a large yolk reserve, which necessitates the need to assimilate externally acquired food in order to meet energy demands required to sustain considerable period of growth, evidenced in the markedly larger PII.

In fact, planktotrophy is inferred in several species of *Bathymodiolus* (s.l.) based on these criteria including the aforementioned *B*. (*s.l*.) *childressi* (Arellano and Young 2009), *B.* (*s.s*.) *thermophilus* (Berg Jr

1985; Craddock et al. 1995a) and *B.* (*s.s.*) *antarcticus* by inference (Johnson et al. 2013); *B.* (*s.s.*) *azoricus,* (Colaço et al. 2006); *B.* (*s.l.*) *securiformis* and *B.* (*s.l.*) *hirtus* (Okutani et al. 2004); *B.* (*s.s.*) *heckerae*, (Gustafson and Lutz 1994; Salerno et al. 2005) and is suggested for *B. mauritanicus* by Arellano et al. (2014). Studied species in the genus *Idas* (*s.l.*) display similar trends, such as in the holotype *Idas* (*de facto*) *argenteus*, Ockelmann and Dinesen 2011; the seep and wood-fall mussel *Idas* (s.s.) *modiolaeformis*, (Gaudron et al. 2012); the seep, whale and wood-fall mussel *Idas* (*s.l.*) *simpsoni* ("*Idas* nov. sp., Marmara", Ritt et al. 2012) and the wood-colonising *Idas*[14] (*s.s.*) *iwaotakii,* (Thubaut et al. 2013a). Similar prodissoconch II sizes are documented in several Mediterranean *Idas* (*s.l.*) morphospecies, however their status as species has yet to be confirmed using molecular analyses (also prodissoconch I sizes were not investigated, Giusti et al. 2012). Finally the sole member of the genus *Tamu*, *Tamu fisheri* also displays similar morphometric characteristics (Gustafson and Lutz 1994; Gustafson et al. 1998).

No exceptions are known, suggesting that planktotrophy is a universal and probably ancestral trait in the Bathymodiolinae (*s.l.*), supported by current paleontological evidence (e.g. *B.* (*s.l.*) *satsopensis* and other examples, Kiel and Amano 2013). The apparent ancestral origins of planktotrophic larval development is of potential importance for the evolutionary history of bathymodiolins (Kiel and Amano 2013) since this trait is restricted for the most part to this group of chemosymbiotic bivalves (accepting certain tropical lucinids, Glover and Taylor 2007), wherein lecithotrophic development is the most ubiquitous larval mode in the Solemyidae, Nucinellidae, Thyasiridae, Lucinidae, and Vesicomyidae (Gustafson and Reid 1988; von Cosel et al. 2001; Oliver et al. 2002; Krylova et al. 2010).

1-9.3.3 Dispersal: larval modes

Planktotrophy furnishes larvae with the potential for an extended planktonic larval duration (Gustafson and Lutz 1994; Arellano and Young 2009) and thus extended transport times (such as that for *B.* (*s.l.*) *childressi*, estimated to be from 13 – 17 months, Arellano and Young 2009). As has already been discussed, while this does not guarantee long dispersal ranges (Shanks 2009), studies examining connectivity and the establishment of vent communities on nascent habitat provide indirect evidence that such far-reaching dispersal likely takes place (Craddock et al. 1995a; Vrijenhoek 1997; Won et al. 2003a; Plouviez et al. 2009; Mullineaux et al. 2010). While planktotrophy is apparently widespread in the subfamily, the dispersal environment may be more variable, not least because of the widely different environmental regimes that impact on vents, seeps, and organic falls. For example, only limited data exists identifying the relative importance of pelagic and demersal larval modes in the Bathymodiolinae, however both have been proposed. Larvae in this subfamily are thought to disperse in sub-surface waters following a relatively brief period of ascent (one of many principal assumptions for dispersal modelling for example), however little

[14] currently considered *A*. [*s.l.*] *iwaotakii* on the WORMS database (accessed 20/07/2014)

data exists to validate this assertion, though it is reasonable. It appears that for *B.* (*s.l.*) *childressi* dispersal is likely to be pelagic within the top 100m of the water column, based on rare occurrences of larvae in this area above seeps in the Gulf of Mexico, and experiments that demonstrated this to be perfectly feasible, physiologically (Arellano and Young 2011; Arellano et al. 2014). However the larvae of *B*. (*s.s*.) *azoricus* have been suggested to be demersal (Trask and Van Dover 1999), though this is based on potentially confounded stable isotopes data. Much has since been established concerning the mixotrophic nature of adults of the species, which calls some of the nutritional reasoning in this paper into question. Evidently this is an area which requires further investigation, though such studies are challenging to perform in these environments.

1-9.3.4 Settlement

Information regarding the processes that determine habitat selectivity in competent larvae has been examined earlier in this chapter, but suffices to say, a great deal remains speculative concerning which environmental cues most influence the timing of larval settlement in deep-sea chemosymbiotic bivalves generally, while rates of mortality during this critical juncture in the lifecycle are not evaluated. Given some of the developmental similarities between shallow-water mytilids and their deep-sea counterparts, it is likely that at least some of the scenarios known to induce settling in shallow-water mytilid pediveligers, probably reflect larval selectivity in reducing habitats. In coastal species from several phyla, it has become evident that rather than being stochastic, larval settlement and metamorphosis are driven both by chemosensory recognition of morphogenic and regulatory molecules in the environment (Morse 1990), and larval behaviour which they use to mediate their position in the water column. The rare accounts of larval development chemosymbiotic bivalves focus on specific shallow-water species for which the entire development is known in detail. The settled plantigrade developmental stage of the lucinid clam *Codakia orbicularis*, whose entire developmental pathway differs considerably from that of mytilids, is heavily influenced by the presence of suitable sandy habitat to complete metamorphosis into a juvenile (Gros et al. 1997). Since the sand was sterilised, the presence of biological cues was however, non-essential (e.g. biofilms, seagrass exudates or potential symbionts). However, since the absence of sandy habitat enforces a delay in metamorphosis in this species, it also directly affects the progression of gill development. Since the gills harbour thiotrophic symbionts in adults, this is thought to have negative fitness consequences for the developing plantigrades.

In shallow-water commercial mytilid species (the mussels most studied) interactions with the physical environment appear to be the most influential, both in terms of current flow and substratum characteristics (e.g. *Mytilus* spp. Pernet et al. 2003). Compound effects with two or more factors are frequently observed. Variable boundary-layer hydrodynamics and either algal waterborne compounds (Dobretsov and Wahl 2008) or elevated oxygen levels (Alfaro 2005) can induce unambiguously positive larval responses. In both cases, sustained reduced-flow regimes increased larval mortality but permitted larvae to act on settlement ‘preferences’, while high-flow regimes hampered re-settlement processes. The

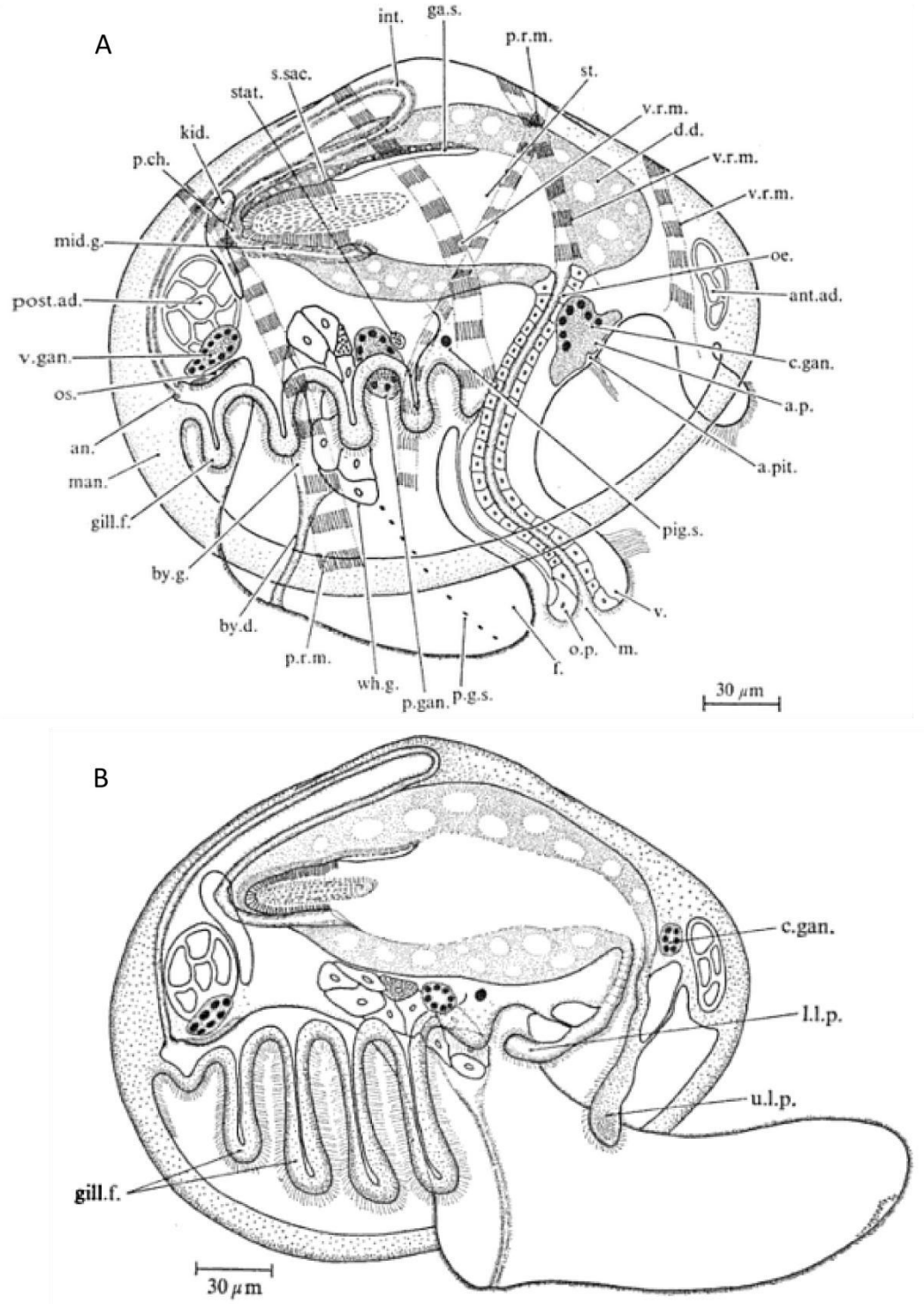

Figure 1.24 A diagrammatic comparison of pediveliger to plantigrade morphoanatomy

A) Diagrammatic reconstruction of pediveliger of *Mytilus edulis.* an *anus,* ant.ad *anterior adductor muscle,* a.p apical plate, a.pit apical pit, by.d byssus duct, by.g byssus gland, c.gan, cerebral ganglion, d.d. didestive diverticulum, f foot, ga.s gastric shield, gill.f gill filaments, int. Intestine, kid. kidney, m. mouth, man. mantle, mid.gut midgut, oe. oesophagus, o.p oral palp, os. osphradium, p.ch posterior chamber of mid-gut, p.gan pedal ganglion, p.g.s. secretions of the phenol (or purple) gland, pig.s pigment spot, post.ad posterior adductor muscle, p.r.m pedal retractor muscle, s.sac style sac, st. stomach, stat. statocyst, v. velum, v.gan visceral ganglion, v.r.m. velar retractor muscle, wh.g white gland. **B)** Diagrammatic reconstruction of plantigrade (post-larva) of *Mytilus edulis* before dissoconch deposition. c.gan cerebral ganglion, gill.f gill filaments, l.l.p lower labial palp , upper labial palp u.l.p . From Bayne (1971).

latter is of particular importance in *M. edulis*, *M. trossulus*, and *Perna canaliculus* (Hunt and Scheibling 1996; Gribben et al. 2011). Gregarious settlement is well known in some coastal benthic species, however its role in mussel settlement is unclear. The biochemical environment can also strongly influence settlement, through the presence of particular biofilms (Ganesan et al. 2010; Wang et al. 2012; Yang et al. 2013), the presence of specific algae or their exudates (e.g. *P. canaliculus* (Alfaro et al. 2006; *M. galloprovincialis,* Yang et al. 2007), or an interactive effect between both algae and biofilms (Dobretsov 1999). Interactive effects have also been identified for the presence of a certain biogenic substratum (e.g. filamentous substrate) and food (microorganisms, Dobretsov and Wahl 2001). Rather more bizarrely some mussels, particularly biofouling species, have been shown to respond positively to sound (or vibrations) at a frequency typical of a large ocean-going vessel (Wilkens et al. 2012). Neurochemical signalling is probably involved in mediating behavioural responses during settlement since the use of targeted neuroactive compounds (e.g. neurotransmitters) have also been extremely effective in inducing (Alfaro et al. 2011; Sánchez-Lazo and Martínez-Pita 2012) and blocking (Yang et al. 2011) settlement behaviour. Thus, in shallow water species the intensity of flow has an overriding effect upon settlement where some minimum velocity is required for survival (presumably for the delivery of oxygen and food) but high flow regimes have

a constricting effect on attempts to resettle after initial contact- i.e. if currents are excessive, larvae will not even attempt to relocate. If mediating their position is possible, larvae appear to respond to an array of cues to maximise their survival. At reducing environments in the deep sea however, the intensities of flow regimes are probably not in the same order of magnitude, and chemical cueing may be a more dominant feature, given the vast array of signature chemicals available to larvae approaching suitable habitat.

In addition to the effects of physical dispersal into unsuitable habitat, several overarching biological and physical factors act to reduce the number of larvae that settle and eventually recruit into the adult population, due to predator/prey interactions, availability of settlement habitat, and post-settlement survival (Cowen and Sponaugle 2009; refer to Figure 1.15). This is particularly relevant where density-dependent biological interactions operate, such as might occur in highly productive reducing environments, e.g. Lenihan et al. 2008). These processes are all thought to be impacted upon by larval condition at time of settlement (i.e. carry-over effects, but also the larval age at the time of settlement, Gribben et al. 2006).

1-9.4. Post-larval development

1-9.4.1 Metamorphosis and post-settlement processes

There is a singular lack of information regarding the dynamics of metamorphosis in deep-sea mussels. However, some classical work has been carried out in great detail in *Mytilus* spp. (particularly *M. edulis*). Following successful identification of and settlement upon suitable habitat, several metamorphic biological processes are initiated in the larvae after which the organism is believed to be committed to their completion physiologically. At the time of settling as a pediveliger, much in the way of its future anatomy has already developed (Figure 1.24). This includes most of the alimentary, excretory and central nervous

systems: for example, a single digestive diverticulum communicates with the stomach along its anterior length in pediveligers (Bayne 1971) and following metamorphosis, the mussel's central nervous system already resembles the adult arrangement of ganglia (Voronezhskaya et al. 2008). A metamorphic rearrangement of organs within the mantle cavity takes place to accommodate new levels of complexity in post-larval anatomy and a more organised feeding mechanism (Bayne 1971; 1976, Figure 1.24a & b). Thus, several morphological changes occur following primary settlement and byssus secretion. The first is the rather rapid loss (e.g. within 48 hours after byssus secretion in *M. edulis*, Bayne 1971) of the velum and the network of muscles that support it, where some structures are adsorbed and others are re-orientated. The apical plate becomes part of the upper labial palps, and the mouth and oesophagus are retained, migrating dorso-anteriorly (Bayne 1971). The prior functional role of the velum is adopted instead by the ciliated, lower and upper labial-palps that form in pairs either side of the mouth, during this time. These will ultimately process inhalant particles transported to the buccal region by coordinated ciliary activity in the gills. However, at the very early stages of post-larval development, the movement of cilia along the ventral surface of the foot also plays an important role in particle transport (Bayne 1976). This is a period during which the larvae are particularly susceptible to mortality, as they tend not to feed, are exposed to predation, and are vulnerable to disease since most of their energy is invested in metamorphosis.

In *M. edulis*, pediveligers can delay metamorphosis for up to forty days at 10 °C or for up to six months in some cases (Lutz and Kennish 1992). In fact, delaying settlement and metamorphosis is a common trait in both asymbiotic and chemosymbiotic shallow-water species (e.g. Pechenik 1990; Gros et al. 1997). The extent of the delay is typically constrained by a lack of substrata (Gros et al. 1997) or temperature, with longer delays possible at low temperature (effects of metabolism suppression), after which larvae appear to be less selective of substrata (Marshall and Keough 2003), termed the "desperate-larva hypothesis". Interestingly, although it was not the goal of the study, Satuito et al. (2005) demonstrated that a period of refrigeration in *M. galloprovincialis* only acted to slow development rather than halt it (or kill more larvae), with survivability being comparable to controls even after a 2-month exposure to refrigeration. In the deep-sea, the concepts of physiological competency and an upper time limit for settlement are not defined. However, low temperatures and their effects on larval metabolism are probably not sufficient explanation for the markedly different rates of development seen with increasing habitat depth (particularly in larvae that ascend to the euphotic zone, e.g. *B.* (*s.l.*) *childressi*), suggesting other as-yet-undetermined factors play a role in overcoming the physiological constraints observed in coastal systems.

1-9.4.2 The developmental path to maturity

The period of development from post-larva to adult has received little attention in deep-sea mytilids, while in shallow water species, contemporary studies have examined more detailed aspects of tissue development (e.g. *in situ hybridisation* with *myvlg* to identify expression patterns in primordial germ-line

cells, Obata et al. 2010; or gill development using SEM, Cannuel et al. 2009). Basic developmental biology in shallow-water species has identified that even as plantigrades, the critical organs necessary to function are in place, with the exception of gonads (Figure 1.24b). Organs then increase in size and complexity as the animal develops, such that in adult heterotrophic mussels, the stomach, the mass of digestive diverticula (forming the digestive gland), gills, and gonads represent the majority of soft tissues.

1-9.4.3 Patterns in overall growth

Mytilid growth rates are subject to considerable variability (e.g. *Mytilus* spp. Holt et al. 1998), due to variations in genotype, multilocus heterozygosity (Gosling 1992) and environmental factors in particular (e.g. include temperature, salinity, resource availability, emersion stress, intraspecific competition for space and food, and parasitism). However growth rates can be 30 – 40mm year^{-1} in length (Seed and Suchanek 1992). *Mytilus* spp. are gregarious, and in situations where larval supply is non-limiting they can form large beds or reefs (Bayne 1976). At high densities *Mytilus* beds become layered, with juvenile mussels taking advantage of the spatial heterogeneity of adult shells and thus further increasing it. The consequence is that the bed continually grows and develops with new recruits eventually replacing those being smothering by faecal waste beneath. These reefs provide complex niche-dense habitat for other species. Bathymodiolin mussels (particularly members of the genus *Bathymodiolus*) are known to form superficially similar reefs when conditions are optimal in diffuse-flow vent systems and in the vicinity of cold-seeps and methane hydrates (Kiel 2010).

Ontogenetic growth rates have rarely been examined in bathymodiolins (Rhoads et al. 1982; Dean 1993; Nedoncelle et al. 2013). In vent and seep habitats, higher rates of growth (or greater longevity) are suspected, with some evidence in the literature that supports this (e.g. *B.* (*s.s.*) *thermophilus*, Rhoads et al. 1982; Nedoncelle et al. 2013). *A posteriori* backcasting from von Bertalanffy growth curves plotted for adult shell measurements of the vent-based *B.* (*s.s.*) *thermophilus* (Nedoncelle et al. 2013) identified an average growth rate of 4.2 cm year^{-1} for post-larval plantigrades immediately after settlement, assuming a larval settling size of 0.04 cm in *B.* (*s.s.*) *thermophilus*. This rate of growth precipitously decreased so that by 10 years old (growth rate of 1.1 cm year^{-1}), mussels had already attained a length approaching 95% of their theoretical asymptotic maximum size (L_∞ = 21.5 cm, at ≈19 years old). These rates of growth were validated in newly established populations of *B.* (*s.s.*) *thermophilus* settling on nascent basaltic substrata following an eruption (unpublished results from Nedoncelle et al. 2013). Interestingly, microvariability in daily shell increments appear to relate to the circalunidian rhythm of the tidal signal for that part of the Pacific Ocean, perhaps as a consequence of near-bottom tidal current variability (Nedoncelle et al. 2013). This is despite the experiment being performed on mussel beds 2,500+ m deep. Such circalunidian timing in growth rates has also been identified for *B.* (*s.s.*) *brevior* (Schöne and Giere 2005) from the North Fiji Basin.

One other study has examined growth in a bathymodiolin species in the deep-sea, in the apparently asymbiotic, wood-inhabiting *I.* (*s.l.*) *argenteus*. Despite colonising sunken wood, this species

appears to lack chemosynthetic symbionts in its gills (Ockelmann and Dinesen 2011). Its growth did not follow typical developmental growth-rate patterns (e.g. such as von Bertalanffy-type growth), which normally include greatly elevated growth rates in the first year, followed by a steep fall in growth by the second year (Dean 1993). Instead, growth was more diminutive in the first year, where rates saw only a slight decline over the following years (compared over 8 years). Consequently, rates of growth were lower in the first year but higher in later years in *I. argenteus* when compared with other species growth rates, available at the time of publication (fresh-water bivalves: *Lampsilis radiata*, *Anodonta grandis*; marine species *Cerastoderma edule*, *Modiolus modiolus*, *Spisula solidissima*, where one, *M. modiolus* is a mytilid). Individuals of *I.* (*s.l.*) *argenteus* only matured a year after settlement, displaying lower maturation rates than shallow-water species, suggesting a degree of food limitation but greater stability in food supply over time (maintaining growth rates in later years), or a reduced investment in other energetically demanding processes (such as gametogenesis). This follows a pattern established by earlier work (e.g. Grassle and Morse-Porteous 1987) which suggests that congeneric trends in developmental rates in organically enriched environments differ between shallow-water and deep-sea species. Examination of gonads revealed that, following maturity, gamete production was relatively high.

1-9.4.4 Organ development: gills and gonads

Two of the organs in greatest need of rapid development in deep-sea chemosymbiotic mytilids are arguably; a) the gills, since it is within the gills that symbionts will be housed in adults, and; b) the gonads, whose speed of development will define the population's reproductive resilience in the face of habitat ephemerality. Regrettably, these processes remain shrouded in mystery in the Bathymodiolinae, where even age or size at first maturity remains poorly defined (one known example: 40–60 mm in *B.* (*s.s.*) *thermophilus* depending on sex, Berg Jr 1985).

These aspects of growth have been examined in shallow water Mytilidae however. Mytilid gills follow a particular developmental template (e.g. *M. edulis*, Cannuel et al. 2009). A number of gill bars initially develop at the pediveliger stage (Figure 1.24a). Consequently, when larvae first metamorphose into plantigrade post-larvae a very small number of filaments are already present (≈ 4-7), forming a gill "basket" (Bayne 1971; Cannuel et al. 2009; Figure 1.24b, Figure 1.25a). As the mussel grows, gill complexity increases, both structurally and functionally. Structurally, the number of filaments and lamellae increases and each of the elements become bound together as a single organ by the formation of various ciliary junctions (Jørgensen 1990; Cannuel et al. 2009). Simultaneously, the frontal cilia of each filament differentiate into three different forms which have differing roles to play during gill activity (Cannuel et al. 2009). Functionally, there is a transition from aeration only (in very young post-larvae) towards autonomously coordinated 'waves' of cilia, which orchestrate particle transport towards the buccal region, and facilitate aeration and respiration (Jørgensen 1981; Beninger et al. 1995; Cannuel et al. 2009). At some point prior to sexual maturation, a definitive pair of gills is ultimately established. Each gill is composed of

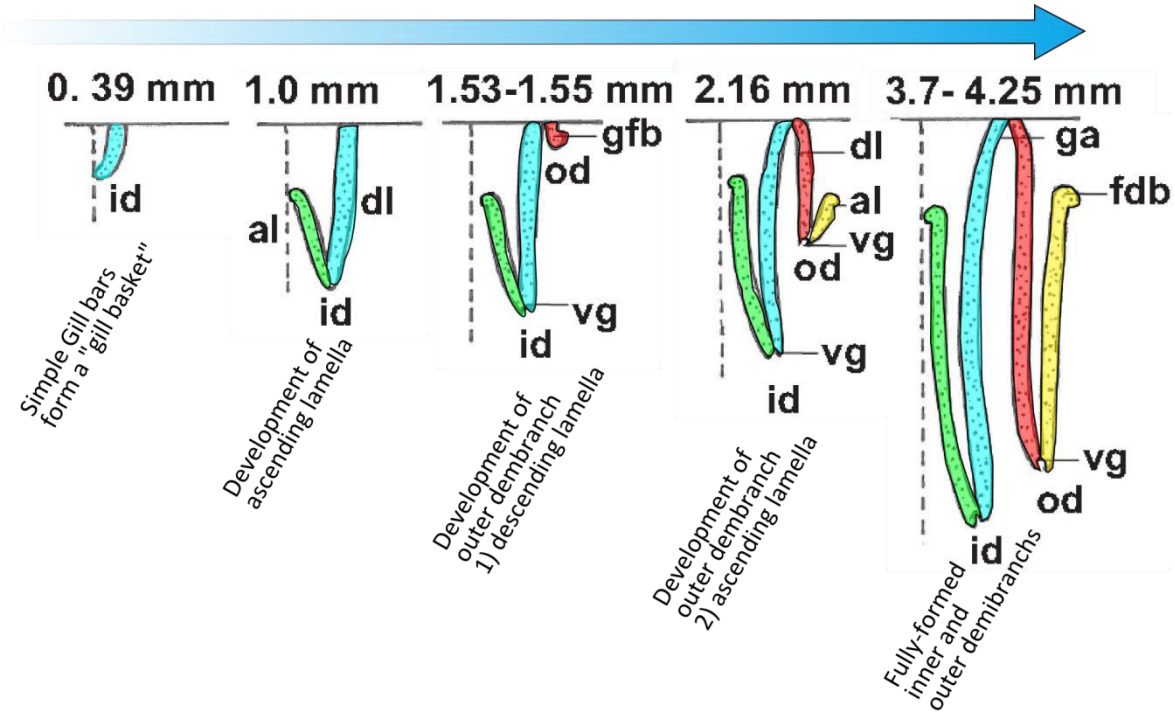

Figure 1.25 Patterns of gill ontogeny in *Mytilus galloprovincialis*
The patterns of development of the gill in *Mytilus edulis.* Following settlement and metamorphosis a simple gill basket is already developed. With increasing size, new filaments are added at the posterior end of the inner demibranch. At < 1mm the second, ascending lamella of the inner demibranch has begun to form. At ≈ 1.53 mm, the descending lamella of the outer demibranch (the outer half of each gill) begins to develop. Finally at just < 2.16 mm, the ultimate ascending lamella of the outer demibranch has begun forming, wherein by 3.7 mm and larger, the gill is fully formed, and filaments continue to proliferate from the posterior budding zone. Dotted line, antero-posterior axis of symmetry. Note process of elongation-reflection in A–D. al, *ascending lamella*; dl, *descending lamella*; fdb, *fused dorsal bend*; ga, *gill arch*; gfb, *gill filament bud*; id, *inner demibranch*; od, *outer demibranch*; vg, *ventral groove*. From Cannuel et al. 2009

an inner – medial – demibranch and an outer – lateral – demibranch, in turn made up of a descending lamella that originates from the gill axis, and an ascending lamella, located along the leading edge of the descending lamella in reflexive juxtaposition (Figure 1.25). The ascending lamellar filaments proliferate in tandem with secondary (not post-larval) descending lamellar filaments, from the distal budding zone of the gill (Cannuel et al. 2009). Cilia eventually begin to form upon the abfrontal filament surfaces as well (i.e. the interior face of each demibranch lamella).

In chemosymbiotic mussels, the gills are thought to develop in much the same way as in shallow water species, though this has not been examined in the Bathymodiolinae. This is an unfortunate oversight, since such a study could also examine symbiotic dynamics in the context of gill development, being predominantly housed in the gills of adult chemosymbiotic mytilids (Wentrup et al. 2013). What is known is that unlike shallow-water species, cilia do not develop on the abfrontal surfaces (Duperron 2010), which are comparatively enlarged by hypertrophy. The increased surface area of non-ciliated epithelia permits the hosting of dense populations of bacteria either inside sub-epithelial bacteriocytes or among microvilli that differentiate from the epithelial surface of bacteriocytes (see previous section for details).

Sexual maturation and the development of gonads in shallow-water mytilids can be traced back to a provision of maternal primordial germ-line cells (PGCs), which are incorporated into oocytes during oogenesis (Obata and Komaru 2012). In developing embryos, maternal germ-line-specific genes are transcribed within specific blastomeres, which ultimately differentiate into PGCs during development (termed PGC preformation, Obata and Komaru 2012). In *M. galloprovincialis*, these have been identified during the juvenile development of the species, tracking their apparent progress towards the ultimate formation of reproductively active tissues (Obata et al. 2010). In small juveniles (< 5 mm), two clumps of germ cells are distributed laterally within connective tissue found between the nephric tubules and posterior byssal retractor muscle (Obata et al. 2010), and along the pericardium. These aggregations appear to proliferate first as the mussels grow, and then migrate anteriorly along the mantle basement. In adults, *Myvlg* expression occurs in various gametogenic stages including spermatogonia, spermatocytes, oogonia, and oocytes, as well as germinal stem cells bordering the acini. In the last case, signals were stronger during the reproductive season (Obata et al. 2010). The preformation of PGCs seems to be a phylogenetically conservative trait in bivalves (Obata and Komaru 2012) suggesting similar patterns are likely to exist in deep-sea mytilids, though this remains to be examined experimentally.

1-9.4.5 Phenotypic plasticity during ontogenetic growth

One of the pervasive traits of coastal mytilids is a certain degree of variability in growth rates and patterns of development, depending on the dynamics of their habitat, on the species, and on the age of the individual. The net result is that shell morphology displays considerable plasticity both intraspecifically across differing habitats and ages (e.g. Seed 1968; Akester and Martel 2000; Peyer et al. 2010), and interspecifically within the same habitat (Suchanek 1981).

Growing evidence in the Bathymodiolinae (*s.l.*) suggests that similar variability in shell morphology exists both within and across species. This is particularly evident in the smaller bathymodiolins (Lorion et al. 2010; Génio et al. 2012) where both cryptic species and monospecific morphospecies are prevalent (Vrijenhoek 2009). Such plasticity probably plays a role in habitat adaptation, such as compensating for constrained space in vacated wooden xylophaginid burrows.

1-10 Overall summary

Despite being bathed in the scientific limelight, deep ocean ecology beyond the continental shelf margins still remains poorly understood (hydrothermal vent systems accepted, Adams et al. 2012). Much has been learned courtesy of the endeavour of early research (e.g. the Challenger expedition, Murray et al. 1891) and the recent advent and rapid development of deep-sea equipment capable of cataloguing the environmental characteristics and ecology of the ocean's depths. However, while these techniques inform us about community-wide biology, distribution and heterogeneity and habitat conditions, they cannot account for

the influence of larval investment, transport and supply upon community dynamics, and the developmental challenges that post-larvae must subsequently face (Levin 2006). As a consequence some of the least studied aspects of deep-sea chemosymbiotic life are the water-borne phases in both the lifecycle of the host and its symbionts, and the earlier juvenile development of the host. In the host this spans the period of embryonic and larval growth to competency and by association, post-larval development prior to maturation. For the bacteria, this involves the free-living stage of their lifecycle which is assumed (and in instances, proven) to exist since they are not obliged to engage in symbiosis, unlike the host in most cases.

The temporal and spatial heterogeneity of physical and biogeochemical environmental parameters characteristic of reducing habitats enforce certain constraints upon the organisms adapted to living in them (discussed in section 1-6.1, p. 28). Reducing habitats are both ephemeral and spatially isolated, but they also provide a rich source of chemical energy, sustaining microbial metabolic processes and in turn, supporting dense aggregations of specialised metazoan life that derive their nutrition directly or secondarily from the abundant microbial assemblages present (Kiel 2010). The key to the success of the chemosymbiotic bivalves that inhabit reducing habitats in the deep-sea is likely to have been a combination of attaining the means to secure symbionts and benefit from their metabolic capabilities, and a predisposition for adaptable life-history traits. Within the chemosymbiotic bivalves it is the Bathymodiolinae (*s.l.*) that contain the greatest symbiont diversity, where examples of multiple chemosymbiotic lineages are frequent. They inhabit a wide range of reducing habitats courtesy of the (predominantly) thiotrophic and/or methanotrophic symbionts that are associated to their gill filaments.

Organisms that engage in bacterial symbioses have evolved to gain maximum benefit from such conditions. However, chemosymbiosis also appears to restrict their distribution to these reducing environments, which are interspersed among vast oligotrophic soft-sediment expanses. Consequently chemosymbiotic organisms must overcome several challenges within their lifecycle in order to persist (discussed in detail in section 1-3, p. 10). According to life-history theory, the spatial and temporal constraints of such reducing habitats (excluding seeps) are likely to favour organisms that mature rapidly, display early age at first maturity (and first reproduction), with high fecundity but minimal energetic investment per egg, short life-spans, and a limited number of reproductive events. Intrinsic to these attributes are elevated offspring mortality rates. Confirming or rejecting such assumptions about their biology requires that the lifecycle of these organisms be examined in full. Adult host biology is reasonably well-documented in select end-state *Bathymodiolus* spp. in the subfamily. With each newly published study, the dynamics of symbiont assemblages in relation to alterations in their chemical environment are becoming better-established. However, as can be seen from the above sections, the integrative 'holobiont' biology requires renewed focus, particularly regarding timing of acquisition, which is thought to be horizontal. Although studies exist which identify the presence of symbionts at various stages of the life cycle, no studies exist that actually identify precisely when it is that bathymodiolin species first become

symbiotic. Symbiont acquisition remains unresolved in nearly all chemosymbiotic species, except in isolated species employing horizontal transmission (e.g. the siboglinid, *Riftia pachyptila* Marsh et al. 2001; *B*. (*s.l*.) *childressi*, Streams et al. 1997; *B.* (*s.s.*) *azoricus*, Salerno et al. 2005; Wentrup et al. 2013; *B.* (*s.s.*) *heckerae*, Salerno et al. 2005; *B*. *puteoserpentis*, Wentrup et al. 2013) and in the vesicomyid and solemyid clams, which appear to inherit their symbionts directly from parental organisms (e.g. Endow and Ohta 1990; Cary and Giovannoni 1993; Cary 1994; Krueger et al. 1996; Szafranski et al. 2014). Thus, in order to fully appreciate the extent to which chemosymbiotic species depend upon these associations, the acquisition and role of symbionts throughout the lifecycle of these organisms must also be established.

More than any other aspect of their biology, details concerning the early developmental life-cycle stages of chemosymbiotic mussels are lacking for nearly all bathymodiolins. Most developmental biology has to be inferred directly from shallow-water species, in the absence of data for the deep sea. Given the degree of variability in life habits within the *Mytilus* genus alone, such extrapolations across more distant clades must be subject to systemic error. Gaps in our knowledge reveal a general lack of understanding regarding the interactive dynamics of development and symbiont acquisition together. Understanding how these prolific species persist in their environment requires that symbiont and host life cycles be considered together for those parts of their lifecycles which are intertwined.

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Chapter 2 SCOPE OF RESEARCH

2-1 Research themes and aims

Since it has been suggested that organic falls in the deep sea may have played a part in the evolutionary transition of many of the species exclusive to deep-sea hydrothermal vents and hydrocarbon 'cold' seeps (e.g. Smith et al. 1989; Distel et al. 2000), the scope of this research was restricted to contemporary species found in association with decomposing vegetal and megafaunal material. The primary goal in developing the research project was to characterise critical junctures in the lifecycle of less-specialised[15] deep-sea chemosymbiotic benthic macrofauna associated with organic falls.

Particular periods in the lifecycle of such organisms are severely lacking across a variety of phyla. The most ubiquitous macrofaunal taxa that routinely settle on organic substrates – distinct from the fauna that colonise the proximal sediments – include several examples of small-sized bathymodiolin (*s.l.*) mussels, and the siboglinid polychaetes. Of these, the bathymodiolins were considered the more suitable model group (discussed below). A *priori* review of the literature identified a dearth of data regarding planktonic and post-larval development, including a near absence of basic biological parameters such as modes of planktonic development, settlement patterns, growth rates, post-larval and juvenile developmental morphoanatomy, and size or age at which juveniles recruit into the adult population (excluding Thubaut et al. 2013a). Given the ephemeral nature of organic falls in the deep sea, these parameters were considered fundamental in describing adaptations of organic-fall chemosymbiotic mytilids to the constraints of their habitat. As nearly all mytilid species from organic-fall communities that have been examined in detail have been shown to engage in symbioses as adults, identifying the role symbionts play during the course of these species' development was equally crucial.

The research presented herein sought to address three principal overlapping themes of organismal biology, which collectively comprise the complex lifecycles of chemosymbiotic mytilids colonising organic falls.

2-1.1. Theme 1: Reproductive biology

2-1.1.1 Research aims

This theme covers the adult period of the lifecycle, from the recruitment into the breeding population onwards. The primary aims of this theme were to describe aspects of reproductive biology recognised to be of particular importance in the context of organic falls. These included spawning patterns (output and frequency); gamete size, as a proxy both for propagule energy investment and larval mode (oocyte diameter) and; size-dependent sex ratios, which might clarify the reproductive 'strategy' being employed. In addition, nutrition in adult specimens would be described within the framework of Theme 3.

[15] In terms of the state of their anatomy and the degree to which symbionts are integrated within the host

2-1.1.2 *A priori* research objectives

To meet the above aim, the research needed to collate newly acquired data on gametogenic trends, sex ratios, fecundity and gamete sizes from live and preserved specimens, and consider these in the context of roughly analogous shallow-water species and in relation to end-state vent and seep fauna.

2-1.2. Theme 2: Developmental biology

2-1.2.1 Research aims

This theme covers the larval, post-larval and juvenile period of the lifecycle, with emphasis on reproductive (linking to Theme 1) and nutritional development (linking to Theme 3) from the moment of gamete fertilisation, up to size/age at first maturity in settled juveniles. It consequently formed the largest part of the envisioned research and was split into larval and post-larval/juvenile subthemes, for increased clarity.

Larval development: The primary aim of this subtheme was first to establish the feasibility of inducing gametes, implementing fertilisation and maintaining the embryos of an aquarium-based deep-sea chemosymbiotic organic-fall species, as they grow and develop into planktonic larvae. If this proved possible, the secondary aims were to examine aspects of early embryonic and larval developmental biology, both alive and in preserved subsamples. These would include embryonic cleavage patterns, rates of growth, larval developmental anatomy during its ontogeny, time until competency to settle, and any behavioural observations that could be performed[16] (under predefined environmental conditions). In addition, feeding behaviour and patterns in symbiotic associations were to be noted within the framework of Theme 3.

Post-larval and juvenile development: The primary aims of this subtheme were to examine larval supply and settlement patterns, shell morphology, and both the detailed and gross anatomical changes taking place in post-larvae right through to pre-adult specimens. Emphasis was to be placed on tracking developing reproductive tissues, in order to characterise the rate at which the chosen taxon might reach sexual maturity, and developing tissues collectively involved in nutrition, within the framework of Theme 3. Though it was not the principal focus of Theme 2, it was hoped that growth rates might be elucidated given the lack of data on growth in deep-sea, organic-fall-associated phyla.

2-1.2.2 *A priori* research objectives

Larval development: To meet the above aims, an ambitious live-animal experimental protocol and customised aquaria were to be designed and developed to accommodate the specific requirements of the chosen organic-fall species, based on what is known of these habitats. This would require deep-sea collection, wet-room facilities, and sample availability to all coincide. Depending on the level of success of this approach, incremental developmental stages were to be subsampled, recorded live and then preserved

[16] No formal plans were made for tracking substrate selection or metamorphosis

for an array of analyses that would best convey the developmental patterns observed. The data that resulted were to be compared with pre-existing data from roughly analogous shallow-water species and in relation to any larval data for end-state vent and seep fauna, be it experimentally acquired or inferred from adult biology.

Post-larval and juvenile development: To meet the above aims, organic larval colonisation devices were either already awaiting sorting, having been deployed for a period in a deep-sea environment, or were deployed during the course of the PhD research and subsequently examined. *N.B. The sorting of pre-existing substrates at the beginning of the PhD ultimately had considerable bearing on the choice of model organism, given that sample availability was an obvious criterion for the research* (detailed in the following section 2-2.1 p. 110 onwards). Specimens of the model organism at various sizes, amassed during substrate sorting (with three predetermined methods for preservation), were first to be measured to detail size-frequency distributions and thus possible patterns in larval supply, based on the presence of surviving post-settlement individuals. Any measurements that could be made on vestigial anatomy of larval origin were to be performed at this stage. Using the multiple sizes available, various aspects of the developing anatomy were to be examined following the embedding and sectioning of either whole animals or select organs if specimen size permitted this. For the formation of reproductive tissues, this would ideally have involved an tracing the migration and proliferation of the germ line into gonad tissues in recently matured adults (thus fusing aims in Theme 1 with this Theme). Size at first maturity was to be assessed based on the smallest size at which gametogenesis was identifiable. A rough minimal average estimate for growth rates was to be determined from substrate deployment length and the maximum specimen size recovered (making some pre-acknowledged assumptions). This entire approach was envisioned to be performed on relatively small organisms, thus requiring the development of techniques for micro-dissection dedicated to the model organism's bauplan, for which little information was available from the literature at the project design stage.

2-1.3. Theme 3: Dynamics of symbiotic and filter-feeding based nutrition

This Theme covers the various means by which nutriments are acquired within the model group's lifecycle. In deep-sea chemosymbiotic faunae that acquire their symbionts as free-living bacteria (or other microbial organisms) from the surrounding environment (i.e. horizontal transmission), ontogenetic variability in the dominant nutritional sources is often postulated, particularly during planktonic dispersal. Therefore, the principal aims sought to collect evidence for shifts in nutritional mode during development. These included identifying the approximate period within the lifecycle at which symbionts are first acquired, the sequence of infection (in terms of localisation and compartmentalisation in tissues, if applicable) within the host, changes in symbiont density (and thus their importance) with ongoing growth, and any observable succession in symbiont phylotypes (host/symbiont mediated nutritional adaptability). In parallel, data that

could indicate alternative means of sustenance other than chemosymbiotically derived nutrition were to be collated in both immature and mature organisms (in collaboration with Themes 2 and 1, respectively).

2-1.3.1 A priori research objectives

To meet these aims, the research would have to combine existing and newly acquired evidence for symbioses at various stages of development and in adulthood, with evidence that implied some level of heterotrophic nutrition (integrating data from Themes 1 and 2). The focus of research would depend upon the model group, since siboglinids are lecithotrophic and chemosymbiotic mytilids are suspected to be planktotrophic (e.g. Lutz et al. 1980; Nussbaumer et al. 2006; Arellano and Young 2009; Arellano et al. 2014). However, essentially, the principal objectives were to use histological and targeted *in situ* hybridisation techniques to identify the presence and distribution of symbionts within the host tissues and infer patterns of phylotype succession, localisation and – using electron microscopy – the degree of integration of symbionts in the host tissues (compartmentalisation), based on how these distributions changed with size of organism. This would require access to a size series of the model organism, both above and below the size at first maturity. Much like Theme 2, samples were to be acquired in the course of sorting preserved colonised substrata from colonisation experiments (small post-settlement specimens) but also longer deployments for larger adult specimens. Availability of post-embryonic and larval samples would depend upon the success of live experiments from Theme 1.

Heterotrophy was either to be witnessed directly in live individuals (i.e. feeding behaviour in larvae, post-larvae and juveniles, and – in mytilids – adults) or inferred. Nutritional inferences would principally be from the ontogenetic or size-specific organisation and state of organs involved in nutrition, such as feeding apparatus and digestive/endocrine tissues, based mainly on observations during specimen dissections and subsequent histological analyses. If specimen availability allowed for it, evidence to support these approaches was to come from chemical analyses of soft tissue, such as wax-esters, fatty-acids and stable-isotopes analysis.

2-2 Studying deep-sea chemosymbiotic lifecycles

2-2.1. Choosing a model taxon

Studying the lifecycle of a species, whose distribution is restricted to deeper depths, continues to be fraught with difficulty. However, advances must be made if deep-sea metazoan developmental biology is to be better understood (Adams et al. 2012). There are two main obstacles which provide the greatest hindrance. The first is the logistical challenge of working under deep-sea conditions characteristic of the larval-source, -transport and -sink environments and the collection and recovery of organisms from these environments at various stages of development. For example, the collection of larvae is severely constrained by the dilution of the larval pool during dispersal, while the collection of benthic lifecycle stages are constrained by high operating pressures, where live recovery often results in sub-lethal to lethal consequences for the organisms retrieved. The second constraint is the recreation of suitable conditions in aquaria (assuming live

animals can be recovered in 'good' health) for the maintenance of multiple lifecycle stages, given the absence of detailed knowledge pertaining to the various environments experienced by organisms throughout its life. Collectively these confound all attempts to study lifecycles in their entirety in living specimens and make informed assertions much more difficult.

2-2.1.1 Keeping it simple

Fortunately, some of the constraints of deep-sea lifecycle studies at organic falls may be circumnavigated by: 1) choosing model organisms with habitat ranges whose shallowest extremes remain below the pressure threshold above which physiological barophilic adaptations or acclimations become obligatory for cellular function and survival (> 100 bar ≈ 985 m, Dixon et al. 2004); 2) employ a sampling strategy that permits the recovery of specimens at multiple stages of development either by time-series sampling of a growing cohort[17], or by examining several cohorts within a single timeframe or better still; 3) incorporating these approaches synergistically.

2-2.1.2 The potential for lifecycle studies in chemosymbiotic organisms

A suitable test organism, which meets the constraints listed, can permit certain questions to be answered that would otherwise be difficult to elucidate, such as those detailed in the research rationale previously (see 2-1, p. 107). From a chemosymbiotic lifecycle perspective, these ought to include aspects of the biology from each stage of development including the role symbionts play therein, so as to truly appreciate the degree to which lifecycles of chemosymbiotic species at reducing environments are adapted to their habitat(s).

If the chosen specimen can be kept alive in the aquarium, it is possible to induce gamete release and so collect data on adult spawning behaviour and timing, processes of gametogenesis, fecundity and fertilisation, and even on larval development. This can yield data on growths rates, developmental patterns, locomotor and feeding performance, the time until competency and thus planktonic larval duration (PLD) under predefined conditions. If competency can be reached, the complex array of behaviours and changes in physiology associated settlement and ultimately metamorphosis itself, may be investigated. In tandem, multiple behavioural aspects of breeding adults can be assessed. Such a study, if performed successfully, can furnish a wealth of data in a brief (but intensive) period of laboratory work.

As an alternative or better still, synergistic approach, studying a range of preserved specimens using standard histological approaches can be highly informative. The availability of multiple sizes (and presumably ages) presents a rare opportunity to examine various stages in a species ontogenetic development, wherein the specimens have been living in similar habitats. Comparisons across such size

[17] Though this presents practical difficulties, particularly if the cohort size is low (subsampling will significantly change density-dependent population dynamics of the remaining individuals, confounding statistical independence)

series from different habitats or species allow for the effects of habitat and evolutionary origin to be considered. For instance, one current area of interest for deep-sea research is in documenting basic biological parameters in 'less-specialised' species that might cast some light on the evolutionary origins of end-state contemporary species, whose adaptations now restrict them to living at hydrothermal vents and hydrocarbon seeps (e.g. Thubaut et al. 2013a).

2-2.1.3 *A priori* considerations

To be able to apply many of these approaches and maximise the data that can be collected from supply-limited samples, some criteria may prove pertinent when choosing an appropriate benthic, deep-sea chemosymbiotic study group. These restrict the suitable model group to: 1) organisms that as adults, are found upon reducing habitats exclusively, including incidences on organic falls; 2) organisms for which larval dispersal is the principal driver of connectivity between habitats; 3) organisms in which symbioses are suspected or known to occur; 4) organisms that may be considered 'less-specialised' (or 'more opportunistic'); 5) organisms that may be kept alive under ambient-pressure conditions in land-based aquaria (particularly organisms for which useful data exists in other related aquaculture species for defining these conditions) and; 6) organisms for which it is possible to acquire specimens at various stages of development, ideally from settlement size right through to maturation as adults.

2-2.1.4 Chemosymbiotic mytilids (Bathymodiolinae, *s.l.*)

The chemosymbiotic mytilids (Bathymodiolinae *s.l.*), which evidently include a considerable number of described and undescribed species, represent a relatively straight-forward model taxon for a lifecycle study for several reasons. First of all, the phylogenetic origins of the Bathymodiolinae are reasonably well understood (though not perfectly). This is a consequence of early molecular studies (e.g. Craddock et al. 1995b; Distel et al. 2000) and an intensive period of molecular systematics which have followed over the last decade performed by several researchers independently (e.g. Jones et al. 2006; Won et al. 2008; Kyuno et al. 2009; Lorion et al. 2010; Miyazaki et al. 2010; Schultz et al. 2011; Fontanez and Cavanaugh 2013; Lorion et al. 2013; Thubaut et al. 2013b). These studies have greatly improved our understanding of the subfamily's possible evolutionary history. The comparison of these cladistics with morphological data for the subfamily, have revealed microstructural shell characteristics to be prohibitively conservative (Génio et al. 2012) and considerable intraspecific phenotypic plasticity (Lorion et al. 2010), perhaps due to neoteny and thus paedomorphism in smaller-sized genera of the subfamily (Génio et al. 2012). Thus alternative evidence concerning the evolutionary status of the subfamily's members (*s.l.*) would aid our understanding concerning the degree of divergence and true speciation in the Bathymodiolinae.

Secondly, as a consequence of their loose phylogenetic affinity with several shallow-water mytilids of commercial and/or ecological value (e.g. the genera *Mytilus* and *Modiolus* respectively), some limited information can already be gleaned concerning gross bathymodiolin biology from the literature and commercial aquaculture. This makes the interpretation of anatomy in juveniles feasible, for example. Thus,

while the conservative nature of chemosymbiotic mytilid morphoanatomy in this subfamily presents difficulties for taxonomy (Lorion et al. 2010; Génio et al. 2012), shallow-water reference species already provide putative 'baseline' data for informing hypothesis testing in members of their deep-water relatives. Such hypotheses could assess anatomical divergence (and convergence) in the bathymodiolins in relation to the most robustly supported phylogenetics available. From a lifecycle point of view, this could include any biological aspect of their development. In addition, the presence of mussels from a multitude of substrates provides context for environmental transitions proposed to have taken place during the subfamily's evolutionary history (Distel et al. 2000). In this way, observed life-history traits in deep sea mussels can be formally assessed in relation to their ancestral background, likely from shallower seas (Jacobs and Lindberg 1998, though demonstrating this with molecular evidence has proven problematic, Jones et al. 2006; Lorion et al. 2013). Other important ways in which baseline data from commercial mussel species can be harnessed for deep sea mussel species is by providing a starting point when designing suitable experimental aquaria, and by providing some reference point for interpreting immature anatomy. Live observations evidently present several avenues for exploring the developmental biology of the Bathymodiolinae (as discussed previously).

Thirdly, let us return to the initial notion of identifying a sub-group of 'less-specialised' mussels from reducing habitats to provide insight into the radiation of the subfamily. High degrees of habitat specialisation are common within the large-sized Bathymodiolinae (*s.l.*), but critically, the degree to which different genera are isolated to specific habitats appears to vary, where some genera are capable of invading several different reducing habitats, across wide depth ranges (e.g. *Idas* and *Adipicola*). The genus *Bathymodiolus* (*s.l.*) has seen the most detailed study both across habitat types and biological disciplines. Paradoxically however, this genus, for which the phylogeny is still under revision (polyphyletic, currently split across 2 – 3 clades depending on interpretation e.g. Lorion et al. 2013; Thubaut et al. 2013b), is comprised of some of the most recently emerged and specialised clades of the subfamily (Lorion et al. 2013). In reality however, with the exception of *Gigantidas* spp., the numerical majority of the Bathymodiolinae (*s.l.*) consist of a diverse range of smaller-sized genera occurring on multiple reducing habitats (Lorion et al. 2013). Some of these smaller-sized genera share recent evolutionary roots with the *B.* (*s.s.*) *thermophilus* group (e.g. *Idas* [*s.s.*]), while several clades emerged much further back in evolutionary time (e.g. the *Benthomodiolus* and *Vulcanidas* clades, and the polyphyletic clade that currently contains the species *I.* [*s.l.*] *simpsoni*, *I.* [*s.l.*] *japonicus and A.* [*s.l.*] *longissima*, Thubaut et al. 2013b).

Several examples from this pool of small-sized mussels, frequently found thriving on organic falls, were cited by Distel et al. (2000) as extant evidence of an evolutionary habitat transition from wood and whale association to vent and seep specialisation in the relatively giant *Bathymodiolus* (*s.l.*) spp.. The deeper, vent- and seep-endemic *Bathymodiolus* (*s.s.*) spp. and the shallower, more-opportunistic wood-, whale-, and occasionally seep- and vent-colonising *Idas* [*s.s.*] spp., probably began diverging as two lineages around 34-

27 Ma ago from a common ancestor (Lorion et al. 2013). The radiation of known contemporary species of *Idas* (*s.s.*) is thought to have started around 24-17 Ma ago, while in *Bathymodiolus* (*s.s.*), contemporary-species radiation for the genus began more recently, around 15-11 Ma (Lorion et al. 2013). Members of the younger clade *Bathymodiolus* (*s.s.*) are thought to be approaching an evolutionary point of no return, as far as habitat specialisation is concerned (Jones et al. 2006; Thubaut et al. 2013b). In contrast, contemporary *Idas* spp. can be found living on one or more types of decomposing organic material in the deep sea often occurring over shallower depth ranges (Lorion et al. 2010). Two sister species are known to occur at methane seeps (*I.* [*s.s.*] *modiolaeformis* and *I.* [*s.s.*] *macdonaldi,* Gustafson et al. 1998; Olu-Le Roy et al. 2004), with one species even occurring at vents (*I*. [*s.s.*] *washingtonius*, Southward 2008). Other deep-branching clades, in which one includes species assigned to *Adipicola* (*s.l.*) and *Idas* (*s.l.*) spp., and the other *Benthomodiolus,* represent additional examples of basal clades frequently with organic-fall associations. *Vulcanidas*, in contrast, may have a quite different evolutionary history; contemporary species occur at shallow vents but fossil records originate from much deeper vent sites. This suggests deep-to-shallow migration, perhaps driven by large-scale deep-sea anoxia during the Cretaceous–Paleogene extinction event (Jones et al. 2006) or competitive exclusion by the emerging *Bathymodiolus* (*s.l.*) spp. (Kiel and Amano 2013).

Considering the entire subfamily's phylogeny (where non-*Bathymodiolus* spp. typically branch deeper in the trees), and the tendency both for less-integrated extracellular symbioses (*s.l.*) and less-hypertrophied gills in the small-sized Bathymodiolinae (*s.l.*), a "stepping stones" hypothesis for habitat use (Distel et al. 2000) remains plausible. This could explain contemporary phylogenetic trends in the Bathymodiolinae (*s.l.*), with isolated habitat reversals in a few species (Miyazaki et al. 2010). The order of habitat transition in terms of type (and depth, Lorion et al. 2013) over evolutionary time remains contentious, since discrepancies exist between evolutionary clock estimates and the appearance of bathymodioliform mussels in fossil records (being rather seeps first, and then almost parallel appearances on wood and whale falls, Amano and Little 2005; Kiel 2006; Kiel and Goedert 2006; Kiel and Amano 2013). Essentially, although it's not clear under which circumstances the rapid radiation of the Bathymodiolinae (*s.l.*) occurred (Lorion et al. 2013), contemporary species which fall outside the current clades containing "*Bathymodiolus*" (*s.l.*) spp. (which includes *Gigantidas* [*s.s*], Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b), seem to be of a less-specialised state.

Finally, having considered all the known biology of the possible model taxa, suitable sample availability had to meet the *a priori* aims of the research envisioned. Several candidates for less-derivative mussels exist in the basal genera of the Bathymodiolinae. Ultimately the genus *Idas* (*s.l.*) was chosen, since it met all of the criteria previously stipulated in 2-2.1.3. (page 112), including access to comprehensive series of post-settlement developmental stages in two species, and rarer still, live organisms for observation and experimental studies. *Idas* (*s.l.*) spp. have been cited previously in the literature as potential model

organisms for investigating the evolutionary origin and dispersal capacity of chemosymbiotic mussels (Distel et al. 2000; Gaudron et al. 2012), as members of this genus (*s.l.*) have been documented to colonise vents, seeps, sunken-wood and whale-falls, sometimes within the same species (Sturany 1896; Marshall 1900; Smith et al. 1989; Dean 1993; Tunnicliffe et al. 1997; Olu-Le Roy et al. 2004; Southward 2008; Lorion et al. 2009; Pelorce and Poutiers 2009; Gaudron et al. 2010; Lorion et al. 2010; Ockelmann and Dinesen 2011; Gaudron et al. 2012; Giusti et al. 2012; Ritt et al. 2012; Thubaut et al. 2013a). A summary of the current knowledge on this genus is thus given below and following that, the various means by which specimens were collected for this research.

2-3 The genus *Idas*

2-3.1. Shell plasticity and polyphyly

As evidenced by the numerous synonyms[18] attributable to this genus over time and the arbitrary misallocation of certain related genera to the genus (for example *AduIa*), the history of *Idas* (*s.l.*) is punctuated with discourse and taxonomic errors (Giusti et al. 2012). It is thus a challenging genus to retrace, particularly since the distinction between *Idas* and *Adipicola* is not a clear one, both morphologically and phylogenetically (e.g. Lorion et al. 2013; Thubaut et al. 2013b). A detailed account of the convoluted phylogeny of this genus may already be found in 1-8.2.4 (page 49), but needless to say, an overhaul of the subfamily as a whole would need to pay particular attention to *Idas* (*s.l.*), which currently contains one of the highest number of putative species within the Bathymodiolinae (*s.l.*). The lack of concomitant genecology and malacology in the genus (e.g. Lorion et al. 2013; Thubaut et al. 2013b) may be due, in part, to phenotypic convergence within the entire family Mytilidae (Distel 2000; Distel et al. 2000). Issues more specific to *Idas*-like mussels have arisen because in some species, intraspecific shell morphology is fairly constant with growth, while in others species the shell outline and sculpture appears to change drastically with increasing age (e.g. *Idas*-like ESUs E, F and S from Lorion et al. 2010; multiple morphospecies from Pelorce and Poutiers 2009; Giusti et al. 2012 and to a lesser extent; *I.* [*s.s.*] *modiolaeformis*, *I.* [*s.l.*] *simpsoni* examined for this research). Shell microstructure appears to be uniform across the genus (Génio et al. 2012). Several characteristics that were thought to be discriminative for the genus such as the microscopic structure of periostracal byssal hairs and the micro-taxodenticulate hinge in particular, now appear to be restricted to juveniles in some species, while seemingly persisting even as adults in others (Giusti et al. 2012), probably as a consequence of paedomorphism (Génio et al. 2012) and polyphyly (Thubaut et al. 2013b). Unfortunately, due to the polyphyly of the genus, it is unclear to what degree these apparent exceptions simply represent incorrectly placed species (*Idas* [*s.l.*] *simpsoni* being an excellent example). Thus any attempts to assign broad morphological characters to the genus are

[18] Includes *Idasola*, Iredale 1915; *Myridas,* Iredale 1939; *Myrinopsis* Nordsieck, 1969; *Habepegris* Bernard, 1978; and indirectly *Myrina* H. Adams & A. Adams, 1854: which was changed to *Adipicola*, since the former was a junior homonym

undermined. Species identification therefore ought to include *as a minimum*: intra- and interspecific anatomical and malacological comparisons, based on samples at comparable ages (or at comparable percentage maximum lengths, at least). This would involve the cataloguing of both inherent interspecific variability and intraspecific plasticity with increasing age, ideally assessing these characters in the context of the most thorough molecular phylogenies available. These are typically most reliable when variability across multiple gene regions is considered collectively in a concatenated tree (Lorion et al. 2013).

2-3.2. Known distribution and habitat use

Idas (*s.l.*) is one of the most ubiquitous bathymodiolin genera (*s.l.*) found in habitats subject to reducing conditions worldwide, both in terms of locality and depth. Current records of members of the genus *Idas* – i.e. described *Idas* [*s.l.*] spp. and *Idas*-like ESUs – indicate a frequent habitat association with sunken, decomposing wood both in relatively shallow, and deeper offshore waters, the greatest known 'species diversity' being from waters < 1000m deep (Samadi et al. 2007; Duperron et al. 2008b; Lorion et al. 2009; Pelorce and Poutiers 2009; Lorion et al. 2010; Giusti et al. 2012). Of course, this may in part be due to sampling bias. Over 20 ESUs have been collected from the South Pacific alone (Lorion et al. 2013), while several putative species from sunken wood and dredged sediments, rich in organic matter, are recorded in the Atlantic (e.g. Southward 2008; Rodrigues et al. 2013) and the Mediterranean (Gaudron et al. 2010; Giusti et al. 2012; Duperron et al. 2013).

To a lesser extent associations with whale skeletons are known, often as a second habitat in species otherwise known for colonising wood (Lorion et al. 2009). These associations appear to occur independent of geography, in the Mediterranean (e.g. *I.* [*s.l.*] *cylindricus*), Atlantic (*I.* [*s.l.*] *simpsoni*, *I.* [*s.s.*] *washingtonius*, Tyler et al. 2009), and Pacific (I. washingtonius, Smith et al. 1989). Bone-associations do not appear to be cetacean-specific, but applicable to megafaunal nektonic carcasses in general (based on the dense colonisation of bovine skeletons in experimental carcass deployments within Portuguese canyons, A. Hilário, personal communication).

A few *Idas* spp. are recorded in moderate densities at hydrocarbon seeps (e.g. *I.* (*s.s.*) *macdonaldi*, exclusively endemic to Garden Banks, Gulf of Mexico, Gustafson et al. 1998; *I.* (*s.s.*) *modiolaeformis* found on MVs in the Gulf of Cadiz, and at the Nile deep Sea Fan, East Mediterranean, Olu-Le Roy et al. 2004; Rodrigues et al. 2013 and; *I.* (*s.l.*) *simpsoni* in the Marmara Sea, North-east Mediterranean, Ritt et al. 2012). One species, *Idas* washingtonius, is even reported from the Mid-Valley vent fields, Juan de Fuca Ridge, northeast Pacific (Juniper et al. 1992; Southward 2008).

The genus *Idas* (*s.l.*) thus displays an intriguing array of habitats for investigation with some species capable of living in association with multiple reducing habitats, including multiple examples wood and bone (Lorion et al. 2009); and then *I.* [*s.s.*] *modiolaeformis* on wood and seeps (Sturany 1896; Olu-Le Roy et al. 2004; Gaudron et al. 2010; Rodrigues et al. 2013; the current research); *I.* [*s.l.*] *simpsoni* on wood, bones and at

seeps (Ritt et al. 2012; Thubaut et al. 2013b; the current research) and finally; *I.* [*s.s.*] *washingtonius* on wood, bones and at vents (Dell 1987; Juniper et al. 1992; Deming et al. 1997; Smith and Baco 2003; Southward 2008; Tyler et al. 2009).

2-3.3. Nutrition and symbioses

The habitat coverage displayed by the genus is thought to be made possible courtesy of unrivalled levels of symbiont flexibility (Duperron 2010). Nutritionally, the limited data available on *Idas* spp. suggests that the genus nestles intermediately between heterotrophic shallow-water mytilids and the relatively giant, predominantly chemosymbiotic, deep-sea vent and seep specialists, *Bathymodiolus* (*s.l.*) spp. From a mytilid perspective, the complexity of the digestive system and labial palps in *Idas* spp. are somewhat basic (based on *I.* [*s.s.*] *macdonaldi*, Gustafson et al. 1998; and *A.*(*s.l.*) *iwaotakii*, Thubaut et al. 2013a), however they retain a higher level of organisation and anatomy-inferred functionality than in *Bathymodiolus* spp. (Gustafson et al. 1998). The exception as always being the larviphage, *I.* (*d.f.*) *argenteus*; the digestive system is typically mytilid-like and labial palps are enlarged and thought to be adapted to handling unusually large particle sizes for the family (Ockelmann and Dinesen 2011). Equally, although the abfrontal surfaces of gills are enlarged when compared with coastal mytilids (Rodrigues et al. 2012), they are far-less hypertrophied than those in *Bathymodiolus* spp. (Southward 2008). Excluding one example (Ockelmann and Dinesen 2011), species from this genus that have previously been examined for symbiosis rely upon chemosynthetically derived nutrition, at least partially (Duperron 2010). The diversity of these symbioses is high, where examples of single, dual and multiple symbioses are known, incorporating phylotypes of various metabolic capacities (e.g. *I.* [*s.s.*] *modiolaeformis*, Duperron et al. 2008a; Rodrigues et al. 2013; see also section 1-7 (p. 39) and 1-8.3.3 (p. 59). It is not known whether an ancestral symbiotic state existed prior to the contemporary absence of symbionts in the atypical larviphage *I.* [*d.f.*] *argenteus* (Ockelmann and Dinesen 2011), as such inferences would require the availability of some molecular data for this species (the type species for the genus). From the point of view of symbiosis, *Idas* (*s.l.*) thus has great potential in elucidating bathymodiolin evolution, especially since the genus (*s.l.*) may be undergoing an evolutionary transition from extracellular to intracellular endosymbiosis (Duperron et al. 2013). Extracellular endosymbioses are known to occur in *I. modiolaeformis* (Duperron et al. 2008a; Lorion et al. 2009), *Idas* (*s.l.*) *simpsoni* (Southward 2008), and several unidentified *Idas* spp. (*Idas* '*woodia*' and ESU C – D respectively, Gros et al. 2007; Lorion et al. 2009). Definitive intracellular endosymbiosis appears to be restricted to *Idas* (*s.s*) *washingtonius,* the only described species of the genus capable of colonising vents (Deming et al. 1997).

The timing and means of acquisition have not been described for any species within this genus. Yet, such processes present interesting areas of study for any chemosymbiotic relationship and especially for *Idas*, given that acquisition is almost certainly horizontal and may differ in dynamic ways depending on species, habitat type, locality, and the symbiont phylotype(s) being 'targeted'.

2-3.4. Growth and development

Bar a single paper examining growth in *I.* (*d.f.*) *argenteus* (Dean 1993), no research has ever examined growth or development in the genus *Idas*, despite the obvious benefits that rapid growth rates may have in their ephemeral habitats. Rates of growth for *I.* (*d.f.*) *argenteus* (see section 1-9.4.3, p. 77) were found to be roughly equivalent to those in *B.* (*s.s.*) *thermophilus* (Kenk and Wilson 1985), although rates of growth retarded rather less than growth curves would typically predict based on the post-larval rates of growth in *I.* (*d.f.*) *argenteus*.

2-3.5. Reproductive and larval biology

Data concerning the reproductive biology of the genus is fragmented, though protandric sequential hermaphroditism has been identified in the three species in which size-specific sex ratios have been investigated (Tyler et al. 2009; Ockelmann and Dinesen 2011; Gaudron et al. 2012). Planktotrophy has been inferred for the larvae of *I. modiolaeformis* and *I. argenteus* (Dean 1993; Ockelmann and Dinesen 2011; Gaudron et al. 2012) based on deductions concerning prodissoconch malacology first postulated for bathymodiolins by Lutz et al. (1980). Egg sizes are small (< 60 µm diameter) and fecundity is thought to be high (Dean 1993; Tyler et al. 2009; Gaudron et al. 2012).

2-4 What remains to be discovered

2-4.1. A lack of basic biological characters

Basic biological understanding is lacking in the lifecycle of species of *Idas* (*s.l.*) including all aspects of anatomical development as post-larvae and juveniles, such as growth rates (except Dean 1993), patterns of tissue development and proliferation and, the origins of, and trends in, symbiont infection. It is also not known how these processes change (if at all) with increasing size prior to, and following maturation. Processes that together comprise the mussel's overall sexual development including size or age at first maturity, sex determination and sex-ratio patterns, post-maturation gametogenesis, spawning behaviour, fecundity and species longevity are not rigorously assessed, or remain entirely unknown.

The genus *Idas* (*s.l.*) evidently displays diverse habitat use, sometimes within a single species. Yet, it is not known how the processes above behave across differing reducing habitats in a single species. Establishing vital baseline data for the various lifecycle components of these species will not only provide insight into chemosymbiotic mytilids that occur on organic falls, but also for the subfamily as a whole, particularly from an evolutionary point of view. By gathering data from multiple habitats and from differing 'intermediate evolutionary species' within the Bathymodiolinae (*s.l.*), our understanding of the evolution of for example, *Bathymodiolus* spp. and their symbioses, may be increased.

2-4.2. Study species for the current research

The three species that featured in this research were *I.* (*s.s.*) *modiolaeformis, I.* (*s.l.*) *simpsoni*, and the type species *I*. (*d.f.*) *argenteus* where the relative commitment of time and resources follow the same order. Rather incidental, putative specimens of *I. argenteus* were found when examined wood substrates for the other two species. The role it played in this research, in collaboration with colleagues, was restricted to the provision of hitherto unavailable soft tissue for molecular confirmation of the type species of the genus, and extension of its known geographic distribution, juvenile morphoanatomy and further confirmation of its asymbiotic state. Outside of Chapter 6 in which this work is presented, it is not discussed further here.

2-4.2.1 Contrasting aspects in the study species' known biology

Two of the most prevalent *Idas* (*s.l.*) species in the Mediterranean and Atlantic are *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*. These two species differ in three fundamental regards: phylogenetically, morphologically and in terms of their respective symbioses (Ritt et al. 2012). Based on what we know of these two species, they are not actually congeneric but instead are distantly related species only, where the latter emerges much earlier in the evolutionary history of the Bathymodiolinae, within a less-derived clade (Thubaut et al. 2013b, Figure 1.19, annotated as '*simpsoni*'). In fact, the two species display genetic dissimilarity to such an extent that they likely diverged long before colonising one of their habitats, the Mediterranean-Sea seeps (Ritt et al. 2012).

Idas (*s.s.*) *modiolaeformis* has repeatedly been shown to host multiple symbionts (e.g. thio-, methano- and methylotrophic symbionts; Duperron et al. 2008a; Duperron et al. 2008b; Southward 2008; Halary et al. 2011), the highest number, six, being in specimens from carbonate crust at methane seeps in the central pockmarks region of the eastern Mediterranean (Duperron et al. 2008a). Such uncommonly diverse symbiont assemblages presumably provide *I*. (*s.s.*) *modiolaeformis* with access to highly adaptable sources of nutrition. Although the metabolic role of a sixth phylotype, Bacteroidetes, remains unknown(Rodrigues et al. 2013), the remaining bacterial assemblage meet their metabolic demands by oxidising sulphur (2 phylotypes), methane (1 phylotype), an undefined methylate substrate (1 phylotype) and via an additional unknown means, in symbiont "G" (Duperron et al. 2008a). The presence of all these symbionts in a single host is so far restricted to this region of the Nile Deep-sea Fan only. For other locations in which the species has been found and from which specimens have been examined, symbioses involve only a subset of this diversity (Duperron et al. 2013).

By way of contrast, only thiotrophic symbionts related to those found in *Bathymodiolus* (*s.l.*) aff. *boomerang* (Duperron et al. 2005) have been identified in *I.* (*s.l.*) *simpsoni* (Ritt et al. 2012). This is despite high concentrations of methane at the study area (Ritt et al. 2010), suggesting that *I.* (*s.l.*) *simpsoni* has either not developed active associations with methanotrophs, or that the methanotrophs in question were overlooked during FISH. The latter seems improbable since FISH upon gill tissue incorporated a comprehensive array of oligonucleotidic probes, targeting bacteria known to occur in both *I*. (*s.s.*)

modiolaeformis (i.e. using all probes from Duperron et al. 2008a available bar the type-T2 thiotroph probe) and *Bathymodiolus* (*s.l.*) aff. *boomerang* (methanotroph and thiotroph phylotypes, Duperron et al. 2005). Thus, while the symbiotic association seen in *I.* (*s.l.*) *simpsoni* resembles that of many other *Idas* spp., it differs considerably from that of *I*. (*s.s.*) *modiolaeformis* (Ritt et al. 2012).

Data available on shell sizes suggest that *I*. (*s.s.*) *modiolaeformis* attains a maximum size (shell length 17mm, Olu-Le Roy et al. 2004) less than half that of *I.* (*s.l.*) *simpsoni*, recorded at 45 mm (cited within species description of *I.* [*s.l.*] *ghisottii*, Warén and Carrozza 1990). This is supported by a recent (unwitting) comparative study between the two species (Ritt et al. 2012), in which "*Idas*-like nov. sp." – now known to be *I.* [*s.l.*] *simpsoni*, based on new COI data for this species (Thubaut et al. 2013b) – was examined in relation to what was known of *I*. (*s.s.*) *modiolaeformis* from the eastern Mediterranean. In that study, similar differences in sizes were recorded (Figure 2.1). In fact, *Idas*-like nov. sp. was assigned a putative new species status, based on comparative malacology against seven other species known to occur in the Mediterranean Sea including *I.* (*s.l.*) *simpsoni* type specimens. This demonstrates the sort of difficulties typically encountered during species description, which plague this highly plastic group of mussels.

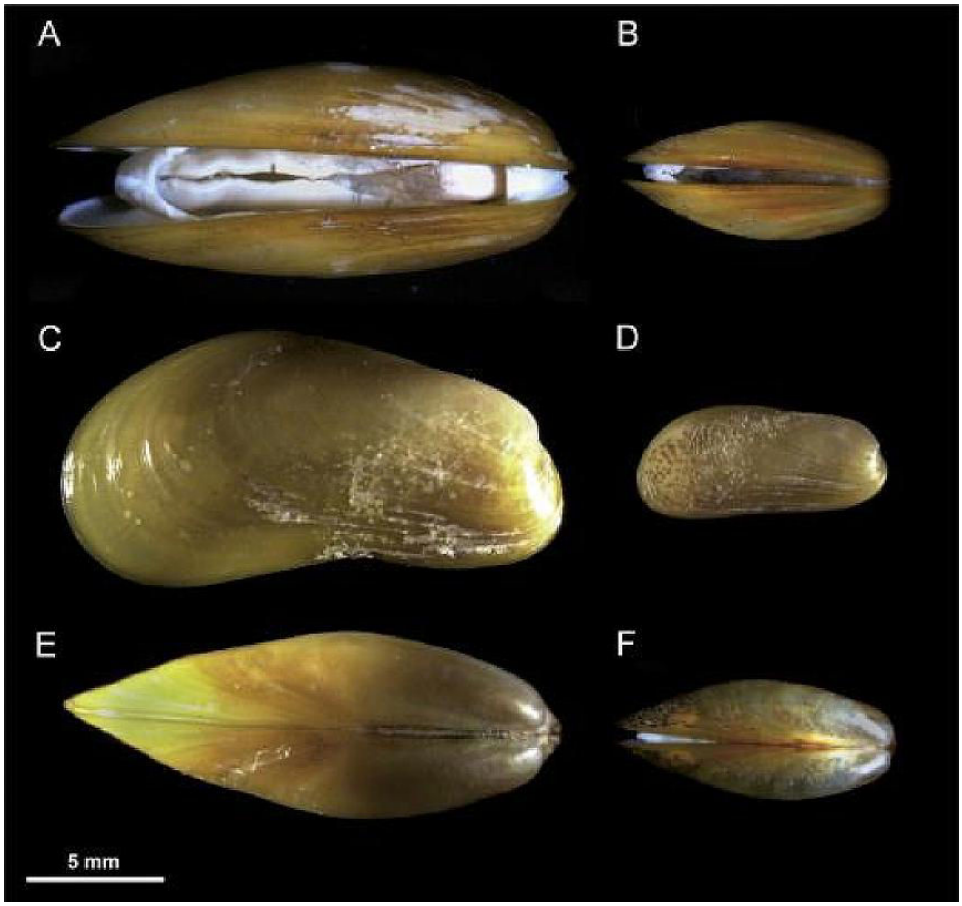

Figure 2.1 *Idas* (*s.l.*) *simpsoni* and *Idas* (*s.s.*) *modiolaeformis* from Ritt et al. 2012
A,C, E are *Idas* (s.*l.*) *simpsoni* , B,D, F are *I*. (*s.s.*) *modiolaeformis.* Views from top: ventral, lateral-right and dorsal

2-4.2.2 Commonality in characteristics

Based on their known distributions, both species display impressive tolerance ranges in terms of depth (and therefore pressure), habitat type and temperature occurring in both in the Mediterranean Sea (i.e. 13+ °C) and the Atlantic Ocean (≈ 2 °C). Consequently, both species occur frequently at reducing habitats in these regions, and have overlapping, conveniently wide depth ranges from 350–3015 m in *I.* (*s.s.*) *modiolaeformis*[19] and from ≈90–1120 m in *I.* (*s.l.*) *simpsoni*[20]. They also have at least two habitat associations in common (sunken wood and hydrocarbon seeps). Thus logically, their distributions ought to overlap geographically and bathymetrically, somewhere within the Mediterranean Sea. Such overlapping distributions are opportune for direct intra-generic[21] comparisons. In addition, as distantly related species only, a comparison of their anatomies may provide insight into hypothesised independent convergences in (or conservation of) morphoanatomy, suspected for the mytilids generally (Distel 2000).

Although symbiont assemblages differ markedly in both species they are suspected to be extracellular in both, where in *I.* (*s.s.*) *modiolaeformis* at least, bacteria are located among a layer of microvilli which extends out from non-ciliated filament epithelia. Since both species represent less specialised species, some filter-feeding function is presumed to remain.

2-5 Overall study area and sampling regime

2-5.1. The Mediterranean and eastern Atlantic

In the past, the Mediterranean Sea has been dubbed a "hotspot" region in terms of land- and coastal-based biodiversity. The deep-sea has often been dismissed under the presumption that biodiversity at greater depths is very low in Mediterranean waters, but theoretical extrapolations from rarefaction curves provide an estimate (excluding prokaryotes) of approximately 2805 species in the overall deep-sea Mediterranean (in that study, considered from 200 – 4000+ m in depth), where 66% remain undiscovered (Danovaro et al. 2010). According to this review, these exist in a wide variety of habitats including: continental slopes; deep-sea basins; submarine canyons and seamounts with deep-water stony corals at both; some isolated hydrothermal vents and; hydrocarbon seeps and deep-hypersaline anoxic basins, particularly in the eastern Mediterranean (Danovaro et al. 2010). Within and around the area of study in the northeast Atlantic Ocean, synonomous habitats exist to those in the Mediterranean Sea, with numerous methane-seeping MVs in the Gulf of Cadiz (e.g. León et al. 2006), an extensive series of vents along the MAR (e.g. Sudarikov and Galkin 1995; Douville et al. 2002; Silantyev et al. 2011), and seamounts (e.g. seamounts Josephine, Erik, Gettysburg (Gorringe ridge), Ampere and Seine, Morato et al. 2013). In addition the continental margins are densely

[19] Depths of mussels found at the Mercator MV, Gulf of Cadiz (Cunha et al. 2013) and at the Cheops Mud volcano, NDSF, (Ritt et al. 2011), respectively

[20] Depth of mussels found on a whale skull, Great Fisher Bank, NE Atlantic (40-50 fathoms, Marshall 1900) and at methane seeps in the northeast Central Basin of the Marmara Sea (Ritt et al. 2012), respectively

[21] The two are sufficiently divergent to assume, as a minimum, placement in two separate genera (Thubaut et al. 2013)

populated by submarine canyons, carved into the continental slope along both the Portuguese and North African coasts (majority are blind canyons, though some cut the shelf break, Harris and Whiteway 2011).

Though frequently overlooked in regional ecosystem-based syntheses, organic falls are known to play host to several species which specialise in colonising organic material undergoing decomposition, making it a habitat type in its own right. Wooden debris is known to occur at a wide range of depths (e.g. in the Caribbean, Wolff 1979). This appears to also be the case for organic debris occurring naturally on the seafloor both in the Mediterranean (e.g. wood, Pailleret et al. 2007b; Giusti et al. 2012; whale bones, Pelorce and Poutiers 2009) and the Atlantic (e.g. wood Rodrigues et al. 2013). The frequency of occurrence of larger, natural wood falls and whale falls in these regions is unknown, but they most certainly occur; artificial organic-fall experiments repeatedly identify rapid colonisation rates by specialist organic-fall species, suggesting relatively local larval sources (typically plant material e.g. Gaudron et al. 2010; Cunha et al. 2013; Romano et al. 2013; Fagervold et al. 2014; but also bovine deployments, CARCACE, project leader-Ana Hilário, preliminary results akin to Jones et al. 2008). It was through such deployments (e.g. as part of the European projects CHEMECO and CARCACE), that many of the samples for this study were collected, summarised below.

2-5.2. Sampling approach

2-5.2.1 Plantigrades, juveniles and smaller adults

Wood- and alfalfa-associated specimens that ranged from newly settled plantigrades to recently matured adults were collected using standardised colonisation devices called CHEMECOLI (Gaudron et al. 2010, Figure 2.2), typically deployed and recovered by remotely operated vehicle during scientific cruises either to vent and seep regions (MAR and NDSF respectively, Figure 2.3; Table 2.1) or while visiting submarine canyons (Lacaze-Duthiers Canyon). These were specifically designed to permit the qualitative collection of newly settled organisms upon various substrata (including two organic substrates), while reducing post-settlement mortality by constraining larger, mobile-predator access.

The housing of these devices consisted of a hollow PVC cylinder (14 cm diameter, 10 cm tall), in which many holes were drilled for water-circulation purposes. Fitted snugly inside this cylindrical casing was a matching basket-like insert constructed from plastic 2-mm garden mesh, into which one of three substratum types was placed. Substrata were either terrigenous carbonate, Douglas-fir pinewood or dried alfalfa grass, where the first two amounted to a total of ≈ 100 cubes per CHEMECOLI (cubes were 2 x 2 x 2 cm) and the last was the volume of grass needed to fill the device entirely (with minimal compaction, standardised volume of ≈ 1.6 dm^3 based on dimensions). Once filled, the basket was fitted with a lid made from the same mesh, so that the contents were sealed inside. In order to counter the buoyancy of the device, the lower edge of the device was weighted using stainless steel chain. Wood-filled devices were given time to soak in cold filtered seawater in order to counter the dry wood's buoyancy. Devices were labelled using conspicuous syntactic foam floats, attached using thick nylon rope to make their recovery straight-forward

by robotic arm (Figure 2.2). As a minimum, at each experiment site one CHEMECOLI with each substratum was deployed. These were at the NDSF (eastern Mediterranean, *I.* (*s.s*) *modiolaeformis* collected only), Rainbow vent-field (MAR, *I.* (*d.f.*) *argenteus* only) and Lacaze-Duthiers Canyon (*I.* (*s.s*) *modiolaeformis* and *I.* (*d.f.*) *argenteus*). Once recovered, substrata were normally spilt and fixed as subsamples immediately. The exception to this was the Lacaze-Duthiers Canyon CHEMECOLI deployed in June 2012 and recovered in April 2013, which were kept intact in aquaria and then sorted 'live', where fixation was on a case by case basis rather than *en masse*.

Bone-associated plantigrades, juveniles and small adults of *I.* (*s.l.*) *simpsoni*, came from remnant bones recovered from 18-month bovine carcass deployments in the Sétubal Canyon (SW Portugal, Figure 2.2; Figure 2.3; Table 2.1)[22]. These had previously been deployed attached to a sampling frame with considerable concrete ballast (Figure 2.2). Densities were extremely high on certain bones, and the subsample of bone mussels used for this research was selected to cover all sizes, thus size-frequency data from this subsample were meaningless.

2-5.2.2 Larger adults and live specimens

The largest adults from both species were collected in the Lacaze-Duthiers Canyon, from small pieces of oakwood (June 2012) and a large section of palm-tree trunk (April 2013) which had been deployed for 11 and 29 months respectively. The palm tree mussels were the individuals predominantly used in live-mussel observations in the Banyuls-sur-mer Marine Observatory (Chapter 3). Live observations were also performed on the limited number of juveniles and small adults collected from the CHEMECOLI from within the Lacaze-Duthiers Canyon (i.e. those mentioned in section 2-5.2.1, p. 122).

The work carried out on live organisms in Banyuls was to be repeated almost immediately, following the beginning of the mobility period in Aveiro, Portugal (starting July 2013), using live mussels from 24-month bone-deployments. However, rather unfortunately bones being recovered on my behalf were subject to excessive mechanical damage due to difficult sampling conditions, where bones were rolling during their transport to shore. The mussels did not survive and were fixed ahead of docking in order to preserve the samples before they spoiled completely. Compared to the preserved samples at 18 months (see section 2-5.2.1, p. 122), the populations densities at 24 months had also almost completely crashed (excessive canyon sedimentation and thus burial).

2-5.2.3 Additional DNA samples

Note that pre-extracted DNA originating from *I.* (*s.s.*) *modiolaeformis* mussels found in CHEMECOLI devices deployed at the Amsterdam MV (eastern Mediterranean), the Meknès and Darwin MVs and Gorringe ridge

[22] Courtesy of Ana Hilário as part of the CARCACE project, Portugal

were also available which, along with the sampling described, were sent for high-throughput 454 sequencing to examine the biogeography of symbiont associations in these two species across the entire Mediterranean and East Atlantic. Unfortunately, delays in the subcontracted analysis mean that this data will not feature in the PhD (but will be processed as part of a 2-month post-doctoral extension).

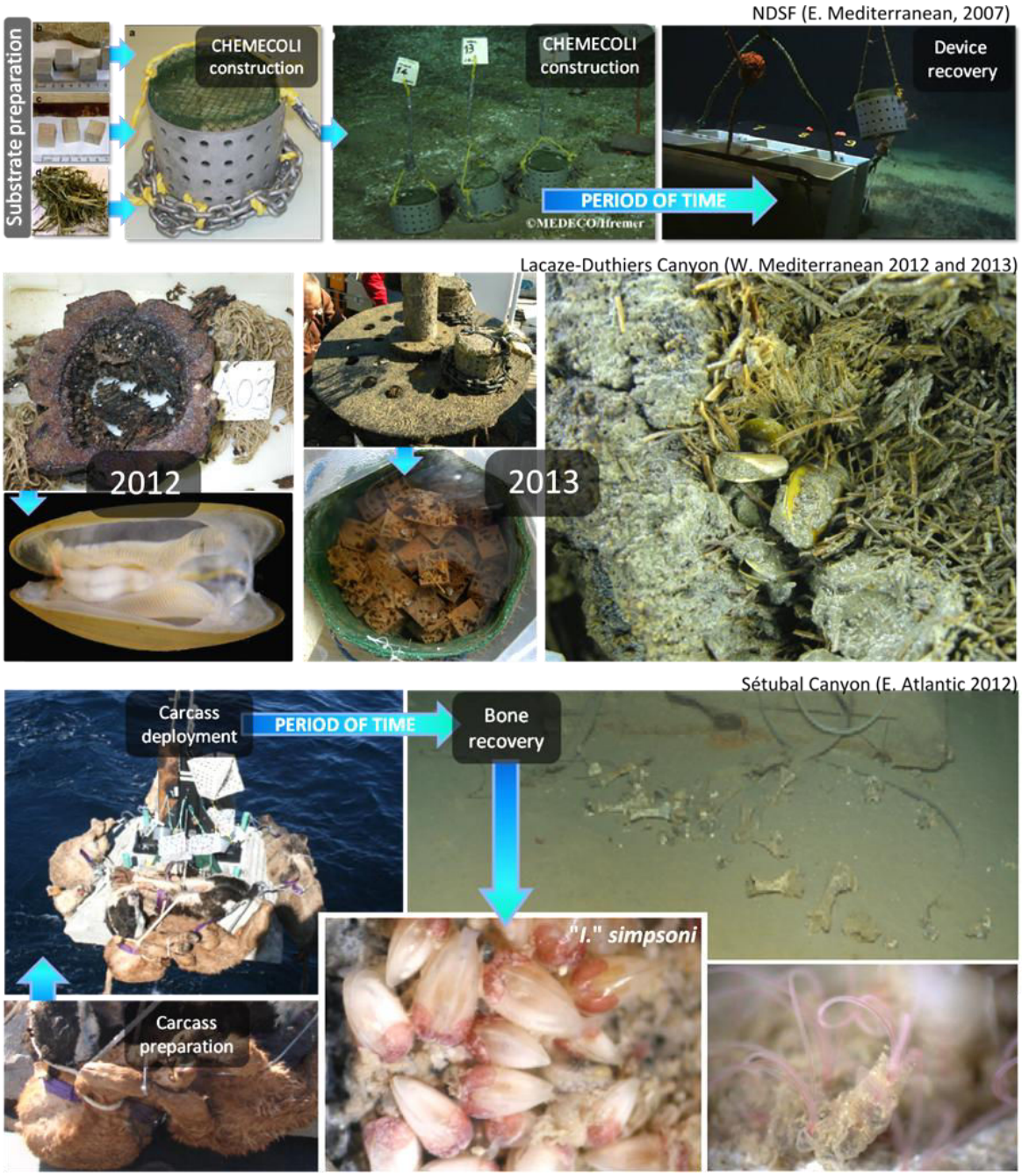

Figure 2.2 Methods employed to collect specimens
Bottom left: CARCACE project deployment in the Setúbal Canyon, Portugal. Middle-left: samples from the Lacaze-Duthiers Canyon, near Banyuls (2012). Centre and mid-right samples collected from following cruise in Lacaze-Duthiers Canyon on wood, 2013. Top row concerns the deployment and recovery of CHEMECOLI in the Eastern Mediterranean (Nile Deep-sea Fan, 2007).

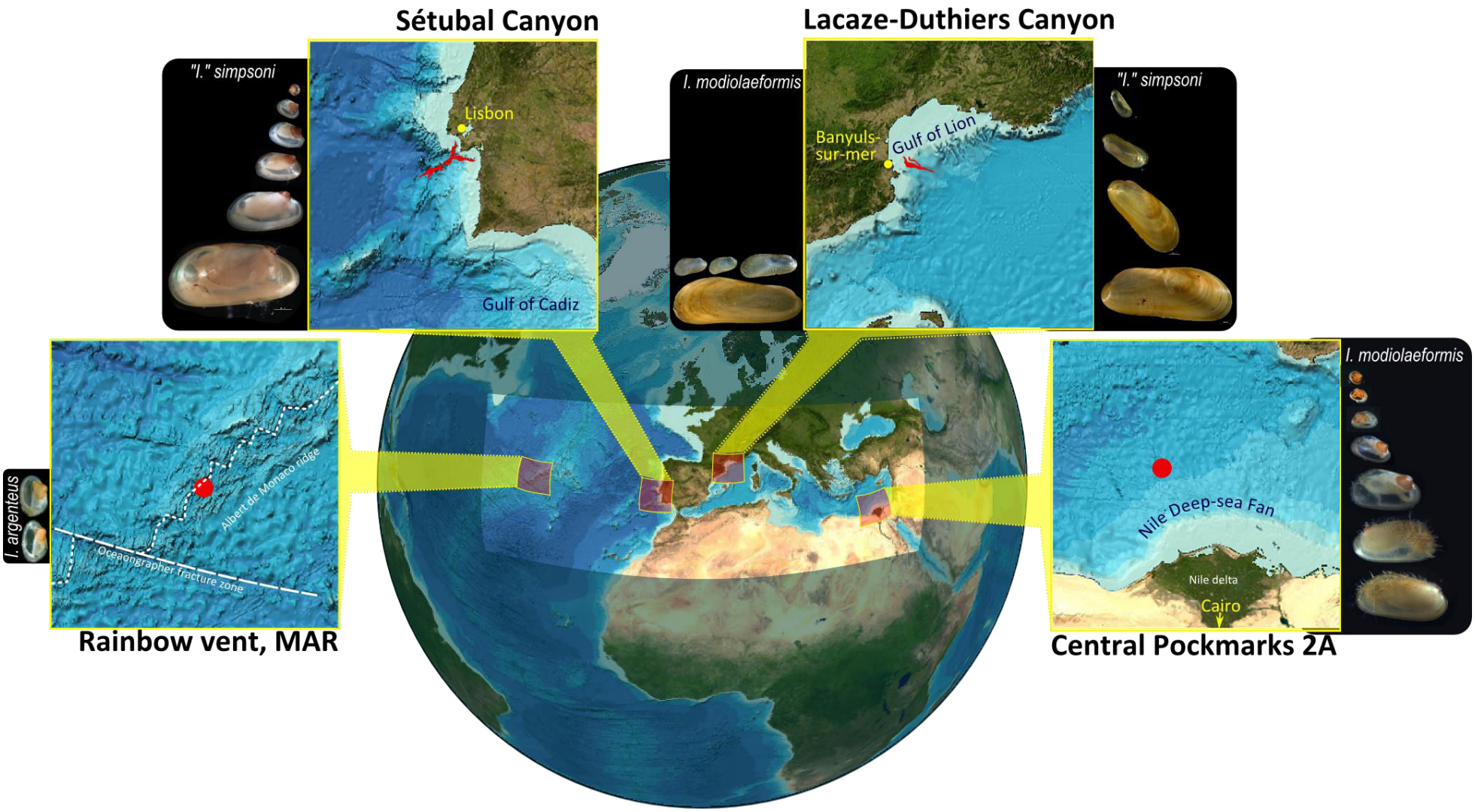

Figure 2.3 Overview of sampling locations
Details are given in Table 2.1

Table 2.1 Site and sampling details

Devices	Substrate	Deployment	Cruise	Collection	Cruise	Site name	Site details	Immersion time (wks)
CHEMECOLI C7	Douglas Pine cubes	14/07/2007	Momardream	31/08/2008	MOMAR08	MAR, Rainbow, EXO2	36°13.7454N, 33°54.0513W 2279 m deep	59
CHEMECOLI C9	Packed alfalfa grass	14/07/2007	Momardream	31/08/2008	MOMAR08	MAR, Rainbow, EXO2	36°13.7454N, 33°54.0513W 2279 m deep	59
Carcass (bovine)	Bone remains	?	CARCACE	?	?	Setúbal Canyon	exact location unknown (1000m)	78
Wood deployment (small and large)	Oak	04/09/2011	LD3	03/07/2012	LD4	Lacaze-Duthiers Canyon	42°32.728'N, 3°25.267'E (525m)	43
CHEMECOLIs x 2	Douglas Pine cubes	06/07/2012	LD4	24/04/2013	LD5	Lacaze-Duthiers Canyon	42°32.728'N, 3°25.267'E (525m)	42
CHEMECOLIs x 3	Packed alfalfa grass	06/07/2012	LD4	24/04/2013	LD5	Lacaze-Duthiers Canyon	42°32.728'N, 3°25.267'E (1000m)	42
Wood deployment (V. large)	Palm incl. cortex	Nov-10	LD1	25/04/2013	LD5	Lacaze-Duthiers Canyon	42°32.728'N, 3°25.267'E (525m)	129
CHEMECOLI C13	Douglas Pine cubes	18/11/2006	Bionil	10/11/2007	Medeco	Pockmark area (centre)	32 31.9772N, 30 21.1779E 1693 m deep	51
CHEMECOLI C11	Packed alfalfa grass	18/11/2006	Bionil	10/11/2007	Medeco	Pockmark area (centre)	32 31.9772N, 30 21.1779E 1693 m deep	51

Chapter 3 *EX SITU* OBSERVATIONS ON NUTRITIONAL FLEXIBILITY AND REPRODUCTION IN TWO CO-OCCURRING DEEP-SEA CHEMOSYMBIOTIC MUSSELS ON WOOD

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3-1 Abstract

The survival of specialist deep-sea benthic species thriving in spatially and temporally finite reducing habitats necessitates long-ranging dispersal, suitably responsive reproductive strategies, and in numerous species also depends upon chemosynthetically derived nutrition from associated bacterial symbionts. However, contrary to the relatively sustained supply of sulphides at hydrocarbon 'cold' seeps and hydrothermal vent fields, sulphide supply at the surface of sunken wood is only intermittent, raising doubts about whether chemosymbiotic organisms rely entirely on their bacterial symbionts for nutrition. To assess this, live observations of feeding and reproduction were examined in two divergent species of chemosymbiotic mussel, *Idas* (*sensu stricto*) *modiolaeformis* and *I.* (*sensu lato*) *simpsoni*, collected from wood-colonisation experiments deployed in the Lacaze-Duthiers Canyon, Western Mediterranean. Both species are probable semi-continuous spawners based on multiple, induced dribble spawning events, and post-spawning retention of mature oocytes. Oocytes display practically neutral buoyancy at atmospheric pressure and 14°C with diameters indicative of planktotrophy in both species. Although both species harboured bacteria in their gills, each retains a capacity for active particle processing based on live observations. The presence of fully-equipped alimentary, digestive and excretory systems, and mixed diet $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios of host tissues support facultative mixotrophy in both species.

3-2 Introduction

Since the discovery of hydrothermal vents (Lonsdale 1977) and the chemosynthetic basis of their productivity, other reducing habitats have subsequently been identified where each is home to their own array of specialist and visiting fauna (Levin 2007). Chemosynthetic bacteria occur as free-living chemotrophs, as symbionts, or as both, supporting communities by deriving metabolic energy from the electron donors and acceptors available at reduction-oxidation interfaces (Dubilier et al. 2008). In the deep sea, these reducing habitats shrouded in darkness, are typically ephemeral over decadal timescales or less and are highly discontinuous in their distribution (Duperron 2010). Thus, the survival of specialist deep-sea benthic species is favoured by long-ranging dispersal, suitably responsive reproductive strategies, and often depends upon taking advantage of the chemosynthetically derived nutrition available (Dubilier et al. 2008). Such constraints in reducing habitats have resulted in a range of contemporary species that engage in chemosymbioses, availing novel metabolic capabilities to the host.

One of the most pervasive chemosymbiotic taxa to inhabit reducing habitat types in the oceans (Duperron 2010; Lorion et al. 2013; Thubaut et al. 2013b) is the mytilid bivalve subfamily Bathymodiolinae (used *sensu lato* hereafter, as with genera enclosed in quotation marks based on the phylogeny in Thubaut et al. 2013b). They colonise hydrocarbon seeps (e.g. Childress et al. 1986; Van Dover et al. 2003), hydrothermal vents (reviewed in Van Dover 2000), the skeletons of decomposing megafauna (e.g. Deming et al. 1997; Tyler et al. 2009; Génio et al. *in press*), sunken wood (e.g. Dean 1993; Gaudron et al. 2010; Lorion et al. 2010; Bienhold et al. 2013; Cunha et al. 2013) and even oily drill-cuttings (Southward 2008). Their success is attributed to suspected long-ranging larval dispersal capacities (Tyler and Young 1999; Mullineaux et al. 2010; Adams et al. 2012; Arellano et al. 2014), and to their capacity to host diverse chemotrophic bacterial associations (Vrijenhoek 2010). Extended larval dispersal has been proposed based on considerable differences between larval shell dimensions (prodissoconch I and II), indicating small hatching size and relatively large settling size respectively (e.g. Lutz et al. 1980; Arellano and Young 2009; Gaudron et al. 2012a). Such small hatching sizes, supported by documented mature oocyte diameters with limited energy reserves (<80 µm, Colaço et al. 2006; Arellano and Young 2009; Gaudron et al. 2012a), suggest that dispersal is planktotrophic (Lutz et al. 1980). Larvae of some small-sized bathymodiolins may be aposymbiotic, excluding the possibility of symbiont-derived nutrition during dispersal (Laming et al. 2014). Data on sex-ratios are scarce, however most bathymodiolins are believed to be gonochoristic, except *Bathymodiolus azoricus*, which displays a mixed mode with limited protandry (Comtet et al. 1999; Dixon et al. 2006). Spawning frequency in the bathymodiolins appears to be predominantly seasonal (e.g. Van Dover et al. 2002; Van Dover et al. 2003; Colaço et al. 2006; Dixon et al. 2006; Tyler et al. 2007), though semi-continuous spawning has been proposed for *B. thermophilus* from the East-Pacific Rise based on gametogenesis (Tyler and Young 1999) and smaller-sized *Idas* spp. based on settlement patterns (Dean 1993; Ritt et al. 2012; Laming et al. 2014).

When documented, bathymodiolin species host symbiotic bacteria within the abfrontal, non-ciliated epithelial surfaces of their hypertrophied gills, which can be extra- or intracellular (reviewed in Duperron 2010). In all cases, symbiont transmission is thought to be horizontal, acquired predominantly from the environment as free-living bacteria (Won et al. 2003; Duperron et al. 2008a; Gaudron et al. 2012a; Fontanez and Cavanaugh 2014; Laming et al. 2014). The timing of acquisition appears to vary between pre- and post-settlement, depending on the species (Streams et al. 1997; Won et al. 2003; Salerno et al. 2005; Wentrup et al. 2013; Laming et al. 2014). The retention of a functional, albeit-reduced digestive system (e.g. Gustafson et al. 1998) has fuelled speculation for mixotrophy, particularly during early benthic life (Cowie et al. 1999; Martins et al. 2008; Laming et al. 2014). For wood-associated mussels, a retained capacity for mixotrophy may represent one way to overcome fluctuations in sulphide supply, which appear to be highly intermittent on sunken wood (Laurent et al. 2009; Laurent et al. 2013; Yücel et al. 2013). Documented variability in symbiont densities within and across species of small-sized bathymodiolins strongly suggests a necessity for alternative nutrition (e.g. Duperron et al. 2008a; Duperron et al. 2008b; Ritt et al. 2012; Rodrigues et al. 2013; Laming et al. 2014), however mixotrophy remains hypothetical. Filter-feeding and assimilation have been demonstrated experimentally in large "*Bathymodiolus"* (*sensu lato*) *childressi*, *B. thermophilus* and *B. azoricus* (Page et al. 1990; Page et al. 1991; Riou et al. 2010).

Several species and clades of small bathymodiolin mussels are deep-branching within the subfamily's phylogeny (Jones et al. 2006; Kyuno et al. 2009; Vrijenhoek 2010; Duperron et al. 2013a; Lorion et al. 2013; Thubaut et al. 2013b). They might thus present more ancestral states for certain characters than the more intensively studied, highly specialised vent and seep species. Many of these mussels are loosely attributed to the genus *Idas*, with pronounced polyphyly in this genus as a consequence (e.g. Lorion et al. 2013; Thubaut et al. 2013b). However, the discovery and molecular analyses of new specimens of *I. argenteus*, the type species, has recently resolved this problem (Rodrigues et al. in press). The mostly extracellular symbioses of members this clade and other *Idas*-like mussels are less-integrated than those in vent and seep species, and this has prompted research into small-sized bathymodiolin biology in order to clarify the evolutionary history of the Bathymodiolinae as a whole (see *Discussion*, Duperron et al. 2008a; Tyler et al. 2009; Ockelmann and Dinesen 2011; Gaudron et al. 2012a; Thubaut et al. 2013a; Laming et al. 2014).

Two such small-sized bathymodiolin species are *Idas modiolaeformis* and *I. simpsoni*. While the species *I. modiolaeformis* truly belongs in its genus, *I. simpsoni* actually places within a deeper-branching clade that predates the later divergence of *Idas* (*sensu stricto*) and *Bathymodiolus* (*sensu stricto*) clades (Lorion et al. 2013; Thubaut et al. 2013b, Rodrigues et al. *in press*). Although *Idas* (*s.s.*) *modiolaeformis* and *I*. (*s.l.*) *simpsoni* belong to markedly divergent lineages, they display some similarities in morphoanatomy and habitat use with sunken wood and hydrocarbon seeps in common (Thubaut 2012). These two species are distributed over a wide variety of reducing environments and depth ranges both in the Mediterranean and the Atlantic (Thubaut 2012), which at their shallowest extremes in are likely to be subject to

considerable vertical flux of particulate organic matter (Palanques et al. 2006). Despite overlap in their distributions, they are only known to co-occur on sunken wood in the Gulf of Lion (Thubaut 2012; Le Bris et al. *in preparation*).

Remarkable symbiont diversity has been demonstrated in *I. modiolaeformis* recovered from hydrocarbon seeps, with six bacterial types identified (Duperron et al. 2008a). Various subsets of these presumptive symbionts have been repeatedly described in other specimens and populations (e.g. Lorion et al. 2010; Lorion et al. 2012; Rodrigues et al. 2013; Laming et al. 2014). In *Idas* (*s.l*) *simpsoni*, only one dominant thiotroph bacterium has been observed in specimens from hydrocarbon seeps in the Marmara sea (Ritt et al. 2012; Thubaut et al. 2013b).

Although data on feeding and spawning behaviour are best collected during the examination of live organisms, only a handful of *in vivo* studies have been published for bathymodiolins, all from *Bathymodiolus* (Page et al. 1990; Page et al. 1991; Colaço et al. 2006; Kádár et al. 2006; Arellano and Young 2009; 2011). This study sought to successfully maintain adult *Idas*-like mussels alive, in order to describe aspects of their nutritional and reproductive biology based upon live observations and histological analyses following dissection and fixation. Specimens were collected from a variety of wood-colonisation experiments, deployed in the Lacaze-Duthiers (LD) Canyon at 525 m depth (Gulf of Lion, France, Le Bris et al. *in preparation*). Live studies were performed at the Banyuls Marine Station (UPMC, CNRS), France.

3-3 Methodology

3-3.1. Sample collection and handling

3-3.1.1 Aquaria

Flow-through experimental aquaria (Figure 3.1) and an additional flow-through container for holding unsorted material, were installed and then acclimated under deep-sea Mediterranean conditions (dark, 13°C ± 1°C) for five days prior to receiving samples. Both were supplied continuously with unfiltered seawater imported from shallower depths around the Banyuls Marine Station. In the aquaria, seawater circulated above an elevated, mesh-covered sample platform, driven by gentle downward inflow from the inlet manifold with corresponding upwelling from aeration at the opposing end (Figure 3.1). The manifold introduced seawater equally across the width of the tank. The sub-platform outflow, equipped with an anti-siphon vent, removed sinking wastewater and material. Aquaria were emptied and cleaned on rotation once week^{-1}.

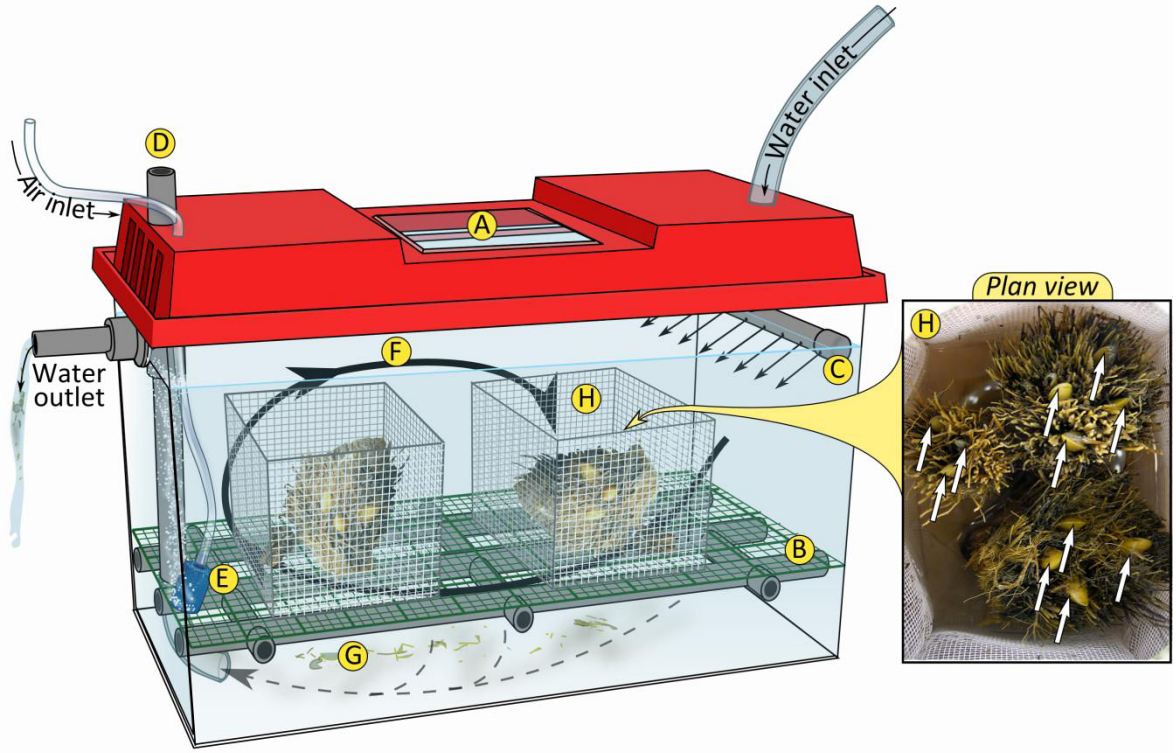

Figure 3.1 Acclimation aquarium for the maintenance of *Idas* spp.
Aquarium (20 x 14 x 14 cm) design was based loosely on Høeg(1984) and Utting and Spencer(1991). Access was via the viewing window [**A**]. The sample platform [**B**] was raised from the floor of the tank which along with the manifold design [**C**], the anti-siphon vent [**D**] and bubbles from aeration [**E**], collectively created a gentle circulatory flow [**F**]. Waster material sank to the base of the tank [**G**] while dissolved waste was removed by the outflow (dashed arrow). Samples were held in plastic 1-mm mesh cages [**H**].

3-3.1.2 Substrate experiments

A multi-annual sunken-wood deployment-recovery experimental programme was established in the Lacaze-Duthiers canyon site at 525 m in October 2010 (42°32.728'N, 3°25.267'E, 5 LD-cruises in total, Le Bris et al. *in preparation*). Using the ROV SuperAchille (COMEX) recoveries and new deployments have since been performed over a series of 4 additional cruises (LD1–LD5, chief scientist: N. Le Bris, R/V Minibex), the last being in April 2013. As part of the LD5 cruise in April 2013, various pre-deployed wood experiments were recovered, including a section of palm-tree trunk (~0.4 m girth) deployed on the seabed during the LD1 cruise (2.5 year deployment), and two pinewood cube-filled larval colonisation devices (CHEMECOLI, Gaudron et al. 2010) deployed on an sampling frame during the LD4 cruise (10 month deployment). Specimens used in live specimen work originated from these substrata (Figure 3.2). The previous year (LD4), some specimens were recovered from oakwood experiments deployed in 2011 (LD3, 11 month deployment). These were used in this study to support stable isotopes analyses and for FISH analysis.

3-3.1.3 Transport and sample sorting

The palm-tree trunk and CHEMECOLIs were transported intact in aerated, filtered seawater (13°C) to the LECOB laboratory facility at the Banyuls Marine Station (UPMC, CNRS), France. Mussels were suspected to

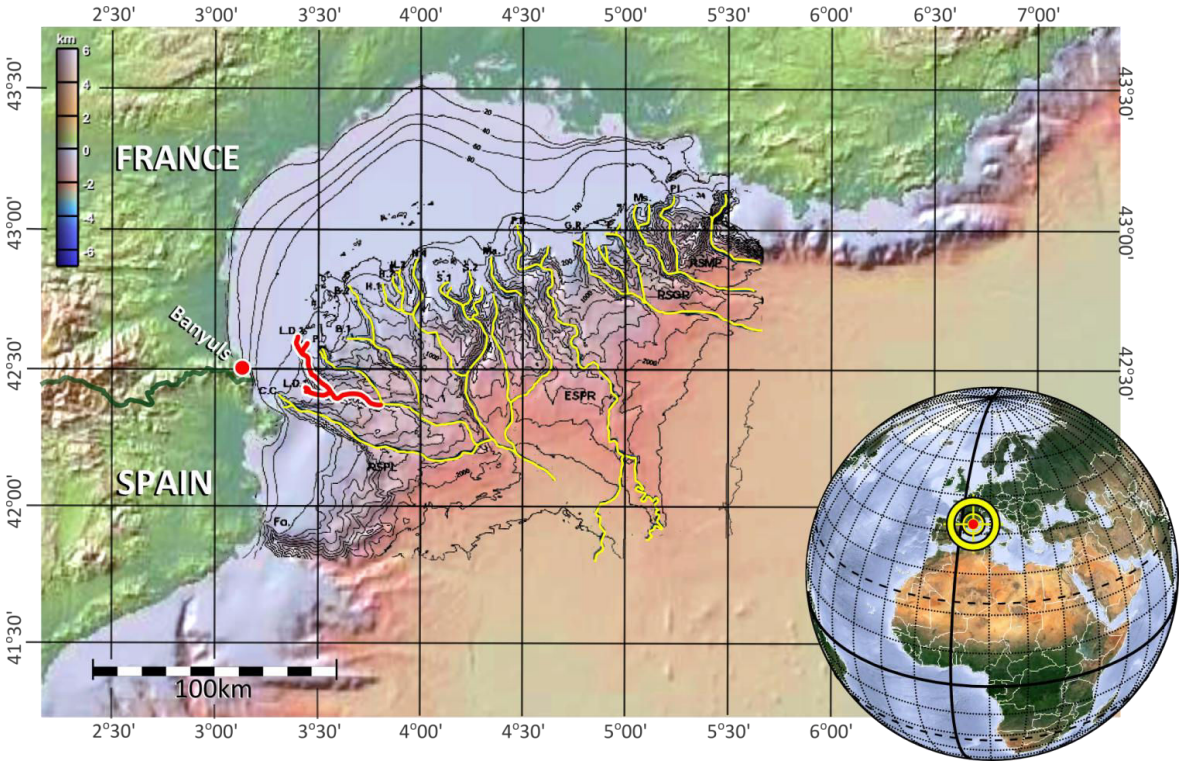

Figure 3.2 Wood-colonisation experiment's location in the Lacaze-Duthiers Canyon, western Mediterranean
Depicted is the Gulf of Lion with the Banyuls Marine Station and Lacaze-Duthiers Canyon in red, where the remaining canyons are in yellow. The deployment/recovery location was near the head of the canyon (42°32.728'N, 3°25.267'E) at a depth of 525 m.

be ready to spawn, since live dissections performed upon *Idas* sp. specimens collected the previous year (LD3-LD4, oakwood substrata) had resulted in the spontaneous release of gametes. The palm-tree individuals were obtained while sorting through subsamples, carefully torn from the trunk after transfer to the onshore laboratory 6-9 hours after sample recovery, while CHEMECOLIs were placed in the flow-through container before sorting. Initially, small samples of substrate (one type per tank, split across two mesh baskets, Figure 3.1) were relocated to the experimental aquaria for acclimation, while samples were sorted. A subsample of *Idas*-like mussels from both substrates were then relocated to experimental aquaria for acclimation, gathered upon pieces of their corresponding recovered wood substrate to provide chemically reduced conditions. Aquaria were kept in the dark (mussels had clearly identifiable pigment spots, suggesting a level of photo-sensitivity), and examined twice daily for maintenance. Only mussels considered to be healthy were retained (i.e. normal resting behaviour: either shells were shut or marginally open with the mantle curled posteriorly forming two pseudo-siphons).

3-3.2. Induction of spawning and fertilisation

Before induction, aseptic seawater (ASW) was prepared by filtration (0.45 µm) and sterilisation (autoclave, 15 min at 121 °C) and then re-oxygenated (steam-sterilised air-stone, ≈50 L air L^{-1} ASW: Supplementary figure 3.1A). After five days, sixteen healthy putatively-adult mussels (i.e. > 2.35 mm in *I. modiolaeformis*, Laming et al. 2014) were selected for spawning (sample code IF#). Those awaiting induction were kept immersed in unfiltered seawater (15–18 °C, running tap-water bath, Supplementary figure 3.1B–C). Mussel

shells were scrubbed and then rinsed in ASW. The most effective methods for induction were shock treatments (mechanical, thermal and saline, discussed in results). Initially, mussels were kept together (to improve induction success, Arellano and Young 2009) in a flat-bottomed container with a black background for contrast. Mussels believed to have begun spawning were relocated to individual compartments in sterile 6-well cell-culture plates (Evergreen Scientific) with renewed ASW, for the collection of gametes separately by pipette (Supplementary figure 3.1E–F). However, a notable number of buoyant waterborne oocytes were found during a post-isolation examination of the communal "induction" ASW after mussel isolation. These were collected separately by filtering (60 µm Nitex mesh) and extracting the oocytes by pipette from the more concentrated ASW eluate.

Sperm concentrations were calculated using single-use haemocytometers (Euromedex Ltd.). Oocyte numbers were calculated from triplicate 1 ml subsample counts. Gametes were kept on ice until fertilisation. Due to apparent dribble-spawning and low resulting fecundity (total ≈2700 oocytes), all oocytes were pooled, while sperm from each individual were kept separate. Subsamples of unfertilised oocytes and sperm were photographed. Fertilisation was carried out in sterile Pyrex Petri dishes, at a density of 20 oocytes ml^{-1} ASW (15mleach, 7 dishes, from 7 males, 1000 spermatozoa : 1 oocyte). After 30 min, each mixture was sieved through a 45-, 60- then 100-µm Nitex mesh to remove organic debris (mainly body fluids and excess sperm, Loosanoff and Davis 1963; Arellano and Young 2009), during which time the ASW was replaced. Air was introduced into each dish via parallel air-lines fitted with a sterile micropipette tip, to aerate the oocytes and reduce sedimentation (Supplementary figure 3.1G). ASW changes were made every 6 h approximately. Oocytes were examined before fertilisation and every 30 min following, for the first 4 hours, with increasing gaps in time thereafter (ranged from 2 up to 8 hours) over the course of the next 3 days, when the experiment was terminated due to halted development. Oocytes were photographed and fixed for further analyses when fertilisation was first identified (presence of a thin fertilisation membrane), and when changes were apparent in fertilised oocytes (Table 3.1)

3-3.3. Anatomical dissections and fixation regime

Following the collection of gametes, mussels were returned to their substrate and relocated to the experimental aquaria to recover (1 wk). During the recovery period, one mussel (IF1) displayed atypical behaviour indicative of being moribund, and was removed and fixed immediately (Table 3.1). Mussels IF2 – IF14 were dissected microscopically to examine anatomy under a dissection microscope and fixed according to intended analyses (Table 3.1). Seeking to identify whether *Idas* spp. truly engage in filter-feeding and thus mixotrophy, detailed observations were made during anatomical examinations and live dissections. Mussel IB1, not induced to spawn due to its impractical size, was also photographed under high-magnification using a digital SLR-mounted compound microscope (Canon EOS 650D, on a Leica Diaplan). Gill filtration activity in this mussel was then video-recorded at high magnification. This mussel and a second individual (species not determined) were fixed whole (Table 3.1).

Table 3.1 Fixation and preservation regime

Tissue	Fixation / storage	Purpose
	IF1	
Shells	96% ethanol	Shell description
Gill (x1)	96% ethanol	DNA extraction
Remaining tissue	4% Formaldehyde (ASW), 2 hrs (RT) / gradient transfer 96% ethanol (4°C)	FISH / Histology
	IF2–IF14	
Shells	96% ethanol	Shell description
Foot	96% ethanol	Isotopes analysis
Gill (x2)	Liquid Nitrogen / Frozen (-80°C)	Host DNA
	4% Formaldehyde (ASW), 2 hrs (RT) / gradient transfer 96% ethanol (4°C)	FISH / Histology
Mantle	Liquid Nitrogen / Frozen (-80°C)	Host DNA (backup)
Gut Gonad	4% Formaldehyde (ASW), 2 hrs (RT) / gradient transfer 96% ethanol (4°C)	H & E staining
Oocytes	4% Formaldehyde (ASW), 2 hrs (RT) / gradient transfer 96% ethanol (4°C)	Hoestch staining
	IF15, IF16, IB1, IB2	
Whole	4% Formaldehyde (ASW), 2 hrs (RT) / gradient transfer 96% ethanol (4°C)	Shell description / Histology / (extended) DNA extraction / FISH

3-3.4. Material analysis

3-3.4.1 Shell morphology

Shells were photographed and shell heights (SH) and lengths (SL) were measured using a standardised approach (Laming et al. 2014) under a camera-mounted dissection microscope (Olympus S2X12 microscope, Japan). Image-stacks of all focal-planes in the Z-axis were aligned in FIJI (Image-J 1.48r, StackReg*,* scaled *rotation)* and extended depths of field were achieved using the *'*EDOF' plug-in (easy mode) in *FIJI (Image-J* 1.48r; Supplementary figure 3.2). Tiled fully-focused images were merged in Photoshop CS3 (without transformation) for the three largest shells. Relationships between SL and SH with increasing size in both species were examined using Pearson's correlation coefficient, with a two-tailed test for significance (homoscedasticity and normality confirmed). A type III sums of squares (SS) ANOVA was employed (to accommodate unbalanced data and low samples sizes, homogeneity of variance and normality confirmed) to identify whether differences in mean shell lengths were significant between the studied species. All analyses were performed in SPSS (17.0).

3-3.4.2 Molecular identification

Host DNA was extracted from frozen gill tissue using the QIAamp® DNeasy blood and tissue kit (QIAGEN, centrifuge protocol). A fragment of the mitochondrial Cytochrome Oxidase I-encoding gene (COI) was PCR-amplified using primers LCO1490 (Folmer et al. 1994) and H691 (Duperron et al. 2008a). The program started at 94°C for 4 min, followed by 35 cycles of 94°C for 40 s, 50°C for 50 s, and 72°C for 1 min, with a final elongation step at 72°C for 10 min. PCR products were sequenced in both directions (GATC Biotech). Sequences assembled and checked using BioEdit v. 7.2.5 (Hall 1999). Only regions for which all residues had

been identified were used (460–534 bp, depending on individual). Sequences were queried against the GenBank nucleotide sequence database using BLAST (Altschul et al. 1997).

3-3.4.3 Stable Isotopes analysis

Foot tissue from mussels collected for this study (n = 14, after pooling individuals where necessary) and a subsample of *Idas modiolaeformis* collected from the canyon in 2012 (LD3-LD4, oakwood substrata) were prepared and analysed according to Gaudron et al. (2012b). C:N atomic ratios were calculated from the percentages of organic carbon and nitrogen.

3-3.4.4 Histological analysis

Viscera (comprising one half of the gut, gonad and mantle tissue, split medially) and gill tissue samples from each specimen were dehydrated in 100% alcohol, transferred incrementally to Histoclear, and embedded in paraffin wax. Slide-mounted (Superfrost plus), 7-µm thick sections cut using a microtome (Jung, Heidelberg) were stained with acidified Harris' haematoxylin (5mL glacial acetic acid L^{-1} solution, 10 s), differentiated with 0.5% acetic acid (aq.), blued in Scott's tap-water substitute (pH >8) and then counterstained with alcoholic Eosin-Y (pH 5, 3 s). Sections were dehydrated (increasing %-ethanol (aq.) series), cleared (Histoclear), and mounted immediately (Eukitt).

3-3.4.5 Fluorescence microscopy

Unfertilised and fertilised oocytes collected during the fertilisation experiment, were stained using the fluorescent dye Hoechst 33258 (Herzog and Schütze 1968, 5 µg ml^{-1}) to first identify whether unfertilised oocytes were in Prophase I or Metaphase I of meiosis, and second the stages of cell division following fertilisation, respectively. To establish whether previously identified bacterial phylotypes occur as symbionts in these mussels, fluorescence *in situ* hybridisations (FISH) were performed on a subsample of unstained 7-µm paraffin-sections of gill tissue, employing all dual-hybridisation combinations (Cy3 and Cy5-labelled probes) of the general eubacterial probe EUB338 (Amann et al. 1990), the non-sense probe NON338 (Wallner et al. 1993), and specific oligonucleotide probes for type-M1 methanotrophs and type-T1 and -T2 thiotrophs (details in Duperron et al. 2008a), following the post-permeabilisation steps of the FISH protocol in Duperron et al. (2005).

3-4 Results and discussion

3-4.1. Species appearance

The genus *Idas* (*s.l.*) remains poorly resolved. However, recent multi-gene molecular analysis of type specimens for *I.* (*s.l.*) *simpsoni* have revealed that it actually belongs within a deep-branching clade of highly-related species in the Bathymodiolinae that predates the later divergence of *Idas* (*s.s.*) and *Bathymodiolus* (*s.s.*) spp. (Lorion et al. 2013; Thubaut et al. 2013b). This clade has been ascribed to a new

but still not formally described genus provisionally named *Nypamodiolus* (Thubaut et al. 2013b); quotation marks are thus employed to emphasise the improper assignment of the *Idas* genus to this species.

Three species of mussel were identified in this study (Supplementary table 3.1). *Idas* (*s.l.*) *simpsoni* dominated numerically (*n* = 11), identified based on shell morphology and 99–100 % COI sequence similarity). All-but-one of the remaining mussels (including IB1) in this study matched *Idas* sp. Med (*n* = 6, 99%; *Idas* (*s.s.*) *modiolaeformis*, Lorion et al. 2012). Mussels recovered from the previous year from oakwood substrata, were dominated numerically by *I*. (*s.s.*) *modiolaeformis* in contrast (n = 25, versus *I.* (*s.l.*) *simpsoni n* = 2, Thubaut 2012, note different wood and deployment conditions). The haplotypes of the two dominant species differed by 86 polymorphic sites in 497 bp (excluding gaps/missing data). This level of divergence warrants classification in two distinct genera, as indicated by Thubaut et al. (2013b). The remaining mussel (IB2), was a third unidentified species (Supplementary table 3.1, not considered further in this paper[23]).

Moderate differences existed between shell characteristics in the dominant species (Figure 3.3, Supplementary figure 3.3), where *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* SLs differed significantly (*n* = 5 and 10 respectively; GLM type III *SS*, $F_{(1, 13)} = 5.53$, $p<0.05$). Mean (±σ) SLs and SHs were 10.3 ± 1.97 mm x 3.8 ± 0.85 mm (*I*. [*s.s.*] *modiolaeformis*) and 18.2 ± 6.18 mm x 6.3 ± 2.24 mm (*I.* [*s.l.*] *simpsoni*). Length-height relationships in *I.* (*s.l.*) *simpsoni* were consistent with size (ρ = 0.99, *N* = 6 <0.001, 2-tailed) and females (ρ = 0.99, *n* = 4, <0.05, 2-tailed). Shells in *I*. (*s.s.*) *modiolaeformis* were elongate by comparison, with a greater degree of shell plasticity (lower value for ρ = 0.93, *N* = 5, <0.05, 2-tailed; Supplementary figure 3.3; Figure 3.3). Periostracal 'byssus hairs' (Choo et al. 2014) were absent on *I.* (*s.l.*) *simpsoni* (except IF4 and IF15) in contrast to *I. modiolaeformis*, (hairs concentrated along the postero-ventral margin). The periostraca were golden-brown in both species, but glossier and more tanned in *I.* (*s.l.*) *simpsoni*. The inner nacreous layer was pearly and off-white in *I.* (*s.l.*) *simpsoni* shells but barely discernible in *I. modiolaeformis*, even in the largest specimen (IF1, Figure 3.3). However, some individuals deviated from these general trends (IF4, IF15, IF16), reaffirming the need for molecular validation when discriminating small-sized bathymodiolin species (Lorion et al. 2010).

3-4.2. Distribution and habitat use

Idas (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* represent the two of most widespread chemosymbiotic mussels documented in reducing environments within the Mediterranean Sea and eastern Atlantic (Thubaut 2012). Including data from the current study, *I. modiolaeformis* is now known to occur in the eastern Mediterranean (Nile Deep-sea Fan, NDSF, on authigenic carbonate crust and siboglinid tubes at

[23] This specimen was later identified as a mature male specimen of *I.* (*de facto*) *argenteus*, the type species for the genus and the subject of Chapter 7, p.)

methane-seeps, at several mud volcanoes and on sunken wood, Olu-Le Roy et al. 2004; Duperron et al. 2008a; Gaudron et al. 2012a; Lorion et al. 2012), in the central Mediterranean (northern Tyrrhenian Sea off Tuscany, Giusti et al. 2012), the western Mediterranean (Gulf of Lion on experimentally deployed wood, Thubaut 2012, *this study*) and the eastern Atlantic (Moroccan slope, Gulf of Cadiz, on experimentally deployed organic substrata in regions of methane-seepage, Cunha et al. 2013; and at the Gorringe Bank on naturally occurring wood, Rodrigues et al. 2013). The known distribution of *I.* (*s.l.*) *simpsoni* now

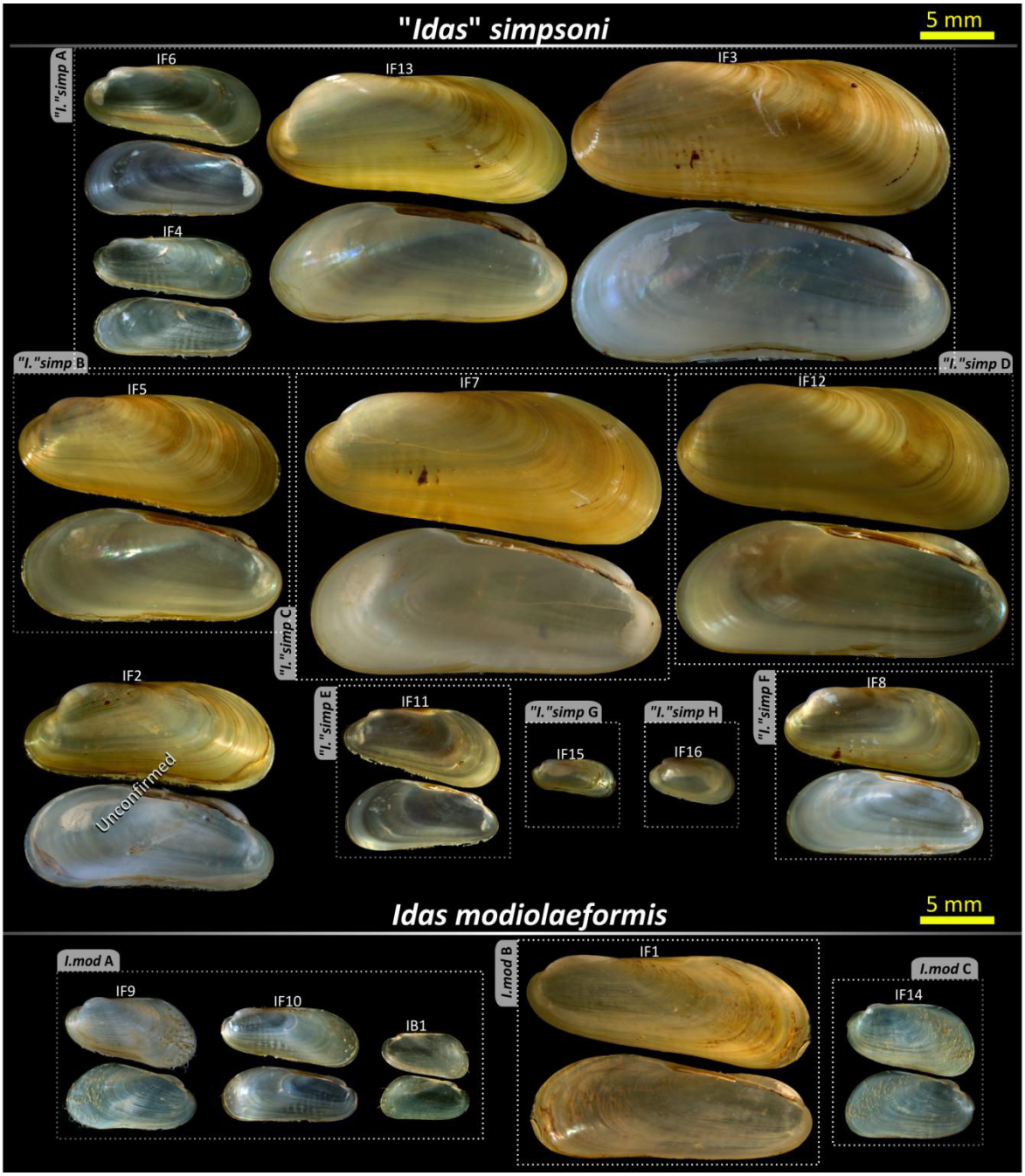

Figure 3.3 Shell morphology of specimens in this study separated by halpotype and species
Haplotypes are based on fragment COI sequence similarities (single haplotypes are 100% similar). Exterior and interior views of the left valve are shown in each case (upper and lower respectively). Note that IF2 is placed with *I.* (*s.l.*) *simpsoni* provisionally as DNA was not successfully sequenced.

comprises the north-eastern Atlantic (several independent cetacean bones at Rockall, Grand Fisher Bank, and off Faroe, Shetland and Orkney Islands, Marshall 1900; 1901; Thubaut et al. 2013b), the eastern Atlantic (on a dolphin skull, Bay of Biscay, Quero 1973; on bone experiments, Iberian margin, Setúbal canyon, Génio et al. *submitted*), the western Mediterranean (on wood experiments, Gulf of Lion, Thubaut 2012, *this study*), the central Mediterranean (northern Tyrrhenian Sea and the Adriatic Sea, Bolotin et al. 2005; Pelorce and Poutiers 2009; Giusti et al. 2012) and the north-eastern Mediterranean (at methane seeps as "*Idas* sp. nov", Marmara Sea, Ritt et al. 2012). It is unclear which combination of factors determines the geographic locations of these species, as both exhibit wide depth ranges and utilise diverse chemically-reduced habitats (*I. modiolaeformis* wood and seeps, 350–3015 m; *I. simpsoni* bone, wood and seeps, <100m –1120 m).

Nothing is known of either species' behaviour in the context of their habitats. The relative similarities (morphologically) and differences (phylogenetic divergence) between these two taxa are particularly intriguing, as their distribution is now reported to overlap marginally: sunken wood in the Gulf of Lion so far represents the only habitat and region in which both species cohabit. In both species, mussels were located beneath the utmost exterior of deployed substrate. In the palmwood experiment, they were entrenched along the circumference of the trunk's sawn cross-section between the cortex and the evidently sulphidic central cylinder (Supplementary figure 3.4). The *I. modiolaeformis* found in the CHEMECOLI (Supplementary table 3.1, "Pine") were sub-peripheral, beneath the outermost structurally-degraded cubes (riddled with empty burrows of *Xylophaga* spp.) but not in direct contact with the device's sulphidic core. Thus both micro-environments were at apparent REDOX transitions (organoleptic observations).

Both mussel species also displayed levels of mobility characteristic of mytilids (Paine and Levin 1981), where in shallow-water coastal species with deterred mobility, such movements reduce intraspecific crowding, mitigate the effects of wave action and can improve filter-feeding efficiency. They often adjusted their positions on aquarium substrata between maintenance checks (<8 hours). Pre-dissection observations revealed this process in more detail. The foot extended and weaved back and forth in a 'searching' motion. Contact with an attachment surface was identified with the tip but temporary attachment with the heal of the, while the tip of the foot continued searching. The tip contained a pit at the distal terminus of the pedal groove which is thought to have a sensory function. The tip itself subsequently attached to a proximal point on the substrate, allowing the heel to detach and the foot to contract, dragging the body. This was repeated process permitted the mussels to crawl using their foot.

3-4.3. Reproduction

The available data for *Idas* spp. generally, indicates probable planktotrophic larval development inferred from larval shell and gamete characteristics (Dean 1993; Ockelmann and Dinesen 2011; Gaudron et al. 2012a; Lorion et al. 2012; Ritt et al. 2012; Laming et al. 2014), a tendency towards protandry based on size-dependent patterns in sex ratios, (Tyler et al. 2009; Gaudron et al. 2012a; Laming et al. 2014), and an age at

first maturation (for males) identified at approximately 4 months following settlement, for *I*. (*s.s.*) *modiolaeformis* in the eastern Mediterranean (51-week wood deployment, no female sex-switching, Laming et al. 2014).

The smallest specimen (SL 5.09 mm) of *I*. (*s.s.*) *modiolaeformis* was male, the remainder (*n* = 5) being female. *Idas* (*s.l.*) *simpsoni* sex ratios approximated 50:50, the two smallest specimens being hermaphroditic, but functionally male (Supplementary table 3.1). Mean diameter for released oocytes (pooled across all mussels) was 59.1 ± 2.31 µm (*n* = 50). Mean diameters for unreleased and potentially-immature, paraffin-embedded oocytes in *I.* (*s.l.*) *simpsoni* (IF12 and IF7, *n* = 75) and *I*. (*s.s.*) *modiolaeformis* (IF14, *n* = 10) were 33.5 ± 3.65 µm and 25.3 ± 2.98 µm respectively. In the latter, the number of available eggs to measure was limited by the volume of the gonad, which was also almost spent. These diameters are comparable to previously-documented vitellogenic oocyte diameters for *I*. (*s.s.*) *modiolaeformis* (32.5 µm), though smaller than sizes for oocytes in fully-ripe gonads (Gaudron et al. 2012a); this is attributed to the release of all fully-formed oocytes during repeated spawning events though diameters may also underestimate true sizes, as oocytes showed signs of heat-shrinkage. Two inductions had been attempted in a subsample of the individuals prior to the success of the third. Initial attempts to induce spawning with 2 mmol l^{-1} serotonin microinjections (Femtojet, Eppendorf) into the anterior adductor muscle (Arellano and Young 2009) proved ineffective, while the batch of oocytes from the second more successful attempt was damaged while being sieved. Successful induction was by mechanical shock (10 min, Sprung and Bayne 1984), saline shock (size-calibrated injections of 0.55M KCl into mantle cavity, Young and Tyler 1993) and then thermal shock (5°C brought to 16°C over ≈20 min, Sprung and Bayne 1984). All but one (IF1) of the adults appeared to recover from these inductions, based on behaviour and visually-assessed body condition.

Repeated induction procedures and (presumably) size-constrained fecundity (e.g. 3rd induction: ≈300–400 oocytes $ind.^{-1}$) may have limited the number of viable oocytes. Difficulties in discriminating between the co-occurring genera without real-time molecular confirmation (and fecundities too low for separate fertilisation treatments) probably led to numerous non-viable cross-fertilisations. Consequently, fertilisation rate was low despite a 1000:1 sperm-to-oocyte ratio. Communally spawned oocytes were also used in the fertilisation experiment, as no premature fertilisation was identified during counts made prior to mixing gametes. Oocytes were white and optically opaque (Figure 3.4A) and very slightly negatively buoyant at ambient pressure and 14 ±1°C. Hoechst staining revealed a subsample of the oocytes to be in Metaphase I of meiosis (evidence of germinal vesicle breakdown), and thus mature (Figure 3.4Bi). During post-experimental dissections, initial adductor-muscle incisions stimulated the release of further gametes (Figure 3.4A). Subsequent histological analysis of reproductive tissue confirmed that all males and several larger females still retained gametes, despite multiple spawning events (Figure 3.5A, D–H). This fact and the repeated release of oocytes in low numbers both hint at semi-continuous spawning behaviour. This could

explain the previously-described larval supply patterns for *I. modiolaeformis* (Laming et al. 2014). Oocyte diameters displayed little variability despite being pooled across both species, suggesting a minimal yolk reserve, typical of planktotrophic development. However, as development stalled ≈11 h at 14 ±1°C after fertilisation at the 4-cell stage in the current study (Figure 3.4B–C), the exact larval mode for each species remains unconfirmed.

In the shallow-water species' *Mytilus edulis* and *Modiolus modiolus*, the swimming ciliated embryo (not reached in the current study) is attained within the first 4-6 hours, depending on temperature (10–19°C, Rattenbury and Berg 1954; Bayne 1965; De Schweinitz and Lutz 1976) placing the second cleavage and thus 4-cell stage within the first 2 hours after fertilisation, five-fold faster than in *Idas* spp. However, early rates of embryonic development in the current study are comparable to those recorded in "*B.*" *childressi,* where the second cleavage typically took place between 7 and 15 h after fertilisation (though at 7–8°C, Arellano and Young 2009). In that study, a small number of larvae developed to D-veligers, with a projected planktonic larval duration (PLD) of up to 13 months. This has recently been validated in both this species and an associated gastropod *Bathynerita naticoidea* as part of an integrated larval study (Arellano et al. 2014). If such a teleplanic PLD was also possible in either *Idas* spp*.,* it might explain how *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* maintain their pan-Mediterranean distributions within reducing habitats, though this assumes favourable transport processes and habitat availability (Shanks 2009). It could

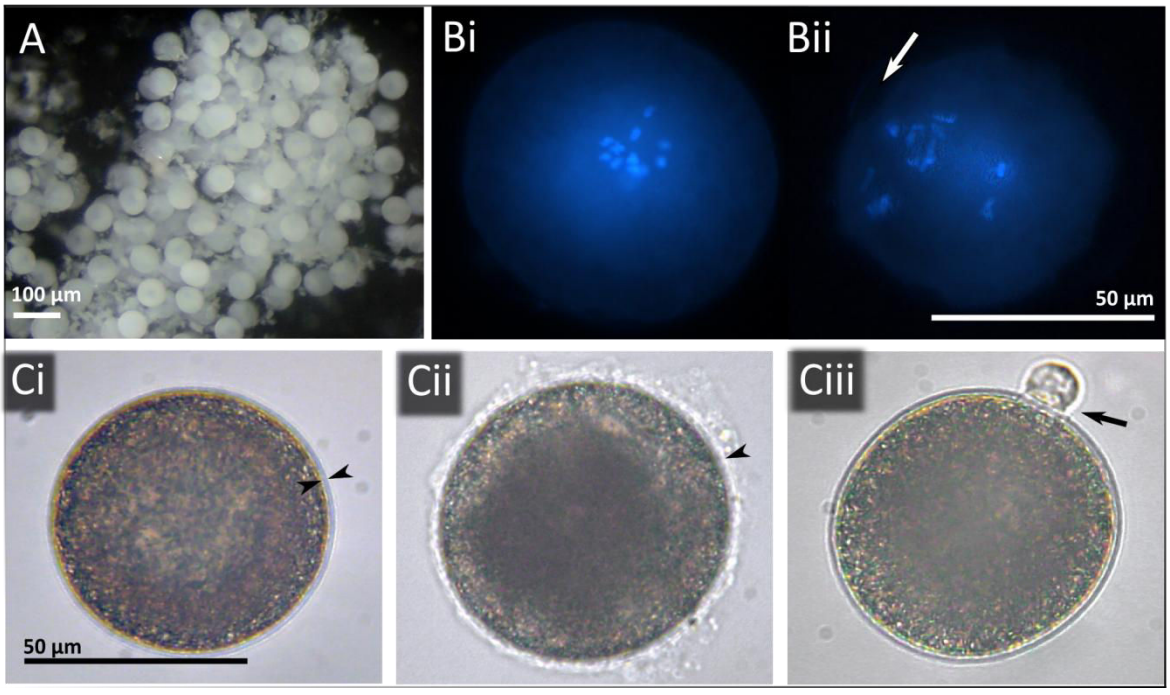

Figure 3.4 Images of liberated ooctes during spawning and following fertilisation (pooled from both species)
A) Oocytes realised by the largest *I.* (*s.l.*) *simpsoni* female IF7 during dissection (thus, not used in fertilisation experiment**). B)** Hoechst Staining identifying **i)** released, but unfertilised, oocyte in Metaphase I; **ii)** 4-cell pre-morula division stage. Arrow identifies detachment of membrane/possible polar body. **C)** Phase contrast microscope images of fertilised oocytes, i) arrowheads delimit fertilisation membrane; **ii)** with jelly coat and; **iii)** following formation of polar body

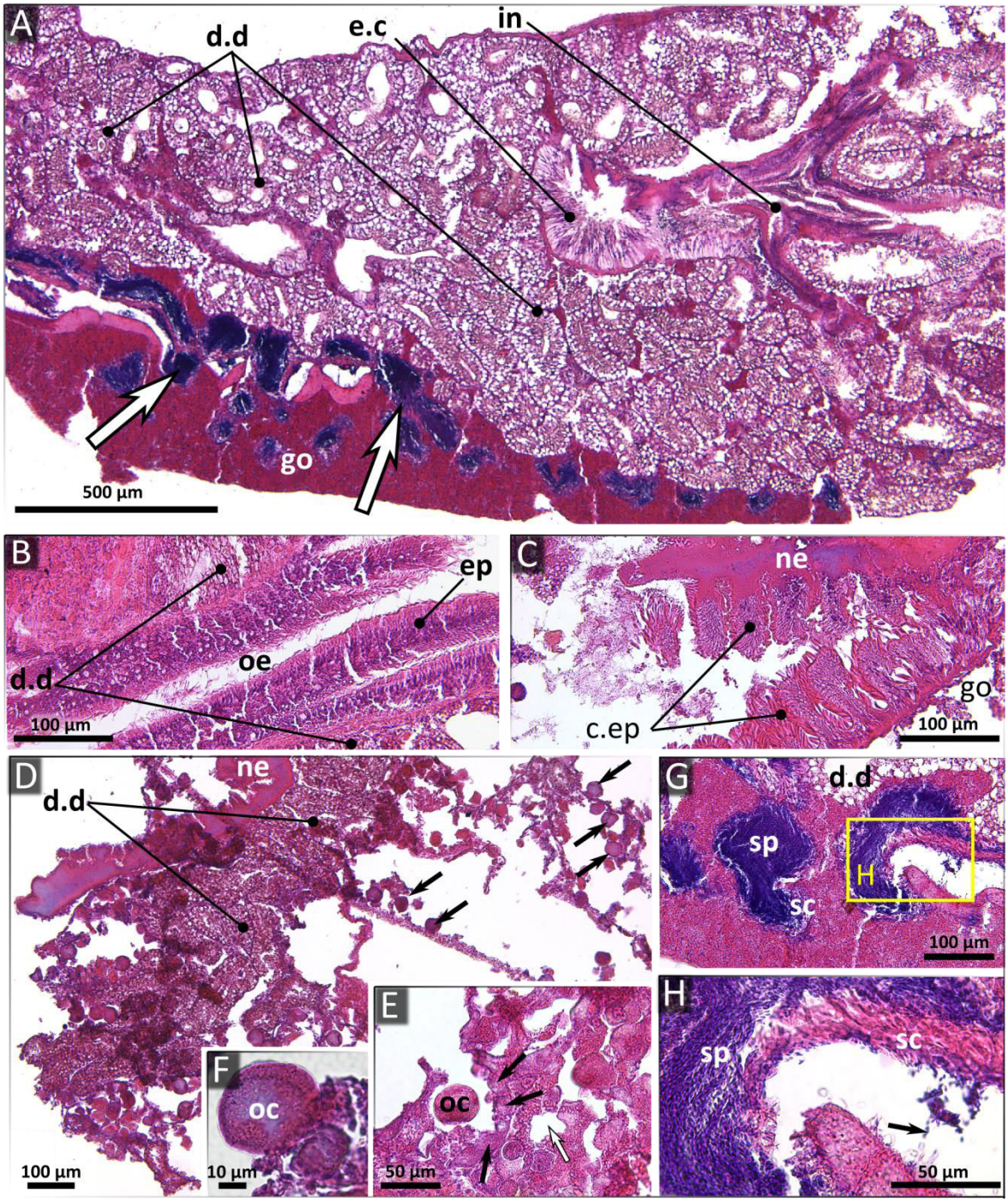

Figure 3.5 Haematoxylin and Eosin Y staining in 7mm histological sections from both species
Note that **A, G, H** are from IF3, male *I.* (*s.l.*) *simpsoni*; **B, C, D, F** are from IF7, female *I.* (*s.l.*) *simpsoni*. **E** is from IF10, female, *I.* (*s.s.*) *modiolaeformis*. **A)** Sagittal view in region where digestive gland and gonad coincide. Large white arrows = spermatogenesis. **B)** Oesophagus in sagittal section, surrounded by digestive diverticula. **C)** Kidney-like nephridia and edge of spent acini in gonad (bottom right of image). **D)** Similar region to C, but focused over gonad. Follicles are spent, though putatively mature oocytes persist (black arrows). **E)** Gonad, with some immature oogonia indicated with black arrows. **G)** View of tubules, some full, some partially depleted (region H). **H)** Magnified view of box in **G**. Empty region is where spermatozoa formerly were, prior to release. c.ep *cuboid epithelial cells*, d.d *digestive diverticula*, ep *epithelium*, e.c *secretory epithelial cells*, go *gonad*, in *intestine*, ne *nephridium*, oc *mature oocyte*, oe *oesophagus*, sc *spermatocytes*, sp *spermatozoa*

also be responsible for the high phylogenetic similarity between *I. modiolaeformis* and its sister-species *I. macdonaldi* (Lorion et al. 2012) found in the Gulf of Mexico, which also harbours chemoautotrophic symbionts (Won et al. 2008). However, the dispersal capacity of *Idas* spp. larvae will only be realised through improvements in the protocol for rearing larvae of this taxa.

3-4.4. Evidence for mixotrophy in adult *Idas* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*

3-4.4.1 Alimentary, digestive and excretory system

Laming et al. (2014) previously examined post-larval to adult anatomical development in *I*. (*s.s.*) *modiolaeformis* from the NDSF, in the eastern Mediterranean (CHAPTER). In that study, many components for a funtioning digestive system were already in place in post-larval plantigrades (SL 0.38–0.42 mm), prior to the acquisition of their symbionts (at SL 0.59 mm). The low symbiont densities prior to maturation, and the presence of an almost fully-formed gut, prompted Laming et al. to suggest a necessity and potential for mixotrophy in *I*. *modiolaeformis*, respectively. In that study, qualitative increases in symbiont densities and gill-specificity with increasing shell-length (SL) suggested that, at the very least, adults were shifting towards increasingly-chemosymbiotic mixotrophy. Mixotrophy had also been postulated recently for a Pacific congeneric, *A.*(*s.l.*) *iwaotakii* based on the simulataneous presence of thiotrophic symbionts and relatively complete digestive anatomy with discernible contents in histological sections (Thubaut et al. 2013a). Anatomical features observed in *I*. (*s.s.*) *modiolaeformis* in the current study, mirrored those previously identified in specimens from the NDSF (Laming et al. 2014). A fully-formed digestive system was apparent in both species (e.g. Figure 3.6, yellow-pigmented digestive diverticula beneath foot of *I*. (*s.s.*) *modiolaeformis*) with a dorsal mid- and hind-gut, visible on account of dark sediment-like contents. In *I*. (*s.s.*) *modiolaeformis* one intestinal loop was typically identifiable; *I.* (*s.l.*) *simpsoni* sometimes had two (not shown). Histological examination of stained 7-µm sections of viscera from *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*, revealed the alimentary route from the mouth (Figure 3.6A: *I*. [*s.s.*] *modiolaeformis*) and heavily-ciliated oesophagus (Figure 3.6B; *I.* [*s.l.*] *simpsoni*) to the stomach and ultimately the mid-gut (partially sectioned, Figure 3.6A; *I.* [*s.l.*] *simpsoni*). All dorsal regions of the mantle cavity unoccupied by other organs, were flush with extensive digestive diverticula in both species (Figure 3.6A), which normally absorb soluble compounds in the stomach. In *I.* (*s.l.*) *simpsoni*, metanephridia were well-developed (Figure 3.5C), and structurally comparable to *Mytilus edulis* (Pirie and George 1979), but in *I*. (*s.s.*) *modiolaeformis* metanephridia were not evident.

3-4.4.2 Particle filtration and ingestion

The morphoanatomy of the gill filaments differed between the two species, where lateral regions of *I.* (*s.l.*) *simpsoni* gill filaments were not hypertrophied, as in *I. modiolaeformis*, instead being delicate and thin. During live examinations under a dissection microscope, the convection of debris in the vicinity of the gills was unidirectional in *I. modiolaeformis* and in most *I.* (*s.l.*) *simpsoni* (not evident in the three largest specimens, IF12, IF7 and IF3). High-magnification observations of this process (Supplementary video 3.1),

taken in the mid-to-anterior region of the ascending lamella for the smallest *I. modiolaeformis* mussel (IB1), confirmed that particle transport was being coordinated by ciliary action, where lateral cilia, latero-frontal cirri, and frontal cilia in the gill filaments could be identified (Figure 3.6D, labels 1, 2 and 3 respectively; Supplementary video 3.1, right inset). Initially, the labial palps –located anteriorly, inflexed posteriorly – appeared to be coupled with the most anterior gill filaments in all specimens (e.g. Figure 3.6A). This coupling disengaged somewhat during dissection, likely as a consequence of stress (Jørgensen 1990).

Initially, particles were captured and transported ventrally between interfilamental lateral cilia, and then anteriorly along the ventral particle groove towards the labial palps and mouth where particles were immediately ingested. Additional particles were entrained in a growing mucous string (Figure 3.6A, C, dashed arrow) on approach to the mouth and teased into a mucous-bound ball by the ciliated labial palps (Figure 3.6A, C, dashed arrow), as in heterotrophic mussels (reviewed in Jørgensen 1990). Due to the presence of the mucous-bound mass additional particle ingestion was difficult to discern; the quantities of

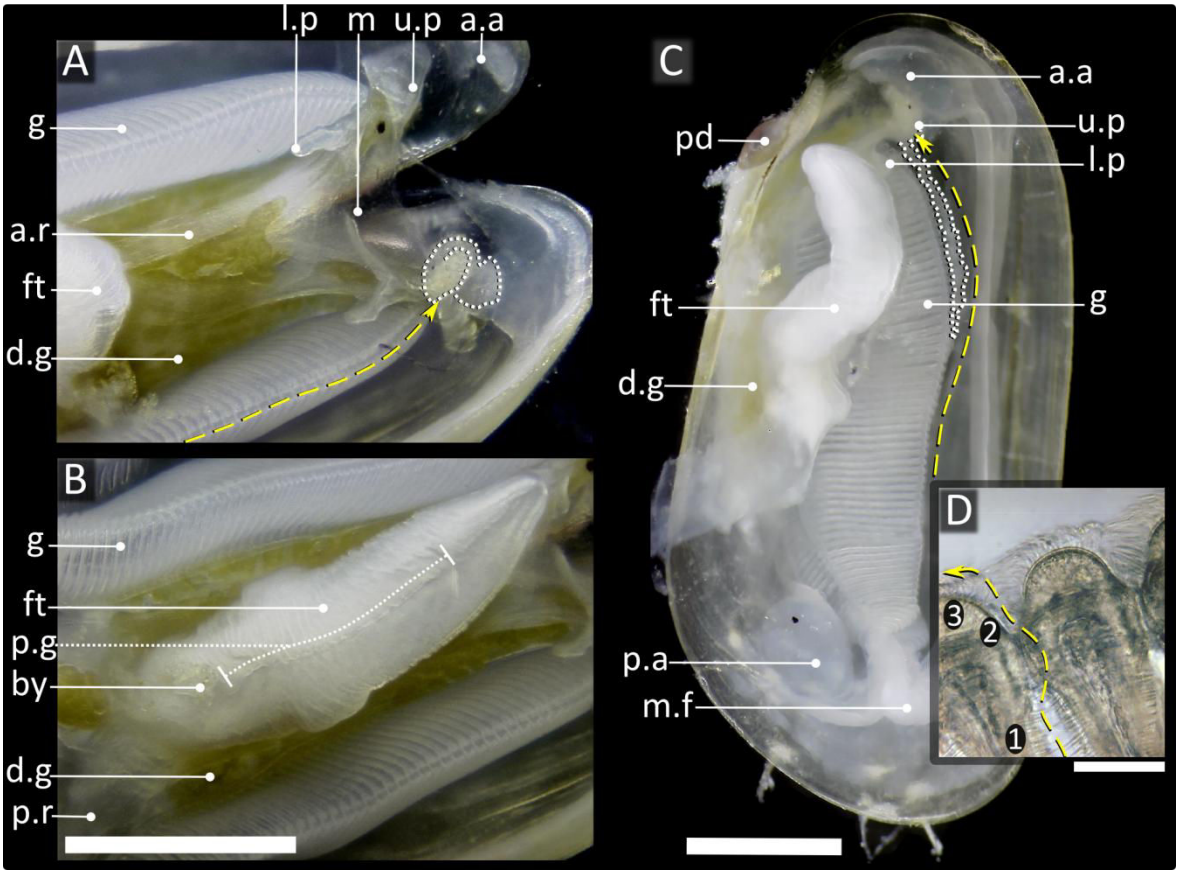

Figure 3.6 Detail of the dissection performed on IB1, a small *I.* (*s.s.*) *modiolaeformis* male
Note that this mussel is the same as that in Supplementary video 1, and was not induced to spawn. **A)** Ventral view of the buccal region during the accumulation of mucus, actively rotating between labial palps (dotted white perimeter), delivered by the feeding groove (yellow path). The foot is curled posteriorly (far left). **B)** Ventral view of the medial region. The pedal groove extends from the byssus gland to the sub-distal pit (left and right stop-marks on dotted line). Note coloration of viscera in both **A** and **B**. **C)** Sagittal view of left lateral region. Mucus string is depicted with a white perimeter, delivered along the ventral particle groove (yellow path). **D)** a high magnification view of the frontal region of filaments, with the lateral cilia (1), laterofrontal cirri (2) and frontal cilia (3), involved in trafficking particles ventrally (yellow path). a.a *anterior adductor*, a.r *anterior retractor*, by *byssus gland*, d.g *digestive gland*, ft *foot*, g *gill*, l.p *lower labial palp*, m *mouth*, m.f *mantle fold*, p.a *posterior adductor*, pd *prodissoconch*, p.g pedal groove, p.r *posterior retractor*, u.p *upper labial palp*. Scale bars 1 mm (inset **D**: 50 µm)

mucous produced may have been abnormally abundant due to physiological stress incurred when opening the mussels (Jørgensen 1990). The ultimate fate of un-ingested mucous was unclear, but it was probably ejected as pseudofaeces and transported away from the mouth, as in other bivalves (Jørgensen 1990). To the author's knowledge, these represent the first direct microscopic observations of particle ingestion in live bathymodiolin mussels providing evidence of filter-feeding behaviour in both species, demonstrated indirectly in some larger-sized members of this sub-family (e.g. tracking assimilation of radio-labelled water-borne bacteria Page et al. 1990; Page et al. 1991; naturally-occurring plankton removal rates, Cowie et al. 1999; or based on a C-flux model, Martins et al. 2008).

3-4.4.3 Evidence of symbiosis

All-but-one of the species of *Idas* previously examined for symbionts, have been shown to be chemosymbiotic (e.g. Deming et al. 1997; Duperron et al. 2008a; Southward 2008; Ritt et al. 2012; Rodrigues et al. 2013; Thubaut et al. 2013a), the exception being the type species *I. argenteus*, a larviphage (Ockelmann and Dinesen 2011). Remarkable symbiont diversity has been demonstrated in *I. modiolaeformis* when recovered from hydrocarbon seeps (Duperron et al. 2008a), in stark contrast to young specimens of the same species in the same region living on wood (Laming et al. 2014). In *I. simpsoni*, a highly dominant thiotrophic bacterium was observed in specimens from hydrocarbon seeps in the Marmara sea (Ritt et al. 2012). FISH performed herein revealed markedly different symbiont patterns depending not only on species, but also on the year of collection. Although *Idas modiolaeformis* collected from oakwood deployments (2012) did not display the level of diversity seen in Duperron et al. (2008a), dense populations of bacteria were identified in the gills, mainly composing of highly dominant type-T1 thiotrophs and a much-reduced presence of type-M1 methanotrophs, established towards the interior regions of the gill (Figure 3.7). This sort of dual symbiosis has been seen before in this species, when settled upon wood (Lorion et al. 2012). General Eubacterial signals mirrored those of T1-thiotrophs, suggesting the thiotrophs accounted for almost all the Eubacteria present. In specimens of *I. simpsoni* from the same substrata and cruise (LD3-LD4, oakwood, recovered 2012), densities were lower (Figure 3.7). However, *I. simpsoni* specimens were less than half the size of the *I*. *modiolaeformis* examined (SL 3.7 mm versus 9.0–9.2 mm). Attempts to elucidate phylotypes using specific oligonucleotide FISH probes were ultimately unsuccessful in *I. simpsoni,* though successful hybridisation with the EUB338 probe confirmed the signal to be eubacterial (Figure 3.7). Note that these mussels were not involved in live fertilisation experiments. Symbionts could not be identified in mussels from the live observational experiments during post-experimental FISH. However, the FISH analyses presented above (specimens from previous year) and the stable isotopes data discussed below for each species (Figure 3.8; Supplementary table 3.2, both years), suggest that this absence reflects a short-term symbiont loss due to the conditions to which these specimens were subjected in the lab. Symbiont loss is a well-known phenomenon in the Bathymodiolinae (e.g. Kádár et al. 2006), and can be rapidly induced using experimental conditions (Duperron, unpublished data).

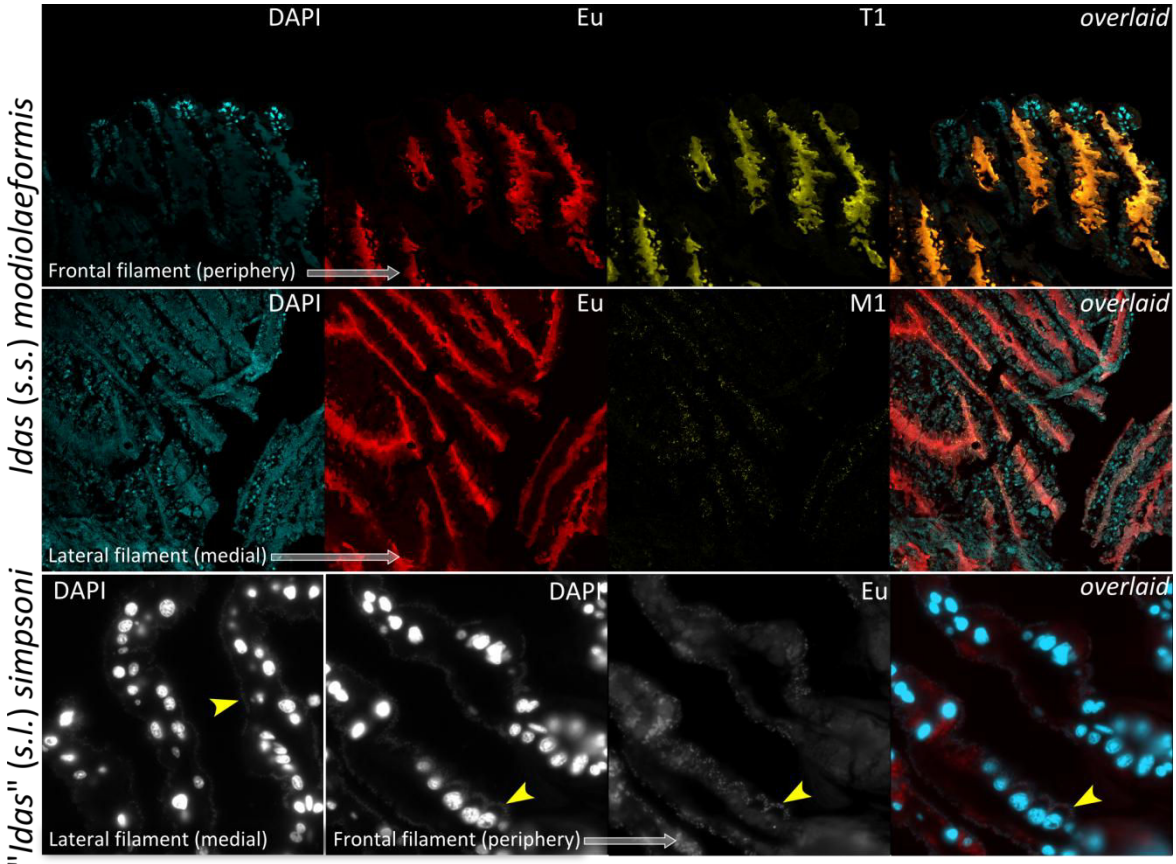

Figure 3.7 Fluorescence *in situ* hybridisations performed on gill tissue of *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*
All micrographs from specimens collected from oakwood in 2012. FISH failed to identify symbionts in IF# mussels (2013) used in live observations (*see main text*). Eubaterial probes confirmed DAPI signals associated with lateral-abfrontal filament surfaces to be Eubacteria in both species (images labelled Eu). In *I. modiolaeformis*, these comprised of thiotrophs (T1) and methanotrophs (M1). Eu-T1 overlaid image identified thiotrophs as highly dominant (Eu-M1 overlaid image has an abundance of eubacteria that were not methanotrophic). In *I. simpsoni*, eubacteria type could not be confirmed with the probes employed.

3-4.4.4 Stable Isotopes signatures in soft tissues

Stable isotopes analyses were performed both on the IF# mussels recovered in 2013, and on the *I.* (*s.s.*) *modiolaeformis* collected from oakwood deployments recovered from the canyon in 2012, recovered from oakwood colonisation experiments (Figure 3.8; Supplementary table 3.2). Samples were categorised putatively by species and by substrate, being pooled where necessary (i.e. insufficient mass individual^{-1}). This was carried out prior to formal COI-confirmation of species, leading to a single instance where a pooled sample resulted in a mixed-species isotopic signature (IF4 and IF10 from palmwood, 0.3 and 0.4 mg contributions respectively). C/N atomic ratio values of foot tissues were similar in all groupings (3.3 – 3.6). Values for $\delta^{13}C$ and $\delta^{15}N$ displayed significantly-reduced variability within experimental groupings than between them, where testable (*I. modiolaeformis* on oak versus *I.* (*s.l.*) *simpsoni* on palm, $\delta^{13}C$: $F_{(1, 10)} = 86.9$, <0.001; $\delta^{15}N$: $F_{(1, 10)} = 609.0$, <0.001; Figure 3.8). Mean $\delta^{13}C$ values for *I. modiolaeformis* found in 2012 on oakwood ($n = 5$) were -27.4 ± 1.05 ‰, while the single pooled *I. modiolaeformis* $\delta^{13}C$ value for 2013 (IF9 and IF14, CHEMECOLI, pinewood) was ^{13}C-enriched by comparison at -20.7 ‰ (Figure 3.8; Supplementary table

3.2). The most ^{13}C-depleted values were identified in the pooled mixed-species sample (IF4 and IF10 on palmwood, δ^{13}C = -29.7 ‰) and in the *I.* (*s.l.*) *simpsoni* from palmwood (*n* = 7) in 2013 (-31.3 ± 0.40 ‰). Mean δ^{15}N values for *I. modiolaeformis* found in 2012 on oakwood were 1.9 ± 0.38 ‰; the pooled *I. modiolaeformis* δ^{15}N value for 2013 (CHEMECOLI, pinewood) was ^{15}N-enriched in comparison at 4.8 ‰. The most ^{15}N-enriched values identified were in the pooled mixed-species sample (IF4 and IF10 on palmwood, δ^{15}N = 9.6 ‰) and in the *I.* (*s.l.*) *simpsoni* from palmwood (*n* = 7) in 2013 (8.7 ± 0.50 ‰).

The δ^{13}C values below -25 ‰ identified for *I.* (*s.l.*) *simpsoni* and the *I. modiolaeformis* 2012 samples, are in accordance with several previously published δ^{13}C values for vent bathymodiolins that derive carbon from associated thiotrophic only and from dual-symbiosis of thiotrophic and methanotrophic primary producers (e.g. -37.1 – -21.3 ‰, Fisher 1990; Cavanaugh et al. 1992; Nelson and Fisher 1995; Trask and Van Dover 1999; Fiala-Médioni et al. 2002; Yamanaka et al. 2003; McKiness et al. 2005; Supplementary figure 3.5). However stable isotope signatures are highly variable depending on the processes that generate the sources of sulphide and methane, and the relative proportions of each when both are available for dual symbioses. One need only examine δ^{13}C-values for mussel tissues from seeps in the Gulf of Mexico (*B.* [*s.s.*]

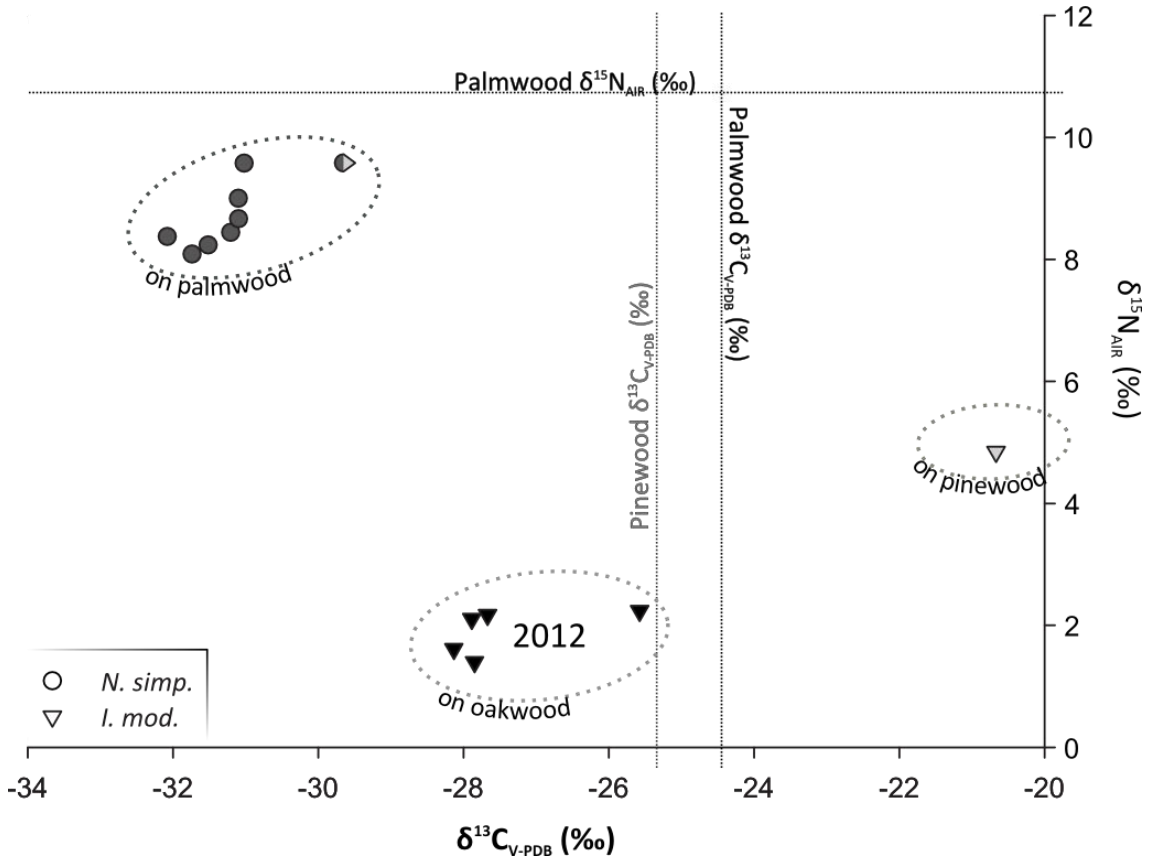

Figure 3.8 Stable Isotopes plot for *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* from various wood substrata
The scatter-plot depicts the δ^{13}C and δ^{15}N values for each of the specimens analysed for stable isotopes ratios. All data are from mussels used in live observations, except points labelled '2012'. The half circle/half triangle point was a pooled sample of the two species (due to initial misidentification of IF4 as *I. modiolaeformis*). Due to very low % nitrogen (0.18 – 0.21 %) in Pinewood, only δ^{13}C value exists for the Pinewood (left-hand vertical line). No data are available for the oakwood substrate.

heckerae, Nelson and Fisher 1995) and the Japan trench (*B*. [*s.l.*] *platifrons*, Barry et al. 2002), which range from -76.4 – -62.3 ‰ (methylotroph dominated symbioses), and compare them with those at ultramafic MAR vent sites (e.g. *B*. [*s.s.*] *puteoserpentis* -37.3 – -32.5, thio-/methanotroph dual symbiosis, Robinson et al. 1998).

In *Bathymodiolus* (*s.l.*) spp. where mixotrophy has been identified (*B.* [*s.s.*] *azoricus*, *B.* [*s.s.*] *thermophilus*, *B*. [*s.l.*] *childressi*, Page et al. 1990; Page et al. 1991; Riou et al. 2010) values are approximately comparable to those of *I.* (*s.l.*) *simpsoni* (palmwood) and *I*. (*s.s.*) *modiolaeformis* (oakwood) in this study (*B*. [*s.s.*] *azoricus* δ^{13}C: -32.6 – -21.3 ‰, δ^{15}N: -10.5 – +0.75 ‰, Trask and Van Dover 1999; Fiala-Médioni et al. 2002; and *B*. [*s.s.*] *thermophilus* δ^{13}C: -37.1 – -30.5 ‰, δ^{15}N: -8.1 – +9.6 ‰). The exception is *B*. [*s.l.*] *childressi* collected from seeps in the Gulf of Mexico, which hosts methanotrophs only (-67.1 – -37.5 ‰, Nelson and Fisher 1995). However, in the Gulf of Mexico, δ^{13}C signatures for methane hydrates are highly variable (Supplementary figure 3.5), depending on the thermogenic or biogenic origins (or a mixture of the two, Brooks et al. 1985). Negative or very slightly positive δ^{15}N values typical of larger chemosymbiotic mussels, are not comparable with those found in this study (except *B*. [*s.s.*] *thermophilus*, Supplementary figure 3.5), however this may reflect an alternative nitrogen source at seeps and vents, or another unknown factor attributable to the larger-sized *Bathymodiolus* (s.l.). In small-sized bathymodiolin mussels for which such data exists, values for both carbon and nitrogen isotopes are much more akin to the current study. In the small undescribed vent mussel (probably[24] *Benthomodiolus* sp., Thubaut et al. 2013b) at the Juan de Fuca ridge Mid-Valley vent fields, values for δ^{13}C and δ^{15}N vary from -30.7 – −26.6 ‰ and 1.5 – 5.19 ‰ respectively (McKiness et al. 2005; Bergquist et al. 2007).

Isotope values for the two species in the current study are only known from seep environments. *Idas* (*s.s.*) *modiolaeformis* found at several mud volcanoes in the Olimpi and Anaximander areas of the NDSF (Olu-Le Roy et al. 2004; Carlier et al. 2010) in the eastern Mediterranean displayed ^{14}C-depleted δ^{13}C signatures of -41.6 – -44.6 ‰. This may however reflect a predominance of methylotrophic symbionts using methane as an energy source, such as in *B*. [*s.l.*] *childressi*, *B*. [*s.s.*] *heckerae* and *B*. [*s.l.*] *platifrons* (Nelson and Fisher 1995; Barry et al. 2002; Olu-Le Roy et al. 2004). Such methylotroph symbionts were either at low abundances (as M1-methanotrophs, *I*. (*s.s.*) *modiolaeformis*), or not identified at all in the current study. Values for δ^{15}N in *I*. (*s.s.*) *modiolaeformis* cited in Olu-Le Roy et al. (2004), were also slightly ^{15}N-depleted, ranging from 0.2 – 0.4 ‰, and lower than those in this study.

Notably for *I.* (*s.l.*) *simpsoni*, δ^{13}C and δ^{15}N values approach those documented for this species in the Marmara Sea, at sites of methane seepage; there, δ^{13}C varied between -37.4 ‰ – -35.5 ‰ while δ^{15}N values ranged from values between 5.7‰ – 6.0‰ (*this study*: δ^{13}C -31.3 ± 0.40 ‰, δ^{15}N 8.7 ± 0.50 ‰). The

[24] this species is sometimes assigned to "*Idas washingtonia*", probably inaccurately

elevated $\delta^{15}N$ values in that study were already unusually ^{15}N-enriched for a chemosymbiotic mussel (perhaps suggesting that nitrogen sources were not strictly chemosynthetic Ritt et al. 2012). In the current study $\delta^{15}N$ were more ^{15}N-enriched again, reflecting a probable input from photosynthetically derived nitrogen, based on the filter feeding behaviour identified in both species.

Mixotrophic diets are thus supported for both species (albeit more biased towards chemosymbiosis in *I.* (*s.l.*) *simpsoni*), although an undetected dual symbiosis cannot be ruled out (as these can have similar $\delta^{13}C$-signatures, e.g. Trask and Van Dover 1999). Subtle distinctions exist between $\delta^{13}C$-values (more negative) and $\delta^{15}N$-values (more negative or equivalent) identified in the current study for *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* and those for organisms whose diets are linked to wood directly (e.g. wood boring bivalve species, wood-associated limpets and chitons, Zbinden et al. 2010; Duperron et al. 2013b). However these distinctions are not such that direct feeding on wood-derived nutrition can be ruled out (though not precedence for this exists in mussels).

^{15}N-enriched values suggest that the diets of both species, but particularly *I.* (*s.l.*) *simpsoni*, are not exclusively of symbiotic or free-living bacterial origin, with $\delta^{15}N$ values approximating those of primary heterotrophs (Carlier et al. 2009; Carlier et al. 2010), rather than other chemosymbiotic species at ≈ -1 – -11 ‰ (Cavanaugh et al. 1992; Trask and Van Dover 1999; Fiala-Médioni et al. 2002), the exception being values for small-sized mussels (McKiness et al. 2005; Ritt et al. 2012) other than *I.* (*s.s.*) *modiolaeformis* (Olu-Le Roy et al. 2004; Carlier et al. 2010). Since the incorporation of a diverse array of nutritional resources could also explain such signatures, the case for a mixotrophy diet cannot be discounted. Accordingly, the notably-deviant values for $\delta^{13}C$ and $\delta^{15}N$ for the 2013-pooled *I. modiolaeformis* found in the 10-month CHEMECOLI deployment (pinewood, Figure 3.8; Supplementary table 3.2), may reflect a diet dominated by photosynthetically-derived material, as might be expected based on the smaller sizes and presumably, younger ages (Cowie et al. 1999; Martins et al. 2008; Laming et al. 2014).

3-4.5. Species comparison and further considerations

3-4.5.1 Reproduction

Spawning appears to be semi-continuous in both *I. modiolaeformis* and *I.* (*s.l.*) *simpsoni*. Oocyte diameters were typical of planktotrophic larval development (Arellano and Young 2009), and display slightly negative buoyancy only. This may affect ascension through the water column to shallower eutrophic waters, where the hatched, planktotrophic larvae – shown to be aposymbiotic (Laming et al. 2014) – are thought to feed and develop, as in *B.* [*s.l.*] *childressi* (Arellano and Young 2011; Arellano et al. 2014). Oocyte properties for both species were so similar as not to be distinguishable, indicating that modes of dispersal in both *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* are likely identical, and may reflect a more general trend in Bathymodiolinae (see *Introduction*). Historically, *I. modiolaeformis* has displayed sex-ratios indicative of protandry (Gaudron et al. 2012; Laming et al. 2014), further corroborated by the size-specific prevalence for

females in the current study at (smallest individual was male only, SL 5.09 mm).*Idas* (*s.l.*) *simpsoni* sex-ratios approximated 50:50 wherein the two smallest individuals at SLs < 6mm were hermaphroditic, but functionally male. While this hints at a similar pattern of protandry, the otherwise equal size-dependent sex ratios show that at these larger SLs in this habitat, both sexes co-occur. The biology behind these patterns remains difficult to decipher as a consequence.

3-4.5.2 Nutritional flexibility

The findings of this study, though preliminary, provide new insights into the nutritional and reproductive biology of two bathymodiolin species. Laming et al. (2014) collated evidence based on the developmental biology of *Idas* (*s.s.*) *modiolaeformis* which strongly suggested not only the capacity for mixotrophy (functional digestive system) but also a probable obligation to filter-feed, as symbiont densities were considered too low to solely support energetic requirements. In the current study, we provide further evidence that this capacity for mixotrophy is retained in adults of *I. (s.s.) modiolaeformis*, and also exists in *I. (s.l.) simpsoni*, from the Gulf of Lion, on wood. This is despite the documented prevelance for dense populations of bacterial symbionts in the gills of these species, based on the current FISH analysis but also previous work (Duperron et al. 2008a; Duperron et al. 2008b; Lorion et al. 2009; Lorion et al. 2012; Ritt et al. 2012). Despite belonging to highly-divergent clades, which would ultimately warrant classification within distinct genera, commonality exists in the biology of *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*. This reflects either conservative or convergent features of their evolution, where such features appear to be widespread within the smaller-sized bathymodiolins.

Idas (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* evidently retain the capacity for filter-feeding and assimilating particulate organic matter (POM), based on live observations of active particle processing, on the presence of considerable volumes of digestive diverticula, and the preservation of excretory function with externally-visible gut contents. However, both species were also documented to engage in symbioses in the current study harbouring dense populations of bacteria in their gills, confirmed to be predominantly sulphur-oxidising in *Idas* (*s.s.*) *modiolaeformis*. Yet to be determined however are how relative contributions of heterotrophy and symbiont-based nutrition vary both ontogenetically, and whether habitat location and type influences nutritional dynamics, as seems likely. This is especially poignant given the well-documented variability in symbiont composition and abundances in *I*. (*s.s.*) *modiolaeformis* at various localities and shell sizes (Duperron et al. 2008a; Lorion et al. 2012; Rodrigues et al. 2013). Disentangling these nutritional components remains a challenge in the absence of targeted nutritional-sources data for this dynamic canyon environment, as evidenced by the complex stable isotopes picture identified. A significant fraction of plume POM is terrestrial in origin within the Gulf of Lion (Darnaude et al. 2004) due to unusually high terrestrial POM loading, originating from the Rhône delta (mean annual flow of 1,700 $m^3 s^{-1}$, catchment area 98,000 km^2, Darnaude et al. 2004; Bourgeois et al. 2011). Recent studies have also identified depth-dependent effects on POM $\delta^{15}N$ (varying across plankton size categories) as POM

degradation increases exponentially with water-column depth, resulting in further alterations in $\delta^{13}C$ and $\delta^{15}N$ signatures (e.g. Mintenbeck et al. 2007). However, the canyon ecosystem itself is subject to complex hydrodynamic and ecological features which prevent a simple assessment of POM origins. These factors, and the additional variables specific to the current study including deployment periods, seasonality, variable substrata type, and unresolved effects of symbiont metabolism (Robinson and Cavanaugh 1995), have rendered further interpretation impossible. The comparison of electron donor availability in different reducing habitats is difficult and is out of the scope of this study. However, as supply of sulphides (and by inference, biogenic methane) at the surface of sunken-wood is subject to considerable temporal variability (Laurent et al. 2009; Laurent et al. 2013; Yücel et al. 2013), sustenance derived solely from engaging in chemosymbioses may not meet the energetic demands of the organism and a degree of nutritional flexibility is advantageous. A capacity for facultative mixotrophy may also explain the multi-habitat distribution of both these species, by taking advantage of varying contributions from heterotrophy and chemosymbiosis.

This study is believed to be the first to report live observations concerning the nutrition and spawning behaviour of *Idas*-like mussels. Be it *in situ* (ideally) or *ex situ* as in the current study, the benefit of carrying out live-organism studies examining deep-sea organismal behaviour, and in particular larval development, has been continually reaffirmed within the deep-sea biology community, emphasising the need for long-term multidisciplinary experimental programmes which permit such work to be undertaken. Aspects of the methodology presented here could benefit from further refinement. In particular, sulphidic conditions were not confirmed electrochemically, with possible consequences for bacterial symbioses in living organisms. Future attempts to keep organisms associated with sulphidic organic-falls, would benefit from a means of monitoring sulphide production (as in Yücel et al. 2013) and a deliberately reduced oxygen supply, as this may have inadvertently oxygenated the supplied 'sulphidic' substrata in the current study (and thus induced symbiont loss). However, the current study has demonstrated that maintaining aquaria for smaller wood-based (and potentially bone-based) chemosymbiotic species need not be costly in terms of space or finances, following the collection of samples, which does remain logistically and financially challenging. It also aims to reopen discussion on larval development in deep-sea organisms, which remains poorly understood (one of the goals of INDEEP, The International Network for Scientific Investigations of Deep-Sea Ecosystems). If specimens are collected from the upper limits of their depth range in the deep-sea, it is possible to keep them alive at ambient pressures (Dixon et al. 2004; Arellano and Young 2009). Seasonal time-series sampling would be of great benefit in empirically demonstrating semi-continuous readiness to spawn, which remains a challenge for deeper habitats, while it is hoped that information presented herein on the nutritional requirements of these species, will aid future attempts to maintain adults, and ultimately grow larvae for further research.

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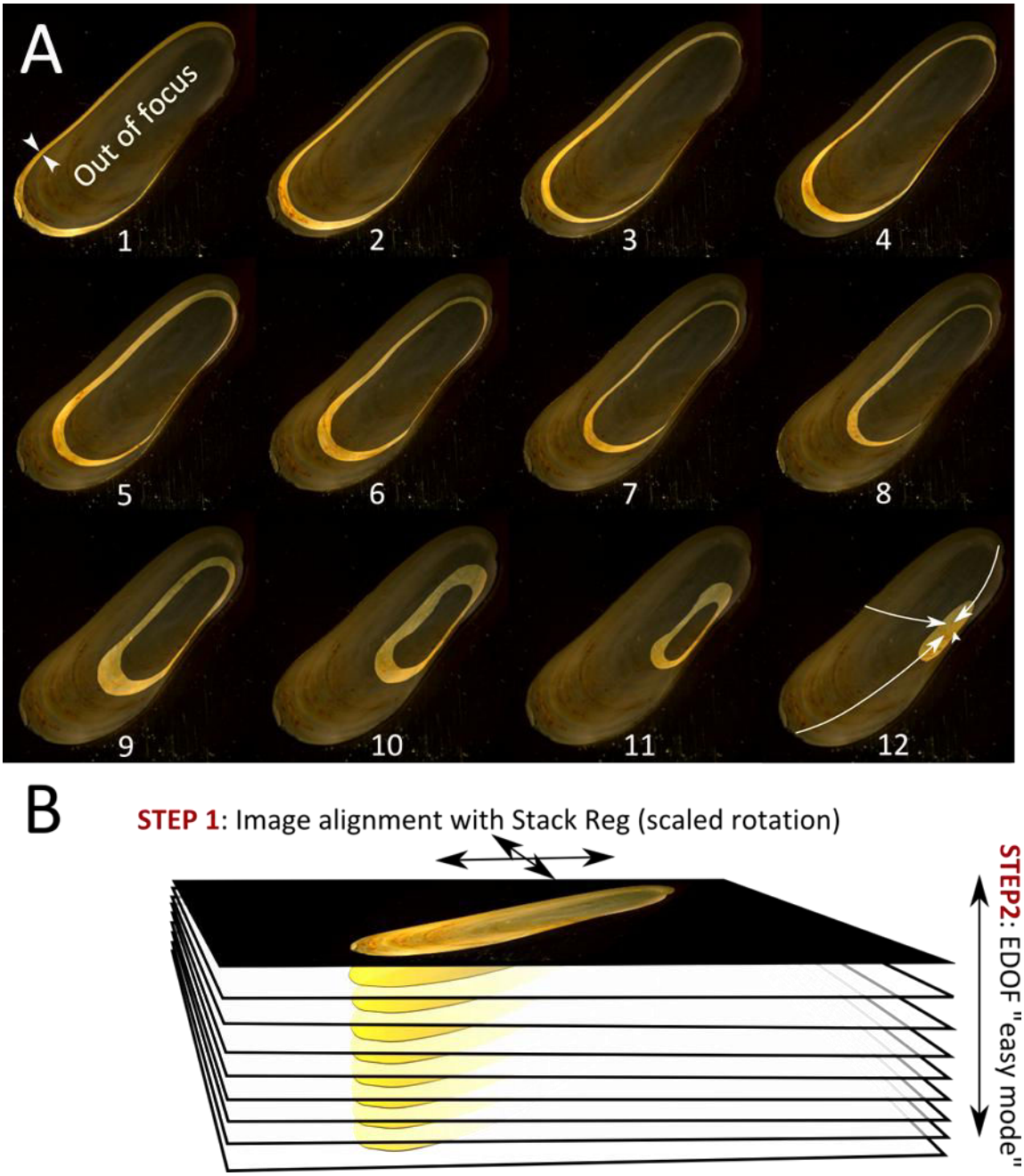
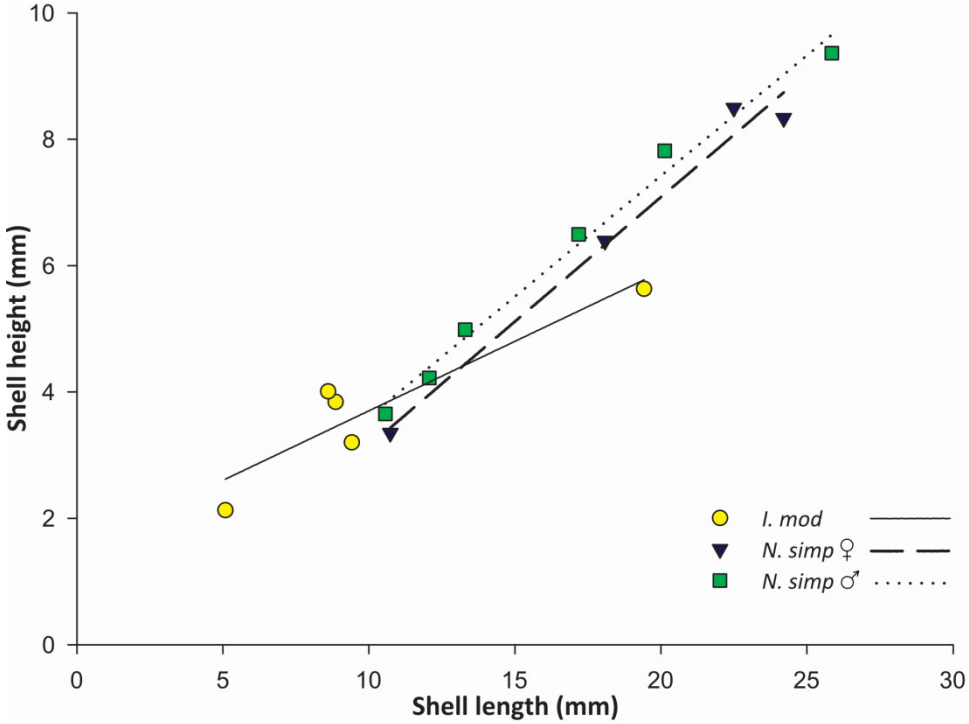
3-7 Chapter 3 Annex 1

3-7.1. Supplementary Material

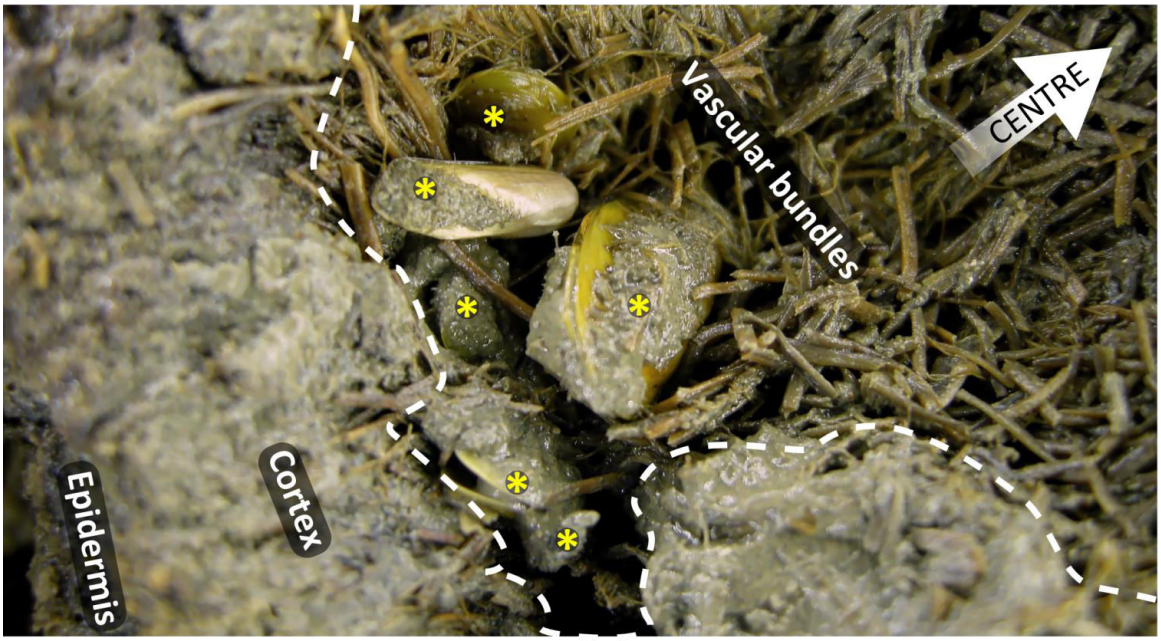
Supplementary figure 3.1 An overview of the fertilisation experiment conditions
A) The use of a tap-water bath to control temperature conditions of the aseptic filtered seawater (blue-lidded flasks, one being aerated in preparation for use) and the animals waiting induction (white baskets in suspended tub). **B-C)** A plan-view of the selected substrates for holding tanks: **B)** pinewood cubes from CHEMECOLI and **C)** palmwood. **E)** Individuals were separated when spawning began **F)** IF7 *I.* (*s.l.*) *simpsoni* in the process of spawning small white eggs. **G)** The conduit for aeration of embryonic development dishes.



Supplementary figure 3.2 Achieving an extended depth of field in micrographs
A) A stack of images are taken at incremental levels of focus in the vertical z axis (the lit area of shell is in focus). **B)** The images are then imported into FIJI (Image-J) either as a stack or as separate images- the stack can be formed form these within the FIJI software. The image stack is then subjected to an alignment (STEP 1) using the turbo-reg/StackReg add-on for FIJI. Scaled rotation worked best. The EDOF algorithm available for FIJI is then used to identify regions of highest contrast density to define focussed slices and a composite image entirely in focus is generated. If necessary, these images were then tiled in Photoshop CS3 (if the focus stacks were of parts of the shell only)

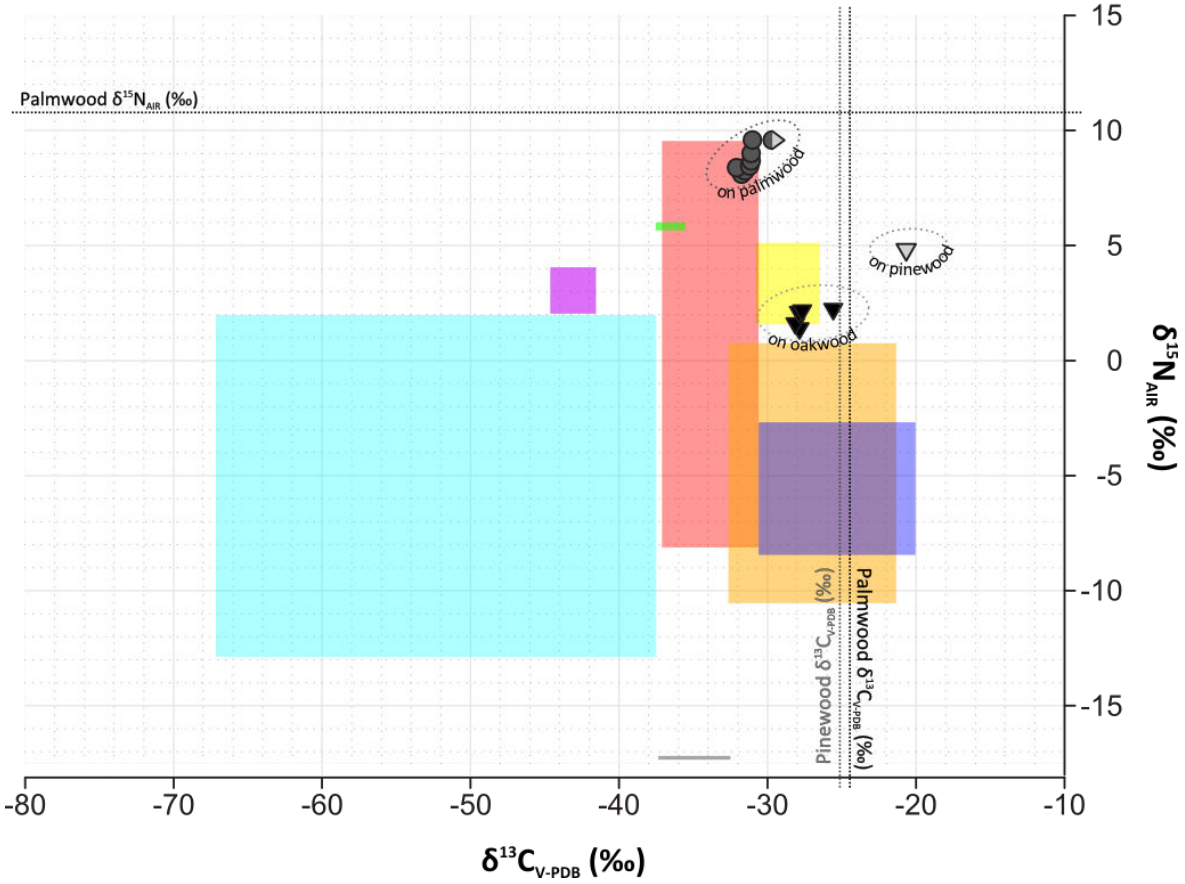


Supplementary figure 3.3 Shell dimensions depending on species and sex
Scatter-plot of shell height as a function of shell length in *I.* (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*.



Supplementary figure 3.4 Location of mussels in the palmwood experiment
Mussels (asterisks) of both species were located in the space between the bark (cortex) and the core water and food transport system, the central cylinder.

		$\delta^{15}N_{AIR}$(‰)	$\delta^{13}C_{V\text{-}PDB}$(‰)	Symbionts	Location
VENTS	*Be.* sp. JdF	+5.2 → +1.5	-26.6 → -30.7	C	Pacific Ocean
	B. thermophilus	-8.1 → +9.6	-30.5 → -37.1	C	Pacific Ocean
	B. brevior	NA	-30.8 → -35.8	C	Pacific Ocean
	B. aff. brevior	-2.7 → -7.5	-20.0 → -30.8	C	Indian Ocean
	B. azoricus	-10.5 → +0.8	-21.3 → -32.6	C, M	Atlantic Ocean
	B. puteoserpentis	-17.2	-32.5 → -37.3	C, M	Atlantic Ocean
SEEPS	*T. fisheri*	NA	-36.2 → -38.1	C	Gulf of Mexico
	B. childressi	-12.9 → +2.0	-37.5 → -67.1	M	Gulf of Mexico
	B. platifrons	NA	-67.5 → -68.1	M	Japan Trench
	B. brooksi	NA	-44.4 → -55.7	C, M	Gulf of Mexico
	B. heckerae	NA	-62.3 → -76.4	C, M	Gulf of Mexico
	I. modiolaeformis	+0.2 → +0.4	-41.6 → -44.6	C, M, G, B	Mediterranean
	I. simpsoni	+5.7 → +6.0	-35.5 → -37.4	C	Mediterranean



Supplementary figure 3.5 Isotopes plot for study species in the context of other bathymodiolins
The scatter-plot depicts the $\delta^{13}C$ and $\delta^{15}N$ values for each of the specimens plotted in figure 8, in the context of values from the literature for both the study species (from methane seeps) and other bathymodiolin mussels from hydrothermal vents and hydrocarbon seeps (see values in the appended table and the coloured polygons). Values mainly from summary table in McKiness et al. 2005 (references therein) except: *I. modiolaeformis* and *I. simpsoni* from seeps (Olu Le Roy et al. 2004; Carlier et al. 2010 and Ritt et al. 2012 respectively) and *Benthomodiolus* sp. from Juan de Fuca vents (Be. sp JdF, Bergquist et al. 2007). *Be*: *Benthomodiolus*, *B*: *Bathymodiolus*, *T*: *Tamu*, *I*: *Idas*. Symbionts, C: chemoautotrophic, M: methanotroph/methylotroph, G: 'symbiont G'; B: Bacteroidetes.

Supplementary table 3.1 General species data for specimens in this study
Haplotypes are defined based on identical fragment COI sequences

Label	Wood	Sex	Species	% similarity	Haplotypes
IB01	Pine	♂	"*Idas* sp. Med" [*I.* (*s.s.*) *modiolaeformis*]	487/488(99%)	
IF09	Pine	♀		504/506(99%)	*I.mod* A
IF10	Palm	♂		505/507(99%)	
IF01	Palm	♀		497/500(99%)	*I.mod* B
IF14	Pine	♀		458/460(99%)	*I.mod* C
IF04	Palm	♀	*I.* (*s.l.*) *simpsoni*	498/498(100%)	
IF03	Palm	♂		504/504(100%)	"*I.*"*simp* A
IF06	Palm	♂		509/509(100%)	
IF13	Palm	♂		522/523(99%)	
IF05	Palm	♀		508/509(99%)	"*I.*"*simp* B
IF07	Palm	♀		507/508(99%)	"*I.*"*simp* C
IF12	Palm	♀		529/532(99%)	"*I.*"*simp* D
IF11	Palm	♂		528/530(99%)	"*I.*"*simp* E
IF08	Palm	♂		509/510(99%)	"*I.*"*simp* F
IF15	Palm	⚥		492/493(99%)	"*I.*"*simp* G
IF16	Palm	⚥		493/494(99%)	"*I.*"*simp* H
IF02	Palm	♂	*I.* (*s.l.*) *simpsoni* ?	~~COI~~	Unconfirmed
IB02	Pine	♂	≈ Idas sp. ESU D	374/436(86%)	Oblong *Idas* sp.

Supplementary table 3.2 A summary of the stable Isotopes data used to plot Figure 3.8

Sample ID	$\delta^{15}N_{AIR}$ (‰)	$\delta^{13}C_{V\text{-}PDB}$ (‰)
Banyuls 2012 (Oakwood)		
CO28D[a]	1.4	-27.9
CO28F[a] & CO28C[a] "alcohol"	1.6	-28.1
CO28A[a]	2.1	-27.9
CO24F[a] & H[a]	2.2	-27.7
CO24E[a]	2.2	-25.6
Mean	***1.9***	***-27.4***
St. Dev.	***0.38***	***1.05***
Banyuls 2013 (Pinewood)		
IF09[a] & 14[a]	4.8	-20.7
Banyuls 2013 (Palmwood)		
IF04[b] & 10[a]	9.6	-29.7
IF07[b]	8.1	-31.7
IF13[b]	8.2	-31.5
IF5[b]	8.4	-32.1
IF8[b]	8.4	-31.2
IF12[b]	8.7	-31.1
IF02[(b)] & 06[b]	9.0	-31.1
IF03[b]	9.6	-31.0
Mean	***8.7***	***-31.3***
St. Dev.	***0.50***	***0.40***
Substrata 2013		
Palmwood	10.7	-24.4
CHEMECOLI Pinewood	---	-25.4

***** Supplementary video 1 may be found on the CD Archive*****

Chapter 4 SETTLED, SYMBIOTIC, THEN SEXUALLY MATURE: ADAPTIVE DEVELOPMENTAL ANATOMY IN THE DEEP-SEA, CHEMOSYMBIOTIC MUSSEL *IDAS MODIOLAEFORMIS*

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4-1 Abstract

The Bathymodiolinae are pervasive in reducing environments in the deep sea, yet data on post-larval and juvenile development and on the process of symbiont acquisition remain elusive. To understand how these opportunistic metazoans survive in ephemeral reducing habitats, individuals of the small bathymodiolin, *Idas* (*s.s.*) *modiolaeformis*, were examined histologically to trace their reproductive development, and with fluorescence and transmission electron microscopy to identify patterns of infection by their environmentally-acquired bacterial symbionts. A size series of these mussels was retrieved from larval colonisation devices containing vegetative substrates, deployed for 51 weeks (November 2006–2007) in the central "Pockmarks" region (site 2A) of the Nile deep-sea fan in the eastern Mediterranean (NDSF), a zone where methane seepage can occur (N 32° 31.97, E 30° 21.18, 1693 m deep). Developmental patterns of germ-line cell migration, size-at-first-maturity, and symbiont acquisition and localisation are presented for the post-larva to adult transition. The smallest mature adult was a male with shell length (SL) 2.35 mm. All larger individuals in the series were male (maximum SL 6.54 mm). Based on the absence of bacterial signals, plantigrades were aposymbiotic, indicating strict heterotrophy in larvae and early post-larvae. During the early stages of dissoconch deposition, extracellular symbiont infection was non-specific. This was followed by increasing specificity on non-ciliated gill epithelia in adults. These observations on early development in *I.* (*s.s.*) *modiolaeformis* represent evolutionary adaptations to their ephemeral, reducing habitats.

4-2 Introduction

Repeated discoveries and descriptions of aphotic communities thriving upon hydrothermal vents (Lonsdale 1977), hydrocarbon seeps (Paull et al. 1984) and whale carcasses (Smith et al. 1989) initiated a new era in deep-sea biology, and altered conventional understanding of deep-sea ecosystems. It was realised that bacteria could metabolise reduced compounds and support whole communities either directly or indirectly through chemosymbioses, courtesy of simultaneous access to electron donors and electron acceptors at anaerobic-aerobic interfaces (Stewart et al. 2005; Cavanaugh et al. 2006). Since then, other organic falls (e.g. other marine mammals, terrestrial vegetative debris) have been postulated as reducing habitats, being colonised by opportunistic chemosymbiotic organisms and sulphur-reducing bacteria (e.g. Bienhold et al. 2013), though anecdotal records of wood-fall communities (Challenger expedition), pre-date records of all known reducing habitats (Murray et al. 1891).

Chemosymbiotic bivalves represent a pervasive and diverse macrofaunal group in these environments. Fifty-three Atlantic and Mediterranean deep-sea species are already confirmed to engage in symbioses (Duperron et al. 2013), with hundreds more worldwide in diverse reducing environments, when all depth ranges are considered (Taylor and Glover 2010). Recent, intensified, ecological study of aphotic habitats driven by chemosynthetic primary production has yet to resolve gaps in our knowledge of basic developmental traits for even the dominant species (Young 2003). The life-histories of deep-sea chemosymbiotic bivalves thus remain speculative (Tyler et al. 2009). Studies have focused on the sex ratios, size and ultrastructure of gametes, and gametogenesis (reviewed by Le Pennec and Beninger 2000; more recently, Le Pennec et al. 2002; Colaço et al. 2006; Dixon et al. 2006; Kádár et al. 2006; Tyler et al. 2007a; Tyler et al. 2009; Gaudron et al. 2012), with one investigation based upon time-series sampling (Tyler et al. 2007a). Larval developmental studies in deep-sea chemosymbiotic bivalves are much rarer (Gustafson and Reid 1986; Arellano and Young 2009), supplemented by inferred larval modes from shell morphometry, where the small prodissoconch I (indicative of hatching size) is compared with the larger prodissoconch II (indicative of settlement size, e.g. Lutz et al. 1980; Gaudron et al. 2012; Ritt et al. 2012). Observations on post-larval and juvenile development in chemosymbiotic deep-sea bivalves are conspicuously absent, other than incidental observations while examining immature symbiont associations (Streams et al. 1997; Salerno et al. 2005; Wentrup et al. 2013). This could be because size-related difficulties encountered during post-larval examination are many (microscopic sample-sorting, identification, dissecting, orienting tissue during embedding, and interpreting juvenile anatomy), while sample availability is limited (larval settlement might at times be supply-limited and sampling often favours larger, generally adult macrofauna). Prior to the incorporation of symbionts, hydrocarbon 'cold' seeps and organic debris probably embodied physiologically-suboptimal habitats during deep-sea mussel evolution. Evolutionary adaptations believed to have facilitated their exploitation, such as the well-documented bacterial symbioses of large deep-sea chemosymbiotic Bathymodiolinae (reviewed in Dubilier et al. 2008; Duperron et al. 2013), now restrict bathymodiolin distributions to these reducing environments. This is evidenced by their conspicuous

absence from 'background' bathyal fauna (Duperron 2010). A time-calibrated molecular phylogeny of this sub-family has demonstrated however, that evolving the capacity to flourish in these habitats facilitated astounding levels of adaptive radiation (Lorion et al. 2013). This occurred over ca. 85 million years (Ma) with peak speciation rates at an estimated 41–32 Ma (0.17 speciation events Ma^{-1}). Extant Bathymodiolinae currently display wide variability in habitat use, shell morphology, adaptive anatomy, developmental and reproductive mode and dependency upon their symbionts.

The bathymodiolin genus *Idas,* (polyphyletic at present, Lorion et al. 2010, 2013; Duperron et al. 2013; Thubaut et al. 2013b), colonises organic falls (Smith et al. 1989; Kiel and Goedert 2006; Samadi et al. 2007; Duperron et al. 2008a; Lorion et al. 2009, 2012; Tyler et al. 2009; Ockelmann and Dinesen 2011; Guisti et al. 2012; Bienhold et al. 2013; Cunha et al. 2013; Thubaut et al. 2013a), authigenic carbonate crusts at methane cold-seeps (Olu-Le Roy et al. 2004; Ritt et al. 2012) and sulphide crusts, bathed in diffuse hydrothermal vent fluids (Juniper et al. 1992; Southward 2008). A then undescribed species, *Idas* sp. Med, was first documented in the Eastern Mediterranean at various hydrocarbon seeps and mud volcano sites (Olu-Le Roy et al. 2004). Several molecular-based analyses of *Idas*-like mussels have revealed *Idas* sp. Med to be one of the most pervasive chemosymbiotic bivalves known in the Mediterranean and eastern Atlantic (Gaudron et al. 2010, 2012; Lorion et al 2010; Cunha et al. 2013; Rodrigues et al. 2013; S.R. Laming, unpubl data from Lacaze-Duthiers Canyon, Gulf of Lyon, western Mediterranean). Lorion et al. (2012) tentatively ascribed this species to *Idas* (*s.s.*) *modiolaeformis* (Sturany 1896) based on morphological characters in the absence of type-specimen organic tissue for molecular analysis (*I*. [*s.s.*] *modiolaeformis* and *Idas* sp. Med are thus considered synonymous herein). This species is presently documented upon authigenic carbonate crust and tubes of *Lamellibrachia* sp. bathed in hydrocarbon seep fluids (Nile Deep Sea Fan (NDSF), Gaudron et al. 2012; Lorion et al. 2012), and upon various types of sunken vegetation. The latter was either 1) deployed as small colonisation experiments, such as alfalfa- or pinewood-filled colonisation devices called CHEMECOLI (Gaudron et al. 2010) placed on mud volcanoes in the Gulf of Cadiz (Cunha et al. 2013; Rodrigues et al. 2013) and at cold seeps in the eastern Mediterranean (Gaudron et al. 2010; Rodrigues et al. 2013, present study) and at the base of submarine canyons in the western Mediterranean (Lacaze-Duthers Canyon, Gulf of Lyon; S.R. Laming, unpubl data); 2) larger wood deployments (NDSF, Lorion et al. 2012; Bienhold et al. 2013; Lacaze-Duthiers Canyon, Gulf of Lyon, S.R. Laming, pers obs, J. Thubaut, pers comm) or; 3) naturally-occurring sunken wood (Gorringe Bank, eastern Atlantic, Rodrigues et al. 2013). The species has a wide depth range, 350–3015 m (Mercator MV, Gulf of Cadiz to Cheops Mud volcano, NDSF respectively).

In a genus associated almost exclusively with thiotrophic bacterial symbionts (Duperron 2010, excludes the asymbiotic *I.* [*d.f.*] *argenteus*), atypically-diverse associations have been documented in *I*. (*s.s.*) *modiolaeformis*, particularly in the eastern Mediterranean. Six bacterial phylotypes have been documented (Duperron et al. 2008a) in adults from authigenic carbonate crust associated with fluid seepage (2130 m

depth; 40 km from current study, NDSF, Mediterranean). Of these, a methanotroph, another methylotroph and two distinct thiotrophs were subsequently confirmed again 4 years later, in mussels from various substrata (Lorion et al. 2012). Relative symbiont dominance shifted from the methanotroph to the two thiotrophs between these two studies, demonstrating a predisposition for diverse, adaptable symbiont assemblages. Considered the prevalent symbionts in *I*. (*s.s.*) *modiolaeformis*, these bacteria can co-occur in mussels from the Nile Deep Sea Fan region, but in the eastern Atlantic only one or two phylotypes associate with this species (e.g. Rodrigues et al. 2013, including strains related to the 5th phylotype, symbiont G, Duperron et al. 2008a). Symbiont acquisition is considered environmental since bacterial symbionts, though present in gills, are absent from gonad tissue in adults (Gaudron et al. 2012). The proliferation of this symbiosis remains undocumented. The larvae of *I*. (*s.s.*) *modiolaeformis* are believed to be planktotrophic based on prodissoconch shell records, and adults are thought to be protandric hermaphrodites (Gaudron et al. 2012). *Idas* (*s.s.*) *modiolaeformis* is prevalent in reducing environments in the Mediterranean suggesting that it has evolved life-history traits to optimise survival. These mussels thus present a fascinating case study for the evaluation of life-history adaptations in highly ephemeral habitats under reducing conditions.

4-2.1. Research aims

The aim of the present study was to describe elements of the life-cycle of *I*. (*s.s.*) *modiolaeformis* likely to be adaptive in its particular habitat. A size series of *I*. (*s.s.*) *modiolaeformis* was collected from larval colonization experiments in a cold seep region of the eastern Mediterranean.

Based on this size-series, this study sought to answer several questions by recording tissue development from metamorphosis to adulthood. 1) What are the patterns of development in the gills and reproductive tissues in *Idas* (*s.s.*) *modiolaeformis*, following settlement? 2) What is the smallest size at which *Idas* (*s.s.*) *modiolaeformis* can reach maturity, in this habitat? 3) Are symbionts already present subsequent to metamorphosis in *Idas* (*s.s.*) *modiolaeformis* or must they be acquired from the environment and, do patterns in the symbiont association change during early development?

4-3 Materials and Methods

4-3.1. Sampling and processing

Post-larval sampling devices (CHEMECOLI, Gaudron et al. 2010) were employed at the cold-seep-rich “Pockmarks area”, central zone 2A of the NDSF, eastern Mediterranean (Cruises: BIONIL 2006; MEDECO 2007, Site: N 32° 31.97, E 30° 21.18, 1693m deep, see Gaudron et al. 2010 for further site details). These provided one of three different substrates for larvae (pinewood, alfalfa, or carbonate). Post-settlement predation and adult migration were minimised (by exclusion, 2 mm mesh), while currents and smaller organisms could still penetrate. A preliminary two-week deployment upon hydrocarbon-derived deposits of authigenic carbonate crust saw wood substrata rapidly colonised by many organisms, including *I*. (*s.s.*)

modiolaeformis post-larvae (see Gaudron et al. 2010). At the same site during this experiment's recovery, one of each substrate (i.e. three devices in total) was deployed by remotely operated vehicle (ROV *Quest 4000*, MARUM, Germany in 2006) for 51 weeks from November 2006 (recovery by ROV *Victor*, Ifremer, France in 2007). The identified individuals ranged from post-larvae to mussels that exceeded 3.6 mm in shell length (SL), the smallest recorded SL at which this species' maturity had been examined (Gaudron et al. 2012).

Once on board, random subsamples were fixed and preserved en masse using protocols specified in Gaudron et al. (2010). In the laboratory, the preserved substrata were sorted microscopically. Post-larval *I*. (*s.s.*) *modiolaeformis* from the two-week wood deployment were not used in this study. Mussels found in the 51-week wood and alfalfa CHEMECOLI deployments (no mussels were identified on carbonate) were fixed in either 4% buffered formaldehyde in twice-filtered seawater (TFSW) for 2–4 h at 4°C with a gradient transfer to 95% ethanol, or 95% ethanol directly (all stored at <5°C). The relative proportions of individuals fixed with each method and resulting analyses is summarised in a supplementary table (Supplementary table 4.1).

Individuals of *I*. (*s.s.*) *modiolaeformis* were identified based on morphoanatomy. Prior molecular studies had identified that all other mussels from these same deployments were of this species (Gaudron et al. 2010; Gaudron et al. 2012). The larval prodissoconch I and II were opaque pearly white and transparent red/orange respectively and, where present, the dissoconch was modioliform, colourless, and almost transparent in all juveniles or more opaque and off-white in individuals >4 mm. Periostracal byssus hairs were already numerous larger juveniles (> 1 mm), most concentrated on the postero-ventral margin flank. Wood- and alfalfa-located mussels were pooled, providing a size-spectrum from post-settlement to putative adults. Thus, it was possible to document key developmental patterns in *I*. (*s.s.*) *modiolaeformis* from the Nile Deep-sea Fan, for the size-range examined.

4-3.2. Preliminary shell and tissue analysis

Individuals were measured where feasible (dissoconch: n = 41, prodissoconch II: n = 35) and photographed (Figure 4.1a–e) using a camera-mounted dissection microscope (Evolution VF camera, Media cybernetics; Olympus S2X12 microscope, Japan; Image-pro v.5.1). Shell height (SH) was the greatest linear distance dorso-ventrally measured from the umbo, perpendicular to the hinge-line, while SL was the greatest linear distance antero-posteriorly measured parallel to the hinge line (Figures 4.1f–g). Prodissoconch II dimensions were measured using the same approach, but with standardised 'larval' hinge-lines, to permit the aggregation of prodissoconch II data across all shell sizes (Figures 4.1f–g). Dissections were performed microscopically using custom-made tools (Supplementary figure 4.1). General anatomy was recorded and tissue was extracted, with minimal disruption. Where gills had remained intact (n = 35), the number of filaments comprising the descending lamella in either inner demibranch - the first to develop - was recorded, as a proxy for gill development.

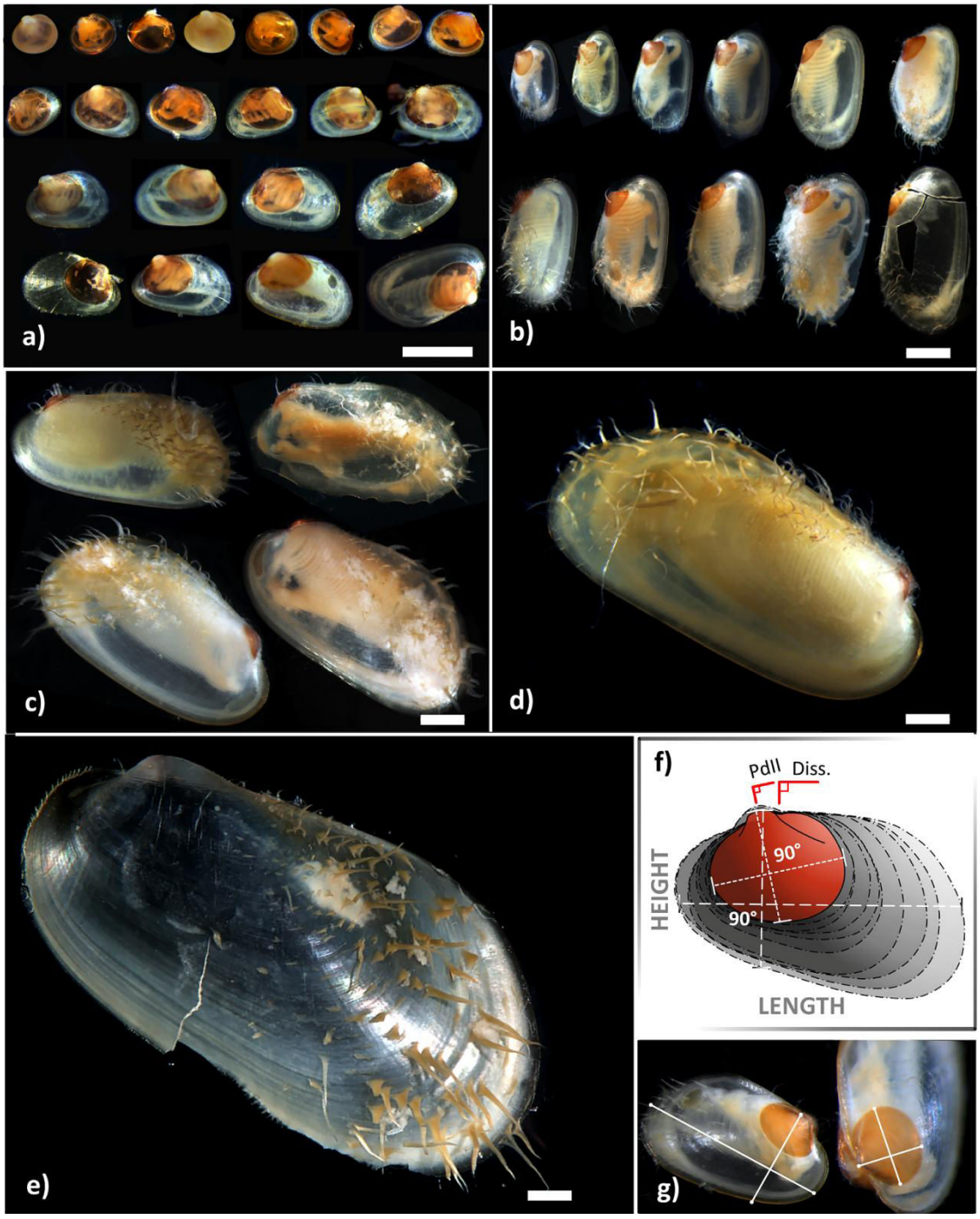

Figure 4.1 Micrographs of *Idas* (*s.s.*) *modiolaeformis* specimens examined
Micrographs are all but two of examined specimens. Grouping based upon morphometric analysis: SL (*a*) 0.38–0.85 mm ; (*b*) 1.22–1.92 mm; (*c*) 2.35–2.94 mm; (*d*) 4.37 mm and; (*e*) 6.54 mm (empty left valve). Figures (*f*) and (*g*) concern measurement protocol. SH dorso-ventrally perpendicular to hinge-line (*f*: right-angles), SL antero-posteriorly parallel to hinge line. Prodissoconch hinge line always considered as post-larval (*PdII*). Dissoconch hinge line was individual-specific (*Diss.*). Growth patterns based on overlaid micrographs, with prodissoconchs approximately aligned. Scale bars 500 µm

4-3.3. Embedding, sectioning and histology

Tissue was blotted dry and infiltrated (8 x 30-min infiltrations) in a gelatine capsule (size 00, Electron Microscopy Sciences, UK) filled with LR White resin (London Resin Company, UK), transferred to a fresh resin-filled gelatine capsule, orientated appropriately, capped, and polymerised at 55°C (20 hours minimum). Gelatine was then removed with hand-hot water.

Semi-thin sectioning maximised structural detail but retained staining contrast and optimised limited tissue availability. Resin blocks were wet-sectioned (glass knife) on a Leica EM Ultra Cut R Ultramicrotome (Germany). Supplementary figure 4.2 depicts the typical orientations. Where possible, 1 µm-thick sections on Superfrost plus slides were used for haematoxylin and eosin-Y staining (H&E), up to 1 µm-thick sections on Superfrost plus slides for fluorescence microscopy, and 350 nm-thick sections on carbon-film, 200-mesh, nickel grids for TEM. Periodic toluidine staining was performed to identify the cutting-axis and location (≈10-µm intervals).

Standard H & E staining procedures for thick sections were modified by extending the staining times and excluding ethanol, which compromises LR white resin. Semi-thin sections of select individuals (Table S1) were rehydrated (distilled water, 5 min), blotted dry and stained (1 hr) in filtered, acidified Harris' haematoxylin solution (5mL glacial acetic acid L^{-1} solution). Slides were 'blued' for 1 min (running-water bath, pH >8). Differentiation was performed with 0.5% acetic acid (aqueous). Slides were rinsed (distilled water) to remove unknown-pH residues, air-dried, stained with 1% aqueous Eosin-Y (pH 5.0, 25 min), rinsed again (3 x separate 1% acetic acid baths, 1 x distilled water), and air-dried. Sections were cleared (Histoclear), and mounted immediately (Eukitt).

Slides were viewed under a camera-mounted compound microscope (Evolution VF camera, Media Cybernetics, USA; Olympus BX61, Japan) and micrographs were processed and measured, where necessary, using Image-pro plus (v.5.1).

4-3.4. Examination of symbiont patterns

4-3.4.1 Fluorescence

Select slide-mounted sections targeting the entire size-spectrum were equilibrated in phosphate buffered saline (PBS 1x), stained with the nucleic acid-specific stain 4',6-diamidino-2-phenylindole (DAPI, 300 nM in PBS for 5 min) and mounted in Slow-Fade Gold (Invitrogen). All body regions were examined. Gill tissue from Lorion et al. (2012) provided the positive control. Negative controls excluded DAPI altogether, to test for auto-fluorescence. Slides were photographed as in the H & E analysis, but with a monochrome filter.

4-3.4.2 Transmission Electron Microscopy (TEM)

A subsample of individuals (Supplementary table 4.1) which harboured putative bacteria, were examined using TEM (JEOL 2100 HC; GIF Tridiem Gatan Imaging Filter). Sections were contrasted with uranyl acetate

(0.05 g mL^{-1} Milli-Q, 7 min in darkness) and lead (II) citrate alkaline solution (2 µg, and 0.01 mL NaOH [10 M] mL^{-1} Milli-Q, 7 min, CO_2-free environment).

4-3.5. Statistical analyses

All graphs were created in Sigmaplot (v.11). Size-class frequency analyses were performed, employing 0.1-mm intervals in shell length (informed by shell dimensions analysis). Differences in both whole-SL and larval, prodissoconch II SL were assessed across presumptive 'cohorts' using general linear models (GLM, unbalanced data: thus type III *SS*) in SPSS v.17.0, having confirmed the distribution's homoscedasticity (Levene's) and normality (Kolmogorov-Smirnov). Gill-filament counts (descending inner-demibranch lamellae only), were plotted against shell size; best-fit analysis was performed in Sigmaplot (v. 11) to assess gill proliferation as a function of SL. A significance level (α) of 0.05 was used.

4-4 Results

4-4.1. General specimen condition and classification

Of the 34 immature mussels found, five were plantigrades (i.e. post-larval mussels in which neither dissoconch deposition, nor gametogenesis was evident), all with shells intact (SL 0.38–0.42 mm). Tissue was damaged or already dehydrated in two cases. The remaining three were used for soft-tissue analysis regardless of fixation (Supplementary table 4.1), to maximise tissue availability. Despite their small size, much of the anatomy pertaining to later development was already evident. Small (<1 mm) and larger (>1 mm) juveniles, in which dissoconch growth was evident but gametogenesis was not, were most numerous (*n* = 29, SL 0.44–1.92 mm). The seven largest individuals (SL 2.35–6.54 mm) were adults with direct evidence of gametogenesis, discussed later. Formalin-fixed individuals were preferentially (but not exclusively) processed for tissue analyses (Supplementary table 4.1).

4-4.2. Shell-size patterns

SH ranged from 0.30–2.93 mm and SL from 0.38–6.54 mm in *I*. (*s.s.*) *modiolaeformis*, when they could be measured (*n* = 41, including plantigrades). Mean prodissoconch II heights ($\overline{\mathrm{PdH}}$) and lengths ($\overline{\mathrm{PdH}}$) ±σ were 0.33 ± 0.017 mm and 0.40 ± 0.018 mm respectively (PdH: 0.30–0.36 mm, PdL: 0.38–0.42 mm, *n* = 35). Figure 4.2 displays a scatter-plot of both measurements.

Size-class frequency analysis (Supplementary figure 4.3) confirmed two numerically dominant groupings of either plantigrades to small juveniles (*n* = 22), or larger juveniles (*n* = 12). Mean SL ($\overline{\mathrm{SL}}$ ± σ) were 0.58 ± 0.143 mm and 1.51 ± 0.208 mm, respectively. Five larger individuals ($\overline{\mathrm{SL}}$ ± σ = 2.61 ± 0.26 mm) and the two largest isolated mussels (4.47 mm and 6.54 mm) represented the remainder of the distribution (Supplementary figure 4.3). Across those presumptive groups for which a mean could be calculated (excludes isolated individuals), differences in SL were found to be statistically significant, having accommodated the unbalanced design (group sizes = 22, 12 and 5; GLM type III *SS*, $F_{(2,\ 36)}$ = 296.43, *P* <0.001). When calculable (i.e. excluding the two largest individuals), $\overline{\mathrm{PdL}}$ (± σ) for the numerically-dominant,

presumptive groups were 0.40 ± 0.014 mm (*n* = 21), 0.38 ± 0.020 mm (*n* = 9) and 0.40 ± 0.019 mm (*n* = 3), listed in order of increasing $\overline{SL}$.

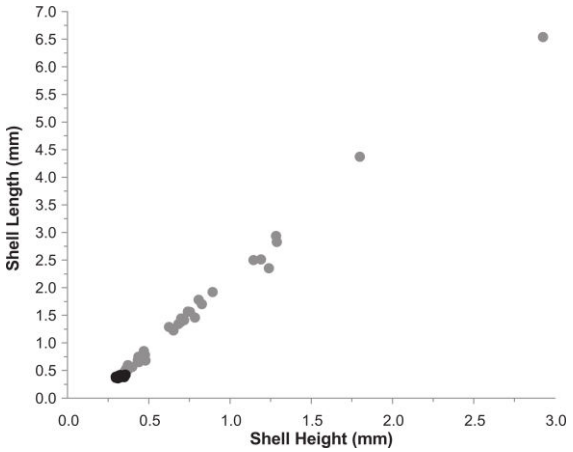

Figure 4.2 Plot of SL against SH of *Idas modiolaeformis*
SL and SH of intact prodissoconch and whole shells (n = 35, black dots and n = 36, grey dots, respectively). Prodissoconch measurements from plantigrades and juveniles/adults. Whole shells exclude plantigrades.

4-4.3. Developmental patterns

General developmental patterns though recorded are not detailed herein, but the digestive system is noteworthy, being already well-developed in plantigrades, with a scaled increase in volume and complexity with increasing shell sizes. Sequential toluidine-stained sections from eight individuals cut in various planes (see Supplementary figure 4.2, for explanatory schematic), ranging from plantigrade-sized to the smallest identified adult, are provided as supplementary material (Supplementary figures 4.4–4.11). Identifiable across the entire size spectrum were a functional digestive system including an oesophagus with a stratified ciliated epithelium and extensive digestive diverticula around a stomach (Figures 4.3c, d and e, Supplementary figures 4.4–4.11), while hypertrophied gills (Figure 4.3e, Figures 4-4a–e, Supplementary figures 4.4–4.11), a style sac (e.g. Figure 4.3e) and a recurrent loop (identified during dissection) in the microvilli-lined intestinal tract were first recorded with certainty in Idas 4 (0.57 mm) and then in all larger individuals examined with histology (preservation permitting).

4-4.4. Reproductive development

At the post-settlement stage in development, gonad tissue was as yet unformed. In addition, at this size it proved impossible to identify primordial germ cells (PGCs), which migrate and differentiate to form germ cells (GCs) and then gametes, later in development. However, putative PGCs were observed in several juveniles and small adults, located between the posterior periphery of the digestive system, the ventral periphery of the pericardium and the dorsal periphery of the organ of Bojanus, the bivalve metanephridium (e.g. Figure 4.3e, encircled). In juveniles of *I*. (*s.s.*) *modiolaeformis* examined, this also placed the PGCs in proximity to the visceral ganglion and the posterior adductor muscle (e.g. Supplementary figure 4.9, upper-right image). In juveniles < 1mm SL, these cells had already formed two small aggregations (≈ 20 µm long, antero-posteriorly) located on either side of the mid-sagittal plane and embedded in vesicular connective tissue (smallest individual, Idas 10, SL = 0.850 mm, Supplementary figure 4.9). In larger specimens, cell proliferation appeared to facilitate PGCs branched migration, towards the utmost dorso-lateral regions of

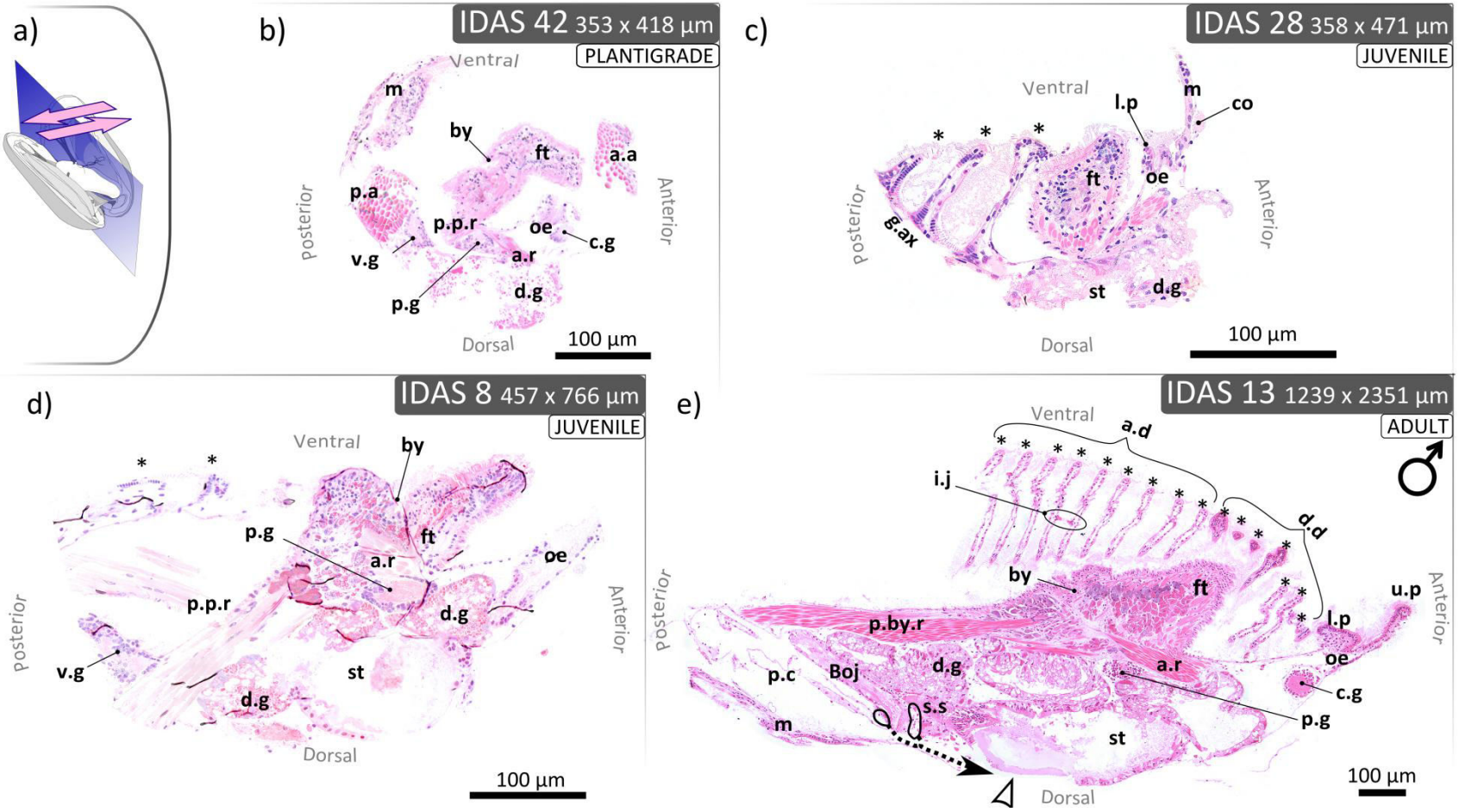

Figure 4.3 Micrographs of Haematoxylin & Eosin-stained mid-sections from *Idas* (*s.s.*) *modiolaeformis* of various sizes

Sections along or parallel to mid-sagittal plane as in (*a*), arrows = direction. A plantigrade (*b*), two juveniles (*c*), (*d*) and the smallest adult (*e*). *a.a.*: anterior adductor muscle *a.d*: ascending inner demibranch, *a.r*: anterior (byssus or pedal) retractors, *Boj*: organ of Bojanus, *by*: byssus gland, *c.g*: cerebropleural ganglion, *co*: neural commissures, *d.d*: descending inner demibranch, *d.g*: digestive gland, *ft*: foot tissue, *g.ax*: gill axis, *i.j*: interfilamentous ciliary junctions, *l.p*: lower labial palps, *m*: mantle, *oe*: oesophagus, *p.a*: posterior adductor muscle, *p.by.r*: posterior byssus-retractor, *p.c*: pericardial cavity, *p.g*: pedal ganglion, *p.p.r*: posterior pedal-retractor, *st*: stomach, *s.s*: style-sac, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments. Dashed arrow, observed developmental path with increasing SL of germ-line cells (encircled) towards gonads (location = arrowhead, see also Figure S11).

the body (Supplementary figure 4.10, upper-left; and based on the location of gonads in young adult specimens, Figures 4-4b and f, Supplementary figure 4.11).

Only following migration to the predominantly posterior, dorso-lateral regions of the body, did PGCs differentiate into GCs and identifiable gametes. In terms of SL, this was first observed at 2.35 mm (Idas 13, male). Unambiguous spermatogenesis was observed in two dorso-ventrally flattened regions of acini (length postero-anteriorly ≈ 100 µm), flush with the mantle epithelium, in dorso-laterally symmetrical posterior positions (Figure 4-4b and f, lateral-left dorsal region; Supplementary figure 4.11, lateral-right dorsal region). All but two of the individuals > 2.35 mm SL were confirmed to be male (e.g. Figures 4-4c and g). The two remaining unsexed individuals still harboured gonad-like interstitial cells and an additional sequence of cells which, based on cell membrane profiles (they lacked contents due to incomplete ethanol-preservation), were undergoing spermatogenesis-type patterns of differentiation.

4-4.5. Gill proliferation and growth

At the post-larval stage, a 'gill-basket' of four to six simple filaments had formed: the earliest incarnation of the inner demibranch's descending lamellae (Supplementary figure 4.4 upper-far-left). The gill axes – the dorsally-located rigid tissue supporting each gill lamella – were not parallel to the hinge-line in post-larvae,

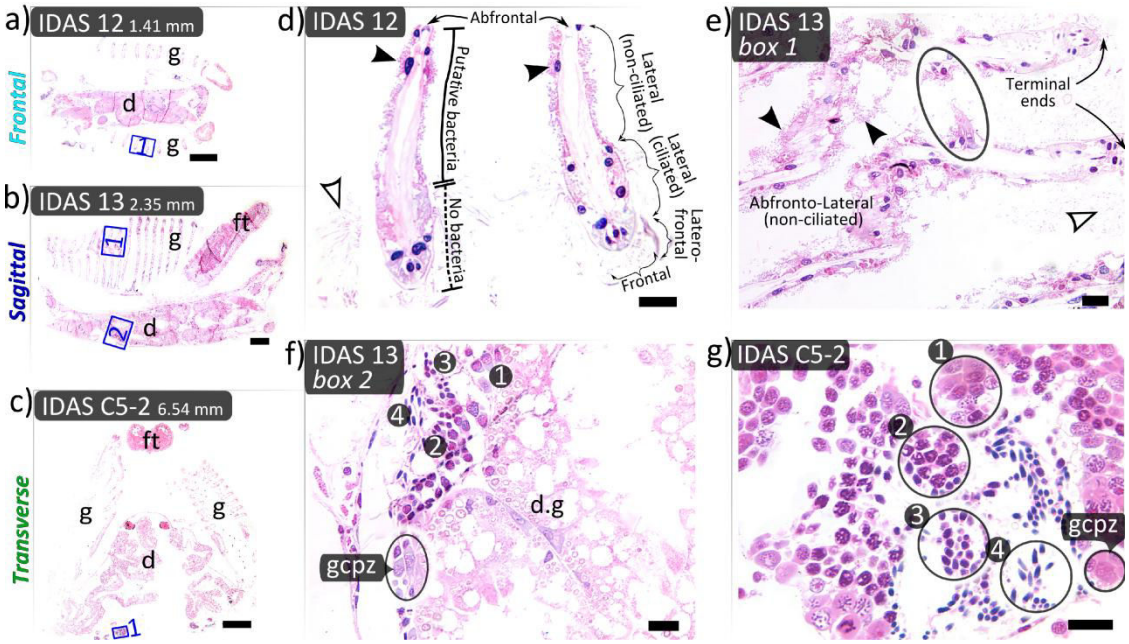

Figure 4.4 High magnification micrographs of Haematoxylin & Eosin-stained gonad and gill tissue from *Idas* (*s.s.*) *modiolaeformis* of various sizes

One juvenile (Idas 12: *a*, *d*) and two adults (Idas 13: *b*, *e*, *f* and Idas C5-2: *c*, *g*). For orientation terms, see Supplementary figure 4.2. Images (*a*) to (*c*), full-organism sections. *d:* digestive system, *g*: gill, *ft*: foot. (*a*) Dorso-medial region in frontal section. (*b*) Left-lateral region in sagittal section. (*c*) Anterior region in transverse section. Images (*d*) and (*e*) gill filaments. Solid arrowheads = putative bacteria, open arrowheads = lateral cilia. (*d*) magnification of box 1 in (*a*). Gill filaments in frontal-section (x100) with putative bacterial distributions (left) and gill nomenclature (right). (*e*) magnification of box 1 in image (*b*). Gill filaments in sagittal-section with gill nomenclature. Note interfilamentous ciliary junction (broken), encircled. Images (*f*) and (*g*), gonad regions undergoing spermatogenesis. (*f*) magnification of box 2 in image (*b*). (*g*) magnification of box 1 in image (*c*). Spermatozoid development is labelled sequentially: spermatogonia, spermatocytes, spermatids and spermatozoids (1, 2, 3 and 4 respectively). Germ-line cell proliferation zones are indicated (gcpz). *d.g.* digestive gland. Scale bars 100 µm (*a*) to (*c*), and 10 µm (*d*) to (*g*).

but elevated from it (≈20°) at the posterior end, towards the ventral margin (identified during dissection). Gill filaments in plantigrades and the smallest juveniles were the least numerous, the shortest dorso-ventrally from the gill axis to the ciliated terminal ends (approximately congruent) and the shallowest along the frontal-to-abfrontal axis (Figures 4.3c & 5g; Supplementary figure 4.4 upper-far-left). Consequently, filaments appeared comparatively stout.

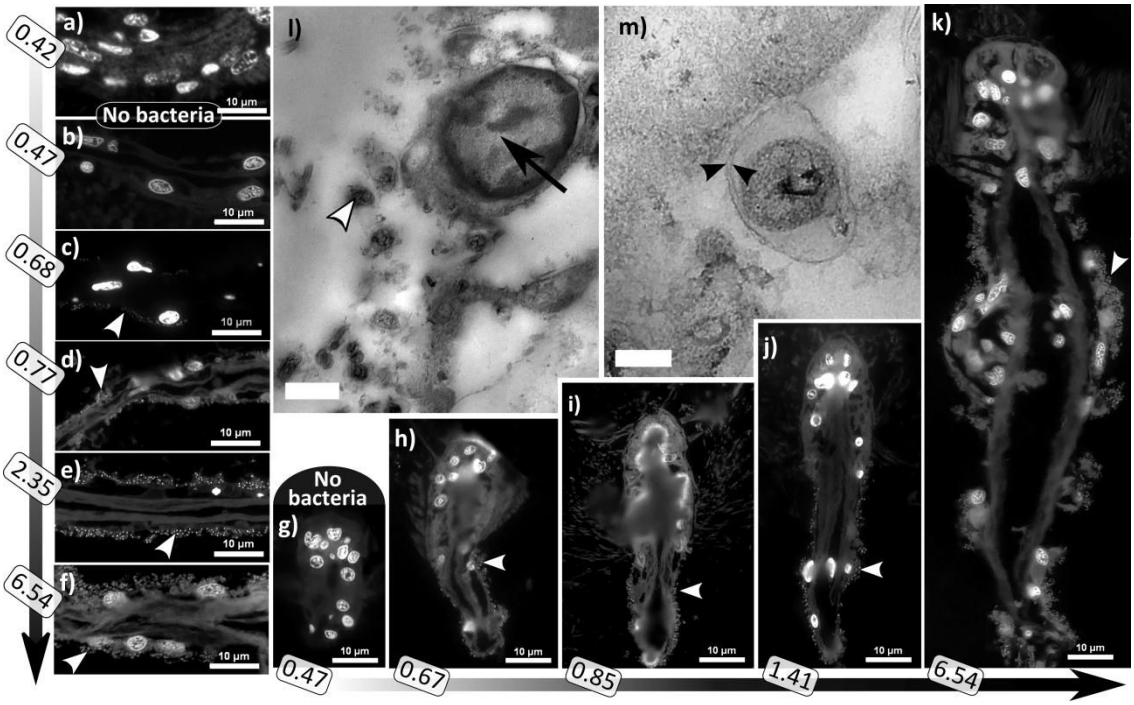

Figure 4.5 Putative bacteria lining the lateral-to-abfrontal regions of gill filaments in *Idas* (*s.s.*) *modiolaeformis*
Images (a) to (k): sagittal (a) to (f) and frontal (g) to (k) gill-filament sections at various sizes, stained with DAPI. Bordering arrows indicate increasing SL (boxes). White arrowheads = putative bacteria (tiny dots). Large nuclei belong to filament epithelia (also mantle epithelia in (a), Idas 42). Pictured; (a) to (f) Idas 42, Idas 28, Idas 3, Idas 8, Idas 13 and Idas C5-2 respectively, along sagittal plane in lateral region of gill filaments; (g) to (k) Idas 28, Idas 9, Idas 10, Idas 12 and Idas C5-2 respectively, frontal gill-filament sections (abfrontal region- bottom of micrograph, frontal- top). Images (l) and (m): TEM images of gill epithelia, Idas 7; (l) bacteria (white arrowhead) in association with gill tissue, with bacteriocyte visible (black arrow); (m) magnified view of similar bacteria, with gram negative-like double-membrane encapsulation (black arrowheads). Scale bars: 1 µm (left), 200 nm (right).

In juveniles and adults, as the inner demibranch's descending lamellae elongated postero-anteriorly and filaments became more numerous with increasing specimen-size (Figures 4-4a–c), dorso-ventral filament length (Figure 4-4e) and frontal-abfrontal filament depths (Figures 4.5h–k) increased. The shortest filaments were always found at the posterior end of lamellae, terminating in bud-like filaments (Idas 7, Supplementary figure 4.6, lower-left). New filaments are thus thought to develop from the posterior ends of the gill axes. In larger individuals, the posterior gill-region "floated" within the mantle cavity except distally, being attached to the inner mantle fold by a band of elastic tissue (repeated observations during dissections). Using all measured individuals, the relationship between filament number in the lamellae and SL was linear in the size range examined (Figure 4.6; Least-squares linear regression, $r^2 = 0.99$, $F_{1,32} = 4213$, $P < 0.001$). Standard curved-line fits (e.g. power-function) resulted in lower r^2 values. Counts ranged from 4–6 gill filaments in post-larvae to 88 filaments in the largest individual.

The reflected, ascending inner lamellae – extending dorsally from the ventral margin of the descending inner lamellae – were first identified at SL 766 µm as heavily-ciliated buds (Idas 8, Supplementary figure 4.6*, lower-centre*). In larger individuals, the ascending lamellae of the inner demibranchs were always evident (Figure 4.3e; Supplementary figures 4.7–11). Like the descending lamellae, filaments became more numerous with increasing size, longer (dorso-ventrally, Supplementary figure 4.8 *vs*. 11) and deeper (fronto-abfrontally, Supplementary figure 4.9 *vs*. 4.10). Ascending-lamella filaments were fused distally forming a continuous leading edge (i.e. the dorsal bend, Supplementary figure 11, upper-left). Inter-filamentous ciliary-disc junctions adjoined some neighbouring filaments laterally within a single lamella in all individuals over 1.5 mm (e.g. Figures 4.3e and 4e). Interlamellar septa however, which in other adult mussel species adjoin opposing paired filaments between descending and ascending lamellae across the exhalant chamber, were not identified in any individual.

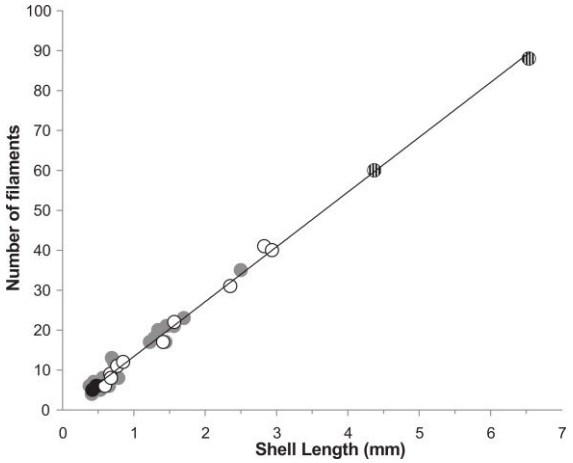

Figure 4.6 Gill-filament counts in the inner demibranch's descending lamella of *Idas modiolaeformis*
Filament counts in one inner-demibranch descending lamella (*n* = 35) as function of SL. Grey dots: not analysed for bacteria. Black: bacteria not identified during fluorescence microscopy. White: bacteria present on all non-ciliated epithelia. Striped: bacteria present on non-ciliated gill epithelia only.

4-4.6. Development of the bacterial symbiosis

Of the three plantigrades examined histologically, two were examined using fluorescence microscopy. Neither revealed conclusive evidence of epithelial bacterial signal (Figure 4.5a), despite positively stained controls. In the smallest juveniles (e.g. Idas 28, SL = 0.47 mm), there remained no bacterial signal upon any epithelia (Figure 4.5b). The smallest SL at which putative bacteria were identified was 0.59 mm (Idas 7, by TEM, Figures 4.5l and m) arranged in a thin extracellular mono-layer only. In this, and larger juveniles, these lined non-ciliated gill epithelia laterally (e.g. Figures 4.5c–f) and abfrontally (e.g. Figures 4.5h–k) but were absent in heavily-ciliated regions such as the entire ventral ciliated zone and the frontal-to-lateral ciliated zones along the length of the filaments (e.g. Figures 4-4d–e, 5h–k). Putative DAPI-stained bacteria, identical in appearance, were identified on other non-ciliated epithelial surfaces, such as the mantle itself, the dorsal side of the foot, epithelial retractor-muscle sheaths and the visceral epithelium (e.g. Supplementary figure 12). Bacterial densities identified in the gills appeared to increase qualitatively in abundance with increasing SL (Figure 4.5), while densities on other epithelia did not. In the two largest mussels examined, Idas 27 and Idas C5-2, bacteria were only identified on non-ciliated gill epithelium (e.g. Figure 4.5k) and could no longer be seen on any other tissue, suggesting increasing specificity with SL. Putative bacterial trends were mirrored in both toluidine-blue (Supplementary figures 4–11) and H & E staining (Figures 4-4d–e). Based on TEM images taken along non-ciliated filament epithelia (e.g. Figures 4.5l and m), bacteria were all similar in

form and size, typically 250–450 nm in diameter, located extracellularly, and usually encapsulated in a double-membrane. Electron-transparent particles, typically considered evidence for sulphur granules, were not visible. Internal stacked membranes, which are indicative of known methanotroph-type bacteria associated with this mussel species, were conspicuously absent.

4-5 Discussion

This study describes two important aspects of early development in *Idas* (*s.s.*) *modiolaeformis*: a proxy for reproductive developmental rate in size at first maturity, and the patterns of acquisition and localisation of symbionts. The size series of individuals from larval collectors, rare in deep-sea studies, made this possible. The results (Figure 4.7) identify pivotal traits which are argued to be adaptive in maintaining populations in highly ephemeral reducing environments.

4-5.1. Shell characteristics

4-5.1.1 Preliminary indications from larval supply patterns

Individuals in the current study are thought to have settled at the latest as plantigrades, since the immigration of juveniles is considered improbable due to the collector's 2-mm mesh cover. The size-frequency analysis suggests semi-continuous settlement, with relatively-high, size-dependent mortality, unrelated to direct predation. However, low sample sizes prohibit further analysis. Apparently continuous larval supply has been documented before in various deep-sea taxa (e.g. Van Dover et al. 1988) and in small bathymodiolin species (e.g. chemosymbiotic *Idas*-like nov. sp. from the Marmara Sea, Ritt et al. 2012), suggesting these patterns may reflect the genus. In contrast, other large bathymodiolins display massive, periodic occurrences of post-larvae (Van Dover 2002; Van Dover et al. 2003), thus no single pattern is characteristic of the subfamily.

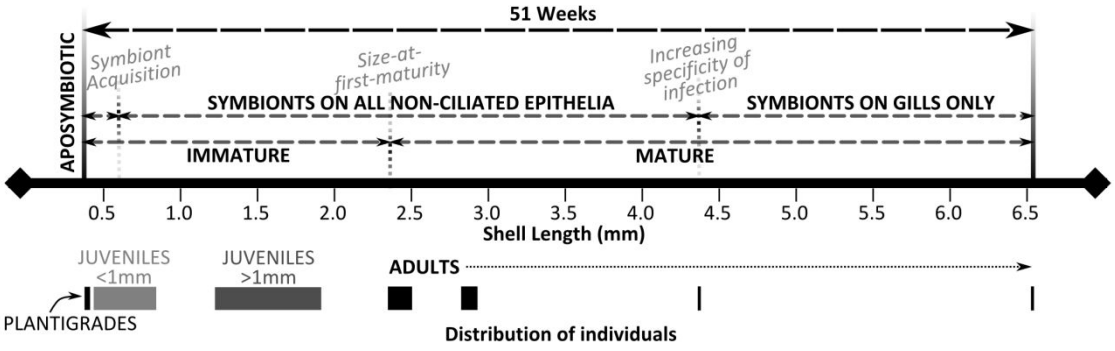

Figure 4.7 Summary of pivotal periods in the development of *Idas* (*s.s.*) *modiolaeformis* as a function of size
Distribution of individuals is based on length-frequency analysis.

4-5.1.2 Rough estimates of growth

By examining dissoconch deposition in the largest mussel recovered from the 51-week deployment, a minimum time-averaged growth rate can be estimated for this individual. It assumes immediate settlement following substratum deployment (Young et al. 2013), considered plausible, given the presence of *I*. (*s.s.*) *modiolaeformis* post-larvae in the two-week experiments (Gaudron et al. 2010). A SL of 6.54 mm thus translates to a rough, minimal time-averaged growth-rate estimate of 0.017 mm d^{-1} following metamorphosis, having corrected for pre-settlement growth (larval prodissoconch II). This estimate is probably conservative, since settlement may not have occurred immediately (Young et al. 2013). This figure is comparable to deep-sea molluscs from wood falls, e.g. the pioneer wood-borer *Xylophaga depalmai* (mean growth rate 0.03 mm d^{-1} over 6 mo, maximum growth rates approached 0.07 mm d^{-1}, Tyler et al. 2007b) or cocculinid and pseudococculinid limpets (over 177–180 days, largest minimum growth rates of 0.025–0.044 mm d^{-1} depending on species, using the current study's approach, Young et al. 2013). Additionally, it is within an order of magnitude of calculated growth rates for the relatively massive bathymodiolin vent mussel, *B. thermophilus*, within the first year of settlement (0.11 mm d^{-1} over the 1st year, reverse forecasted from a von Bertalanffy curve, falling to 0.03 mm d^{-1} at the asymptotic length, L_∞, Nedoncelle et al. 2013).

4-5.2. Mature and exclusively male

4-5.2.1 Size at first maturity *versus* maximum recorded size

The minimum size at which gonads develop is documented for the first time in *I*. (*s.s.*) *modiolaeformis*, from the migration of putative, medial, posterior germ-line cells in juveniles to the dorso-lateral regions of body. In Gaudron et al. (2012), the smallest specimen examined at 3.6 mm (greatest postero-anterior length, regardless of hinge-line) had already reached sexual maturity. In the present study, two larger individuals and several smaller were sexually mature. The threshold of 2.35 mm identified here for size at first maturity is supported by the patterns of germ-line migration towards the dorso-lateral regions of smaller specimens, based upon classic histological descriptions (Lubet 1959), which have since been corroborated by *vasa*-like gene-marker techniques where *vasa*-like gene expression is restricted to the germ cells and is undetectable in somatic tissues (Fabioux et al. 2004; Obata et al. 2010).

Size at first maturity is not an ideal interspecies index for comparing developmental rates, on account of wildly different maximum sizes and growth rates. However, in deep-sea reducing environments such examples in relation to maximum size are: ≈110 mm in *Calyptogena magnifica* (Berg 1985, representing ≈40% of largest recorded SL, SL_{max} = 263 mm, from Boss and Turner 1980) and; 40–60 mm in *B. thermophilus* depending on sex (Berg 1985), representing ≈19.5–29% of SL_{max} (205 mm, from Nedoncelle et al. 2013). *I*. (*s.s.*) *modiolaeformis* falls below these two at ≈14% of SL_{max} (17 mm, Olu-Le Roy et al. 2004).

4-5.2.2 Approximate age at first maturity

Age at first maturity is generally considered a more robust measure of comparative reproductive developmental rate, being less sensitive to variable growth rates (though not always, e.g. Nakaoka 1994). The shallow-water genus *Mytilus* displays highly variable sizes at first maturity (≈2–7 mm SL), but less variable ages-at-first-maturity of 1–2 mo in *M. trossolus*, 4–8 mo in *M. californianus* and ≈1–2 y in *M. edulis*, depending on habitat (Seed 1969; Suchanek 1981; Seed and Suchanek 1992). Additionally, *B. thermophilus* may mature at ≈8–13 mo, when documented size at first maturity (Berg 1985) and Von Bertalanffy growth analyses (Nedoncelle et al. 2013) are integrated. The time-averaged growth rate of 0.017 mm d^{-1} in the current study, may be cautiously applied to the size-at-first-maturation for comparison, where a SL of 2.35 mm, 36% of the largest SL (6.54 mm), represents an age of ≈4 mo. This conversion assumes that early growth rates in *I.* (*s.s.*) *modiolaeformis* are less variable than in coastal species. The lack of obvious growth discontinuities in the shell may reflect a reduced dependency on an external food supply (Seed and Suchanek 1992). The second is that compound size-scaling effects upon daily increments of growth are limited; since the size range examined here covers <40% of the range recorded for the species, this is considered only a moderate source of error.

This rough estimate of ≈4 mo for age-at-first-maturity, is half the age of the relatively massive Bathymodiolinae example available, younger than most heterotrophic mussels of the genus *Mytilus* (except *M. trossulus*), and considerably younger than many other benthic invertebrate species (listed in Gosselin and Qian 1997, appendix 1). This, and the fact that the associated size at first maturity accounts for only 14% of the known L_{max}, are thought to represent evolutionary adaptations maximising reproductive developmental rate.

4-5.2.3 Protandric hermaphroditism in reducing habitats

All individuals >2.35 mm were confirmed male. Based on a larger number of adults, Gaudron et al. (2012) demonstrated that sex ratios are size-dependent in *I.* (*s.s.*) *modiolaeformis* from authigenic carbonate crust in the same region. In sexually functional “transition” males 3.6–11.6 mm SL, female acini were frequently present but non-functional (disintegrated oocytes). Females were exclusively > 7 mm in length, with a single size-intermediate hermaphrodite. This information provided compelling evidence for protandric hermaphrodism with a proposed intermediate period of abrupt sex-switching, similar to *I. washingtonia* (Tyler et al. 2009, since redescribed as *I. washingtonius*, Coan and Valentich-Scott 2012), where 7 mm also represented the lower female size threshold. Neither mature females nor female–sized empty shells, were identified during sample-sorting in the current study, providing evidence that maturation in this species is towards becoming predominantly male, and that the first sex alternation may not occur within the first 51 weeks (or <6.54 mm). For the local population to reach size/age at first reproduction, a period in excess of 51 weeks is thus necessary, under the experiment’s environmental conditions.

Protandry is often argued as an adaptation to finite nutritional resources (Ghiselin 1969, where the costs of reproductive activity as a female at a given size (or age) may be prohibitive in contrast to being male (or *vice versa*). In the case of protandry in *I*. (*s.s.*) *modiolaeformis* (Gaudron et al. 2012; this study) and *I. washingtonius* (Tyler et al. 2009), investment in oogenesis are evidently minimal below a threshold size or age (based on the absence of females < 7mm), where larger females would be more fecund. This would maximise somatic growth in smaller mature individuals, by partitioning a minimal amount of energy for spermatogenesis as males, and may also promote survival by reducing the period during which juveniles are most susceptible to predation. After a period of growth, and when nutritional resources allow, a switch in sex may then occur. With the staggering of sex-alternation, an array of small but numerous males, and larger fecund females would be reached. Tyler et al. (2009) cite variability in microhabitat as a potential determinant for the diverse range of sizes over which sex-switching occurred, a pattern also seen in *I*. (*s.s.*) *modiolaeformis* . Microhabitat heterogeneity within the CHEMECOLI may therefore have contributed to the sex-switching patterns in Gaudron et al. (2012).

4-5.3. Gill development

4-5.3.1 Building a bacterial home

Demibranch proliferation and elongation appeared to follow patterns documented in other mussel species (e.g. *Mytilus edulis* post-larvae, Bayne 1971; post-larvae-to-adults, Cannuel et al. 2009). The long, developed profile of frontal and lateral cilia and the latero-frontal cirri on gill filaments for example is more typical of heterotrophic mytilids than larger Bathymodiolinae (Southward 2008, figure 1 therein).

The specificity and appearance of bacterial distributions (DAPI and TEM) on the non-ciliated lateral and abfrontal surfaces of gill-filament epithelia, were the same as in extracellular symbionts repeatedly identified in adult *I*. (*s.s.*) *modiolaeformis* (Duperron et al. 2008a, b; Rodrigues et al. 2013), its congeners (Gros et al. 2007; Southward 2008) and other bivalve species (e.g. Dufour 2005). In our TEM observations, their small size and the absence of concentrically-stacked intracellular membranes (Cavanaugh et al. 1987) indicate that they were not methane oxidisers. Although preservation limitations do not permit the bacteria to be identified further, they are believed to be thiotrophic symbionts since *I*. (*s.s.*) *modiolaeformis* has previously been demonstrated to harbour multiple symbionts, where thiotrophs and methanotroph phylotypes dominate (Duperron et al. 2008a; Lorion et al. 2012; Rodrigues et al. 2013). Preliminary fluorescence in-situ hybridisation (Laming, unpubl. data), employed upon the same organisms using thiotroph-specific oligonucleotidic probes, appears to support this, along with the fact that sulphide emissions from these samples were detected using autonomous potentiometric sensors (Gaudron et al. 2010) and that free-living sulphur-reducing bacteria are now known to colonise CHEMECOLI substrata (Khelaifia et al. 2011). Duperron et al. (2008a), identified 6 bacterial phylotypes, where methanotroph-like and unspecified methylotroph-like bacteria collectively appeared to dominate other symbiont types. In the 2012 study, thiotroph-type bacteria were dominant, indicating a level of symbiont flexibility (Lorion et al.

2012). A fifth *Gammaproteobacteria* phylotype 'symbiont G' of unknown metabolism has been identified less predictably (Duperron et al. 2008a; Rodrigues et al. 2013). In the study by Gaudron et al. (2012), methanotroph-like bacteria were identified using FISH in the gill tissues of all 29 *I*. (*s.s.*) *modiolaeformis* collected. However tissue was not analysed for the remaining known symbionts. The absence of methanotroph-like bacteria in the TEMs of the mussels in this study was unexpected for the NDSF given that methane seepage was thought to occur in the area. Such exclusively-thiotrophic associations have previously been identified in *I*. (*s.s.*) *modiolaeformis* from the Gulf of Cadiz (Rodrigues et al. 2013). Considering all the evidence, sulphide emissions due to anaerobic substrate degradation (e.g. Laurent et al. 2013) likely predominated in the immediate environment of the organic substrata provided, which would have permitted thiotrophic symbionts to dominate. However the possibility that different symbionts are acquired at different stages of development cannot be ruled out.

4-5.3.2 If not symbiotic, then what?

Specimens in this study <0.595 mm appeared to be either devoid of bacteria, or bacterial signals were undetectable. When infected, putative bacteria identified upon non-ciliated epithelia in individuals from 0.595–<4.37 mm in SL (confirmed with TEM, except in the smallest infected individuals) were comparable to gill-associated bacteria. This infection became more gill-specific in adults (specifically ≥4.37 mm). Although this pattern of general-to-specific tissue infection is based on signals of putative symbionts only, it has already been shown in juveniles (4–21 mm) of two considerably larger bathymodiolin species, *Bathymodiolus azoricus* and *B. puteoserpentis* (Wentrup et al. 2013). In that study the two symbiotic phylotypes infecting the tissues were found to be the only detectable bacteria present. However unlike the current study, size-at-first-acquisition could not be established, since post-larval specimens were not examined and aposymbiotic juveniles were never identified, which is not always the case (e.g. Streams et al. 1997). The increase in symbiont density with size probably reflects a developing association which has yet to attain stable densities, unlike the symbiont densities described for post-larvae onwards in *B. heckerae* and *B. azoricus* (Salerno et al. 2005), where symbiont morphotypes were observed in all specimens regardless of life-history stage and remained relatively unchanged in density regardless of size.

Gaudron et al. (2012) proposed that symbionts in *I*. (*s.s.*) *modiolaeformis* are probably acquired horizontally (i.e. environmentally) rather than vertically (parentally), since bacterial symbionts were never detected in parental germ cells or unreleased oocytes. The current study provides additional evidence to further support this hypothesis, by isolating the probable period of acquisition to the post-settlement phase of development. The absence of bacteria in plantigrades, whose digestive system is well-developed, indicates that planktotrophic larvae and post-larval plantigrades are both exclusively heterotrophic.

4-5.4. Summary of findings

Although much of the anatomical developmental patterns in this species are parallel to those of shallow water mytilids, key developmental traits are viewed as adaptive. *Idas* (*s.s.*) *modiolaeformis* is capable of

colonising and reaching maturity well within a year, at just 14% of its SL_{max}. Whether this trait was selected for directly or is a by-product of selection for a reduced body size (e.g. though paedomorphism, Génio et al. 2012), the net effect is the same: it permits *I.* (*s.s.*) *modiolaeformis* to colonise ephemeral habitats and attain maturity well within the constraints of the habitat's finite existence (particularly for males). Equally, the adaptive advantage of protandric, sequential hermaphroditism is in maximising early investments in growth (which are almost certainly underestimated in the current study), and ultimately attaining an optimised ratio of sexes across sizes (Ghiselin 1969). In larger wood falls, sulphide production is greater and the substratum persists for longer (Laming, unpubl. data). Once colonised, the population's stratified size-distribution and sex-ratio structure would increase the likelihood of colonising nascent habitats in subsequent generations, by maximising the population's potential reproductive output (Ghiselin 1969).

The symbiotic partnership between *I.* (*s.s.*) *modiolaeformis* and its bacteria is perhaps the most evident adaptation in the species' development, in habitats that might otherwise be sub-optimal or intolerable physiologically. In the earliest stages (<1 mm) during filter feeding for nutritional requirements, hypertrophic gills would facilitate symbiont acquisition. Flexibility in nutritional mode would promote survival in juvenile individuals in which bacterial densities remain low during the early symbiotic period (qualitatively observed in the current study) and when infection is not mediated. The presence of bacteria on nearly all types of non-ciliated epithelia during the early symbiotic period is believed to augment the limited surface area available upon the gills at this size. As the gills increased in length and depth, the non-ciliated regions soon represented a far greater surface area in cross section and longitudinal section. The linear relationship between descending lamellae filament counts and SL suggest that gill development was allometric (as in other Filibranchia), once increases in filament dorso-lateral length, fronto-abfrontal depth and the initiation of ascending lamella development at sizes ≥ 0.77 mm in length are considered. This allometric development would increase the symbiont carrying capacity considerably. It is unknown whether heterotrophic nutrition remains important at this stage, however the apparent increase in the relative volumes of gill tissue versus digestive tissue in larger specimens of the same species found upon carbonate crust (Gaudron et al. 2012), may indicate a switch in nutritional mode from heterotrophic to predominantly-chemosymbiotic with increasing size.

The eventual isolation of bacteria to the gill regions only (presumably mediated by the host), places these symbionts in the best possible location to gain access to both electron donors such as H_2S, and electron acceptors (O_2 in this case), thus maximising potential metabolic activity (Cavanaugh et al. 2006). Assuming the host has a means to assimilate the resulting bacterial organic carbon, this proves mutually-beneficial, particularly if the bacteria also indirectly confer a fluid detoxification role (Duperron 2010).

The larval colonisation devices (CHEMECOLI) employed permitted the collection of a comprehensive size-series from substrata providing habitat for the hosts and reduced compounds for their symbionts. The fact that *I.* (*s.s.*) *modiolaeformis* larvae can settle and grow to maturity successfully in these artificial

environments validates their use. The findings of previous CHEMECOLI colonisation experiments (e.g. Gaudron et al. 2010; Cunha et al. 2013; Rodrigues et al. 2013) and wood deployments (e.g. Bienhold at al. 2013) all demonstrate that *I*. (*s.s.*) *modiolaeformis* is capable of rapidly and repeatedly colonising finite decaying organic debris in the Mediterranean and eastern Atlantic. The present study has identified that early post-larvae have a functional gut and no detectable symbionts, which suggests strict heterotrophy at the larvae and immediate post-settlement stages. Extracellular bacterial infection occurs shortly after metamorphosis, on various non-ciliated epithelia, but bacteria are only retained on non-ciliated gill epithelia following maturation, where densities increase. In much less than one year, juveniles reach maturity as males, which in addition to previous data (Gaudron et al. 2012), supports protandric hermaphrodism. Individuals in the present study were likely below the threshold size (or age) at which the first female alternation occurs. This study closes a previous gap of knowledge of post-settlement and juvenile development, a critical period for survival in the life-cycle of any species (Duperron et al. 2008a; Gaudron et al. 2012). Intuitively, selective pressures, early in the evolutionary adaptation of organic-fall-associated Bathymodiolinae are likely to have favoured species with a predisposition to grow rapidly and quickly reach maturity, in part by harnessing chemosynthetic symbionts. Together, these adaptive traits would have facilitated their persistence in patchy and ephemeral habitats. This study's findings, and the evident success of *I*. (*s.s.*) *modiolaeformis* on organic falls and cold seeps, are testament to this fact.

4-6 Acknowledgments

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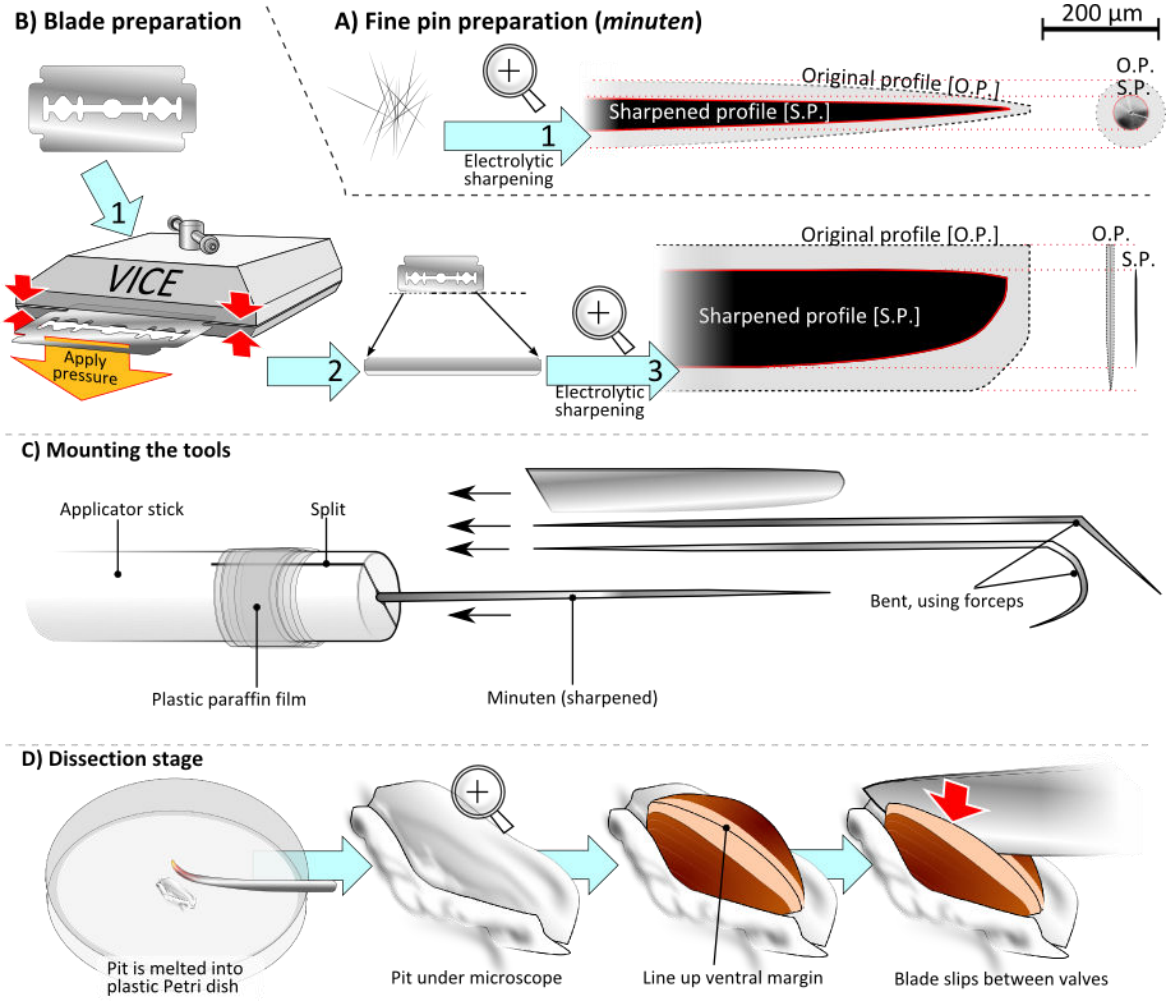
4-8 Chapter 4 Annex 1

4-8.1. Supplementary material

Supplementary table 4.1 Summary of the fixation employed and analysis performed upon specimens in this study
41 individuals were used in this study: 5 post-larvae, 29 juveniles and 7 adults. Regardless of fixation, preservation was in 96% ethanol at <5 °C, except C5-2 (see footnote). Abbreviations for analyses are S- Shell dimensions measured, H- standard histological analyses, D- DAPI staining, T- transmission electron microscopy, Formald. – Formaldehyde in twice-filtered seawater.

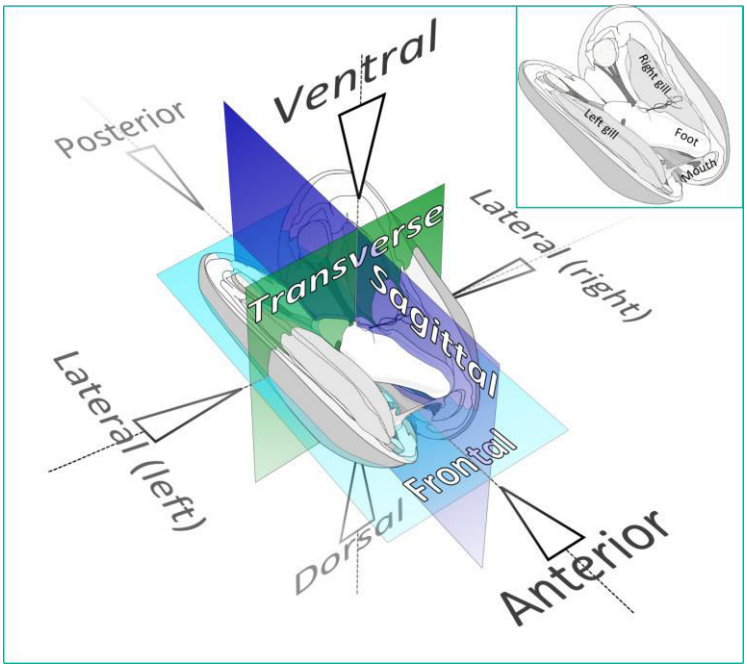
Sample ID	Life stage	Substrate	Fixation	SL mm	Analyses	Mature?	Bacteria?
IDAS 6	Post-larva	Alfalfa	Formald. 4%	0.38	S, H, D	No	No
IDAS 41	Post-larva	Wood	Ethanol	0.41	S, H		
IDAS 34	Post-larva	Wood	Ethanol	0.41	S		
IDAS 5	Post-larva	Alfalfa	Formald. 4%	0.42	S		
IDAS 42	Post-larva	Wood	Ethanol	0.42	S, H, D	No	No[4]
IDAS 36	Juvenile[1]	Wood	Ethanol	0.44	S		
IDAS 28	Juvenile	Wood	Formald. 4%	0.47	S, H, D	No	No
IDAS 37	Juvenile	Wood	Ethanol	0.48	S, H, D	No	No[4]
IDAS 18	Juvenile[1]	Alfalfa	Ethanol	0.52	S		
IDAS 4	Juvenile	Alfalfa	Formald. 4%	0.53	S, H	No	No
IDAS 35	Juvenile[1]	Wood	Ethanol	0.53	S		
IDAS 44	Juvenile[1]	Wood	Ethanol	0.57	S		
IDAS 7	Juvenile	Alfalfa	Formald. 4%	0.59	S, H, T	No	Yes
IDAS 2	Juvenile	Alfalfa	Formald. 4%	0.65	S, H	No	
IDAS 9	Juvenile	Alfalfa	Formald. 4%	0.67	S, H	No	Yes
IDAS 3	Juvenile	Alfalfa	Formald. 4%	0.68	S, H, D	No	Yes
IDAS 17	Juvenile[1]	Alfalfa	Ethanol	0.69	S		
IDAS 16	Juvenile[1]	Alfalfa	Ethanol	0.72	S		
IDAS 20	Juvenile[1]	Alfalfa	Ethanol	0.75	S		
IDAS 8	Juvenile	Alfalfa	Formald. 4%	0.77	S, H, D	No	Yes
IDAS 1	Juvenile	Alfalfa	Formald. 4%	0.78	S, H	No	
IDAS 10	Juvenile	Alfalfa	Formald. 4%	0.85	S, H, D	No	Yes
IDAS 31	Juvenile[1]	Wood	Ethanol	1.22	S		
IDAS 22	Juvenile[1]	Alfalfa	Ethanol	1.29	S		
IDAS 25	Juvenile[1]	Alfalfa	Ethanol	1.34	S		
IDAS 24	Juvenile[1]	Alfalfa	Ethanol	1.38	S		
IDAS 12	Juvenile	Alfalfa	Formald. 4%	1.41	S, H, D	No	Yes
IDAS 29	Juvenile	Wood	Formald. 4%	1.44	S		
IDAS 23	Juvenile[1]	Alfalfa	Ethanol	1.46	S		
IDAS 38	Juvenile[1]	Wood	Ethanol	1.56	S		
IDAS 14	Juvenile	Alfalfa	Formald. 4%	1.57	S, H, D	No	Yes
IDAS 32	Juvenile[1]	Wood	Ethanol	1.70	S		
IDAS 11	Juvenile	Alfalfa	Formald. 4%	1.78	S, H, D	No	Yes
IDAS 15	Juvenile	Alfalfa	Formald. 4%	1.92	S, H, D, T	No	Yes
IDAS 13	Adult	Alfalfa	Formald. 4%	2.35	S, H, D, T	Yes	Yes
IDAS 26	Adult [2]	Alfalfa	Ethanol	2.50	S, H	Yes	
IDAS 43	Adult	Wood	Ethanol	2.51	S, H	Yes	
IDAS 30	Adult	Wood	Formald. 4%	2.83	S, H	Yes	
IDAS 33	Adult [2]	Wood	Ethanol	2.94	S, H	Yes	
IDAS 27	Adult	Alfalfa	Ethanol	4.37	S, H, D	Yes	Yes
IDAS C5-2	Adult	Wood	Formald. 4%[3]	6.54	S, H, D	Yes	Yes

[1]Presumptive juveniles based on their size only. [2]Sex could not be determined but poorly preserved reproductive tissue was identified. [3]Fixation *and preservation* (i.e. no transfer to 96% Ethanol after 4 hr of fixation). [4]DAPI analysis was performed during which bacteria were not identified, however the sample was ethanol-fixed so epithelial preservation was not complete.



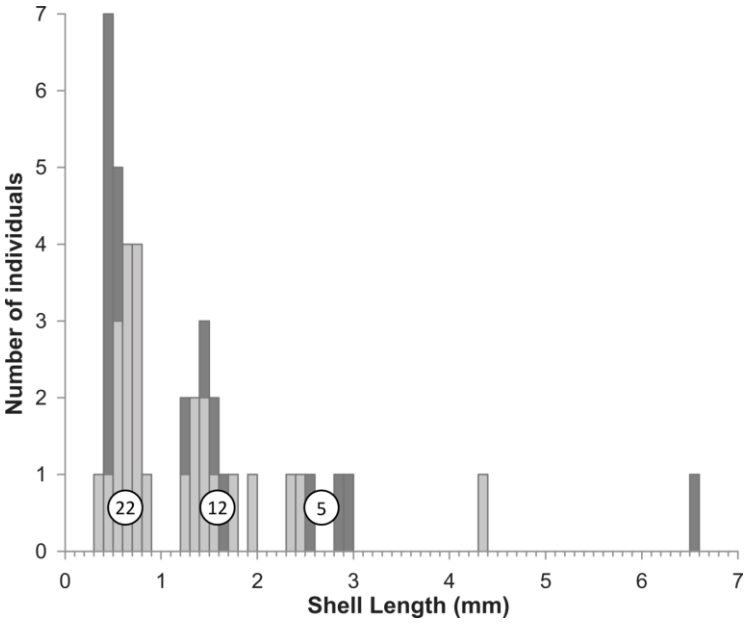
Supplementary figure 4.1 Brief description of the methodology for constructing micro-dissection tools and their subsequent use

Micro-dissection pins and pliable metal scalpels (< 10 µm point/blade) were manufactured by electrolytic sharpening of either steel entomological minuten pins (**A**) or feather-blade razors (**B**) respectively (0.5M NaCl solution, 4.5V DC current), wherein the blade or pin which needed to be reduced in size formed the anode, and a 1-mm thick electrical copper wire (immersed length stripped of plastic coating) formed the cathode (not depicted). Pins were 'sharpened' directly using this set-up (with resulting silhouette profile). However, blade-edges were first snapped off the razor along their length in a vice (**B1**), to create shallow blades but maintain their straight edge-profiles (**B2**), and then the razor edge was dipped evenly into the electrolytic solution (not depicted). Blades and pins, which were often bent into specific forms using forceps (**C**), were mounted on applicator sticks (or toothpicks). Valve-margin orientation was facilitated by placing the mussels in 'dissection wells' of varying sizes and depths that had been melted into the upper surface of sterile, plastic Petri-dish bases prior to dissection (**D**). Using the highly pliable fine blade, it was possible to part the valves of even the smallest plantigrades, open shells, with minimal damage to the organism, having cut the anterior and posterior adductor muscles.



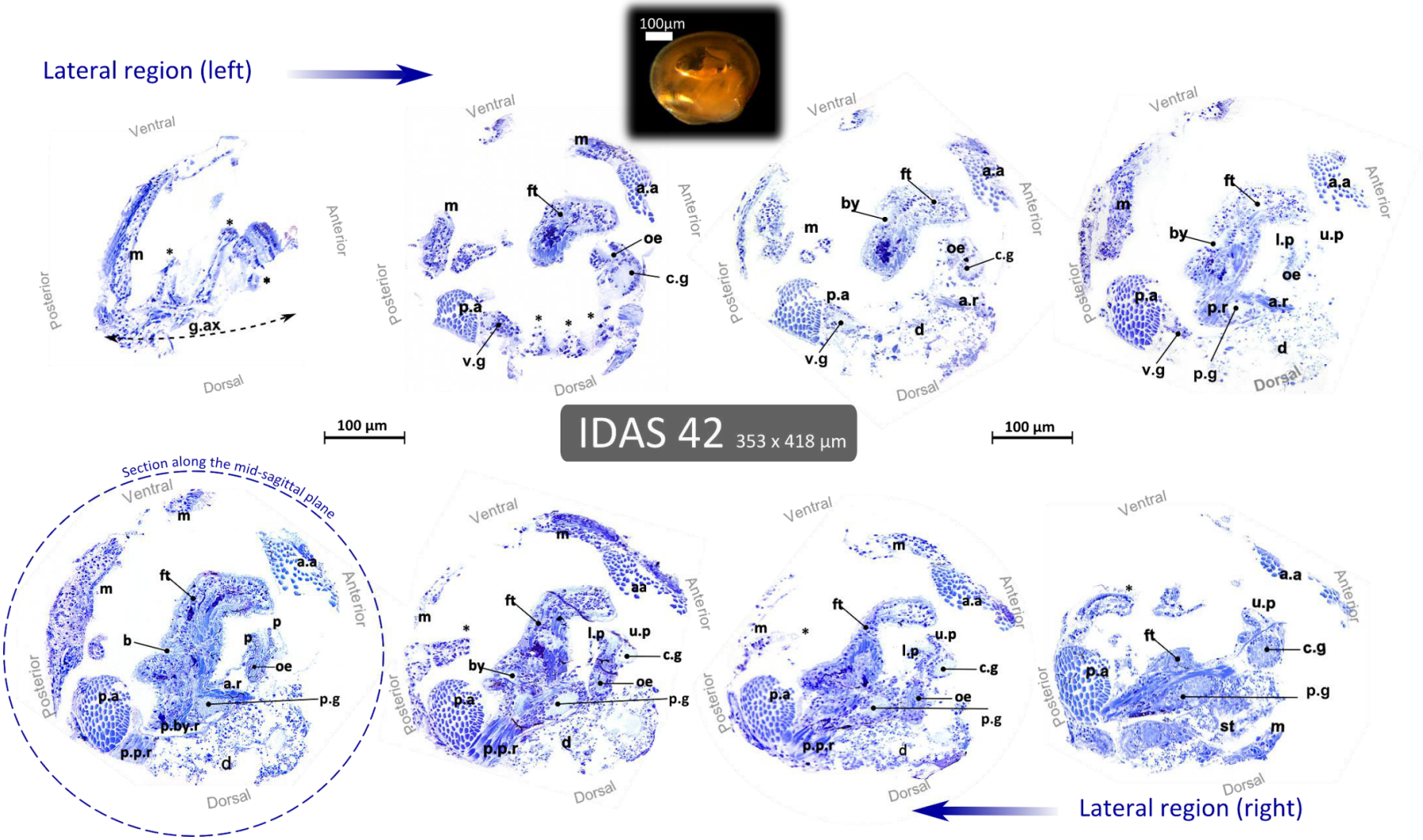
Supplementary figure 4.2 Summary schematic to help visualise the orientation of organisms during sectioning

Pictured, is an axonometric drawing of a *Idas modiolaeformis*, based upon the animals dissected for this study. Axes of orientation are the dashed lines. Arrowheads indicate the direction of view for each region of the animal. Planes are labelled in white print. Serial sections cut from the animal (in either direction) along the lateral (left)-lateral (right) axis, would be in the sagittal plane (royal blue), they would be in the transverse plane (green) if cuts were made along the postero-anterior axis, and along the dorso-ventral axis, they would be in the frontal plane (turquoise).



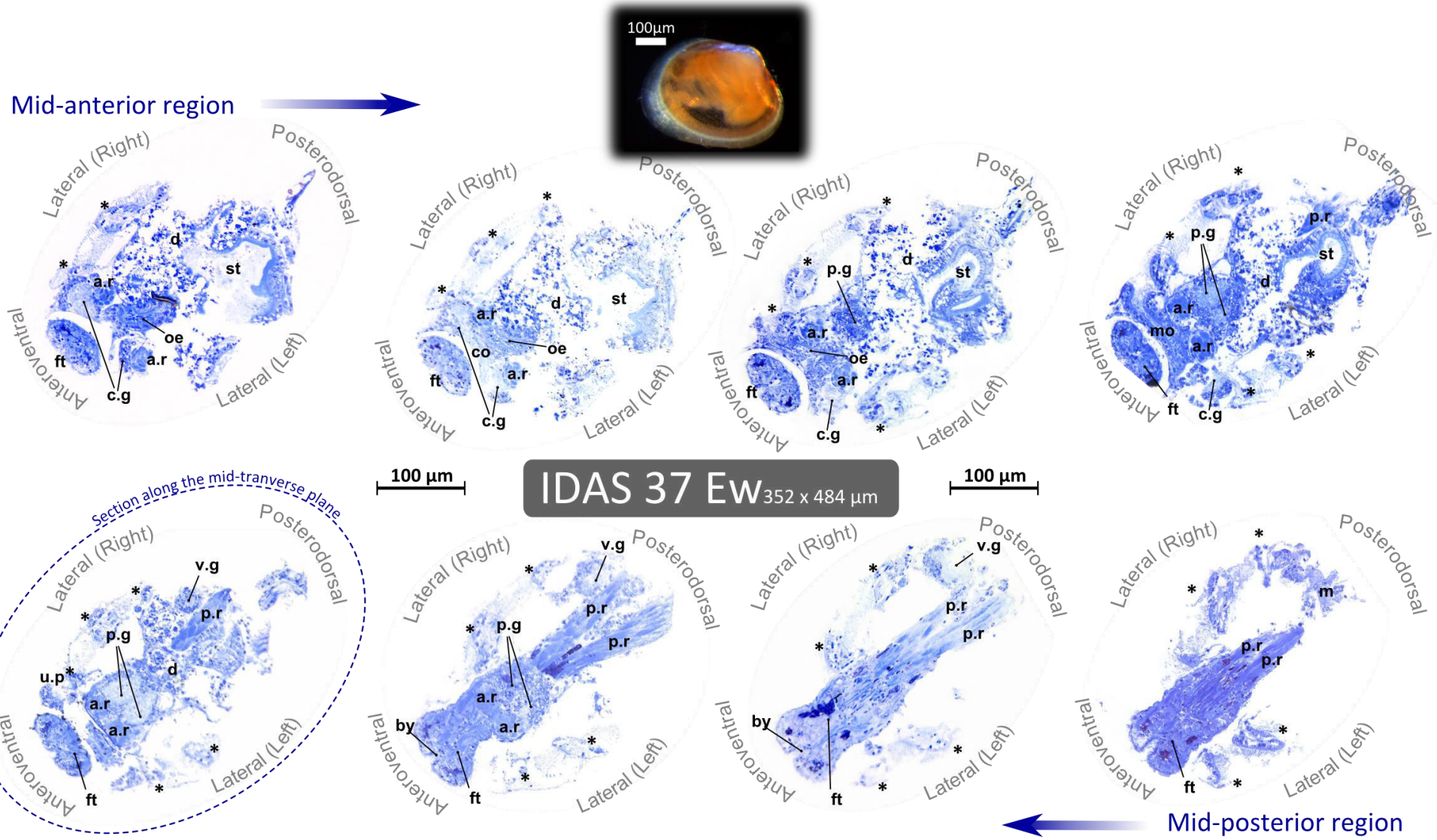
Supplementary figure 4.3 Length-class frequency histogram of *Idas (s.s.) modiolaeformis*

The histogram displays the number of individuals that occurred in consecutive 100 µm length-class intervals over the entire dataset (sample sizes are encircled). Dark grey counts are individuals from wood substrate, while light grey counts are those from alfalfa, in which counts are stacked e.g. the highest frequency for a size class at 7 individuals (400 - <500 µm) is comprised of 1 individual from alfalfa and 6 from wood.



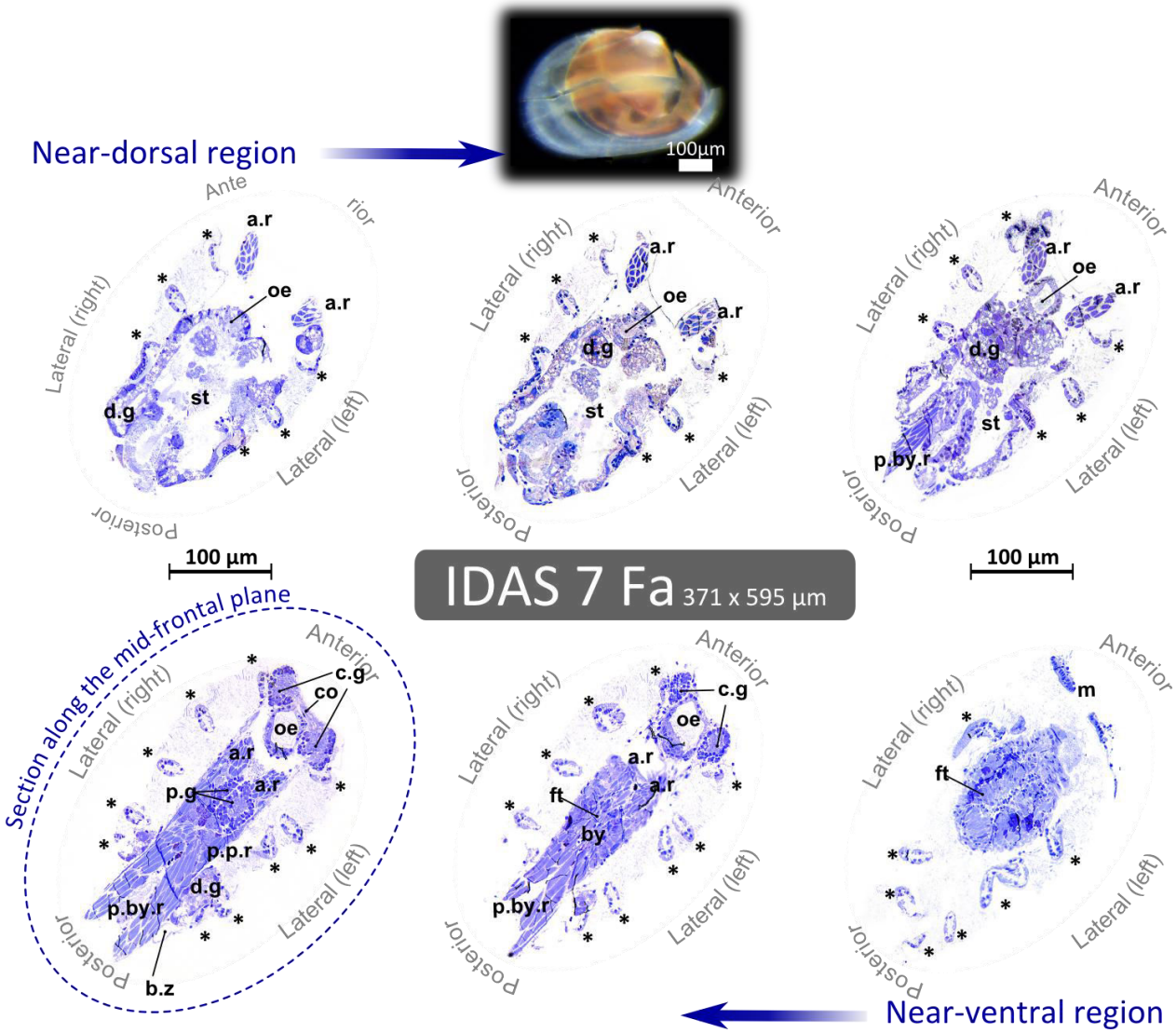
Supplementary figure 4.4 Toluidine-blue-stained sagittal serial sections from a plantigrade (S_L = 418 µm)

Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the lateral-lateral (left-right) axis in the plantigrade, Idas 42. Shell dimensions are cited in the grey box. *a.a.*: anterior adductor muscle *a.r*: anterior (byssus or pedal) retractors, *by*: byssus gland, *c.g*: cerebropleural ganglion, *d*: digestive system, *ft*: foot, *g.ax.*: gill axis (along arrow), *l.p*: lower labial palps, *m*: mantle, *oe*: oesophagus, *p.a.*: posterior adductor muscle, *p.by.r*: posterior byssus-retractor, *p.g*: pedal ganglion, *p.p.r*: posterior pedal-retractor, *p.r* (undefined) pedal retractor, *st*: stomach, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments.

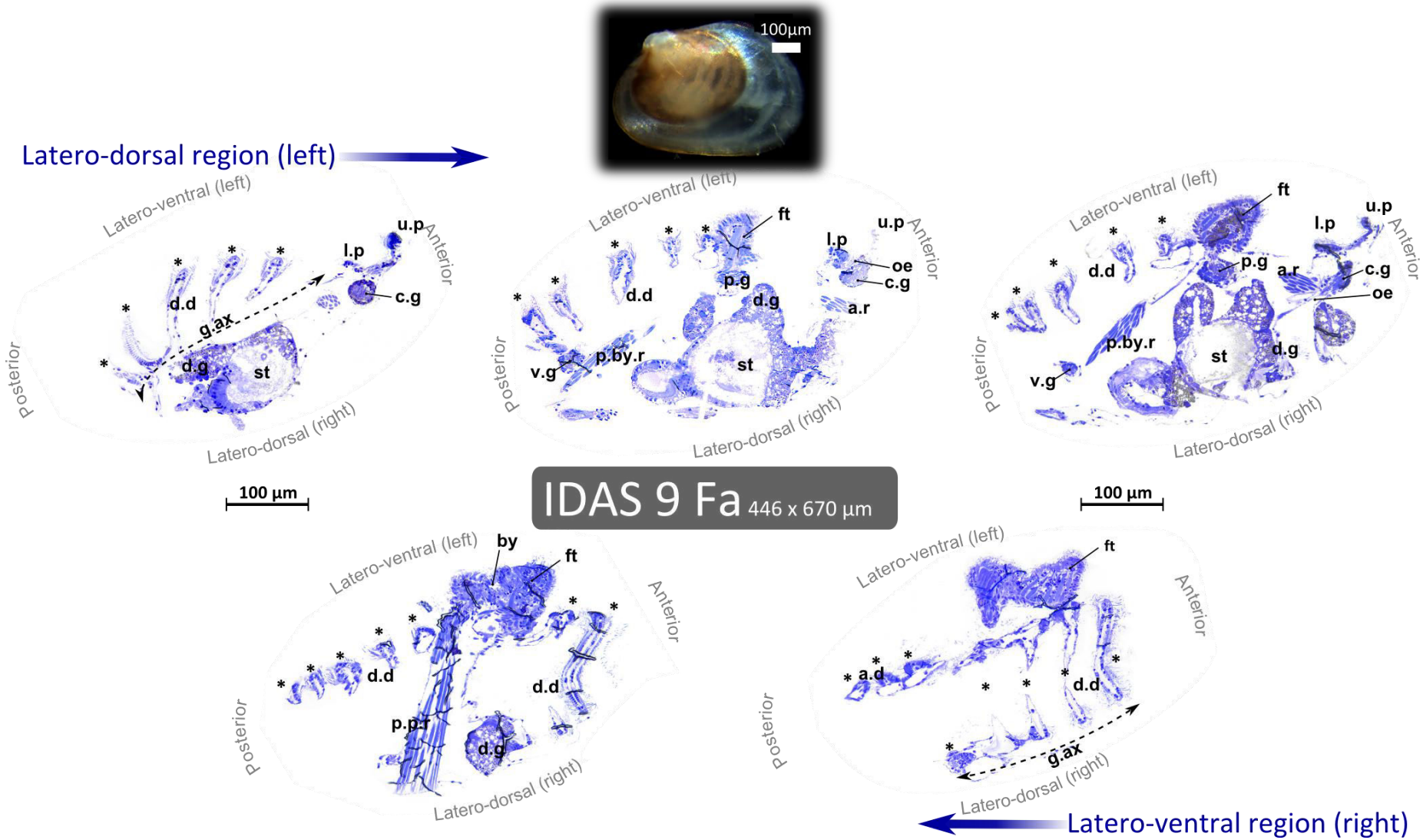


Supplementary figure 4.5 Toluidine-blue-stained serial sections from a small juvenile (S_L = 484 µm)

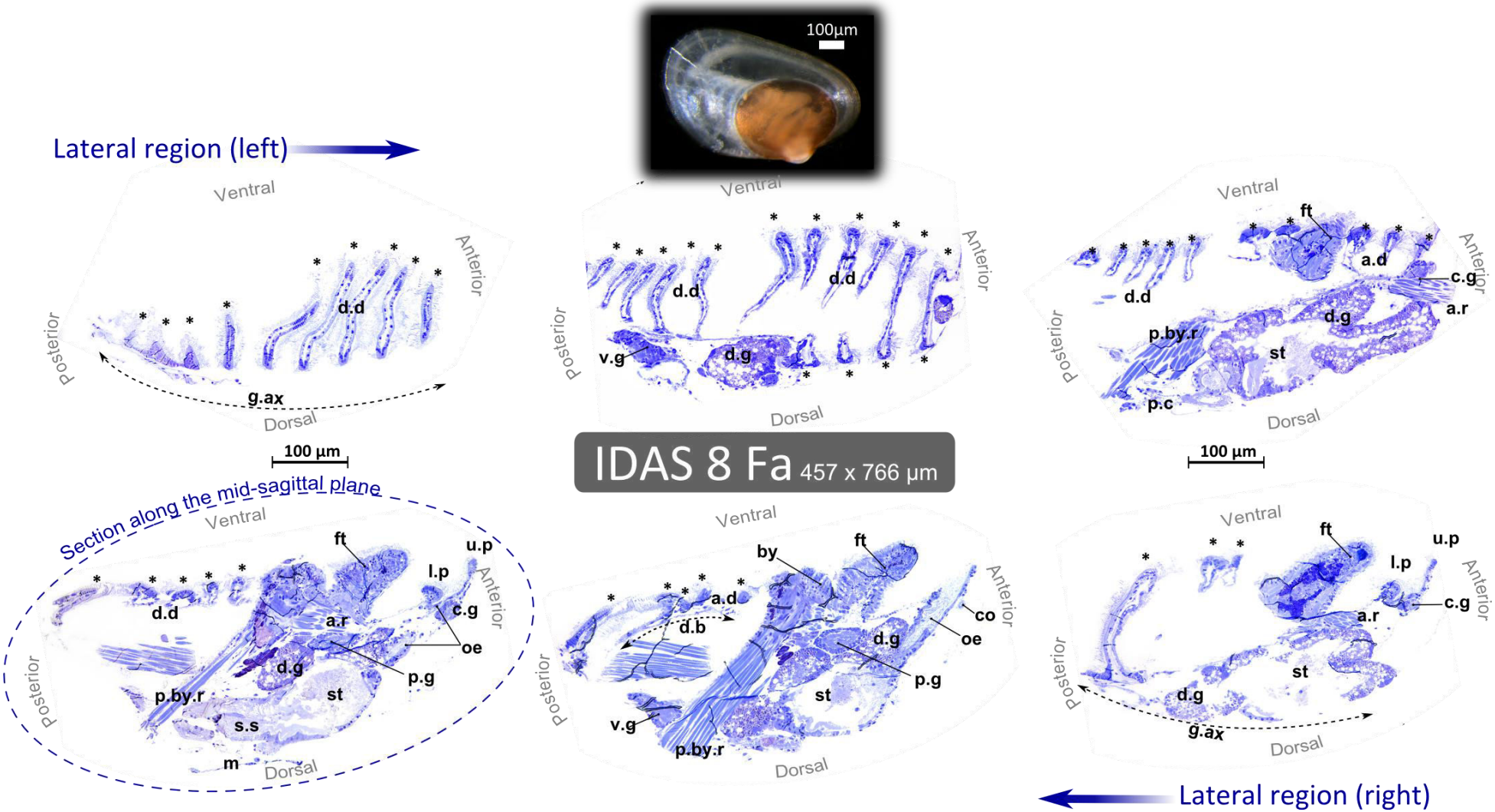
Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the antero-posteriror axis in the small juvenile, Idas 37. Shell dimensions are cited in the grey box. *a.r*: anterior (byssus or pedal) retractors, *by*: byssus gland, *c.g*: cerebropleural ganglion, *co*: neural commissure *d*: digestive system, *ft*: foot, *mo*: mouth, *oe*: oesophagus, *p.g*: pedal ganglion, *p.r* (undefined) pedal retractor, *st*: stomach, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments.



Supplementary figure 4.6 Toluidine-blue-stained serial sections from a small juvenile (S_L = 595 µm)
Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the dorso-ventral axis in the small juvenile, Idas 7. Shell dimensions are cited in the grey box. *a.r*: anterior (byssus or pedal) retractors, *b.z*: budding zone, *by*: byssus gland, *c.g*: cerebropleural ganglion, *co*: neural commissures, *d.g*: digestive gland, *ft*: foot, *m*: mantle, *oe*: oesophagus, *p.by.r*: posterior byssus-retractor, *p.g*: pedal ganglion, *p.p.r*: posterior pedal-retractor, *st*: stomach, * = gill filaments.

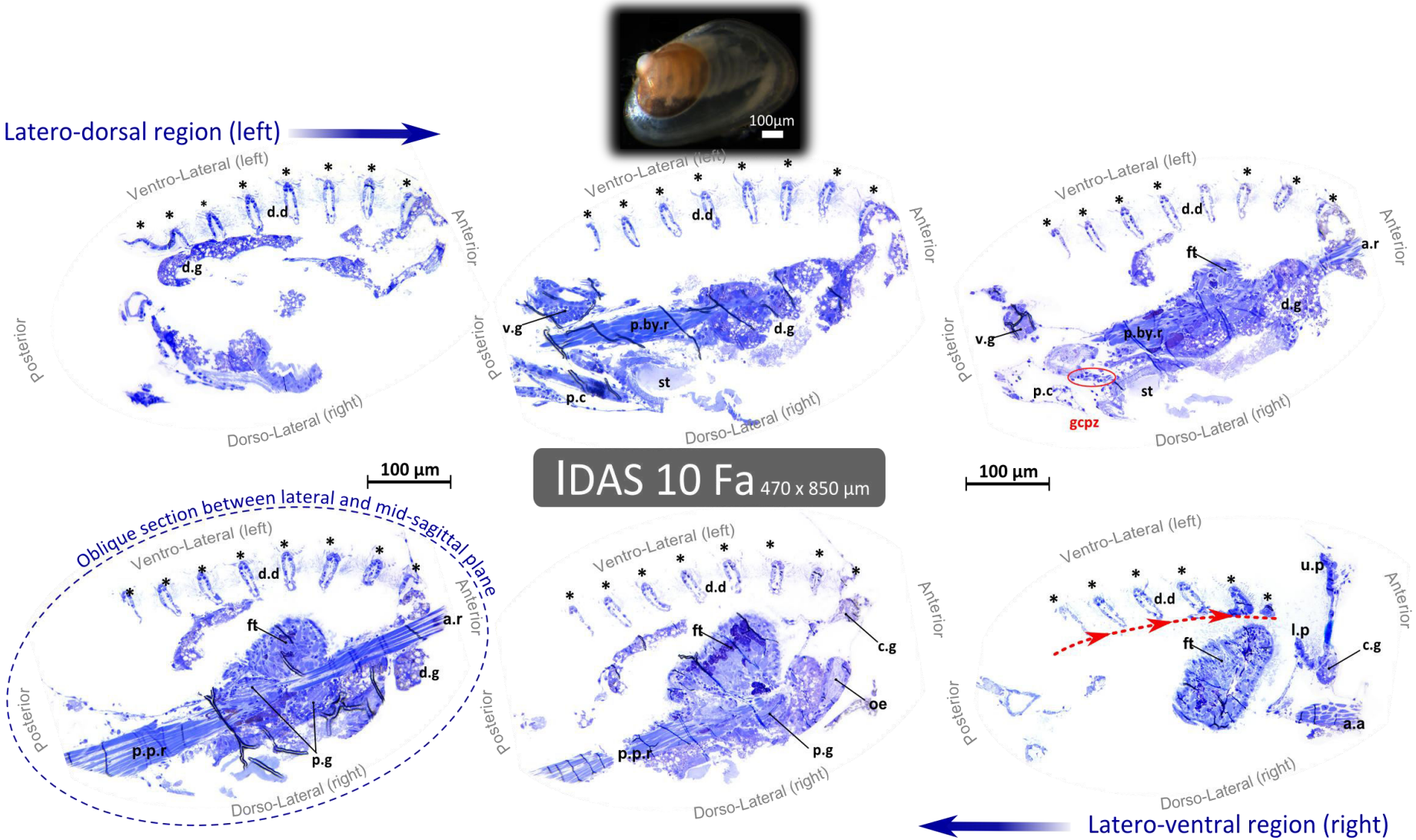


Supplementary figure 4.7 Toluidine-blue-stained serial sections from a small juvenile (S_L = 670 µm)
Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the latero-dorsal (left)-to-latero-ventral (right) axis in the small juvenile, Idas 9. Shell dimensions are cited in the grey box. *a.r*: anterior (byssus or pedal) retractors, *by*: byssus gland, *c.g*: cerebropleural ganglion, *d.d*: descending demibranch, *d.g*: digestive gland, *ft*: foot, *g.ax*.: gill axis (along arrow), *l.p*: lower labial palps, *oe*: oesophagus, *p.by.r*: posterior byssus-retractor, *p.g*: pedal ganglion, *p.p.r*: posterior pedal-retractor, *st*: stomach, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments.



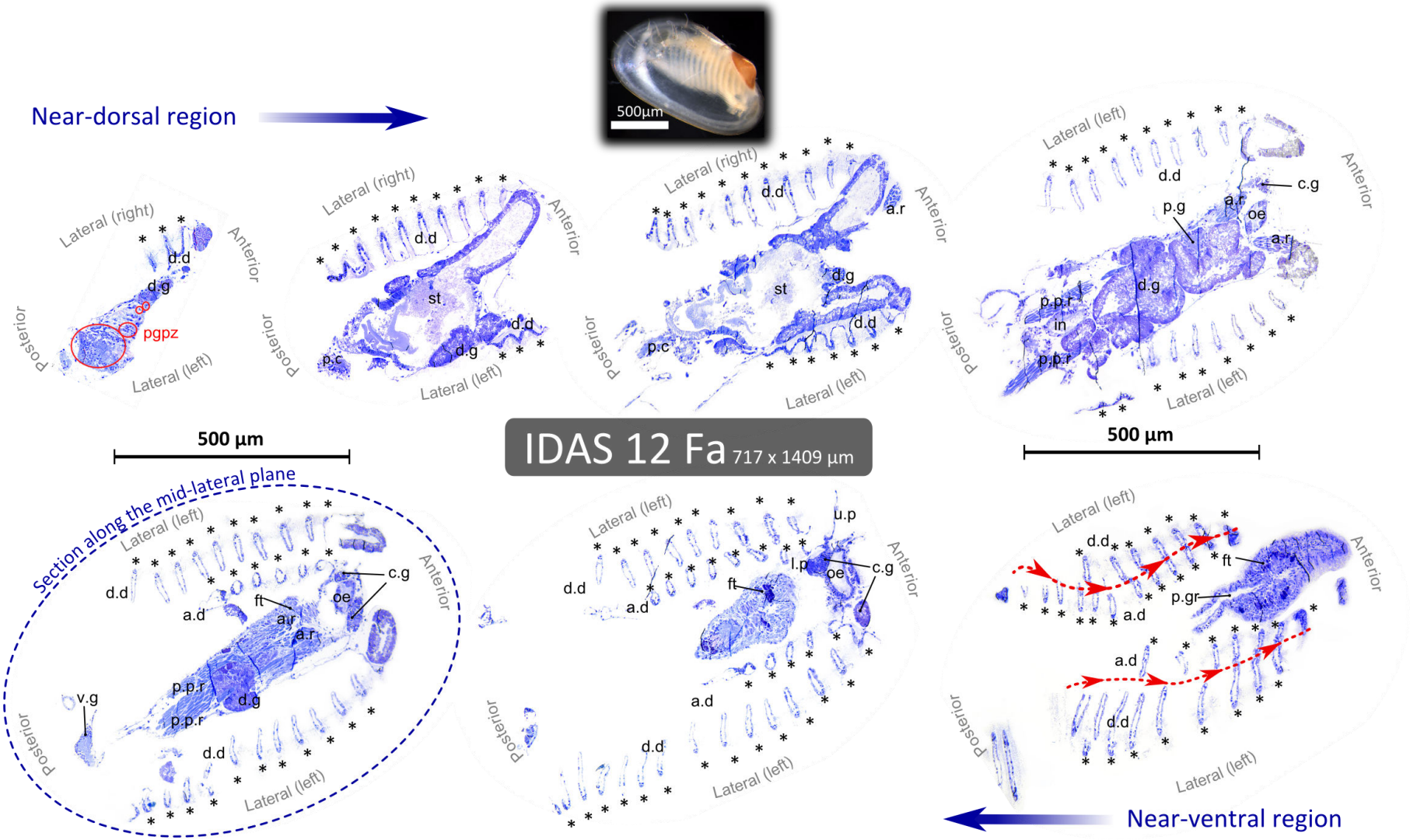
Supplementary figure 4.8 Toluidine-blue-stained serial sections from a small juvenile (S_L = 766 µm)

Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the lateral-lateral (left-right) axis in the small juvenile, Idas 8. Shell dimensions are cited in the grey box. *a.d*: ascending demibranch, *a.r*: anterior (byssus or pedal) retractors, *by*: byssus gland, *c.g*: cerebropleural ganglion, *d.b*: dorsal bend (along arrow), *d.d*: descending demibranch, *d.g*: digestive gland, *ft*: foot, *g.ax*.: gill axis (along arrow), *l.p*: lower labial palps, *m*: mantle, *oe*: oesophagus, *p.by.r*: posterior byssus-retractor, *p.c*: pericardial cavity, *p.g*: pedal ganglion, *st*: stomach, *s.s*: style-sac, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments.



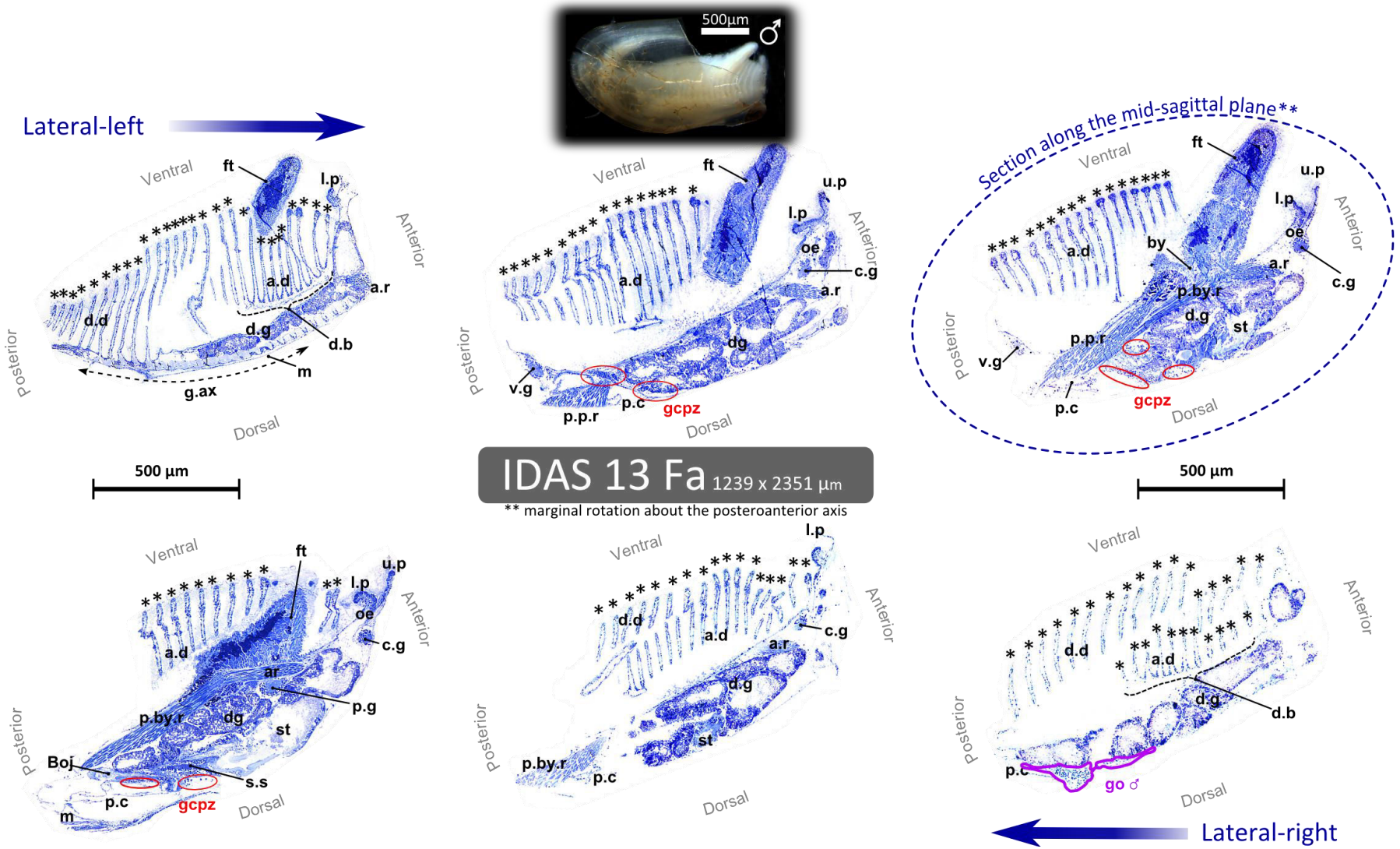
Supplementary figure 4.9 Toluidine-blue-stained serial sections from a small juvenile (S_L = 850 µm)

Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the latero-dorsal (left)-to-latero-ventral (right) axis in the small juvenile, Idas 10. Shell dimensions are cited in the grey box. Annotation (alphabetically): *a.a*: anterior adductor, *a.r*: anterior (byssus or pedal) retractors, *c.g*: cerebropleural ganglion, *d.d*: descending demibranch, *d.g*: digestive gland, *ft*: foot, *gcpz*: germ-cell proliferation zone (encircled in red), *l.p*: lower labial palps, *oe*: oesophagus, *p.by.r*: posterior byssus-retractor, *p.c*: pericardial cavity, *p.g*: pedal ganglion, *p.p.r*: posterior pedal-retractor, *st*: stomach, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments. Red path indicates the ventral particle groove (direction of particle flow).



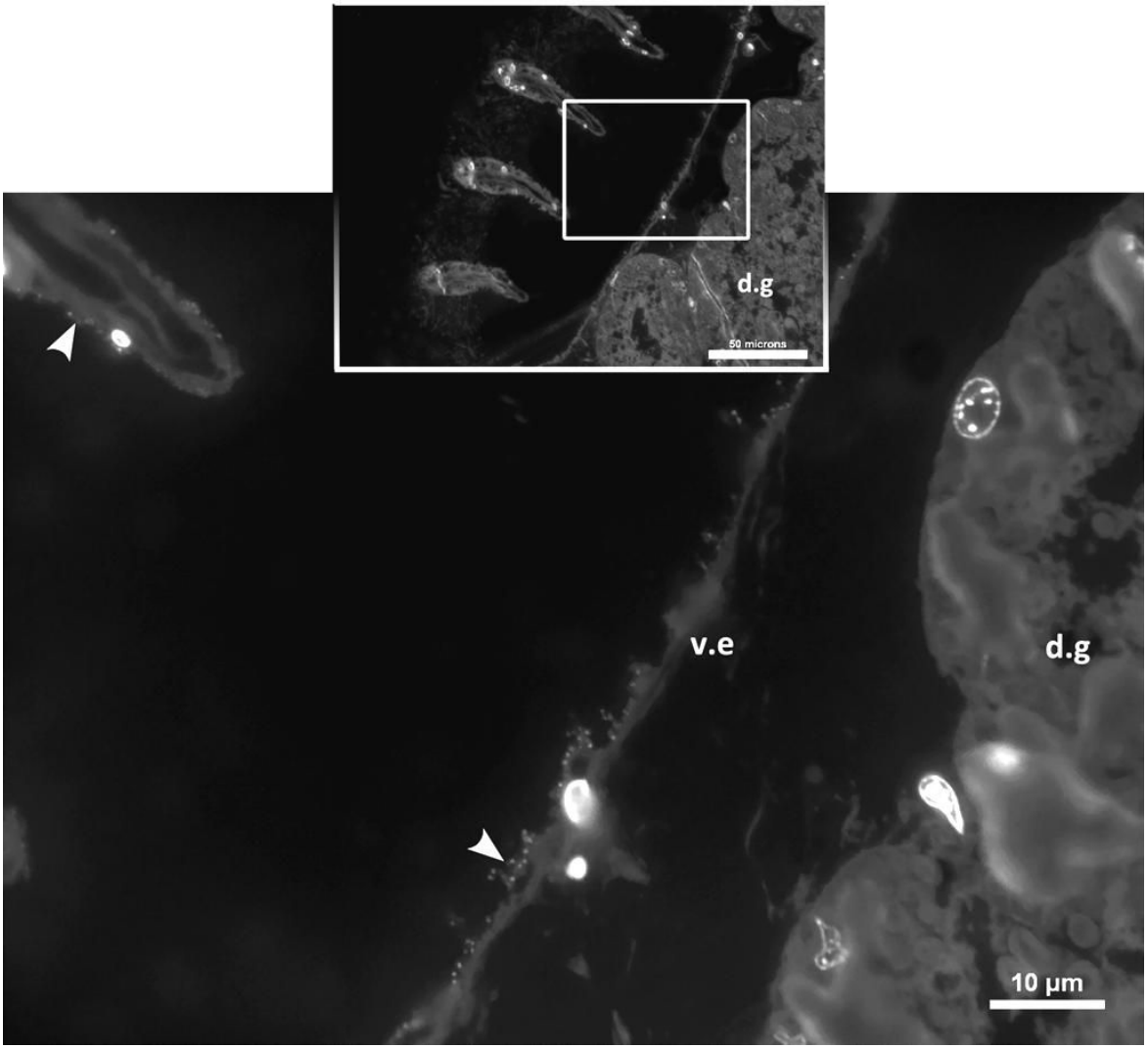
Supplementary figure 4.10 Toluidine-blue-stained serial sections from a large juvenile (S_L = 1409 µm)

Micrographs of 600 nm semi-thin sections stained with toluidine blue at incremental locations along the dorso-ventral axis in the large juvenile, Idas 12. Shell dimensions are cited in the grey box. *a.r*: anterior (byssus or pedal) retractors, *a.d*: ascending demibranch, *c.g*: cerebropleural ganglion, *d.d*: descending demibranch, *d.g*: digestive gland, *ft*: foot, *gcpz*: germ-cell proliferation zone (encircled in red), *in*: intestinal tract, *l.p*: lower labial palps, *p.c*: pericardial cavity, *oe*: oesophagus, *p.g*: pedal ganglion, *p.gr*: pedal groove, *p.p.r*: posterior pedal-retractor, *st*: stomach, *u.p*: upper labial palps, *v.g*: visceral ganglion, * = gill filaments. Red path indicates the ventral particle groove (direction of particle flow).



Supplementary figure 4.11 Toluidine-blue-stained serial sections from a small adult male (S_L = 2351 µm)

Micrographs of 600-1000 nm semi-thin sections stained with toluidine blue at incremental locations along the lateral-lateral (left-right) axis in the small adult male, Idas 13. Shell dimensions are cited in the grey box. a.d: ascending demibranch, a.r: anterior (byssus or pedal) retractors, Boj: organ of Bojanus, by: byssus gland, c.g: cerebropleural ganglion, d.b: dorsal bend, d.d: descending demibranch, d.g: digestive gland, ft: foot, g.ax.: gill axis (along arrow), gcpz: germ-cell proliferation zone (encircled in red), go: gonad (delineated in purple), l.p: lower labial palps, m: mantle, oe: oesophagus, p.by.r: posterior byssus-retractor, p.c: pericardial cavity, p.g: pedal ganglion, st: stomach, s.s: style-sac, u.p: upper labial palps, v.g: visceral ganglion, * = gill filaments.



Supplementary figure 4.12 Presence of putative bacteria on visceral epithelia in Idas 12
Micrographs of 350 nm semi-thin section stained with DAPI cut in the frontal plane along the dorso-ventral axis, in the large juvenile, Idas 12 (between the upper- central-right and far-right sections in Supplementary figure 10). General region is depicted in inset image (white box is magnified). Bacteria = white arrowheads. *d.g*: digestive gland, *v.e*: visceral epithelium. Scale (inset) = 50 µm.

4-9 Chapter 4 Annex 2

4-9.1. Full protocol developed for histological staining of semi-thin LR white sections

The following is an adapted histological protocol for the staining of semi-thin (350nm) methylacrylate sections of *Idas* sp. for Harris' Haematoxylin and Eosin-Y:

4-9.2. Scope and application

This protocol, which has been adapted from classical histological staining techniques used in reproductive histology, is for use upon unusually thin sections (350nm) of animal tissue, previously embedded in a methacrylate resin (e.g. LR White) which is incompatible with alcohol-based reagents. The principal adaptations are therefore the removal of any ethanol-dependent steps (with substitution, where possible)

and the elongation of staining steps to retain sufficient contrast in semi-thin sections. In addition, since the embedding media is not removed, initial steps involving a solvent to dissolve the embedding media and, if necessary, subsequent removal of solvent by replacement using alcohol (such as is the case in paraffin embedding), are not required.

4-9.3. List of equipment and consumables

- Extraction Hood
- Oven or hot-plate at <60 °C
- Compound Microscope (20x to 40x objective lens as a minimum)
- Silicone pen
- Superfrost slides
- Adjustable micropipettes
- Filter paper
- 20ml syringe with 0.2 µm filter
- Glacial Acetic acid
- Distilled water-source
- Running tap-water (ideally with a pH above 7)
- Suitable bath with slide-rack insert
- **Harris' Haematoxylin (working-stock):**

  Mix well. Pour off a given volume of standing-stock into a clean vessel and filter (0.2 µm) to remove particulates. Add 2-4ml glacial acetic acid per 100ml of stain immediately prior to use, to increase the precision of nuclear staining. ***Use immediately***.
- **Scott's tap-water (bluing) solution:**

  Magnesium sulphate ($MgSO_4$)- 30.0 g; Sodium bicarbonate- 2.0 g; Tap water- 3 litres
- **Aqueous Eosin-Y (working-stock):**

  Mix well. Pour off a given volume of standing-stock into a clean vessel. Add glacial acetic acid drop-wise (using a 10 or 20 µl micropipette) to attain a pH of between of 5.0, monitored using a pH-meter. Be conservative!

  Too acidic (< pH 4), and the Haematoxylin stain contrast is reduced (as this stain turns increasingly pink with lowering pH) and Eosin staining is "cloudy". Too alkaline (> pH 5) and the Eosin contrast will be drastically reduced.

4-9.4. Principal procedure

4-9.4.1 Hydration of sections

Sections which have been air-dried onto Superfrost plus slides previously are rehydrated for 3 minutes in a Milli-Q-filled slide-holder. Sections are typically encircled with a silicone PAP-pen.

4-9.4.2 Harris' Haematoxylin staining (regressive stain)

1) Working-stock Haematoxylin stain (see above) is applied by aliquots (30-50µl) to each section under examination.
2) Leave for 30 mins (prolonged staining step)
3) Rinse slides with distilled-water.
4) Arrange the running tap-water bath (no slide-rack) so that a gentle flow of tap-water overflows from the bath (with minimal turbulence).
5) Place slides in the slots of the bath rack, and submerge in bath so that sections face AWAY from the direction of current (reduces risk of stripping).
6) Run for one minute or until water runs clean (no evidence of streaking with stain) and then remove slide-rack to bench on tissue-paper.
7) Examine slide under microscope. Check for degree of bluing (will depend on tap-water alkalinity, see step 10)), and evidence of over-staining (see step 8)).
8) If differentiation of stain is necessary[25] (i.e. nuclear detail is obscured by over-staining and cytoplasm is strongly stained), the brief application (1-2 seconds, or 2 – 3 dips) of 0.25 – 0.5% aqueous glacial acetic acid will act to remove excessive stain.
9) Return immediately to tap-water bath for a further minute to halt differentiation and rinse acid.
10) Examine slide under microscope: the cytoplasm has only a faint stain but the sharp nuclear stain still remains. Nuclei will probably appear reddish. In the case of over-differentiation (too faint), steps 1) - 9) can be repeated with a reduced exposure/concentration (or both) for the differentiating agent (step 8)).
11) If nuclei appear reddish, dip slide-rack (3-6 times) in a second bath with Scott's tap-water bluing solution until specimens are "blue-jean blue".
12) Return immediately to tap-water bath for a further minute to remove traces of bluing solution and then remove slide-rack to bench on tissue-paper

N.B. Some degree of Haematoxylin over-staining is desirable, as the Eosin-Y solution which is acidified, will somewhat differentiate the Haematoxylin (i.e. remove excess) during its subsequent application. In 350 nm semi-thin sections, the excessive over-staining of tissue - to the point of obscuring detail completely - has yet to be identified.

[25] This step may not always be necessary for semi-thin sections

13) Examine slide under microscope again. If satisfied, proceed to Eosin staining.

4-9.4.3 Eosin-Y staining (aqueous)

14) Blow-dry slides rapidly (this step was included to prevent alteration of Eosin stain pH by dilution from precious rinse step
15) Working-stock Eosin-Y stain (see above) is applied by aliquots (30-50µl) to each section under examination.
16) Leave for 20 mins (prolonged staining step)
17) Rinse slides for 10 seconds with aqueous, glacial acetic acid (pH adjusted to same range as eosin, pH 4.3 – 5).

N.B. Originally a rinse of distilled water (and on one occasion, running tap-water) was used, but this may have been removing or altering Eosin staining as results were poor

4-9.4.4 Dehydration

IMPORTANT: this step is compounded by methyacrylate resins such as LR White being sensitive to alcohol- it compromises the resin integrity and can lift sections as a consequence

Dehydration is necessary to ensure that all water is removed prior to application of a suitable mounting medium (if not, the Eosin stain tends to be leached). Options consist of:

18) Air-dry only at <60 °C
19) N.B. Vacuum-assisted dessication diminished eosin staining
20) Reduced-length alcohol series step (this seems to cause irrevocable damage to sections (shrinkage) but results in better contrast in staining (probably due to complete removal of water)

4-9.4.5 Clearing and mounting of sections

N.B. Clearing of slides using Histoclear II: this clearing medium is not compatible with many xylene-based permanent mounting fluids. If such a mounting medium is being used, replacing the last histoclear step with Xylene will removed problems associated with mixing and bubbles. An alternative is the use of Omnimount© (Electron Microscopy Sciences RT 17997-01), formulated to be used in conjunction with Histoclear II. If using xylene to clear slides, most permanent mounting fluids are suitable, however xylene (and toluene) are both highly-toxic.

21) Place slides in holder with Histoclear for 30 seconds.
22) Transfer to second bath of Histoclear for a further 30 seconds.
23) If necessary (see note above), an additional bath of Xylene for 1 minute.
24) Without drying slides, mount in a sufficient volume of suitable permanent mounding medium (see note above) to just cover samples upon addition of a cover-slip
25) Place cover-slip and seal with nail varnish.
26) View under compound microscope (plan-fluorite objective-lens as a minimum).

4-9.5. Notes on method

4-9.5.1 Benefits

The sub-cellular detail which can be realised with semi-thin sections is superior to that of thicker preparations, singular-layer nature of the tissue under examination. Tissue loss should be minimised due to the retention of the embedding media on the slide (assuming the use of Superfrost slides)

4-9.5.2 Constraints

At this thickness (350 nm), expected contrasts associated with thicker paraffin-embedded specimens are unrealistic and difficult to achieve with aqueous Eosin-Y, which is less variable in intensity with variable tissue-types when compared to alcohol-based alternative, particular in the absence of Phloxine. The retention of the embedding media may be contributing factor to variable staining intensity, though trials are on-going.

Chapter 5 RAPID AND PROLIFIC SYMBIONT ACQUISITION IN NEWLY SETTLED, FAST-DEVELOPING, BONE-ASSOCIATED MUSSELS

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N.B. This manuscript is still in the early stages of preparation, wherein exact details for site location (i.e. GPS coordinates) remain unconfirmed. However several of the publications that have been written based on the preliminary findings of the CARCACE project (Colonisation of mammal carcasses in the deep Atlantic Ocean) have been aimed at Marine Ecology, as part of a special issue.

5-1 Abstract

On the scale of the habitat, the arrival of whale falls to the seafloor provides massive influxes of labile organic matter to localised areas of the seabed. Sulphidic conditions can last from a year or two in small shallow-water whale falls to many decades in deep large carcasses. The benthic chemosymbiotic fauna that specialise in colonising these organic substrates must be adapted to dynamic variability in their habitat which simultaneously provides their principal source of food. This study thus investigates two such adaptations, rate of maturation and the patterns of developmental symbiosis within the context of general development towards adulthood. A size series of mussels that spanned post-larvae to confirmed adults was used. Aposymbiosis in post-larval plantigrades provides evidence for entirely heterotrophic larvae. Acquisition of symbionts was found to be rapid and in abundance, almost immediately following settlement: shells had only increased in size by less than 10 % when bacteria were first detected. Maturation was at less than 2.6mm shell length, or the equivalent of < 6% of this species maximum known size. These results are discussed in relation to its habitat and in relation with other chemosymbiotic species for which data is available.

5-2 Introduction

The discovery rate of new highly productive habitats in the deep sea has risen exponentially (Ramirez-Llodra et al. 2010) following the initial descriptions of dense megafaunal aggregations thriving in the vicinity of hydrothermal vents (Corliss and Ballard 1977; Lonsdale 1977) and 'cold' hydrocarbon seeps (Paull et al. 1984). Some of the most startling were the discoveries of specialized fauna found at large organic falls such as vegetative debris and megafaunal nektonic carcasses (whale falls being the best documented). Inexplicably high species abundances on and around whale skeletons were initially surprising until the resident species' loose affiliations to vent fauna were realised, suggesting that organic falls formed similar sulphide-based reducing habitats (Smith et al. 1989), but with elevated levels diversity in comparison with hydrothermal vents (Baco and Smith 2003). Following the habitat's formal description (Smith et al. 1989), the earliest studies carried out upon both natural- and experimental-whale-fall communities identified commonality with some vent and seep organisms, albeit as minor contributors to species richness (Bennett et al. 1994; Deming et al. 1997; Feldman et al. 1998; Smith et al. 2002; Baco and Smith 2003; Smith and Baco 2003; Schuller et al. 2004). Accordingly, extant or ancestral whale fauna have been implicated in the evolution of vent and seep fauna (Smith et al. 1989), based on the apparent similarities in taxa between the different types of reducing habitat; ancestral vent- and seep- species already developing symbiotic associations are believed to have inhabited large organic falls as dispersal pathways over their evolutionary history. Whilst most researchers in reducing habitats tend to agree that this hypothesis is plausible, the order of habitats remains contested due to somewhat conflicting patterns between fossil records and current multi-gene phylogenies, Kiel and Goedert 2006; Lorion et al. 2013). However, a similar hypothesis was subsequently put forward for sunken wood (Distel et al. 2000), suggesting that organic falls may have played a crucial role in the eventual adaptation of vent and seep[26] fauna to their respective habitat conditions.

Collectively, whale falls at various stages of degradation are estimated to cover 35 km^2 of the ocean floor (Ramirez-Llodra et al. 2010). This is based on the knowledge that a 30t whale fall can enrich surrounding sediments up to 9 m from the site of the carcass (i.e. approximately 50 m^2 area of seafloor, Baco and Smith 2003; Treude et al. 2009), and an estimated 690 000 whale falls are decomposing on the seafloor at any one moment in time (from Ramirez-Llodra et al. 2010, using data from Baco and Smith 2003; Smith 2006; Treude et al. 2009). It follows that when averaged over the entire deep-sea floor's surface area (3.6×10^8 km^2), whale falls worldwide probably only represent ≈0.1% of the background particulate organic carbon (POC) flux to the deep-sea even under the most oligotrophic central gyre waters (Baco and Smith 2003, details and assumptions therein). However the reality at the habitat scale is that whale falls provide massive influxes of labile organic matter to localised areas of the seabed (Baco and Smith 2003).

[26] This is under debate, given the fossil records for seep associated bathymodiolins predate other habitats

In the case of megafaunal nektonic carcasses, four stages of community succession have been recognised during decomposition, of which the first three are expedited by biological processes (Bennett et al. 1994), though the depth of the carcass appears to dictate to what extent each phase applies (Lundsten et al. 2010). These are the *mobile scavenger* stage, the *enrichment-opportunist* stage, the *sulphidic* stage and finally, when all organic sources of energy are depleted, a skeletal *reef* stage. Following the arrival of the carcass at the seafloor, mobile scavengers quickly consume the labile protein and lipid rich flesh. Applying an estimated removal rate of 40–60 kg d^{-1} (Smith 2006), the very largest carcasses in the deep sea (e.g. 160 t Blue whale *Balaenoptera musculus*) could provide tissue in this way for up to a decade, though for an average-sized baleen whale (e.g. 35 t) this period is in the range of 1.5 years (Smith et al. 2002). Due to settling particles of carcass flesh ('messy' feeding) and rapid input of faecal waste, underlying sediments typically become greatly enriched, supporting an array of organic-enrichment type opportunistic species (e.g. dorvellid polychaetes, cumaceans and oligochaetes, Smith and Baco 2003). Enrichment of this sort has been documented to last 0.3 – 4.5 years, depending on carcass size (5 – 35 t, n = 3, Smith et al. 2002). Sediment enrichment is relatively rapid, occurring within days to weeks of the addition of whale biomass due to microbial sulphate reduction and methanogenesis (based on *in vitro* studies Treude et al. 2009). The levels of sulphide and methane found in sediments appear to depend on proximity of underlying sediments to the carcass, but at 0.5-m distance, values of 717 and 99 mmol m^{-2} d^{-1} of *ex situ* sulphate reduction and *in vitro* methanogenesis have been recorded (Treude et al. 2009). Approximately 10% of the total sulphides produced are as free sulphides, while sediments subject to direct whale biomass enrichment, can see rates of methanogenesis equivalent to 20 – 30% of those for sulfate reduction (Treude et al. 2009). Decomposing whalebones are thus thought to create a habitat intermediate to that of vents and seeps (Treude et al. 2009).

Once bones are exposed, the resultant efflux of sulphides (Treude et al. 2009) can fuel the metabolism of chemoautotrophic bacteria, both free-living (e.g. microbial mats) and as symbionts in specialist chemosymbiotic metazoans that colonise the hard surfaces of organic falls. The semi-porous nature of bone permits limited release of sulphides from decomposing lipid-rich marrows internal to bones of whale skeletons (Higgs et al. 2011b). Sulphide reduction rates are considerably lower in bones than the surrounding sediments (e.g. ≈ 10 mmol total sulphide d^{-1} versus ≈1.5 – 2 mol d^{-1} in sediments). However, the release of sulphides can be accelerated by the erosive action of microbial mats on the bone's surface and through the digestion of bone directly by the 'bone-eating' polychaete species (genus *Osedax*) in the course of accessing internal lipid-rich marrow (Higgs et al. 2011a). This is made possible by employing symbiotic heterotrophic bacteria housed in bacteriocytes located in the characteristic root tissue of this genus, typically buried into the bone (possibly aided by acidic mucopolysaccharide secretion through the carbonic anhydrase-catalysed hydration of CO_2, Rouse et al. 2004; Higgs et al. 2011a; Katz et al. 2011; Tresguerres et al. 2013). In addition to the organic-fall siboglinid polychaetes specialists (Jones et al. 2008; Kiel et al. 2011; Rouse et al. 2011), small-sized chemosymbiotic bathymodiolins such as *Adipicola* (*s.l.*)

pacifica, *Idas* [*s.s.*] *washingtonius* and *I.* [*s.l.*] simpsoni are often present on bone surfaces during the sulphidic stage (e.g. Marshall 1900; 1901; Southward 2008; Fujiwara et al. 2010), taking advantage of the release of free sulphides and methane (Treude et al. 2009) used by their bacterial symbionts. The first indication that members of the genus *Idas* (*s.l.*) might harbour bacteria in their gills, already known to be the case in larger bathymodiolin (*s.l.*) mussels at that time, was when Gram-negative bacteria were identified in the gills of *I*. (s.s.) *washingtonius* from whale bones (Deming et al. 1997). Evidence for the symbiotic nature of these bacteria was based upon gills testing positively for the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and adenosine 5'-phosphosulfate reductase (APS), which support evidence of sulphide oxidation by the bacteria. Assimilation by the host was argued, since $\delta^{13}C$ values in the tissues of these mussels were characteristically depleted in ^{13}C (–33.7 to –26.8 ‰). Studies since, that have examined the presence of symbiotic bacteria in *Idas*-like mussels have invariably identified their occurrence (Smith and Baco 2003; McKiness et al. 2005; Duperron et al. 2008a; Duperron et al. 2008b; Southward 2008; Gaudron et al. 2012; Lorion et al. 2012; Rodrigues et al. 2013; Thubaut et al. 2013a; Laming et al. 2014; except the types species *I.* [d.f.] *argenteus,* Ockelmann and Dinesen 2011).

Considering the ca. 690000 whale carcasses distributed globally at a given time, only around 0.005% (approximately 30 carcasses) have been studied in detail. This reflects both the random way in which natural whale falls are discovered and the logistical (and ethical) problems associated with securing experimental carcasses. Thus, furthering our understanding of mammalian fall community dynamics is hindered by a lack of suitable organic substrate. One way in which this problem may be circumnavigated is by using analogous mammalian alternatives which are readily accessible to researchers. Precedence for this approach exists in a study by Jones et al. 2008, in which bovine femur bones stripped of meat were deployed in an area proximal to documented whale falls (Monteray Bay, USA). That study, which was focused upon collecting and identifying *Osedax* spp. found such deployments to be highly effective for this purpose, despite lower lipid contents in smaller mammalian bones compared to those of intact whalebone (Evershed et al. 1995). The evident success of such deployments opens up a new avenue for colonisation experiments in the deep sea which can approximate natural nektonic falls.

Funded by Fundação para a Ciência e a Tecnologia (FCT), the CARCACE project was devised (project coordinator: Ana Hilário), bringing together a multidisciplinary international team of scientists with experience in various aspects of deep-sea chemosynthetic benthic ecology. Within the framework of the CARCACE project , entire bovine carcasses attached to concrete ballast, were deployed in Setúbal Canyon on the west Portuguese margin and at the Condor Seamount (near the Azores), with subsequent recoveries following 18- and 24-month durations (Génio et al. *submitted*). The principal aim was to study the community response to the arrival of large organic falls to deep-sea floor in the Atlantic Ocean and to gauge the importance of this localised enrichment as a sulphide-rich habitat. As part of this overall aim, a random size-stratified subset of specimens of the chemosymbiotic mussel "*Idas.*" (*s.l.*) *simpsoni,* collected from

bones recovered after 18 months in the Setúbal Canyon (≈1000m), were selected to assess post-larval to adult development in terms of general anatomy, rates of maturation and patterns of symbiont acquisition.

5-3 Methodology

5-3.1. Sampling and processing

Details concerning the deployment of cow carcasses and their recovery may be found in Génio et al. *submitted*. On board the ship, bones were sorted immediately and samples were fixed and preserved for a variety of analyses (Génio et al. *submitted*). Mussels for the current study were fixed in 4% formaldehyde (in filtered seawater) for 2-4 hours and then preserved in 96% ethanol, by serial transfer (70%, 80%, 96%). Samples were stored at room temperature. In the laboratory, the preserved substrata were sorted microscopically.

Individuals of *Idas* (*s.i.*) *simpsoni* were identified based on morphoanatomy and partial Cytochrome Oxidase I data. The larval prodissoconchs I and II were opaque, pearly white and transparent, red/orange respectively and, where present, the dissoconch was modioliform, colourless (but iridescent), glossy, and almost transparent in all juveniles or translucent and fleshy-pink in individuals >2 mm (examples displayed in Figure 5.1). Bones recovered at 18 months were colonised by these mussels at prolific densities, along with at least one species of *Osedax* polychaete, such that the recovery resulted in 1000s of *I.* (*s.l.*) *simpsoni* individuals in total (Ana Hilário, personal observation). The subset of mussels used in this study was selected randomly from several bones, but with the requirement that the resulting size-spectrum was sufficiently wide to cover post-settlement to putative adults (in order to repeat procedures in Laming et al. 2014). Thus, it was possible to document key developmental patterns in *I.* (*s.l.*) *simpsoni* from the Setúbal canyon, western Portuguese margin, using the size-range selected.

5-3.1.1 Shell and preliminary soft tissue analysis

Individuals were measured (dissoconch: n = 38, prodissoconch I: *n* = 24, II: *n* = 38) and photographed using a camera-mounted dissection microscope (Nikon Elements camera and software, Japan), as in Laming et al. (2014). Shell height (SH) was the greatest linear distance dorso-ventrally measured from the umbo, perpendicular to the hinge-line, while SL was the greatest linear distance antero-posteriorly measured parallel to the hinge line. Prodissoconch II dimensions were measured using the same approach, but with standardised 'larval' hinge-lines, to permit the aggregation of prodissoconch II data across all shell sizes. Prodissoconch I SLs, measured parallel to the vestigial provinculum, were estimated using their coloration to define margin limits. Prodissoconch II shells were oddly blistered on their surfaces, in contrast to *I.* (*s.s.*) *modiolaeformis* in Laming et al. (2014). Size-class frequency analyses were not performed here as the subset was an artificially size-stratified sample (such analysis will be included in Génio et al. *submitted*).

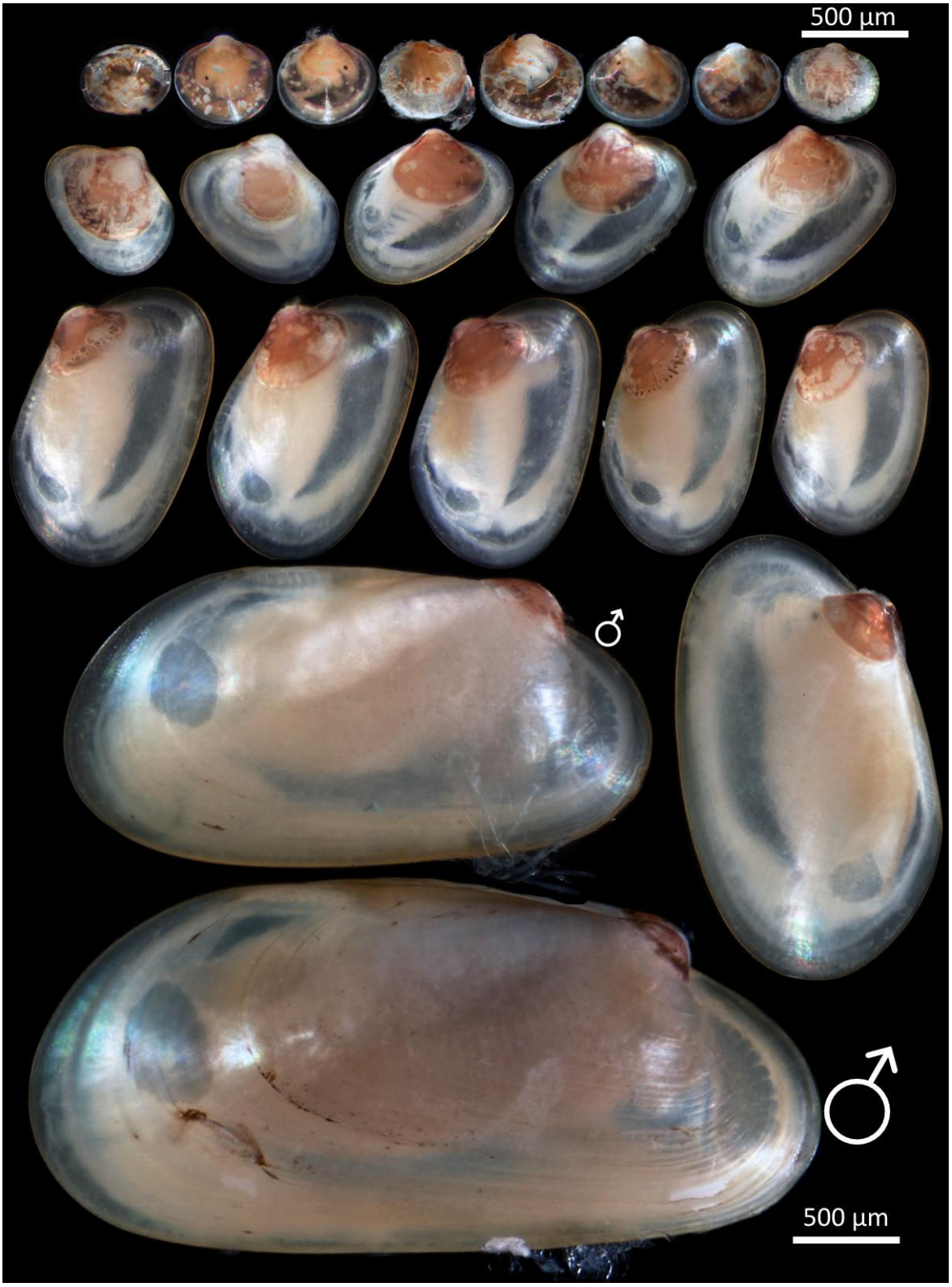

Figure 5.1 Micrographs of *Idas (s.l.) simpsoni* specimens examined
Micrographs are representative subsample of the total number of specimens examined for this study (*n* = 38). The top 8 specimens are the smallest individuals examined at < 500 µm. The smallest mature individual and largest specimen examined are also pictures (small and large ♂ respectively).

However, SLs and SHs were plotted against one another to examine relative changes in dimensions with increasing size (Sigmaplot v. 11). Dissections were performed microscopically using custom-made tools (see supplementary figure 4.1 of Chapter 4). General anatomy was recorded and tissue was extracted, with minimal disruption. Where gills had remained intact (n = 31), the number of filaments comprising the descending lamella in either inner demibranch was recorded, as a proxy for gill development, as in Laming et al. (2014). Counts were plotted against shell size (Sigmaplot v. 11); best-fit analysis was performed in Sigmaplot (v. 11) to assess gill proliferation as a function of SL.

5-3.2. Embedding, sectioning and histology

Tissue was blotted dry and infiltrated (8 x 30-min infiltrations) in a gelatine capsule (size 00, Electron Microscopy Sciences, UK) filled with LR White resin (London Resin Company, UK), transferred to a fresh resin-filled gelatine capsule (orientated appropriately), the capsule was capped, and polymerised at 55°C (20 hours minimum). Orientations (sagittal, frontal and transverse, see Supplementary figure 4.2, Chapter 4, for visual explanation) were achieved using a hair to roll the tissue into position, where plane orientation was alternated sequentially along the size spectrum, i.e. each aspect was equally represented in similarly sized organisms. Transverse orientations required double-polymerisation. The first was a catalysed polymerisation at room temperature, so that a flatbed mould could be used in the presence of oxygen. The mould was afloat on ice water to draw away exothermic heat generated during the 10-min reaction. Surface resin was trimmed (removing resin polymerised imperfectly at the oxygenated surface), submerged vertically in a LR white-filled gelatine capsule, and polymerised as with sagittal and frontal orientation, above. Gelatine was removed with hand-hot water.

Semi-thin sectioning had previously been used in a similar developmental study with great success (Laming et al. 2014), so this approach was also employed in this species. Resin blocks were wet-sectioned (glass knife) on a Leica EM Ultracut UC6 Ultramicrotome (Germany). 1 µm-thick sections on Superfrost plus slides were used for haematoxylin and eosin-Y staining (H&E) and 2 µm-thick sections on Superfrost plus slides for fluorescence microscopy (to mitigate previously encountered poor signal to noise ratios, Laming et al. 2014). Periodic toluidine staining was performed to identify the cutting-axis and location (≈10-µm intervals).

Standard H & E staining procedures for thick sections were modified for semi-thin LR-white sections (according to Laming et al. 2014; Chapter 4). In the rare instances where contrast remained poor and alcoholic Eosin-Y had to be used, section edges were clamped with a border Eukitt hard mounting medium, in order to mitigate resin distortion. Slides were viewed under a camera-mounted compound microscope (Evolution VF camera, Media Cybernetics, USA; Olympus BX61, Japan) and micrographs were processed and measured, where necessary, using Image-pro plus (v.5.1) or during post-processing in Photoshop CS3.

5-3.3. Examination of symbiont patterns

5-3.3.1 Fluorescence

Select slide-mounted sections targeting the entire size-spectrum were equilibrated in phosphate buffered saline (PBS 1x), stained with the nucleic acid-specific stain 4',6-diamidino-2-phenylindole (DAPI, 300 nM in Milli Q for 3 min) and mounted in Slow-Fade Gold (Invitrogen). All body regions were examined. Gill tissue from Lorion et al. (2012) provided the positive control. Negative controls excluded DAPI altogether, to test for auto-fluorescence. To establish whether previously identified bacterial *Idas*-symbiont phylotypes occur as symbionts in these mussels, fluorescence *in situ* hybridisations (FISH) were performed on a subsample of unstained 1–2-µm whole-specimen LR white sections, employing dual-hybridisation combinations (Cy3 and Cy5-labelled probes) of the general eubacterial probe EUB338 (Amann et al. 1990), the non-sense probe NON338 (Wallner et al. 1993), and specific oligonucleotide probes for type-M1 methanotrophs and type-T1, -T2 and Bang-T[27] thiotrophs (details in Duperron et al. 2008a) following the post-permeabilisation steps of the FISH protocol in Duperron et al. (2005). Slides were photographed as in the H & E analysis, but with a monochrome filter.

5-4 Results

5-4.1. General specimen condition and classification

Of the 38 mussels, 35 were still immature. Five of these were plantigrades (i.e. post-larval and immature, no dissoconch deposition evident: SL 0.405–0.479 mm). Two of these were unfortunately lost during the dissection-embedding-infiltration process (tissue was ≈ 300µm long). The remaining three were used for soft-tissue analysis. Though small, much of the anatomy pertaining to later development was evident. Small (<1 mm) and larger (>1 mm) juveniles, in which dissoconch growth was evident but gametogenesis was not, were most numerous (*n* = 30, SL 0.43–2.45 mm). The three largest individuals (SL 2.61–3.74 mm) were adults with direct evidence of gametogenesis, discussed later.

5-4.2. Shell-size patterns

SHs ranged from 0.32–1.54 mm and SLs from 0.41–3.74 mm in *I.* (*s.l.*) *simpsoni* (*n* = 38, including plantigrades). Mean prodissoconch I lengths ($\overline{\mathrm{PdIL}}$) ±σ were 95.9 ± 6.3 µm. Mean prodissoconch II heights ($\overline{\mathrm{PdIIH}}$) and lengths ($\overline{\mathrm{PdIIL}}$) ±σ were 0.40 ± 0.028 mm and 0.46 ± 0.026 mm respectively (PdH: 0.32–0.46 mm, PdL: 0.40–0.52 mm, *n* = 38). Figure 5.2 displays a scatter-plot of both measurements.

[27] Previously identified in this species (Ritt et al. 2012)

5-4.3. Developmental patterns

5-4.3.1 Nervous and sensory system

Nerve ganglia were easily identified in all individuals across all size ranges, on account of their distinctive appearance and conservative size. Ganglia ranged from ≈50 µm in diameter in post-larval plantigrades, to ≈100 µm in mature specimens. The respective locations of cerebropleural-, pedal-, and visceral paired-ganglia were, immediately dorso-lateral to the mouth, centrally within the proximal region of the foot, and in proximity to the posterior adductor muscle (Figures 5.3–5.5). Prior to dissection, pigment spots were actually visible within the translucent tissue under the microscope as two pairs of tiny black dots. These were subsequently identified in Haematoxylin and Eosin (HE) stained sections, coupled directly with the cerebropleural ganglia, either side of the mouth and very close to the anterior-most gill filament.

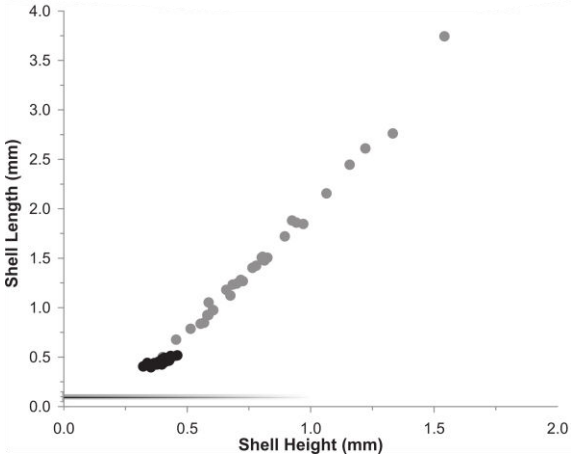

Figure 5.2 Plot of SL against SH of *"I." (s.l.) simpsoni*
SL and SH of prodissoconch and whole shells (n = 38, black and grey dots, respectively). Prodissoconch measurements from plantigrades and juveniles/adults. Whole shell measures exclude plantigrades. Prodissoconch SL range marked horizontally

In serial sections, these three paired ganglia were elliptical, comprising of a peripheral layer of neuronal cell-bodies (axons being directed inwardly towards the nerve centre) with a point of exit for the nerve bundle (e.g. Figure 5.3B). It was not possible to identify connective nervous cords with the staining techniques used in this study, but cerebropleural commissures were identified in specimens sectioned along the dorso-ventral axis in the sagittal plane (not shown).

5-4.3.2 Digestive system

The digestive system was already well-developed in plantigrades with a scaled increase in volume and complexity with increasing shell sizes (Figure 5.3A, C). In plantigrades examined histologically (*n* = 3), ciliated labial palps were already well-developed (Sequential toluidine-stained sections in Supplementary figure 5.1), with a ciliated oesophagus, stomach and digestive gland. The oesophagus was practically flush to the utmost anterior region of the digestive system with most of its length orientated perpendicular to the hinge-line (Supplementary figure 5.1). Identifying whether palps were associated with the anterior region of the gill-basket directly, proved infeasible at this size with the techniques employed. The entire alimentary system typically occupied about a third of the area of the soft tissue in mid-sagittal section (Figure 5.3A).

In juveniles, the moderate increase in shell capacity was occupied, in part, by an expanding digestive system. Sequential HE sections with detailed annotation from two similarly sized juvenile individuals are presented, cut in the transverse (Figure 5.4) and sagittal (Figure 5.5) planes. These

individuals were representative of general trends observed in developing specimens (summarised using colour coded schematics, in part C of Figures 5.4 and 5.5 respectively: full-page versions of these schematics can be found in Supplementary figures 5.2 and 5.3). Hypertrophied gills were already loaded with bacteria (Figure 5.3–5.5), analysed in detail in section 5-4.4, p. 215). Labial palps were considerably more elongate than in plantigrades, coupled with the most anterior gill filaments (Figure 5.5A; and observed during dissections). The oesophagus was longer and orientated at a more acute angle to the hinge-line than in

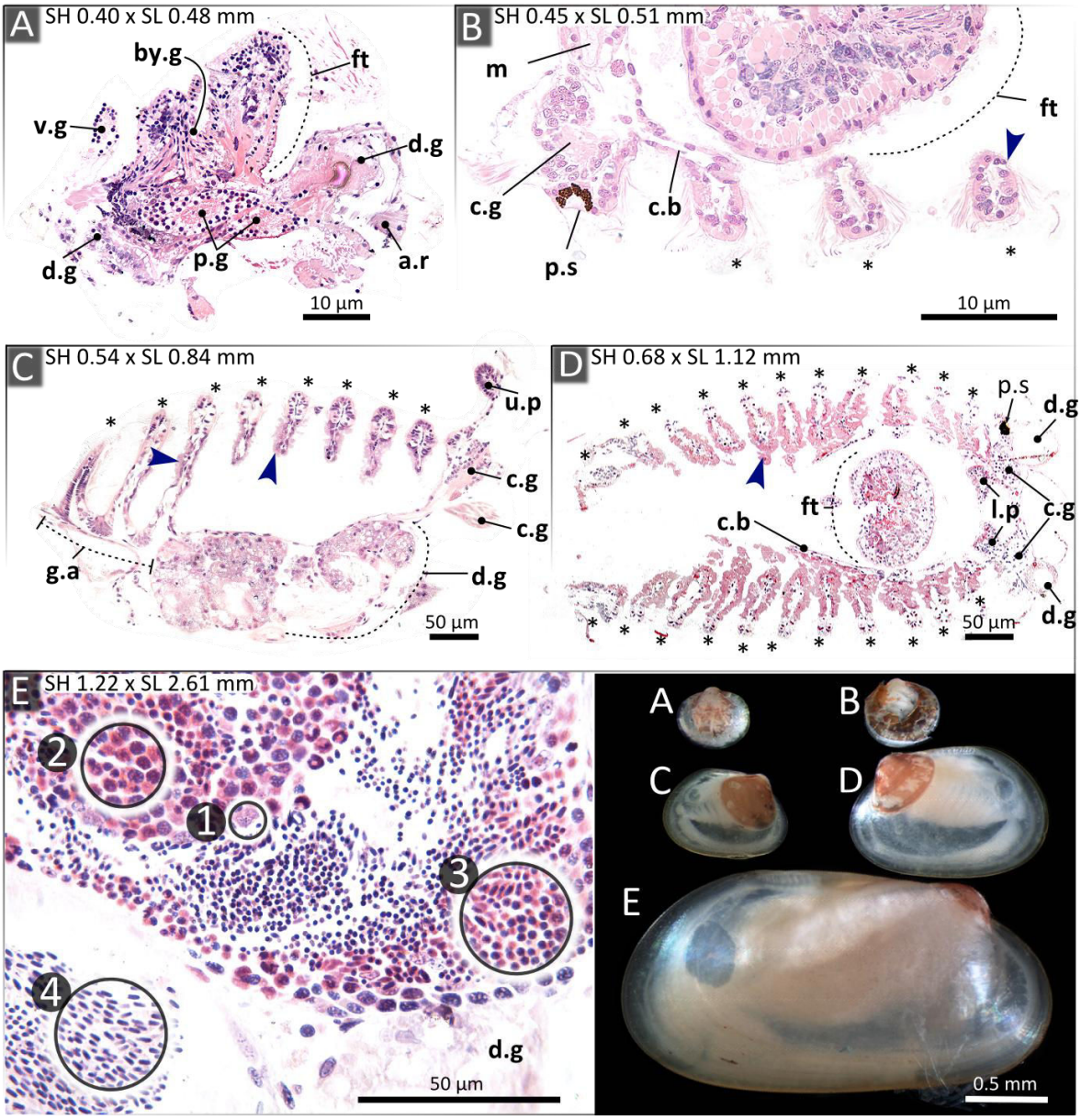

Figure 5.3 Overview of Haematoxylin and Eosin-Y developmental histology in *Idas* (s.*l.*) *simpsoni*
A) Post-larval plantigrade in mid-sagittal section. **B)** Right lateral half-view of of ventral region of small juvenile in frontal section. **C--D)** Larger juveniles in both laterally displaced sagittal [**C**] and ventrally displaced frontal [**D**] section. Arrowheads indicate bacteria identified during HE staining. **E)** High magnification view of gonad tissue in the smallest mature individual. Four stages (1,2,3,4) of spermatogenesis are identified (spermatogonia, spermatocytes, spermatids and spermatozoids, respectively). **E)** Shell micrographs prior to dissection of specimens [**A**–**E**]. a.r *anterior retractor*, by.g *byssus gland*, c.b *connecting band*, c.g *cerebropleural ganglion*, d.g *digestive gland*, ft *foot*, g.a *gill axis*, l.p *lower labial palp*, m *mouth*, p.g *pedal ganglion*, p.s *pigment spot*, u.p *upper labial palp*, v.g *visceral ganglion*. * = gill filament.

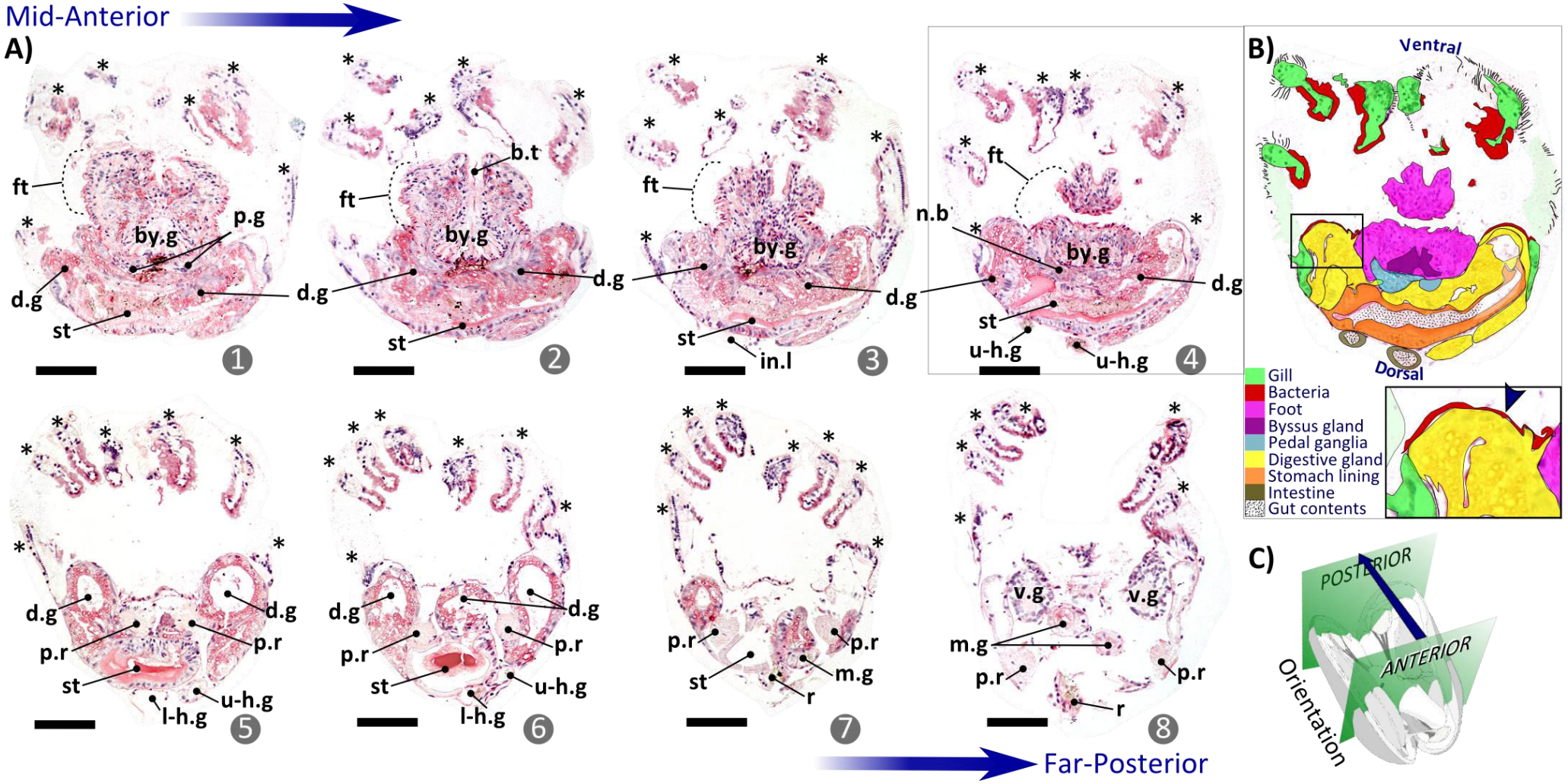

Figure 5.4 Serial Haematoxylin and Eosin-Y stained transverse sections of a juvenile *Idas* (s.*l.*) *simpsoni* (SH 0.59, SL 0.92)

A) Series of sections taken at sequential locations along the antero-posterior axis [❶= mid-anterior, ❽ = Far-posterior]. **B)** A schematic that segregates the various organs and tissues identified in ❹. Inset: magnification of region inside box, highlighting the location of non-gill bacteria (arrowhead). An enlarged version is available in Supplementary figure 5.2. **C)** A generic *Idas*-like mussel drawing, with region of sections displayed (approximate). by.g *byssus gland*, b.t *byssus thread,* d.g *digestive gland*, ft *foot*, in.l *intestinal loop*, l-h.g *lower hindgut*, m.g *midgut*, n.b *nerve bundle*, p.g *pedal ganglion*, p.r *posterior retractor*, r *rectum*, st *stomach*, u-h.g *upper hindgut*, v.g *visceral ganglion*. * = gill filament

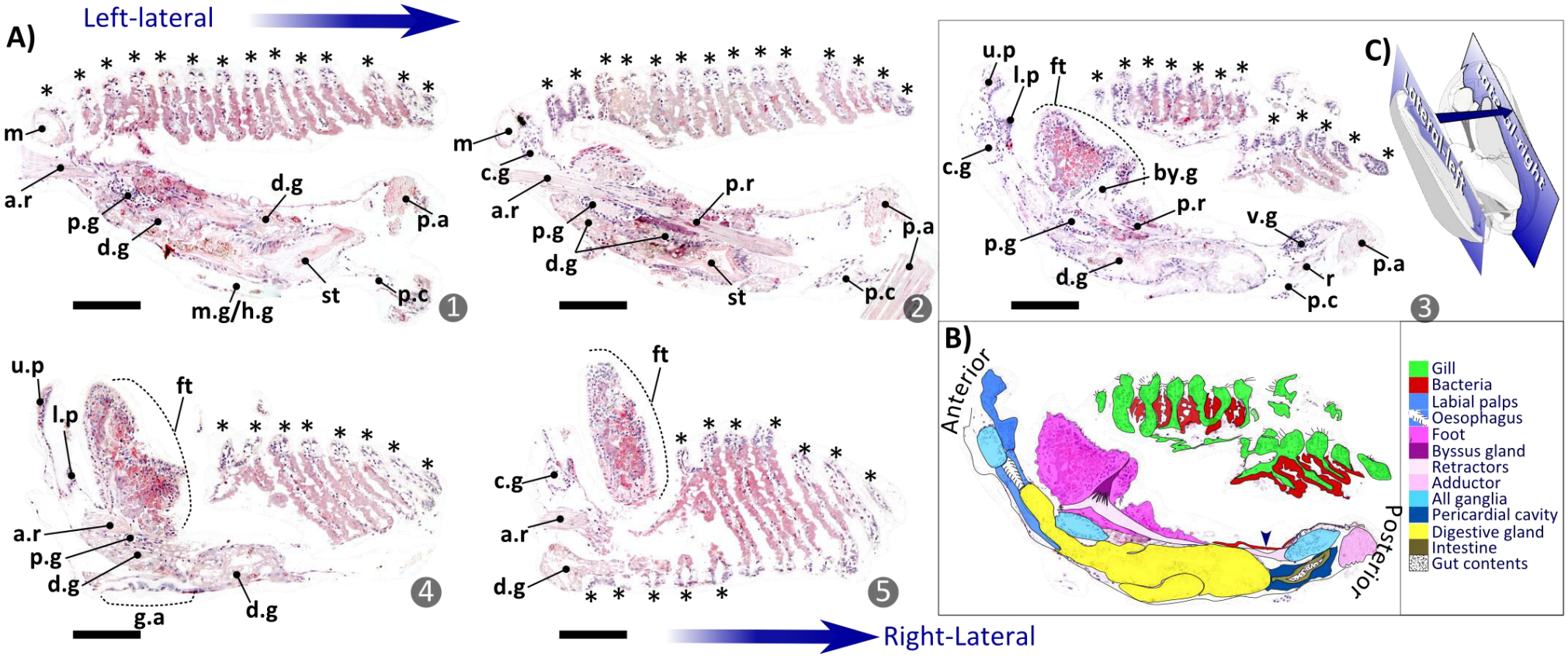

Figure 5.5 Serial Haematoxylin and Eosin-Y stained sagittal sections of a juvenile *Idas* (s.*l.*) *simpsoni* (SH 0.59, SL 1.05)

A) Series of sections taken at sequential locations along the latero-lateral axis [❶= Left-lateral side, ❺= Right-lateral side]. **B)** A schematic that segregates the various organs and tissues identified in ❸, highlighting the location of non-gill bacteria (arrowhead). An enlarged version is available in Supplementary figure 5.3. **C)** A generic *Idas*-like mussel drawing, with region of sections displayed (approximate). a.r *anterior retractor*, by.g *byssus gland*, c.g *cerebropleural ganglion*, d.g *digestive gland*, ft *foot*, g.a *gill axis*, h.g *hindgut*, l.p *lower labial palp*, m.g *midgut*, m *mouth*, p.a *posterior adductor*, p.c *pericardial cavity*, p.g *pedal ganglion*, p.r *posterior retractor*, r *rectum*, st *stomach*, u.p *upper labial palp*, v.g *visceral ganglion*. * = gill filament

plantigrades (Figure 5.5A, B). In an efficient use of space, regions of digestive diverticula flanked the oesophagus on all sides, but particularly laterally and dorsally. Digestive diverticula also surrounded the stomach (Figure 5.4A, B; Figure 5.5A) with at least one recurrent intestinal loop in the upper hindgut (Figure 5.4A, B). A style-sac was not knowingly observed. The stomach and digestive gland combined, occupied the majority of the dorsal region (Figure 5.4–5.5). In Figure 5.4, the ascending (labelled midgut: m.g) and the descending intestine (hindgut/rectum: h.g / r respectively) could be tracked along the length of the specimen.

Adults possessed all the attributes of juveniles, but scaled-up in line with shell capacities (not shown, for brevity). Alimentary organs and tissues were identified more easily on account of their characteristic structure, typical of descriptions available in the literature for related, shallow-water species (e.g. Bayne 1976). The stomach and digestive diverticula still represented a vast portion of the visceral mass in the dorsal region.

5-4.3.3 Attaining maturity

Maturation rate was estimated based on the size at first maturity. In *I.* (*s.l.*) *simpsoni*, the first individual to be identified as a mature male had a SL of 2.61 mm, based on clear evidence of spermatogenesis (Figure 5.3E). However, the gonad generally, and the structuring of the tissue particularly were already relatively developed, suggesting that this individual had been mature for a marked period of time prior to fixation. The reproductive status of preceding mussel sizes was examined in as much detail as possible to assess whether earlier sizes showed signs of gametogenesis, but none was found[28]. Thus a cautious size at first maturity of ≤2.6 mm as a male, is given. The two larger specimens were also identified to be male.

5-4.4. Development of the gills in the context of symbiosis

5-4.4.1 Gill development

Gill development followed similar trends to those in Laming et al. (2004) for *I*. (*s.s.*) *modiolaeformis* and more generally for *Mytilus edulis* (Cannuel et al. 2009) and are consequently discussed briefly herein. Gill filaments in plantigrades and the smallest juveniles were the least numerous, the shortest dorso-ventrally and densely ciliated, with exception to the minimal abfrontal region present, on the interior face of filaments (Figure 5.3A, B; Supplementary figure 5.1). Filaments became more numerous with increasing specimen-size, were more elongate dorsoventrally, and of greater depth fronto-abfrontally. The extension in depth was mainly through the hypertrophic proliferation of the abfrontal zone, when comparing filaments in plantigrades (Figure 5.4A, Supplementary figure 5.1) to juveniles of increasing size (from Figure 5.3B to D and from Figure 5.4 to Figure 5.5). Shortest filaments were located distally in the posterior regions of the gill lamellae, suggesting that these regions are the site of new filament formation (e.g. Figure 5.3D).

[28] N.B. at the preceding specimen size, SL 2.45 mm, the tissue were bad damaged during a difficult dissection

An unusual morphological trait in this species was the presence of an apparent thin connecting band of tissue along the antero-posterior length of the gill filaments (*connecting band* in Figure 5.3B and D). This band may also have been the dorsal bend of a developing ascending lamella, however no filaments of these secondary lamellae were identified with confidence, perhaps suggesting their relatively delayed development in this species. Gill filament numbers recorded during dissection increased linearly in *I.* (*s.l.*) *simpsoni* with increasing SL (Figure 5.6; Linear regression, r^2 = 0.98, $F_{1,31}$ = 1236, P <0.0001). In post-larval plantigrades, these numbered between 5 and 7 simple, stout, gill bars, while 46 filaments were identified in one descending lamella of the largest specimen examined (SL 3.74 mm).

5-4.4.2 Patterns in symbiont acquisition, proliferation and diversity

No evidence existed for the pre-acquisition of bacteria prior to settlement and metamorphosis. Signals were entirely absent in post-larval plantigrades during DAPI analysis (Figure 5.7B), corroborated by a lack of putative bacterial staining both in HE and toluidine blue histology (Supplementary figure 5.1). In one of the smallest developing juveniles (SL 0.50 mm, PdII settlement size 0.44 mm, 13.8% increase in SL after settlement), there remained no evidence for bacteria based on the same analyses (Figure 5.7B). The smallest specimen in which bacteria were identified (of those analysed) was actually slightly smaller at SL 0.43 mm, where the settlement size was also smaller at PdII SL 0.40 mm (8.3% increase in SL, after settlement). Other than the aforementioned aposymbiotic juvenile at SL 0.50 mm, all larger specimens analysed using DAPI and FISH (*n* = 15 in total), were found to house high densities of symbionts on non-ciliated regions of gill filaments (Figure 5.7–5.8) and at much lower densities on dorsal non-ciliated visceral epithelia (<10 bacteria deep, see schematics in Figure 5.4B, Figure 5.5B: regions indicated with arrowheads). Little or no mantle epithelia survived sample processing, so bacterial colonisation of these epithelia could not be confirmed.

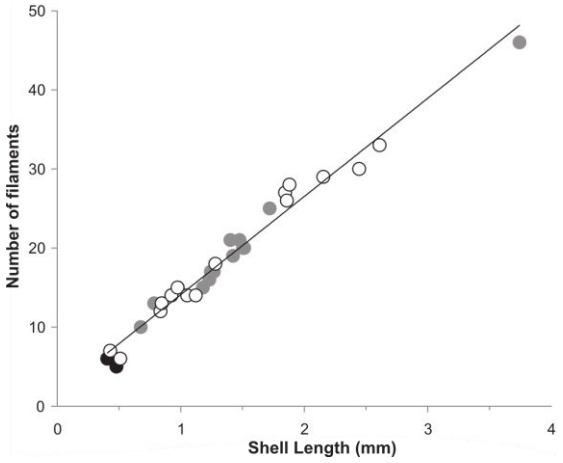

Figure 5.6 Gill-filament counts in the inner demibranch's descending lamella of *Idas modiolaeformis*
Filament counts in one inner-demibranch descending lamella (*n* = 33) as function of SL. Grey dots: not analysed for bacteria. Black: bacteria not identified during fluorescence microscopy. White: bacteria present on non-ciliated epithelia, but most dense on abfrontal and lateral non-ciliated gill filament surfaces (DAPI and FISH, *n* = 15)

Initial densities on gill epithelia were relatively low, however densities increased rather dramatically with increasing SL (Figure 5.7), so that some larger juveniles (>1 mm) had deep layers of bacteria between filaments (Figure 5.3, 5.5, 5.7 and 5.8). In some instances, the latero-abfrontal regions of gill filaments loaded with bacteria appeared to have undergone atrophy, based on their relatively thin

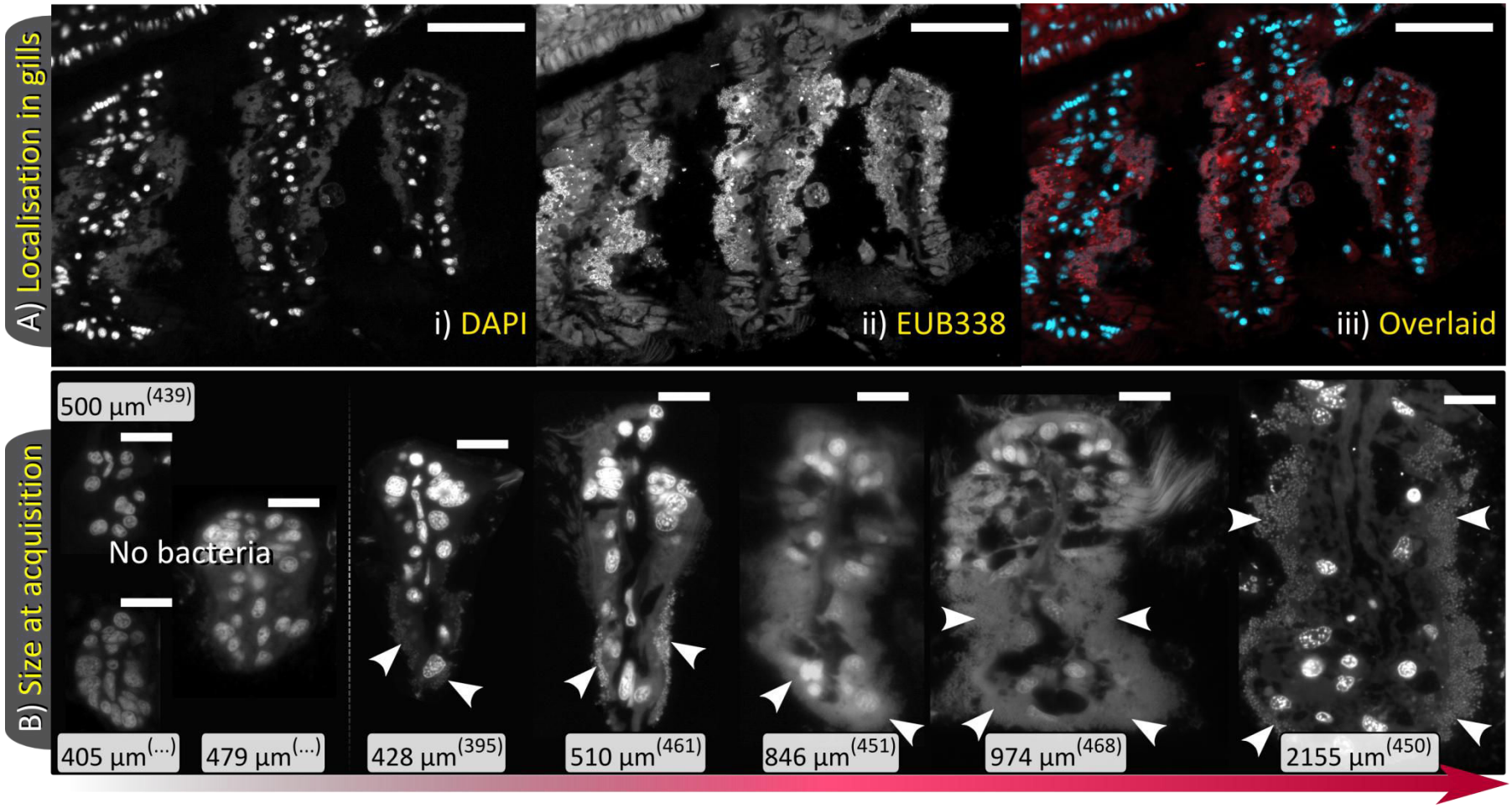

Figure 5.7 Analysis of symbiont localisation and the period of acquisition using fluorescence microscopy

A) A sequence of images taken in succession using multiple epifluorescence source frequencies, which targeted nucleic acid-labelling [i] DAPI and generic eubacterial 16S RNA-labelling [ii] EUB338. These are overlaid for comparison in [iii], with DAPI in blue and EUB338 in red. **B)** DAPI staining over a subset of the entire size series available for this study. Vertical dotted lines marks boundary between aposymbiotic and symbiotic sizes. Whole shell lengths are indicated in the grey boxes with the prodissoconch II (≡ settling size) superscripted in parentheses if it differs (i.e. (...) indicates a post-larval plantigrade). Arrowheads identify bacterial signals. The largest specimen in [**B**] is the same as that of [**A**].

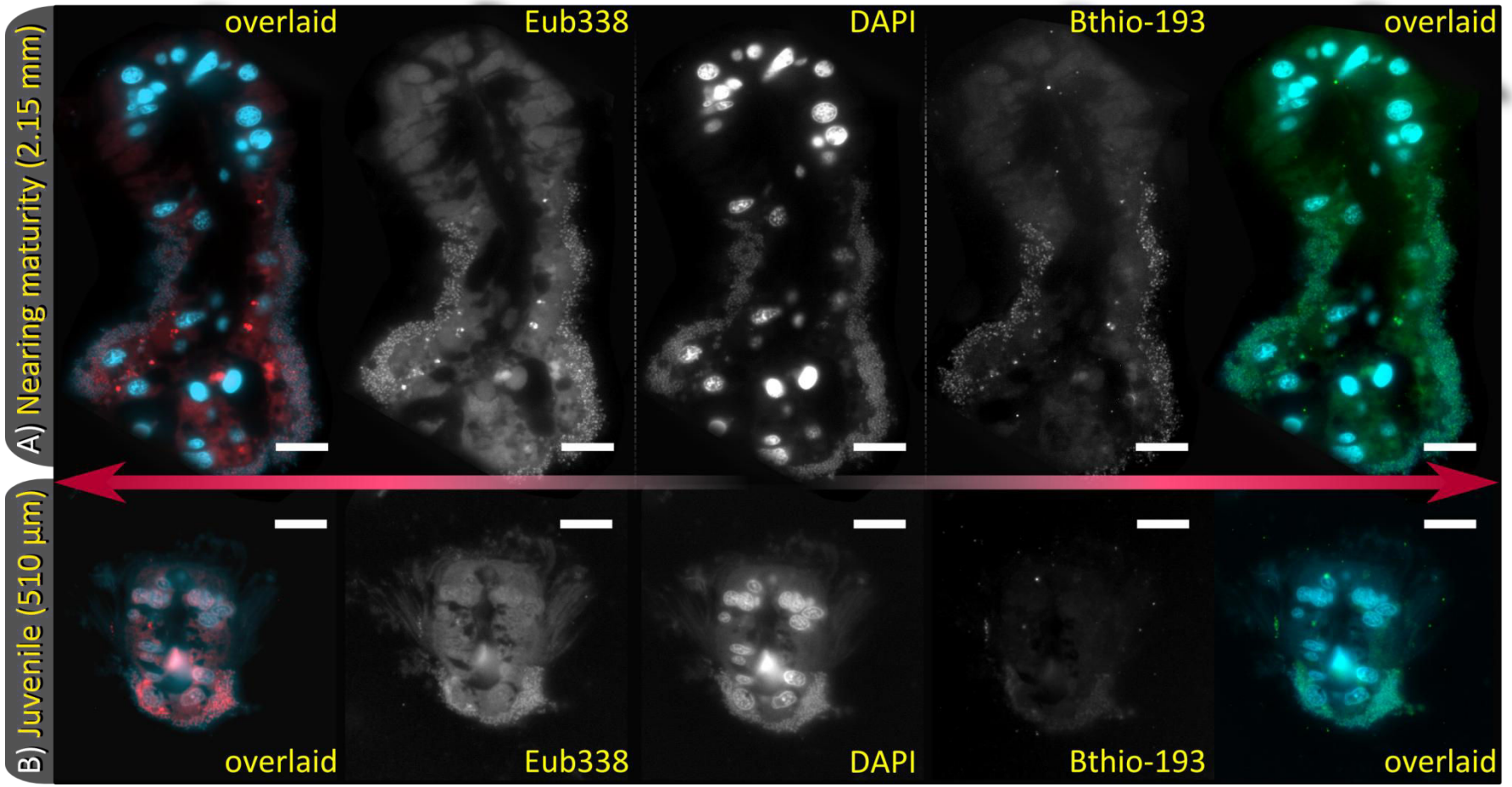

Figure 5.8 Fluorescence *in situ* hybridisations of one of the largest specimens not confirmed as an adult, and a small juvenile
In both specimens, the starting point is the DAPI staining in the centre. **A)** DAPI (targets nucleic acids indiscriminately), EUB338 (on Cy3: targets most Eubacteria) and Bthio-193 (on Cy5: targets T1-thiotrophs) signals from the same filament in a mussel approaching maturity. Signals from DAPI and EUB338 are superimposed on left, DAPI and Bthio-193 are superimposed on right. Signal fidelity was high, though DAPI signal was more densely populated. **B)** Applying the same approach, similar results were obtained for smaller juveniles (frontal filament section in one of the smallest mussels to have any bacterial symbionts).

sagittal cross-sectional area (e.g. Figure 5.3C). This may however be an early state in gill development before the effects of hypertrophy are truly witnessed (e.g. size series in Figure 5.7B).Despite employing a wide range of targeted oligonucleotidic probes during FISH analysis, only T1-thiotrophs were confirmed to reside on the filaments of *I.* (*s.l.*) *simpsoni* from the Setúbal Canyon. Superimposed signals from DAPI and the general Eubacterial probe EUB338 revealed complete overlap in bacteria (Figure 5.7A; Figure 5.8), although DAPI signal appeared more densely populated. A similar approach for the specific thiotroph probe Bthio-193, revealed high signal overlap also, but again extraneous signal in the DAPI could not be confirmed with the targeted probe's signal. This may reflect the fact that DAPI and the oligonucleotide probes are targeting DNA and ribosomal RNA respectively, meaning the latter will fail to hybridise with those bacteria that were not very active prior to fixation. Signal from the FISH probes in smaller mussels was quite weak but distinguishable (e.g. Figure 5.8B: Bthio-193 in particular), or non-existent, suggesting that some issues remained with an unknown aspect of the method. This may be partly related to focal depth of the frequency of light (this was noticeably shallower for Cy3 and Cy5).The least ambiguous signals (including those pictures in Figure 5.8) were from transverse-sectioned specimens that also happened to have been subjected to a different polymerisation protocol (see methodology).

5-5 Discussion

By examining size-specific trends in the developmental biology of *Idas (s.l.) simpsoni*, this study has considerably advanced our understanding of early post-settlement life all the way through to sexual maturation in a bathymodiolin species colonising bone remnants in the deep sea. This was made possible using relatively short term (for carcass falls) deployments: specimens, at previously undetermined key points along their developmental path to adulthood, were acquired and analysed. The results identify crucial developmental junctures which are believed to maximise this species survival in spatially and temporally finite environments: in this instance mammalian bones.

5-5.1. Nutrition

5-5.1.1 Pre-settlement inferences

Morphometric analysis of larval shells revealed the prodissoconch I to be around 96 ± 6.3 µm. A study by Ritt et al., (2012) described the phylogeny and symbiosis of a putative new species of *Idas* (*s.l.*), "*Idas* nov sp." which was ultimately identified as *I.* (*s.l.*) *simpsoni*, based on partial sequences released subsequently for the gene that codes for Cytochrome Oxidase I in this species (Thubaut et al. 2013b). Examination of the scanning electron micrograph of the prodissoconch for *I.* (*s.l.*) *simpsoni* presented in Ritt et al. (2012) reveals the prodissoconch I to be approximately equivalent, at almost 100 µm. Values for related species are typically slightly smaller than this (*Adipicola* (*s.l.*) *iwaotakii*: PdI SL [±σ], 74.05 ± 9.35 µm, *n* = 15, named "*Idas iwaotakii",* Thubaut et al. 2013a; *I.* (*d.f.*) *argenteus*: ≈ 98 µm, Ockelmann and Dinesen 2011 and *Idas* (*s.s.*) *modiolaeformis*: 78.6 ± 3.4 µm, *n* = 6 from seeps, Gaudron et al. 2012; and 80.3 ± 5.14 µm NDSF

samples on wood/alfalfa (*n* = 20), measured after submission of Chapter 4 as a manuscript). Prodissoconch II dimensions (PdII SH x SL [±σ], 459 [± 26.4] x 403 [± 27.9] µm, *n* = 38) were also very similar to the single value available in the literature for this species (PdII SH x SL, 450 µm x 404 µm, Ritt et al. 2012). In descending order, values for related species were PdII SL (±σ) 544.33 ± 37.58 µm, *n* = 15, (*A.* [*s.l.*] *iwaotakii*: Thubaut et al. 2013a), ≈ 459 µm in *I.* (*d.f.*) *argenteus* (Ockelmann and Dinesen 2011), 379 [± 11.7] x 344 [± 5.0], *n* = 14, *n* = 27 in *Idas* [*s.s.*] *modiolaeformis* from seeps (Gaudron et al. 2012) and 398 [± 17.5] x 322 [± 17.4], *n* = 35 in *Idas* [*s.s.*] *modiolaeformis* from NDSF samples on wood/alfalfa (Laming et al. 2014).

Given that PdI SL roughly corresponds to the size of the "D"-veliger following initial shell formation, it is also thought to relate to oocyte diameter (Lutz et al. 1980). Diameters such as those above would thus be equivalent to oocytes at <96 µm, which is a small-sized oocyte and thus could only hold a small yolk reserve. The PdII provides a proxy for pelagic larval duration, which in this case argues for an extended larval phase (*M. edulis* has a PdII SL of 120-252 µm by comparison, Sprung 1984). As yolk reserves are likely to be low, researchers often cite these data together as evidence for planktotrophy, though this overlooks to the possibility of chemosymbiotic larval nutrition (e.g. as suggested for *B.* (*s.s.*) *azoricus*, Trask and Van Dover 1999). Fortunately, since bacterial signals were entirely absent from all epithelia in post-larval plantigrades regardless of means of analyses, no evidence exists to support symbiotic nutrition prior to settlement and metamorphosis. Taken with the larval shell data this argues for obligatory planktotrophy during larval dispersal, though the pre-acquisition of a very low seeding population of bacteria can not be entirely ruled out.

5-5.1.2 Acquisition of symbionts following settlement

It has been shown that, once settled and metamorphosed on bone material, *I.* (*s.l.*) *simpsoni* appears to spend minimal energy on growth before it acquires its first symbionts (8% increase in SL, from PdII SL 0.40 mm, to 0.43 mm with the deposition of a thin margin of dissoconch shell). A large proportion of these symbionts were certainly Eubacteria, and probably thiotrophic (T1-type), based on Bthio-193-specific bacterial FISH signals in host gill tissues (though bacteria were also identified on non-ciliated visceral epithelia). Following their initial appearance in very small juveniles, bacteria appeared to qualitatively increase in numbers unit^{-1} gill-space within a narrow range of shell lengths; in fact they rapidly achieved densities that seem to approach saturation, at least visually (e.g. Figure 5.5; Figure 5.7B even at SLs 846 and 974 µm). However, an increase in specificity towards the gill filament surfaces only, observed in other bathymodiolins (Wentrup et al. 2013; Laming et al. 2014), did not occur within the size range examined. In the largest individual, traces of bacteria could still be seen on the ventral surfaces of visceral epithelia. This probably reflects the fact that even the largest adult examined was less than 10% of the maximum size recorded for this species (SL_{max} 45mm, from Warén and Carrozza 1990).

The only other chemosymbiotic *Idas* mussel to which this rate of acquisition can be compared directly is in *I.* (*s.s.*) *modiolaeformis,* colonising wood and alfalfa substrata (Laming et al. 2014). When the two are

examined in parallel, it is evident that *I.* (*s.l.*) *simpsoni* acquires its symbionts at smaller sizes, following only marginal changes in length by comparison. In *I*. (*s.s.*) *modiolaeformis*, not only is size at first acquisition at a larger SL of 0.60 mm (Laming et al. 2014), this represents just less than a 50% increase in SL following settlement (in contrast to 8% in *I.* [*s.l.*] *simpsoni*). What's more, the acquisition of symbionts is a gradual process in *I*. (*s.s.*) *modiolaeformis* living on wood substrata, but appears to be rapid in *I.* (*s.l.*) *simpsoni* occurring on bone substrata. Thus bacterial associations proliferate on *I.* (*s.l.*) *simpsoni* gills at a rate far beyond that seen in *I*. (*s.s.*) *modiolaeformis* (Laming et al. 2014), developing over a relatively small host size-range.

5-5.1.3 Symbiont affinities and relative abundances

Variability in symbiont assemblages both in terms of diversity and abundances has been demonstrated previously for *I*. (*s.s.*) *modiolaeformis*, where specimens have been found to harbour anything from one (Laming et al. 2014) to several differing combinations of symbionts (Duperron et al. 2008a; Lorion et al. 2012; Rodrigues et al. 2013), where signal intensity can be highly variable between mussels from different habitat conditions (e.g. between carbonates and wood, Lorion et al. 2012), and at different sizes/ages (Laming et al. 2014). The bacterial symbiont identified in this study, which is related to the T1-trhiotrophs found in *I*. (*s.s.*) *modiolaeformis*, *B.* (*s.s.*) *brooksi* and *B.* (*s.s.*) *heckerae* has not yet been documented as a dominant symbiont in *I.* (*s.l.*) *simpsoni.* Previously identified dominant bacteria are restricted to the *B*. aff. *boomerang* Bang-T-like symbiont (Ritt et al. 2012). However this bacterium was not present in sufficient densities to elicit a signal during FISH using the Bang-T probe in the current samples from bones *I.* (*s.l.*) *simpsoni*. In fact, with the exception of the T1 thiotroph (and EUB338), no other phylotype combinations revealed signal characteristic of bacteria in *Idas*-like mussels (these included ImedM-138, ImedT2-193, BhecM2-822, and Bang-T, for details see Duperron et al. 2005; Duperron et al. 2008a).

5-5.1.4 Reproductive energetics

Symbiont associations deliver novel metabolic capabilities to the host (Dubilier et al. 2008). Such patterns of symbiont acquisition and proliferation can ultimately provide the host with a plentiful food supply which might be harnessed to fuel augmented growth rates or drive the development of new host tissues (e.g. early pre-maturation acini formation). Perhaps reflecting the metabolic advantages of acquiring symbionts earlier and at higher densities as young juveniles, size at first sexual maturity as a male, was at – or likely preceded – SL 2.61 mm, which represents < 6 % of the current maximum size recorded for *I.* (*s.l.*) *simpsoni*. Therefore, although *I.* (*s.l.*) *simpsoni* matures at a greater SL than *I*. (*s.s.*) *modiolaeformis* (according to this study and Laming et al. 2014), the percentage-SL_{max} is lower for *I.* (*s.l.*) *simpsoni* (corresponding value for *I*. (*s.s.*) *modiolaeformis* ≈ 12% SL_{max}). This may simply be a result of variability in maximum size however; *Idas* (s*.l.*) *simpsoni* can reach sizes over double that of *I*. (*s.s.*) *modiolaeformis* (Ritt et al. 2012). Most members of the *Idas* (*s.s*). clade, which includes *I*. (*s.s.*) *modiolaeformis* and *I*. (*s.s.*) *washingtonius*, have unusually small sizes for the Bathymodiolinae, a possible by-product of paedomorphism (Génio et al. 2012) during these

species' ancestral adaptation to organic falls (space limited in comparison with other reducing habitats, Lorion et al. 2013). In fact, this appears to have taken place around the same time that *Bathymodiolus* (*s.l.*) spp. evolved their contemporary levels of gigantism (Lorion et al. 2013).

5-5.1.5 Developmental anatomy

Finally, this study has described some general aspects of development in *I.* (*s.l.*) *simpsoni* in terms of organismal anatomy. In particular, it identified a functional digestive system in post-larvae of *I.* (*s.l.*) *simpsoni,* with increasingly developed organisation at later stages nearing adulthood. The persistence of a fully-formed gut is intriguing in a species where symbionts seem abundant (at least for this habitat). However, the retention of a fully-formed digestive system appears to be the norm in organic-fall-colonising small-sized bathymodiolins (*s.l.*), based on the limited data available (e.g. Gustafson et al. 1998; Thubaut et al. 2013a; Laming et al. 2014; Chapter 3, p. 127). This is despite evidence for intracellular chemoautotrophs in the bone-colonising species *I.* (*s.s.*) *washingtonius* (Smith et al. 1989; Deming et al. 1997), extracellular thiotrophs in *I.* (*s.l.*) *simpsoni* in the current study (and Ritt et al. 2012, "*Idas* nov. sp."), and various extracellular phylotypes in closely related mytilids found on organic falls more generally: several thiotrophic phylotypes, methanotrophs, other methylotrophs, and regularly occurring bacteria of unknown nutritional significance (e.g. symbiont G, Bacteroidetes, Duperron et al. 2008a; Duperron et al. 2008b; Fujiwara et al. 2010).

In a group of mussels in which chemosymbiosis presents the most common nutritional state described in adults (except the carnivorous larviphage, *I. argenteus*, Ockelmann and Dinesen 2011), the retention of a functional gut could be the result of ongoing selective pressures (i.e. a gut remains a requirement for survival) or the presence of the gut is neutral to survival, and thus is simply an echo of evolutionary history. However, in the large bathymodiolins, obligatory chemosymbiosis (i.e. at most deeper hydrothermal vents and hydrocarbon seeps) typically coincides with a moderate reduction in the gut, now known to have occurred independently multiple times (Gustafson et al. 1998; Lorion et al. 2013; Thubaut et al. 2013b). In all bathymodiolin species, the chemosymbiotic mode is believed to have evolved relatively recently (Lorion et al. 2013), and thus varying degrees of symbiont dependency in the subfamily may represent several stages in an evolutionary transition towards further holobiont integration, as in the pliocardiin vesicomyid clams. If this were so, *Bathymodiolus* spp. would currently be the most 'derived' state (more reduced digestive system, intracellular – as opposed to extracellular – bacteria, and stable isotopes data supporting a high dependency on chemosynthetically derived nutrition in some species, Cavanaugh et al. 1992; Dubilier et al. 2008). However, there are no arguments *per se*, to suspect that bathymodiolins aren't already suitably adapted to their habitats, i.e. no evidence for active selection towards a more integrated holobiont state. In fact, there is growing evidence in the literature that the retention of a partially or fully formed digestive system in bathymodiolins (including many *Bathymodiolus* [*s.l.*] spp.) reflects a real need; a retained capacity for – and active engagement in – filter-feeding is postulated for several species (e.g. Page et al. 1990; Page

et al. 1991; Chapter 3). Species that colonise whale falls may similarly rely on retaining a level of mixotrophy. *Idas* (*s.s.*) *washingtonius* in particular displays wildly variable $\delta^{13}C$ and $\delta^{15}N$ isotope ratios, depending on the habitat it occurs on, ranging from values that may be considering reliably indicative of chemosymbiosis (at vents on the Juan de Fuca Ridge and larger whale falls in Monteray Bay Smith and Baco 2003; McKiness et al. 2005; Bergquist et al. 2007), to those which mirror signatures of benthic secondary consumers (e.g. small whale carcasses Smith and Baco 2003) with specimens of *I.* (*s.s.*) *washingtonius* on wood falling between the two extremes (Smith and Baco 2003).

The rather divergent phylogeny of *I.* (*s.l.*) *simpsoni* (Thubaut et al. 2013b) in comparison with *I*. (*s.s.*) *modiolaeformis*, *I.* (*s.s.*) *washingtonius*, *I.* (*s.s.*) *macdonaldi* and the various *Idas* (*s.l.*) ESUs that colonise sunken wood in the *Idas* (s.s.) clade (as in Thubaut et al. 2013b) is reflected somewhat by morphological characteristics and digestive anatomy. No style-sac could be identified in the stomach (the region in coastal mytilids where enzymatic and mechanical digestion is most active, putatively identified in *I*. (*s.s.*) *modiolaeformis*, Laming et al. 2014). However, *I.* (*s.l.*) *simpsoni* has a slightly more convoluted intestinal tract when compared with *I*. (*s.s.*) *modiolaeformis*. In terms of patterns of symbiont acquisition and diversity, principal differences include the rate and the inherent abundance of symbiont acquisition in *I.* (*s.l.*) *simpsoni*, versus the still-unrivalled symbiont diversity of *I*. (*s.s.*) *modiolaeformis*, though the localisation of signal during FISH in both suggest that they are both extracellular. Oligonucleotide probes that are designed based on bacterial sequences identified from the gill tissue of *I.* (*s.l.*) *simpsoni* (and other *Idas*-like species), may reveal hitherto unknown levels of symbiont diversity, and thus flexibility. The almost immediate appearance of bacteria in the gills of *I.* (*s.l.*) *simpsoni* raises questions about the origins of these bacteria. Such rates of acquisition suggest one of two immediately obvious hypotheses. The first is that the relative speed with which *I.* (*s.l.*) *simpsoni* became symbiotic, reflects a greater abundance of free-living symbiont candidates in the vicinity of bones versus those around the wood habitat on which *I.* (*s.s.*) *modiolaeformis* has settled in Laming et al (2014). An alternative explanation could be a tendency for 'leaky' vertical transmission in *I.* (*s.l.*) *simpsoni*, where a 'seeding' population of bacteria, at densities too low to detect, were transported from the natal site and thus were already present on arrival as larvae to the cow bones. In other words, larvae were pre-equipped with a very low density starter population of bacteria, removing the need for environmental acquisition and possibly accelerating the patterns of symbiont expansion in the gills. This could explain site-specific bacteria assemblages in *I*. (*s.s.*) *modiolaeformis* (Rodrigues et al. 2013), which suggest acquisition is truly after settlement. However, both scenarios remain plausible, and the argument is rooted in relative rather than absolute rates of acquisition. A future hypothesis to be tested would be whether the symbiont assemblages in *I.* (*s.l.*) *simpsoni* hosts display phylogenetic affinities with bacteria living at the host's natal site, rather than its source.

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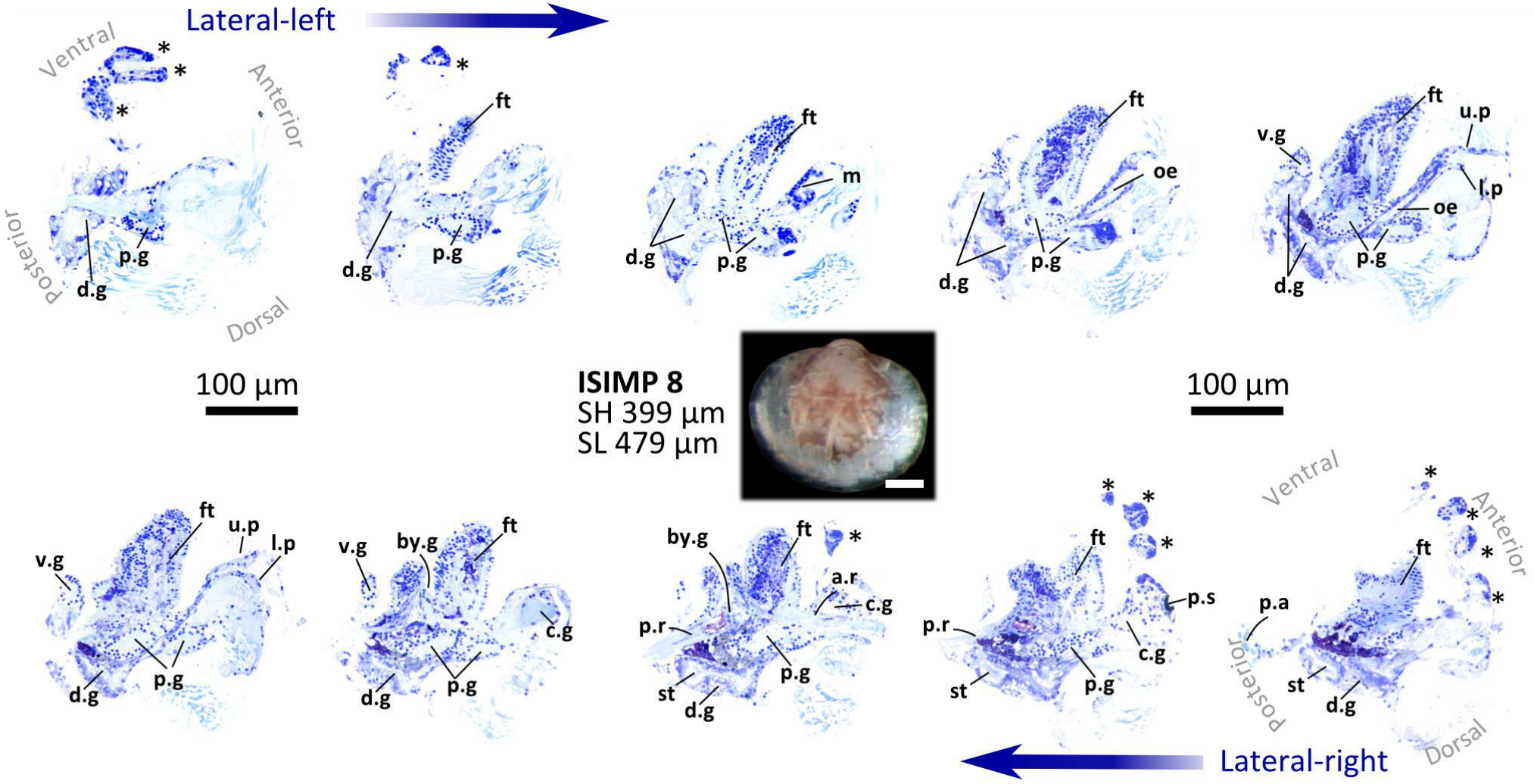
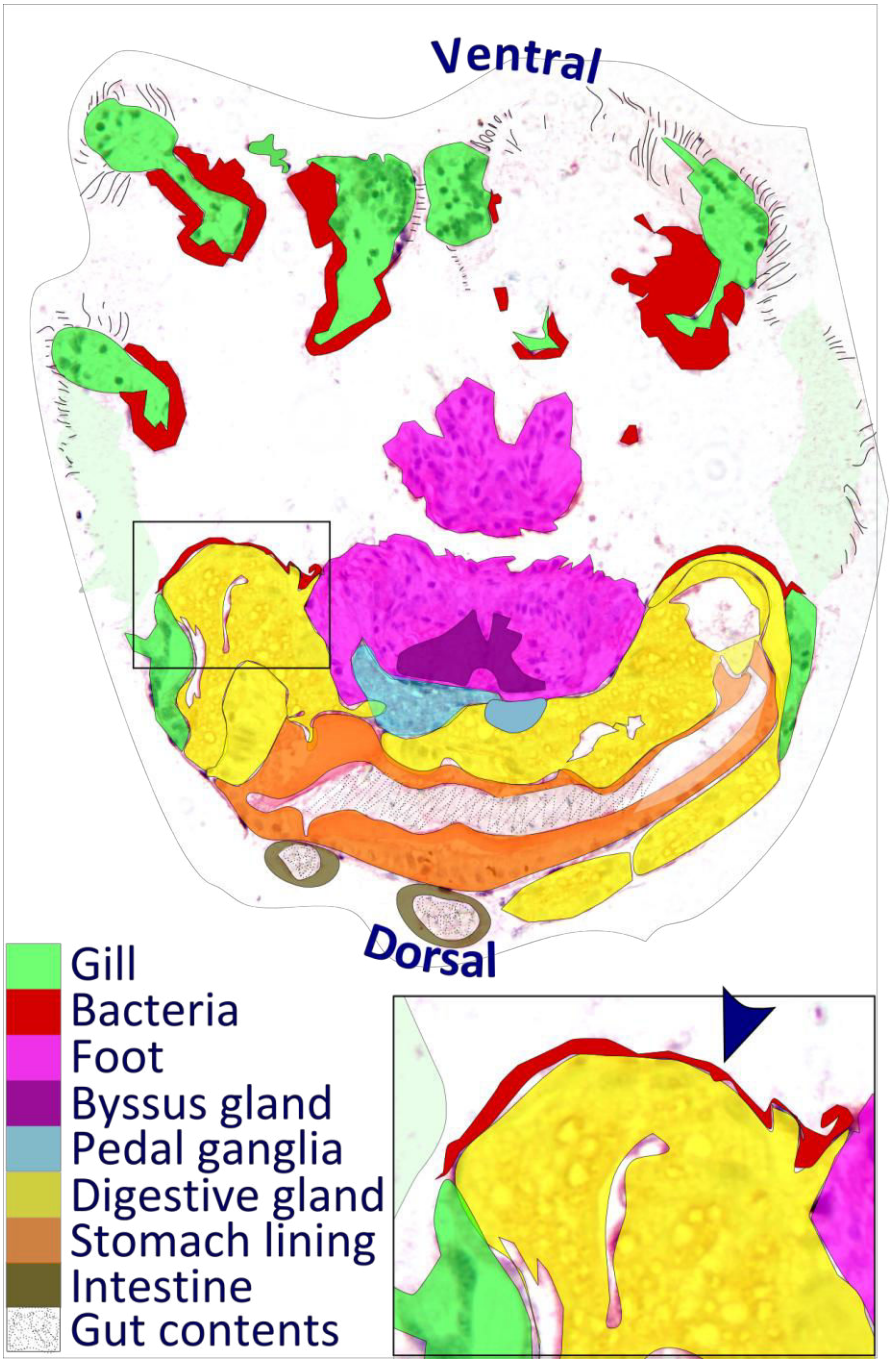
Supplementary table 5.1 Summary of the fixation employed and analysis performed upon specimens in this study
38 individuals were used in this study: 5 post-larvae, 30 juveniles and 3 adults. Preservation was in 96% ethanol at <5 °C, following serial transfer. Abbreviations for analyses are S- Shell dimensions measured, H- standard histological analyses, D- DAPI staining, F- FISH, Formald. – Formaldehyde in twice-filtered seawater.

Sample label	Life stage	PdI SL	PdII SH	PdII SL	SH	SL	Analyses	Mature?	Bacteria?
ISIMP 4	Post-settlement	-	0.32	0.41	0.32	0.41	S, H, D, F	no	no
ISIMP 7	Juvenile	-	0.35	0.40	0.37	0.43	S, H, D, F	no	yes
ISIMP 1	Post-settlement	-	0.34	0.44	0.34	0.44	lost	no	-
ISIMP 2	Post-settlement	-	0.43	0.46	0.43	0.46	lost	no	-
ISIMP 3	Post-settlement	-	0.43	0.47	0.43	0.47	S	no	-
ISIMP 8	Post-settlement	-	0.40	0.48	0.40	0.48	S, H, D, F	no	no
ISIMP 6	Juvenile	-	0.37	0.44	0.40	0.50	S, H, D	no	no
ISIMP 5	Juvenile	-	0.42	0.46	0.45	0.51	S, H, D, F	no	yes
ISIMP 9	Juvenile	-	0.38	0.45	0.46	0.68	S, H	no	-
ISIMP 11	Juvenile	105	0.42	0.46	0.51	0.78	S, H	no	-
ISIMP 15	Juvenile	96	0.38	0.44	0.55	0.84	S, H, D, F	no	yes
ISIMP 10	Juvenile	85	0.41	0.45	0.57	0.85	S, H, D, F	no	yes
ISIMP 16	Juvenile	85	0.41	0.46	0.58	0.92	S	no	-
ISIMP 13	Juvenile	-	0.41	0.46	0.59	0.92	S, H, D, F	no	yes
ISIMP 12	Juvenile	98	0.41	0.47	0.61	0.97	S, H, D, F	no	yes
ISIMP 14	Juvenile	91	0.37	0.43	0.60	0.98	S	no	-
ISIMP 17	Juvenile	98	0.41	0.46	0.59	1.05	S, H, D, F	no	yes
ISIMP 18	Juvenile	94	0.39	0.43	0.67	1.12	S, H, D, F	no	yes
ISIMP 19	Juvenile	92	0.42	0.46	0.66	1.18	S, H	no	-
ISIMP 20	Juvenile	100	0.41	0.45	0.68	1.23	S, H	no	-
ISIMP 21	Juvenile	-	0.41	0.44	0.70	1.24	S	no	-
ISIMP 24	Juvenile	103	0.41	0.47	0.73	1.27	S	no	-
ISIMP 22	Juvenile	105	0.38	0.42	0.72	1.28	S, H, D, F	no	yes
ISIMP 28	Juvenile	91	0.43	0.49	0.76	1.40	S, H	no	-
ISIMP 27	Juvenile	101	0.46	0.52	0.78	1.42	S	no	-
ISIMP 25	Juvenile	-	0.42	0.49	0.81	1.48	S	no	-
ISIMP 29	Juvenile	103	0.43	0.49	0.82	1.50	S, H	no	-
ISIMP 26	Juvenile	91	0.43	0.49	0.80	1.51	S	no	-
ISIMP 23	Juvenile	104	0.41	0.49	0.81	1.51	S	no	-
ISIMP 30	Juvenile	89	0.40	0.42	0.90	1.72	S, H	no	-
ISIMP 31	Juvenile	88	0.40	0.44	0.97	1.84	S, H, D, F	no	yes
ISIMP 32	Juvenile	88	0.42	0.49	0.94	1.86	S, H, D, F	no	yes
ISIMP 34	Juvenile	101	0.41	0.47	0.92	1.88	S, H, D, F	no	yes
ISIMP 33	Juvenile	-	0.40	0.45	1.06	2.15	S, H, D, F	no	yes
ISIMP 35	Juvenile	98	0.43	0.51	1.16	2.44	S, H, D, F	no	yes
ISIMP 36	Adult male	99	0.42	0.49	1.22	2.61	S, H, D, F	yes	yes
ISIMP 37	Adult male	-	0.37	0.44	1.33	2.76	S, H	yes	-
ISIMP 38	Adult male	96	0.42	0.46	1.54	3.74	S, H	yes	-

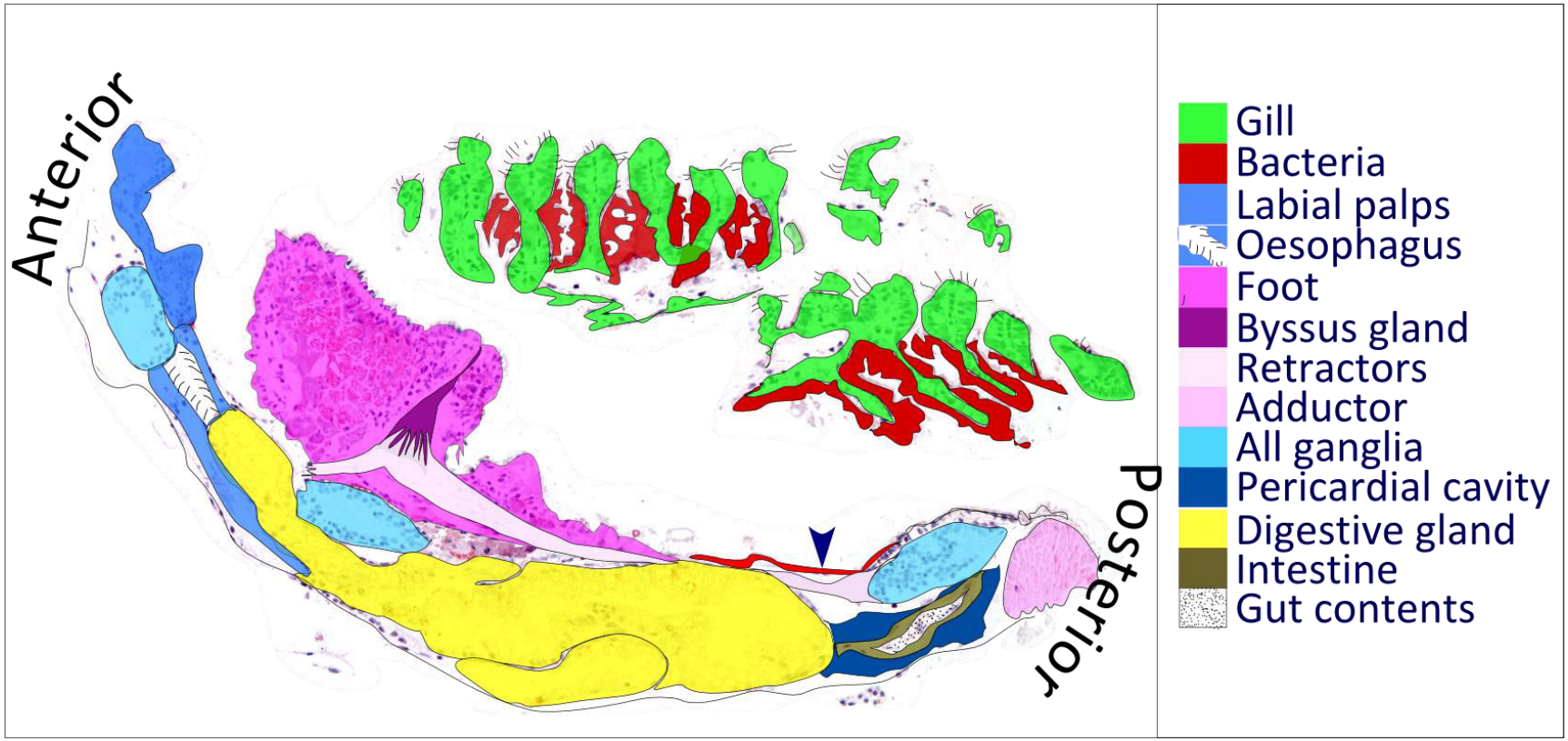


Supplementary figure 5.1 Serial sagittal sections of a plantigrade stained using Toluidine blue

This post-larval specimen is the same featured in the main-text, figure 5.3A. Axis of cutting is from the left side of the mid-sagittal plane to the right side of the mid-sagittal plane, along the latero-lateral axis. by.g *byssus gland*, c.g *cerebropleural ganglion*, d.g *digestive gland*, ft *foot*, l.p *lower labial palp*, m *mouth*, oe *oesophagus*, p.a *posterior adductor*, p.g *pedal ganglion*, p.r *posterior retractor*, p.s *pigment spot*, st *stomach*, u.p *upper labial palp*, v.g *visceral ganglion*. * = gill filament



Supplementary figure 5.2 Enlarged version of the main-text figure 5.4B
Please see main text legend for Figure 5.4



Supplementary figure 5.3 Enlarged version of the main-text figure 5.5B
Please see main text legend for Figure 5.5

Chapter 6 A SAD TALE: HAS *IDAS ARGENTEUS* LOST ITS SYMBIONTS?

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N.B. This manuscript has been submitted to *Biology Letters* (which has a strict 2500 overall word limit, hence the 'telegram-style' writing). Contributions from S.R. Laming include: sample sorting; the initial putative identification of the species (confirmed by G. Oliver, based on shell characteristics); live dissection and characterisation of behaviour; shell morphology and photography; soft-tissue processing; embedding, sectioning and histology; DNA extraction, and the methodology/results/discussion paragraphs dedicated to these areas. C.F Rodrigues: PCR; sequencing; tree-construction and; methodology/results/discussion paragraphs related to these analyses, plus entire Introduction. Editing and comments by all authors.

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6-1 Abstract

Idas argenteus belongs to the Bathymodiolinae, a clade of deep-sea mussels which typically harbour chemosynthetic bacterial symbionts. In contrast, this species was documented to lack symbionts. In this study, new specimens were assigned to *I. argenteus* based on shell and soft parts analysis. Molecular data and histology confirm the absence or low abundance of symbionts. Phylogeny based on 5 genes indicates that the symbiont-bearing *I. washingtonius* is the closest relative of *I. argenteus*. Symbiosis loss or extreme reduction is thus inferred to have occurred since the speciation event, 11 to 13 million years ago (Ma). This is the first report of loss of symbiosis within the Bathymodiolinae.

6-2 Introduction

Symbiosis between bacteria and metazoans is key to the productivity of deep-sea cold seeps and hydrothermal vents, in which most of the primary production is ensured by chemoautotrophic symbionts (Dubilier et al. 2008). Symbiosis is usually facultative for the bacteria and several shifts between free-living and symbiotic lifestyles during the life of a bacterium or during evolution of bacterial groups are reported (Dubilier et al. 2008; Duperron et al. 2013; Wentrup et al. 2013). Taxa within the annelids and bivalves on the other hand live in obligate symbiosis with bacteria which contribute to their nutrition, and symbiosis is considered the key synapomorphy that allowed efficient colonization of reducing habitats (Won et al. 2008; Duperron 2010). Habitat and depth shifts (and reversals) can occur (Won et al. 2008; Thubaut et al. 2013). It is thus reasonable to propose that symbiosis may disappear, or become less significant to the host's metabolism under particular circumstances. Recent examples within the bathymodiolin mussels (Mytilidae: Bathymodiolinae) indicate the presence of extracellular symbionts, sometimes in low abundances and possibly, intra-specific variability in symbiont types depending on sites (Duperron 2010; Rodrigues et al. 2013; Thubaut et al. 2013). In at least one case, *Idas argenteus* Jeffreys, 1876 from sunken wood in the north Atlantic, microscopy-based evidence supported the absence of bacterial symbionts and a larviphagous regime (Ockelmann and Dinesen 2011). This mytilid is of particular taxonomic importance, as it is the type species of its genus (Lorion et al. 2013; Thubaut et al. 2013). Whether *I. argenteus* is a relict species belonging to a deep-branching lineage emerging before the acquisition of symbiosis in Bathymodiolinae or belongs to a recently emerging lineage in which symbiosis was secondarily lost, has not been investigated due to the lack of material for molecular studies.

We recently collected specimens resembling *I. argenteus* during colonization experiments deployed in the Lacaze-Duthiers (LD) Canyon (Gulf of Lion, Mediterranean) and at the Rainbow hydrothermal vent site in the Mid-Atlantic Ridge (MAR). Multi-locus sequencing is used to ascertain the phylogenetic position of these specimens. Assignation to *I. argenteus* is based on the comparison of shell and anatomy with original and subsequent descriptions (Jeffreys 1876; Dean 1993; Oliver and Holmes 2009; Ockelmann and Dinesen 2011). Tissue histology and PCR amplification of bacterial genes are employed to test for the presence of symbionts and the presence of a functional digestive tract.

6-3 Material and Methods

6-3.1. Sampling and histology

Mussels were collected from CHEMECOLI colonization devices deployed for 414 days (n=2) at the Rainbow hydrothermal vent (MAR, 36º13.74'N, 33º54.05'W, 2279m, Gaudron et al. 2010) and 382 days (n=1) in the LD canyon (42°32.72'N, 03°25.27'E, 513m). The LD specimen was observed alive under compound microscope. Specimens were preserved in 4% formaldehyde (2-4hrs, gradient transfer to 96% ethanol).

6-3.2. Molecular characterization

DNA was extracted from one specimen from each site using the QIAamp DNA MicroKit (QIAGEN). Fragments of three mitochondrial loci (encoding COI, NADH4 and 16S rRNA) and three nuclear loci encoding 18S, 28S rRNA and Histone 3 were amplified for phylogenetic analysis. About 610 bp of COI, 650 bp of NADH4, 480 bp of 16S, 1650 bp of 18S, 1000 bp of 28S and 300 bp of H3 were amplified using primers and PCR amplification profile summarized in Table 1. PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN) and send to sequence in both directions at GATC Biotech (United Kingdom). DNA sequences obtained during this study were complemented with data from GenBank and following available datasets (Lorion et al., 2013; Thubaut et al., 2013) (Table S1) and aligned using ClustalW (Thompson et al., 1997). Phylogenetic reconstructions were performed for single and concatenated genes using maximum likelihood (ML) and a General Time Reversible model with Gamma distribution of rates and a fraction of invariant sites using MEGA 6 (Tamura et al., 2011).

The presence of bacteria was tested by PCR of fragments of genes encoding 16S rRNA, 23S rRNA and APS reductase using previously described protocols (Table 1; Rodrigues et al., 2013).

Table 1- Primers and PCR parameters used for amplifications and sequencing reactions

	Gene		Primer names	Primer sequences (5'→3')	Reference
Host	COI mtDNA	50ºC (35)	H691	GTRTTAAARTGRCGATCAAAAAT	Duperron et al. 2008b
			LCO1490	GGTCAACAAATCATAAAGATATTGG01	Folmer et al. 1994
	NADH4 mtDNA	50ºC (35)	NADP2H	TGGAGCTTCTACGTGRGCTTT	Arevalo et al. 1994
			ArgBl	CAAGACCCTTGATTTCGGCTCA	Bielawski and Gold 1996
	16S rRNA	55ºC (35)	16SA	GGARGTASGCCCTGCCCWATGC	Baco-Taylor 2002
			LRJ	CTCCGGTTTGAACTCAGATCA	Ratnasingham and Hebert 2007
	18S rRNA	55ºC (35)	1F	ACCTGGTTGATCCTGCCAGTAG	Giribet et al. 1996
			5R	CTTGGCAAATGCTTTCGC	Giribet et al. 1996
			3F	GTTCGATTCCGGAGAGGG	Giribet et al. 1996
			9R	ATCCTTCCGCAGGTTCACCTAC	Giribet et al. 1996
			Bi	GAGTCTCGTTCGTTATCGGA	Okuzu et al. 2003
			A2	ATGGTTGCAAAGCTGAAAC	Giribet et al. 1996
	28S rRNA	55ºC (35)	C1prime	ACCCGCTGAATTTAAGCAT	Hassouna et al. 1984
			C4	TCGGAGGGAACCAGCTACTA	Hassouna et al. 1984
	Histone 3	55ºC (35)	F1	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 1998
			R1	ATATCCTTRGGCATRATRGTGAC	Colgan et al. 1998
Bacteria	16S	45°C (27)	27F	AGAGTTTGATCATGGCTCAG	Lane 1991
			1492R	GTTACCTTGTTACGACTT	Lane 1991
	23S	53°C (35)	3505F	GACCGTCAGCTAAGGTCCCAA	Stewart and Cavanaugh 2009
			4761R	CCAGTCAAACTACCCACCATG	Stewart and Cavanaugh 2009
	APS reductase	58°C (25)	APS1-FW	TGGCAGATCATGATYMAYGG	Meyer and Kuever 2007
			APS4-RV	GCGCCAACYGGRCCRTA	Meyer and Kuever 2007
	V5-V6			CAAACAGGATTAGATACCCTG	Wang and Qian 2009
				TGTTGGGTTAAGTCCCGRAACG	Wang and Qian 2009

6-4 Results

6-4.1. Shell morphology

The largest specimen (2.13mm length) matched *Idas argenteus* descriptions, (Jeffreys 1876; Dean 1993; Oliver and Holmes 2009; Ockelmann and Dinesen 2011) of a delicate, iridescent, silvery-white, nacreous shell with a rounded oblong, submodioliform, plain edged outline, the anterior slightly narrower than the posterior (Figure 1). External sculpture of close-set commarginal lines (Figure S1), with short periostracal hairs over the posterior ventral area. Ligament thin, external, posterior of the beaks. Hinge plate minutely denticulate, posterior series twice the number of the anterior. Prodissoconch 2, very large (549µm), red-brown in colour. Juvenile specimen was similar to other small-sized bathymodiolins, but more oblong in form (Laming et al. 2014).

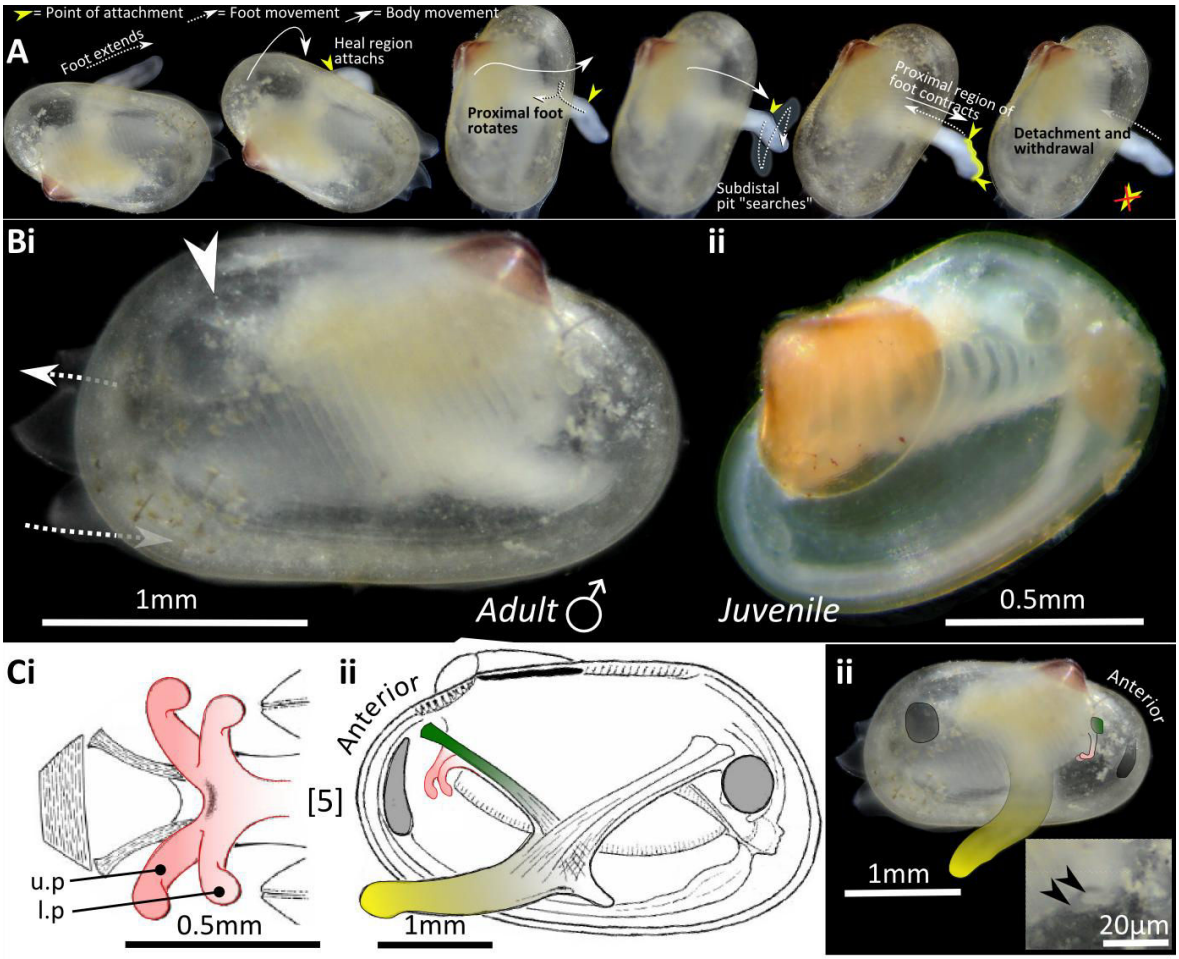

Figure 6.1 Live observations, shell morphology and anatomical analysis of *Idas argenteus*
A) Locomotion in *I. argenteus* is driven by the extension, attachment and contraction of foot. **B)** Shell micrographs: **i)** adult male from Lacaze-Duthiers canyon; **ii**) juvenile from MAR. Dashed arrows: seawater current. Arrowheads: i) location of visibly-beating pericardium; **C)** comparative analysis of anatomy described in Ockelman and Dinesen (2011) adapted for **Ci – ii**, and **Ciii)** discernible anatomy through the shell of the adult male *I. argenteus*. Pink = labial palps, magnified and indicated by arrowheads in the inset of **Ciii**. Yellow = foot; black/grey = adductor muscles; green = anterior retractor bundle, or point of attachment of retractor, as in **Ciii**.

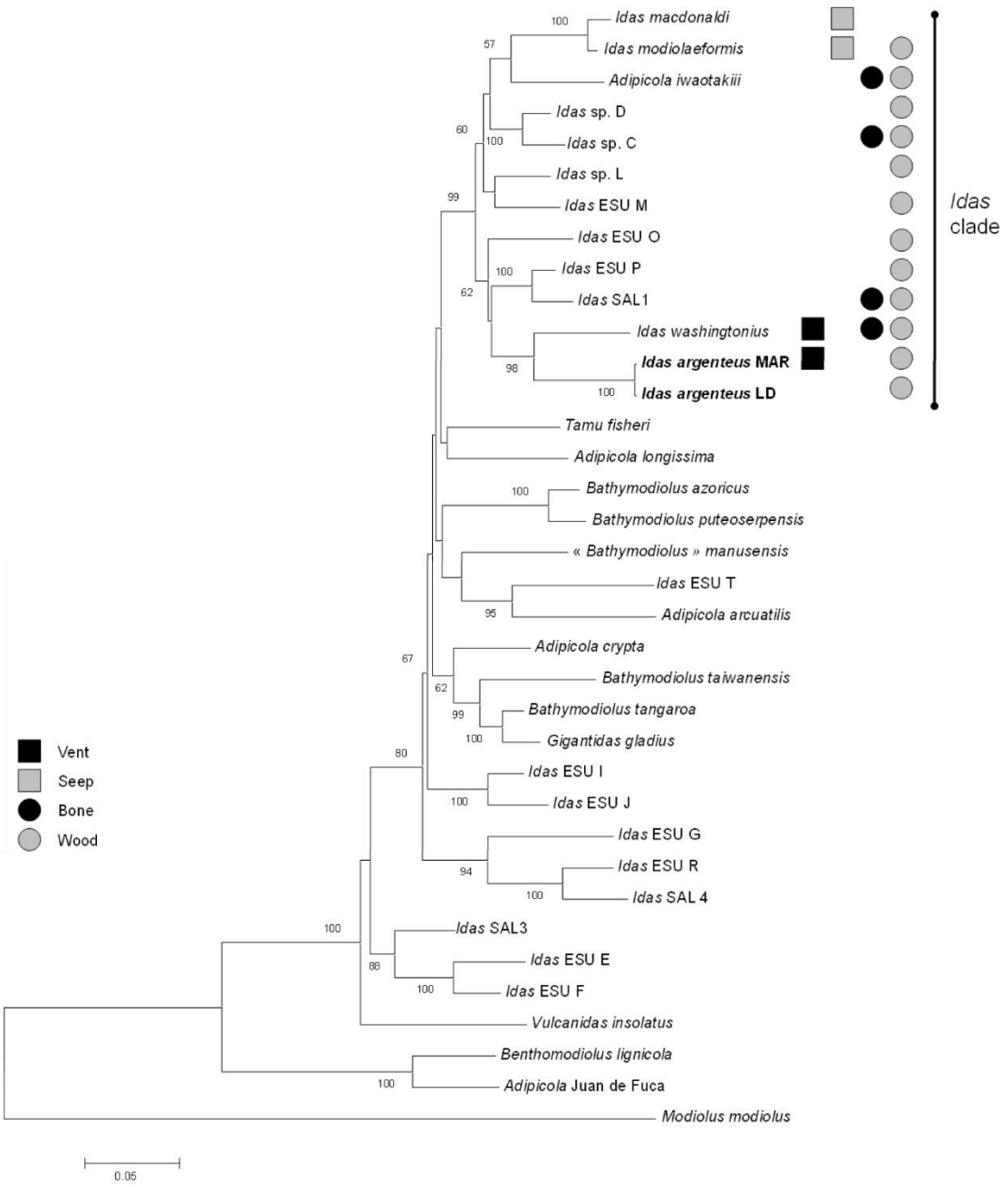

Figure 2. Phylogenetic relationships among Bathymodiolinae
Obtained by maximum-likelihood (ML) analysis of the multigene data set (2 mitochondrial and 3 nuclear genes), details in Table S2

6-4.2. Phylogenetic relationships

Sequences from LD and MAR specimens displayed similarities between 99.4 to 99.9% for the six studied genes. Phylogenies based on COI (Table S2, Figure S2), on concatenated datasets of 5 genes (including closest relatives, Figure 2) and on all 6 genes (excluding *I. washingtonius* and *I. macdonaldi*, not shown) presented similar topologies and well-supported nodes using ML methods. *I. argenteus* was most closely related to *I. washingtonius* (K2P COI distance: 19.7 to 20.0%) and belonged to a clade that included *Idas* ESU O, *Idas* sp. P, *Idas* SAL1 (Figure 2) but also *Idas* ESU K and *Idas* ESU N (COI tree, 2S).

PCRs on all 4 bacterial genes using various DNA concentrations and PCR conditions including nested PCR failed to yield any product despite success with similar-sized symbiotic *Idas* spp. from the same experiment.

6-4.3. Live observations and anatomy

The largest LD individual liberated sperm during dissection, confirming it to be male. Ctenidial ciliary action was evident and a ventral particle groove was present. Rotating mucous-bound debris was identified within the ciliated labial palps, which were not obviously plicate, but enlarged (Figure S1). The adult male was highly active during observations, moving around the Petri dish using it's vermiform foot (Figure 1). The pericardial vale could clearly be seen during these observations, where beat rate increased during movement, in comparison to resting entirely still.

6-5 Discussion

Idas argenteus were found associated with sunken wood colonization experiments on the Rainbow MAR vent field and the LD canyon. Although *Bathymodiolus azoricus* occurs at Rainbow and *I. simpsoni and I. modiolaeformis* are reported at LD (Thubaut 2012; *this research*, Section 3-4.2, p. 136), this is the first report of *I. argenteus* at these two sites.

This study provides the first phylogenetic analysis of *I. argenteus*. Its closest relative is *I. washingtonius* found in organic falls and cold seeps in the Eastern Pacific. All other *Idas* species in the clade were found in the Western Pacific, mainly associated with wood. The only two Atlantic species (*Idas macdonaldi* and *I. modiolaeformis*) displayed COI K2P distances with *I. argenteus* between 19.3 and 20.4%, in the upper range of distances measured among members of the clade (9.3 to 23.1%). Because *I. argenteus* is the type species of the genus *Idas*, this clade is actually the true *Idas* clade, and our results confirm that this genus is polyphyletic.

Ockelmann & Dinensen 2011 reported no evidence of bacteria in the gills of *I. argenteus*. However, this could be due to a site-specific rarity or absence of symbionts. Without exception, symbiont acquisition in other bathymodiolin mussels occurs long before sexual maturation (Laming et al., 2014; Wentrup et al., 2013). The fact that symbiont genes were not detected by PCR tests in the specimens studied here is thus not size- or age-related. Even if present in small numbers, below the detection limit of PCR approaches used herein, symbiont rarity would imply a negligible contribution to host nutrition.

From the 13 *Idas* species belonging to the *I. argenteus* clade in the COI tree (Figure S2, Table S1), symbiosis is documented in only five. *I. macdonaldi*, *I. washingtonius*, *Idas* sp. C, and *Idas* sp. D have sulfur-oxidizing symbionts, meanwhile *I. modiolaeformis* has up to 6 different phylotypes with relative dominance of methanotroph or thiotrophs depending on time of sampling or habitat (Duperron et al., 2008; Lorion et al., 2012; Rodrigues et al., 2013; Thubaut et al., 2013). Symbiosis in other species is not yet documented. At this stage, it can be assumed that the *Idas* clade had a common ancestor with thiotrophic symbionts, most likely extracellular as in all species but *I. washingtonius* (Deming et al., 1997). Extracellular thiotrophic

symbionts were reported as an early acquisition dating back to the stem of the group, almost 30 Myr (Lorion et al., 2013). Since *I. washingtonius* still harbors thiotrophic symbionts, it can be assumed that the loss (or considerable reduction) of symbiosis is a derived state and occurred within the branch leading to *I. argenteus*. A clock analysis using known calibrations (Lorion et al., 2013) estimated that *I. argenteus* diverged from *I. washigtonius* between 11.1 and 13 Myr. Loss or reduction of symbiosis is not unexpected given the predisposition of *Idas* mussels for diverse, adaptable symbiont assemblages (Lorion et al., 2012; Rodrigues et al., 2013). Absence of symbionts in an otherwise symbiotic group has been observed in the Thyasiridae where some species have symbionts while others do not. A recent study even demonstrated that a single (potentially two cryptic) species may either harbor or lack symbionts in two distinct populations (Batstone et al., 2014), a flexibility that could potentially also exist in deep-sea mussels.

The results described here concur with previous studies suggesting the absence of symbionts in *Idas argenteus* (Ockelmann & Dinesen, 2011). This is to date the only reported case of symbiosis loss or strong reduction within the Bathymodiolinae. This shift to strict heterotrophy could be linked with the organic-enriched habitats, where larviphagy or filter-feeding may alone sustain the animal nutrition. Whether this will remain a unique example, or a convergent trend in different mussel clades will require further investigation of symbiosis in smaller mussels.

6-6 Acknowledgments

We thank the chief scientists, captains, crew and ROV pilots of the two cruises.

6-7 Data accessibility

The sequences were deposited in GenBank (accession numbers: XXXXXX-XXXXX).

6-8 Funding statement

This research was supported by CHEMECO-ESF-EURODEEP, and TOTAL foundation. CFR and SRL were supported by a Portuguese FCT grant (SFRH/BPD/64154/2009) and an EU MARES grant (FPA 2011-0016) respectively.

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6-10 Annex 1

6-10.1. Supplementary material for Chapter 6

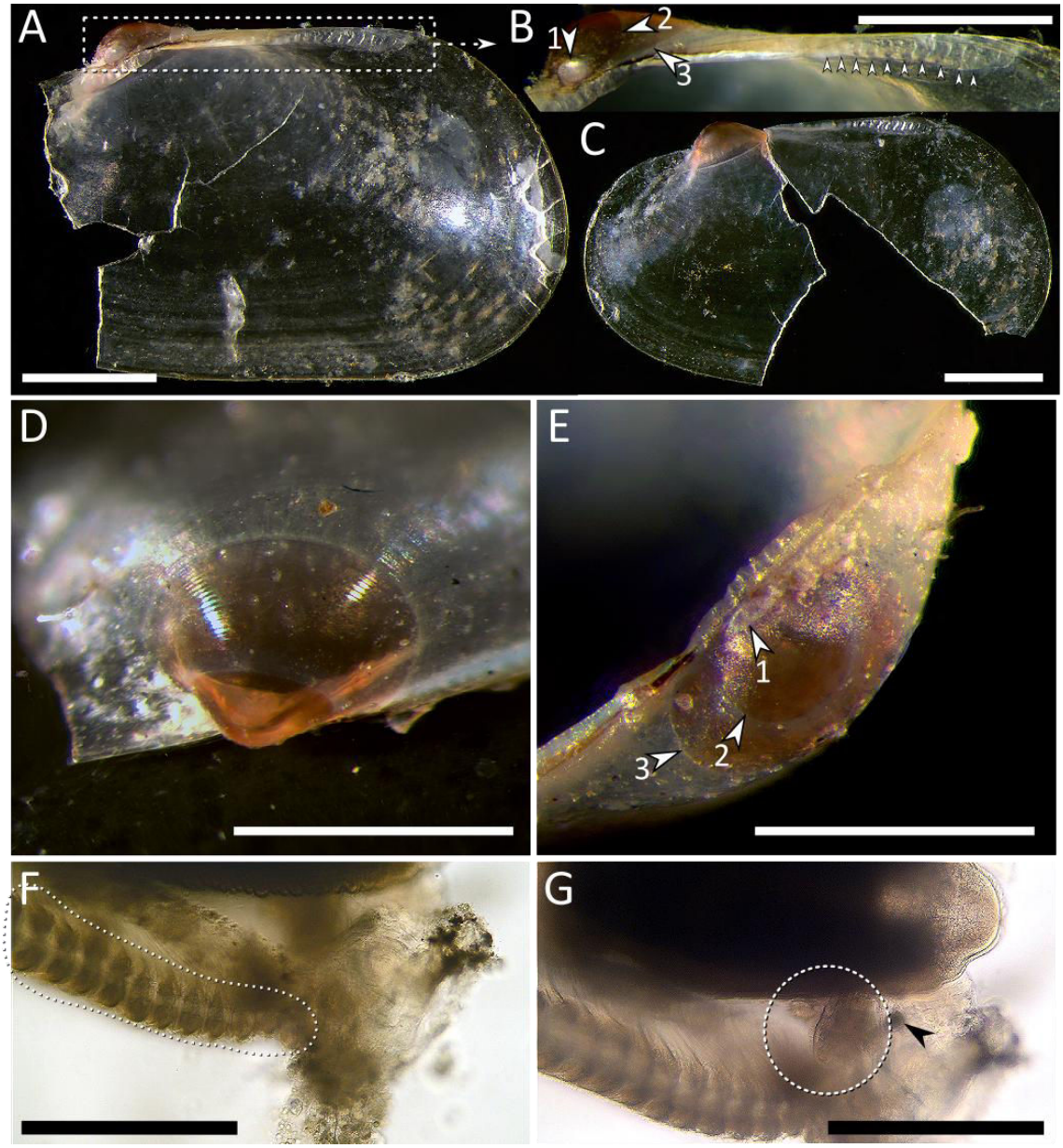


Figure S1 Morphometrics and live observations of the adult male from Lacaze-Duthiers Canyon
A) Interior view of the right valve. B) Boxed region in A displaying larval shell and hinge plate: Numbered arrowheads are the prodissoconch I, IIa and IIb respectively. Row of arrowheads denote hinge-plate denticulation. C) Exterior view of the left valve. D–E) Dorsolateral and dorsal view of larval shell respectively. Numbered arrowheads are as in B. F–G) Left anterior region of specimen during live dissection at two focal planes identified the encircled ventral 'feeding' groove and enlarged – for the genus – ciliated upper labial palp. Scale bars = 500µm

Table S1 Specimen collection sites and GenBank acession numbers for Mytilidae included in the phylogenetic analyses.

Table built after Lorion et al. 2013; Thubaut et al. 2013. Distribution: A, Atlantic; WP, Western Pacific; EA, Eastern Atlantic; EP, Eastern Pacific. Habitat: I, intertidal; W, wood; B, Bone; S, seep; V: vent. Genus1, as originally described; Genus2 as proposed Thubaut et al. 2013b.

Genus1	Species	Distribution	Depth range	Habitat	COI	ND4	16S	18S	28S	H3	Genus2
Modiolus	Modiolus	A	10–300	I	FJ890501a	EF526453	KF611732	KF611701	EF526455	KF720595	Modiolus
Benthomodiolus	Lignocola	WP	1180	W, B	AY275545	AY649817	KF611733	AF221648	AY781131	KF720596	Benthomodiolus
Adipicola	sp. Juan de Fuca	EA	2420	V	KF611694	HF545177	KF611734	KF611702	KF611699	KF720597	Benthomodiolus
Benthomodiolus	sp. South Atlantic	A	3900	B	KF611691		KF611735	KF611703	KF611698	KF720593	Benthomodiolus
Benthomodiolus	geikotsucola	WP	4020		HF545103	HF545180	HF545049		HF545023	HF545149	Benthomodiolus
Idas	ESU E	WP	150–785	W	FJ937079	HF545198	KF611736	KF611704	GU065791	KF720598	Vulcanidas
Idas	ESU F	WP	275–560	W	FJ937127	HF545205	KF611737	KF611705	GU065809	KF720599	Vulcanidas
Idas	SAL3	WP	400–1085	W	DQ340772	DQ863949	KF611738	DQ340800	DQ863946	KF720600	Vulcanidas
Vulcanidas	Insolatus	WP	140–504	V	FJ767936	HF545189	KF611739	KF611706	FJ767937	KF720601	Vulcanidas
Tamu	Fisheri	A	546–650	S	AY649803	HF545181	HF545065	AF221642	AY781132	HF545148	Tamu
Idas	ESU S'	WP	230–380	W	FJ937240	HF545194	HF545061		GU065816	HF545146	Lignomodiolus
Idas	ESU S"	WP	190–400	W	FJ937258		KF611740	KF611707	GU065829	KF720602	Lignomodiolus
Idas	SAL4	WP	94–886	W	DQ340776	HF545179	KF611741	DQ340796	DQ863947	KF720603	Lignomodiolus
Idas	ESU R	WP	490	W	FJ937239	HF545191	KF611742	KF611708	GU065877	KF720604	Lignomodiolus
Idas	ESU Q	WP	100–130	W	FJ937230		KF611743	KF611709	GU065875	KF720605	Lignomodiolus
Idas	ESU G	WP	150–670	W	FJ937161	HF545206	KF611744	KF611710	GU065778	KF720606	Lignomodiolus
Gigantidas	sp1 broken bay	WP	361–750	S	KF611693		KF611745		KF611696	KF720607	Gigantidas
Bathymodiolus	taiwanensis	WP	200–355	V	GU966638	HF545215	KF611746	KF611711	GU966641	KF720608	Gigantidas
Bathymodiolus	mauritanicus	A	540–2222	S	FJ890502	HF545214	KF611747	KF611712	FJ890504	KF720609	Gigantidas
Bathymodiolus	Tangaroa	WP	920–1205	S	AY608439	HF545203	KF611748	AY649820	AY781149	KF720610	Gigantidas
Gigantidas	Sp. 2 Broken Bay	WP	361–750	S	KF611692		KF611749	KF611713	KF611697	KF720611	Gigantidas
Gigantidas	Gladius	WP	300–460	V	AY649802	AY649813	HF545085	AY649821	AY781134	HF545174	Gigantidas
Adipicola	crypta (B')	WP	441	W, B	EU702319	HF545182	KF611750	KF611714	EU683298	KF720612	Gigantidas
Adipicola	Crypta (B")	WP	431–493	W, B	EU702315		KF611751		EU683301	KF720613	Gigantidas
Gigantidas	horikoshii	WP	486	V	HF545113	HF545190	HF545086		HF545043	HF545150	Gigantidas
Bathymodiolus	Platifrons	WP	1028-1523	V, S	HF545106	HF545183	HF545082		HF545029	HF545162	Gigantidas
Bathymodiolus	japonicus	WP	908-1180	V, S	HF545108	HF545185	HF545081		HF545039	HF545154	Gigantidas
Bathymodiolus	securiformis	WP	641	S	HF545109	HF545186	HF545079		HF545037	HF545166	Gigantidas
Gigantidas	nsp Ashizuri	WP	575	S	HF545120	HF545202	HF545088		HF545042	HF545130	Gigantidas
Bathymodiolus	nsp Sissano 1	WP	1646-1881	S	HF545125	HF545217	HF545076		HF545045	HF545168	Gigantidas

Bathymodiolus	nsp Sissano 2	WP	1881	S	HF545122	HF545204	HF545078		HF545047	HF545169	Gigantidas
Gigantidas	nsp Aitape	WP	470	S	HF545119	HF545201	HF545087		HF545044	HF545129	Gigantidas
Bathymodiolus	nsp Kikaijima	WP	1430	S	HF545112	HF545188	HF545077		HF545046	HF545155	Gigantidas
Adipicola	longissimus	WP	400–1767	W	DQ340773	HF545175	KF611752	DQ340798	DQ863945	KF720614	Nypamodiolus
Idas	ESU J	WP	360–370	W	FJ937189	HF545195	KF611753	KF611715	GU065842	KF720615	Nypamodiolus
Idas	ESU I	WP	190–567	W	FJ937188	HF545209	KF611754	KF611716	GU065774	KF720616	Nypamodiolus
Idas	ESU H	WP	220–560	W	FJ937073		KF611755	KF611717	GU065856	KF720617	Nypamodiolus
Idas	Simpsoni	WP	162	B	KF611695		KF611773	KF611731	KF611700	KF720594	Nypamodiolus
“Bathymodiolus”	manusensis	WP	1627	V	GU966637	HF545184	HF545059	KF611718	GU966642	KF720618	“Bathymodiolus”
“Bathymodiolus”	aduloides	WP	1378-1451		HF545118	HF545200	HF545060		HF545036	HF545128	“Bathymodiolus”
Adipicola	Arcuatilis	WP	880	B	FJ937033	HF545207	KF611756	KF611719	GU065879	KF720619	Terua
Idas	ESU T	WP	800–1060	B	FJ937283	HF545197	KF611757	KF611720	GU065804	KF720620	Terua
Adipicola	Pacifica	WP	220-230	B	HF545115	HF545192	HF545066		HF545040	HF545161	Terua
Bathymodiolus	puteoserpentis	A	3023–3510	V	AY649796	HF545176	HF545053	AF221640	AY781151	HF545163	Bathymodiolus
Bathymodiolus	Azoricus	A	866–2330	V	AY649795	AF128534	KF611758	AY649822	AY781148	KF720621	Bathymodiolus
Bathymodiolus	heckerae	A	3314	S	AY649794			AF221639	AY781138		Bathymodiolus
Bathymodiolus	boomerang	A	1000–3170	S	FJ890503	HF545213	KF611759		FJ890505	KF720622	Bathymodiolus
Bathymodiolus	Brevior	WP	3589	V	AY649799			AY649824	AY781150		Bathymodiolus
Bathymodiolus	thermophilus	EA	2460–2747	V	GU966639	AY649808	KF611760		GU966640	KF720623	Bathymodiolus
Bathymodiolus	aff. thermophilus	EA	2331	V	AF456317	AY649809			AY781140		Bathymodiolus
Bathymodiolus	Brooksi	A	2222–3314	S	AY649798	HF545178	HF545056		AY781135	HF545133	Bathymodiolus
Bathymodiolus	septemdierum	WP	3589	V	HF545111	HF545187	HF545055		HF545031	HF545132	Bathymodiolus
Idas	SAL 1	WP	408–1356	W, B	DQ340775	DQ863951	KF611761	DQ340794	DQ863944	KF720624	Idas
Idas	ESU P	WP	180–1390	W	FJ937222	HF545211	KF611762	KF611721	GU065846	KF720625	Idas
Idas	ESU O	WP	473–890	W	FJ937211	HF545199	KF611763	KF611722	GU065763	KF720626	Idas
Idas	ESU K	WP	500–540	W	FJ937192		KF611764	KF611723	GU065868	KF720627	Idas
Idas	japonicus	WP	220-560	W	FJ937190	HF545210	HF545072		GU065841	HF545151	Idas
Idas	washingtonius	EP	960–1910	V, W, B	AY275546	AY649815	HF545073	AF221645	AY781146		Idas
Idas	sp. D	WP	556–1724	W	EU702357	HF545193	KF611765	KF611724	EU683275	KF720628	Idas
Idas	sp. C	WP	275–1285	W, B	EU702376	HF545208	KF611766	KF611725	EU683260	KF720629	Idas
Idas	ESU M	WP	590–720	W	FJ937202	HF545212	KF611767	KF611726	GU065845	KF720630	Idas
Idas	ESU L	WP	450–1010	W	FJ937193	HF545196	KF611768	KF611727	GU065767	KF720631	Idas
Idas	ESU N	WP	800–1290	W, B	FJ937205		KF611769	KF611728	GU065843	KF720632	Idas
Adipicola	iwaotakii (A')	WP	441–1866	W, B	EU702333	HF545218	KF611770	KF611729	EU683288	KF720633	Idas
Adipicola	iwaotakii (A")	WP	490–2307	W	EU702322		KF611771		EU683295	KF720634	Idas
Idas	macdonaldi	A	650	S	AY649804	AY649816	HF545092	AF221647	AY781145		Idas
Idas	modiolaeformis	A	2129	S	FJ158585	HF545216	KF611772	KF611730	FJ159555	KF720635	Idas

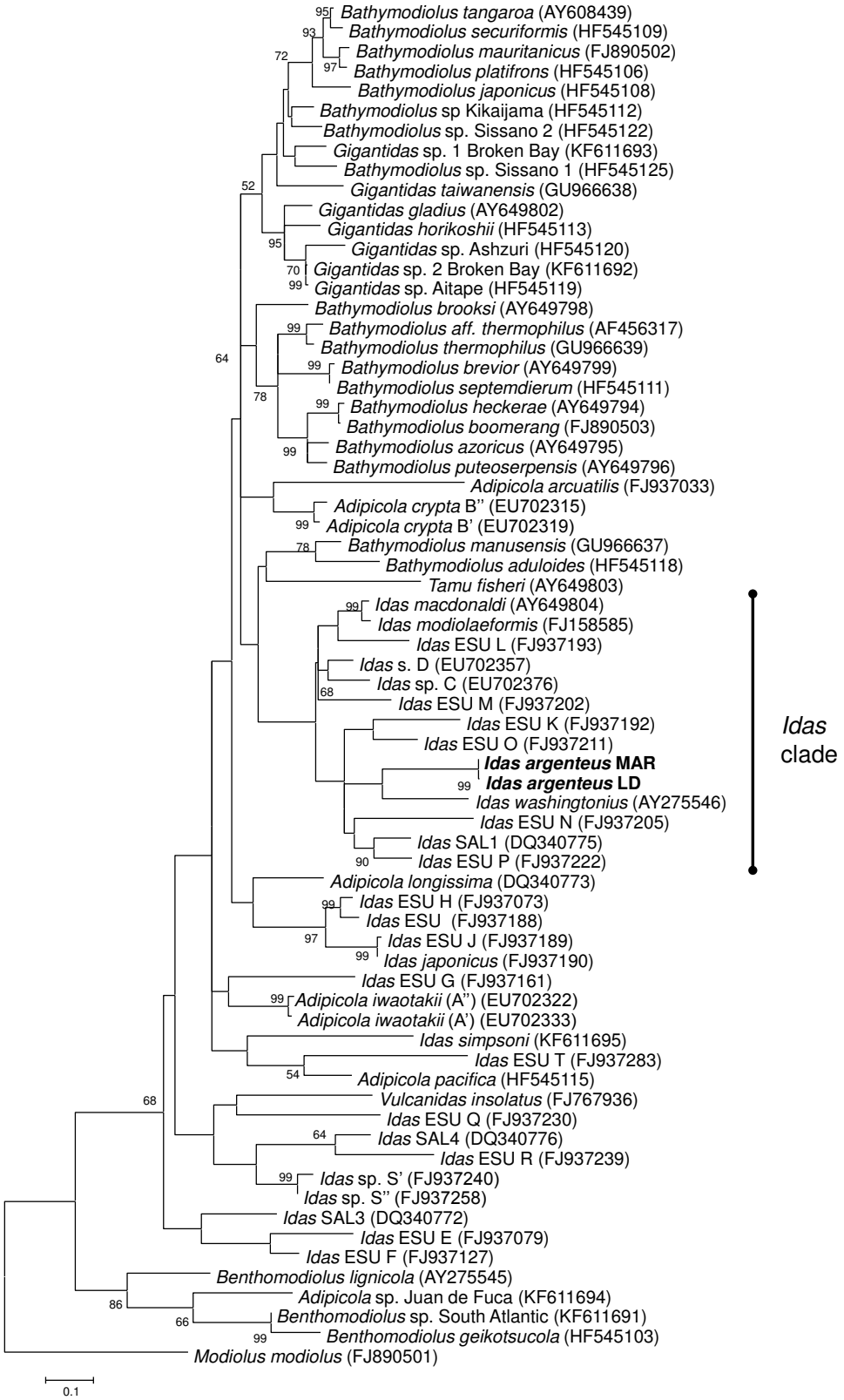


Figure S3 ML tree obtained from the analysis of the Cytochrome Oxidase I (COI) mtDNA data set (table S2)
Only bootstraps higher than 50% are given

6-10.2. References for supplementary material

Lorion J, Kiel S, Faure B, Kawato M, Ho SYW, Marshall B, Tsuchida S, Miyazaki J-I, Fujiwara Y. 2013. Adaptive radiation of chemosymbiotic deep-sea mussels. Proceedings of the Royal Society B: Biological Sciences 280.

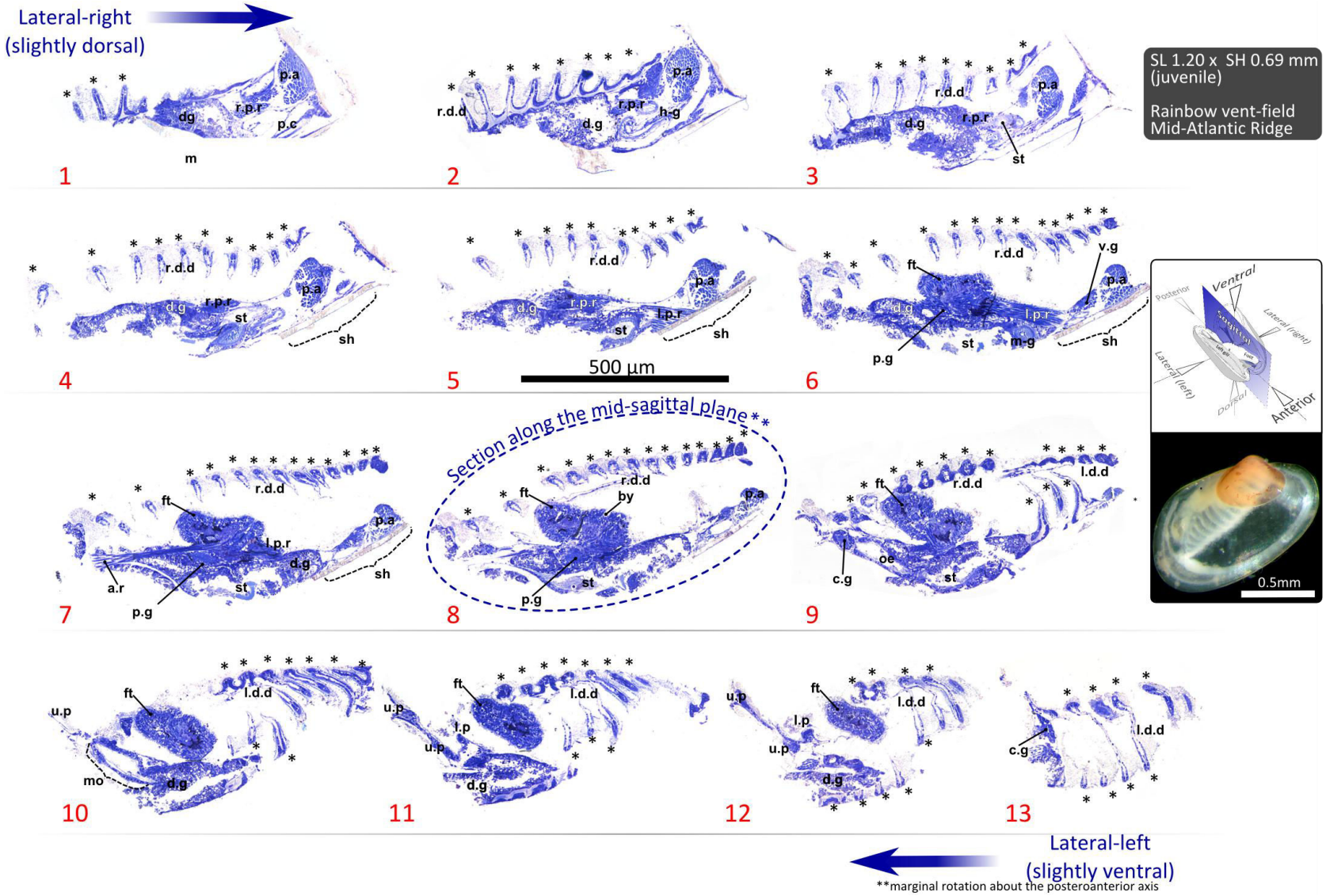
Thubaut J, Puillandre N, Faure B, Cruaud C, Samadi S (2013). The contrasted evolutionary fates of deep-sea chemosynthetic mussels (Bivalvia, Bathymodiolinae). *Ecology and Evolution*, **3**(14): p. 4748–66

6-11 Annex 2

6-11.1. The aposymbiotic *Idas* aff. *modiolaeformis/macdonaldi*

On specimen was found from the Mid-Atlantic Ridge which was initially included in this paper (hence the figure following) but was subsequently removed when it was realised that it is an intermediary species to both *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.s.*) *macdonaldi*. Below is the histology demonstrating that it lacks symbionts, rather unusually.

(*following page*) Figure S2 Toluidine-blue-stained serial sections of juvenile from MAR.
Micrographs of 1-µm semi-thin sagittal sections of I. argentus (specimen in micrograph, lower-rght) stained with toluidine blue at incremental locations along the right-lateral to left-lateral axis (#1–13): sectioning plane is depicted (mid-right box). Specimen details are cited in the grey box (upper-right). a.r: anterior retractor, by: byssus gland, c.g: cerebropleural ganglion, d.g: digestive gland, ft: foot, h-g: hing-gut, l.d.d: left descending demibranch, l.p: lower labial palps, l.p.r: left posterior retractor bundle, m: mantle, m-g: mid-gut, mo: mouth, oe: oesophagus, p.a: posterior adductor muscle, p.c: pericardial cavity, p.g: pedal ganglion, r.d.d: right descending demibranch, r.p.r: right posterior retractor bundle, sh: shell, st: stomach, u.p: upper labial palps, v.g: visceral ganglion, * = gill filaments.


Lateral-right
(slightly dorsal)
SL 1.20 x SH 0.69 mm
(juvenile)
Rainbow vent-field
Mid-Atlantic Ridge
1
2
3
4
5
6
7
8
9
10
11
12
13
500 µm
Section along the mid-sagittal plane**
Ventral
Posterior
Anterior
Dorsal
Lateral (right)
Lateral (left)
0.5mm
Lateral-left
(slightly ventral)
**marginal rotation about the posteroanterior axis

Chapter 7 GENERAL DISCUSSION

7-1 The genus *Idas*: who's in and who's out

7-1.1. A brief note on the updated phylogeny of the genus *Idas*

The history of nomenclature for wood- and bone-associated mussels has been complex, with members of the genus (*s.l.*) as it currently stands having been assigned one or more of the following genera over time *Adula*; *Idasola*; *Myridas,*; *Myrinopsis*; *Habepegris*; *Myrina, Adipicola Idas*, *Terua*. For scientists working on these small-sized mussels this has been highly frustrating, since making assertions about the evolutionary origins of the various genera is, by definition, confounded by ongoing debate concerning their taxonomy and more recently, phylogeny. Some researchers have however contributed considerable advances in the cladistics of the subfamily (e.g. Jones et al. 2006; Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b; Rodrigues et al. *in press*, Chapter 6, p. 232).

7-1.1.1 Observations on the type species, *Idas argenteus*

As part of the research carried out within the framework of this PhD, (Rodrigues et al. *in press,* Chapter 6, p. 232), key characteristics of the adult-shell sculpture, umbo placement, larval prodissoconch size and hinge-plate denticulation were used to provisionally identify specimens collected as the species *I.* (*d.f.*) *argenteus* (Figure 7.1). Digitate labial palps characteristic of the species (Ockelmann and Dinesen 2011), were discernible from live video observations of the small male from the Lacaze-Duthiers Canyon, though grabbed still images were not of sufficient quality to demonstrate this feature formally in publication (see Figure 6.1). Unfortunately, prior to the realisation that this specimen was *I. argenteus*, the soft-tissue had been used in its entirety for DNA extraction and thus a very detailed anatomical investigation was never carried out on this adult specimen. Ockelman and Dinesen (2011) predicted that the inner muscular folds of the mantle edge could probably extend a considerable degree beyond the ventral margins of the shell valves, based on the musculature of preserved specimens. During live observations while the mussel was resting, it was indeed possible to see a curtain of mantle extending along most of the length of the ventral margin, and in particular where pseudo-siphons formed at the posterior region of the specimen. The entire inner fold then briefly extended further still, prior to complete valve-closure when the specimen was accidentally disturbed (vibrations of microscope). This behaviour may flood the mantle cavity with oxygenated water in preparation for closure. However, during periods when the foot was extended during movement, the mantle curtain was retracted within the shell. The absence of the incomplete and modified outer demibranchs (Ockelmann and Dinesen 2011) was probably a question of size. The adult specimen from the Lacaze-Duthiers Canyon (SL 2.13 mm) fell well below the minimum size identified for outer demibranch formation in this species (> 4 mm, Ockelmann and Dinesen 2011). Based on all these observations, the evidence available indicated that these specimens were indeed the type species for the genus. This was subsequently confirmed by Graham Oliver of the National Museum of Wales.

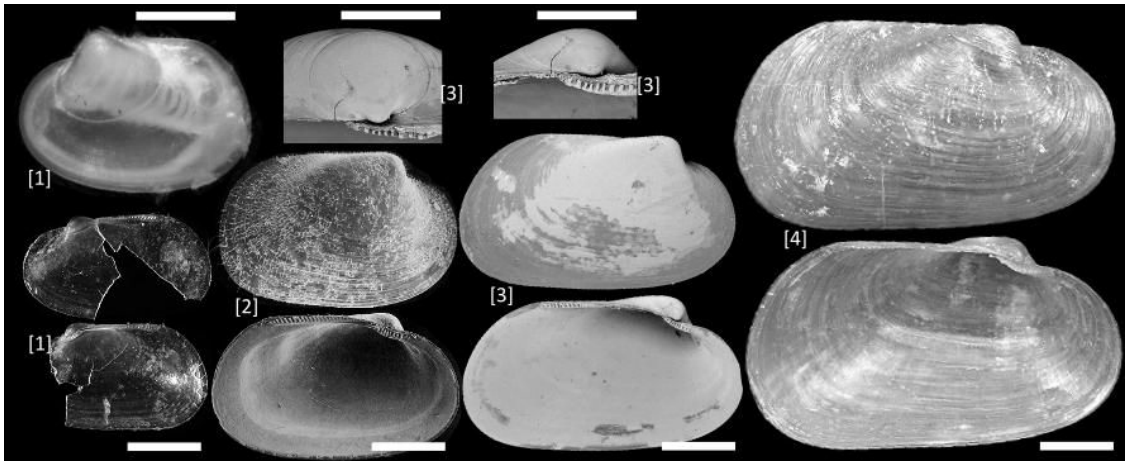


Figure 7.1 *Idas argenteus* morphometry with increasing size (multiple studies and sample sites), and its anatomy
Depicted is the full record of shells used to assess the species, based on the description in Ockelmann and Dinesen (2011), and on the original holotype [4]. Note that the valves from source [3] bear a strong resemblance to those of *Idas lamellosus*. Scale bars are 500 µm (when above image); and 1 mm (when below image). Image sources are: [1] this PhD research; [2] Dean 1993; [3] Oliver et al. 2010; [4] Jeffreys 1881; Oliver et al. 2010.

7-1.1.2 Consequences for the phylogeny of the Bathymodiolinae (*s.l.*)

Concerted efforts have been made to establish more robust concatenated trees for the Bathymodiolinae (s*.l.*) subfamily (e.g. Jones et al. 2006; Miyazaki et al. 2010; Lorion et al. 2013; Thubaut et al. 2013b; Rodrigues et al. *in press*, Chapter 6, p. 232). The use of multigene concatenated tree construction has resolved some of the incongruence typical of single-gene trees (seen most recently in Fontanez and Cavanaugh 2013), greatly increasing bootstrap support for many the more recently emerging nodes (Lorion et al. 2013). One example is the study by Thubaut et al. (2013b), in which some of the most drastic alterations to the current phylogeny are made. A multi-gene concatenated Bayesian tree, identified 10 distinct clades with robust support for 8 based on the posterior probabilities and bootstraps values cited (two clades terminate in genera for which only one species is known). The analysis examined 51 taxa/ESUs and 5912 base pairs (bp) from two mitochondrial and five nuclear genes. These were fragment sequences for mitochondrial genes which code for cytochrome oxydase I (mtCOI) and the 16S region of ribosomal RNA (16S), the nuclear genes 18S rDNA (18S) and 28S rDNA (28S), which code for the 18S and 28S region of ribosomal RNA respectively, the nuclear gene histone H3 (H3), the nuclear gene that codes for the 70-kDa heat-shock protein (HSP70), and the gene that codes for adenosine nucleotide (ADP/ATP) translocase (Ant).

However, these studies rarely make concerted efforts to acknowledge any divergence, or conservativeness/convergence in morphoanatomy, where the erection of new genera in particular, appears to be based upon genetics alone (Thubaut et al. 2013b; but Lorion et al. 2010; Thubaut et al. 2013a). Additional criticism might be levelled at the revision of the nomenclature proposed in Thubaut et al. (2013b), for the re-introduction of the genus *Terua* Bartsch & Rehder (1938), previously synonymised with *Adipicola* Dautzenberg, (1927), since *Adipicola* takes precedence chronologically. Equally, the amalgamation of members of the "*childressi*" group with *A. crypta* and the limited number of Gigantidas sequences (one described species only) to form a single clade ranked at the genus level (*Gigantidas*) is also questionable

(low bootstrap support, Thubaut et al. 2013b, Figure 1.19). Retaining *A. crypta* in its own genus would improve clade support for the "Bathymodiolus"-Gigantidas genera complex. The sequences used by Lorion et al. (2013), available for *Gigantidas* from Japanese waters would potentially lend considerable support to this clade.

Idas (s.s.) *argenteus* is now known to fall within the largest of the clades containing described and undescribed *Idas*-like mussels. This clade is the same one that was retained as the genus *Idas* (s.s.) by Justine Thubaut and colleagues, a decision that is now validated by the molecular characterisation of *I. argenteus*. This confirms that all other species outside of this clade which are provisionally assigned to the genus *Idas* based on their DNA, merit placement in other existing or new genera, as suggested by Thubaut et al. (2013b). Thus the research herein concerning *I. argenteus* has succeeded in resolving the nomenclature of at least a subset of the species assigned to this genus. Obstacles to the revision of the subfamily as a whole, would benefit from the molecular characterisation of other type species, such as in *Adipicola* (type taxon formerly *Myrina danhmai* H. Adams & A. Adams, 1854, but synonymised with *A. pacifica* Forbes in Woodward, 1854). In effect, such treatment should be extended to all morhospecies (and particularly *I.* [*s.l.*] species from the Mediterranean), which are yet to be assessed molecularly (demonstrated by the case of *I.* [*s.l.*] simpsoni, Thubaut et al. 2013b). For example, at least two of the morphospecies in the Mediterranean may simply be paratypes of *I.* [*s.l.*] *simpsoni* and *I*. (*s.s.*) *modiolaeformis*, based on their gross malacology (*I.* [*s.l.*] *cylindricus* and *I.* [*s.l.*] *ghisottii* respectively). In fact, even for the purposes of the current research, a certain level of intraspecific nucleotide variability was accommodated for individuals of *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*, resulting in several haplotypes (e.g. Section 3-4.1, p. 135)

7-1.2. Two phylogenetically divergent reducing-habitat species

From an evolutionary point of view, *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* provide an interesting pair of species for lifecycle comparisons. The molecular characterisation of *I.* (*s.l.*) *simpsoni* by Thubaut (2013b) identified this species to have emerged relatively early in the evolutionary history of the Bathymodiolinae (*s.l.*), and certainly earlier than *I*. (*s.s.*) *modiolaeformis*. Unfortunately the molecular clock presented in Lorion (2013), which incorporated three fossil calibrations, does not include data for this species. However, the earlier branching of species common to both analyses and to which *I.* (*s.l.*) *simpsoni* is most closely related (i.e. "*Nypamodiolus*" clade of *I. japonicus*, ESU I, and ESU J and *A. longissima*: the four species which group with *I.* (*s.l.*) *simpsoni*, in Thubaut et al. 2013b), provide support for the earlier emergence of *I.* (*s.l.*) *simpsoni*. According to Lorion (2013), this lineage (which is missing *I.* (*s.l.*) *simpsoni*) emerged about 32–24 Ma ago (diverging from the monospeciate *Tamu* lineage (Lorion et al. 2013). The divergence of the two lineages which ultimately contained *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* (i.e. the *Bathymodiolus-Idas*-clade (s.s.), and the remainder of the "*Nypamodiolusi*" clade, Thubaut et al. 2013b), appears to have been from 34–27 Ma ago, at the preceding node (Lorion et al. 2013). However, the trees differ considerably

in their arrangement between the two papers. The clade containing the other "*Nypamodiolus*" members (*I. japonicus*, ESU I, and ESU J and *A. longissima*), is not as deep branching in Lorion (2013) as the equivalent clade in Thubaut (2013), which also includes *I.* (*s.l.*) *simpsoni*. Consequently, depending on which analyses one trusts, *I.* (*s.l.*) *simpsoni* would either be in a clade that is most closely related to the *Idas* (*s.s.*) and *Bathymodiolus* (*s.s.*) clades but branching later than the "*B.* (*s.l.*) *childressi*" group (Lorion et al. 2013) or it emerged much earlier, prior to all *Bathymodiolus* (*s.l.*) and *Idas* (*s.s.*), as in Thubaut et al. (2013b). This discrepancy exists in a region of both trees where low posterior probabilities and boot-strap support are common place. Although fewer species (and a slightly different array of genes) were used, the concatenated tree presented in Chapter 6 (Rodrigues et al. *in press*) appears for the most part to agree with the structure of the tree from Lorion (2013). Despite these discrepancies, in all instances the lineage which terminates in the *Bathymodiolus* (*s.s.*) and *Idas* (*s.s.*) clades, is highly divergent from the clade to which *I.* (*s.l.*) *simpsoni* appears to belong. Thus, an opportunity presented itself to assess the corresponding degree of divergence in the morphoanatomy of these species, to examine whether such differences might also be apparent in their biology.

7-2 Synthesis of current research

The research results presented in the previous chapters (excluding Chapter 6) are summarised in Table 7.1, which also provides some data on habitats occupied by *I.* (*s.s.*) *modiolaeformis* and *I.* (s.*l.*) *simpsoni* that were not examined during this study (i.e. seeps). Note that this table effectively represents a walkthrough guide for the sections below.

7-2.1. General species information

An updated version of known distributions of the two species is presented (Figure 7.2). Occasional records cited in the literature (e.g. some within Pelorce and Poutiers 2009) could not be confirmed directly (in particular, the location of the largest recorded specimen of *I.* (s.*l.*) *simpsoni*, from Warén and Carrozza 1990). When viewed in conjunction with Table 7.1, habitat associations can be placed in the context of geographical location. Notably, through the course of the current research, new records of *I.* (*s.s.*) *modiolaeformis* include specimens found associated with various deployed wood experiments both in the Lacaze-Duthiers Canyon and at the Rainbow vent field (*I.* aff. *modiolaeformis*/*macdonaldi*), while new records of *I.* (s.*l.*) *simpsoni* are from deployed wood experiments in the Lacaze-Duthiers Canyon. Rather surprisingly, the juvenile *I.* aff. *modiolaeformis*/*macdonaldi* at the Rainbow Vent Field appeared to be possess few or no symbiotic bacteria, based on histological evidence (toluidine blue, DAPI, FISH) and a failure to amplify bacterial DNA in soft tissues (PCR). The distribution clearly demonstrates a considerable overlap in habitat distribution and yet co-occurrence of the two species is apparently restricted to the wood recovered from the Lacaze-Duthiers Canyon in the Gulf of Lion, western Mediterranean. This is probably due to the unavoidably sporadic nature both of deep-sea sampling and reducing habitat distributions (based on current knowledge). It seems likely however that with a greater sampling effort – focussed

Table 7.1 Synthesis of the results from this research integrated with those from other habitats

In red, are the data that have been elucidated from the work undertaken within the framework of this PhD. Themes, broken down into multiple aspects of the research are listed in the first columns. Data contributed by the PhD research are in red. Known distribution notation refers to the distribution map presented in Figure 7.2.

			I. modiolaeformis		*"I." simpsoni*		
CONTEXT	**Benthic environment**	Habitat types	Seep	Wood	Wood	Seep*	Bone
		Known distribution (map)	[1][2][3][4][9][10]	[1][8][11][17vent]	[7][8]	[5][16]	[12][13][14][15]
	Species information	Max. shell length SL_{max} (mm)	17	19.4	24.9	22	45
		Species authority	Sturany 1896 (*Idas sensu stricto*)		Marshall 1900 (genus undergoing revision)		
REPRODUCTION	**Sex determination**	Sex-ratio patterns	Protandric	Protandric	1:1 (SL 5.6–24.2 mm)	...	Males only (≤ 3.74 mm)
		Median hermaphrodite size (mm)	3.6–11.6	9.3	N/A *at these sizes*	...	if applicable, > 3.74 mm
	Gamete characteristics	Mature oocyte ⌀ (µm)	40.7 ± 7.8	25.3 ± 2.98	33.5 ± 3.65		N/A
		Released oocyte ⌀ (µm)	...	59.1 ± 2.31		...	...
		Spermatids ⌀ (µm)	3.2 ± 0.4	2.1 ± 0.29	...	...	...
		Spermatozoid acrosomes (µm)	4.4 ± 0.6	3.0 ± 0.34	3.0 ± 0.28		3.0 ± 0.27
	Spawning	Fecundity	...	≈ 300 oocytes ind.$^{-1}$		...	...
		Periodicity	...	semi-continuous	semi-continuous	...	...
DEVELOPMENT	**Embryonic development**	Cleavage	...	Probably spiral		...	...
		Time to 4-cell	...	≈ 11 hours		...	...
	Larval biology	Larval mode	Planktotrophy (see text)				
		Settlement patterns	...	semi-continuous	Not elucidated	semi-continuous	semi-continuous?
		Prodiss. I **SL** (µm) ± σ (*n*)	78.6 ± 3.4 (6)	80.3 ± 5.14 (20)	Not measured	≈95 (1)	95.9 ± 6.34 (24)
		Prodiss. II **SL** (µm) ± σ (*n*)	379 ± 11.7 (14)	398 ± 17.5 (35)	Not measured	450 (1)	459 ± 26.4 (38)
		Prodiss. II **SH** (µm) ± σ (*n*)	344 ± 5.0 (27)	322 ± 17.4 (35)	Not measured	404 (1)	403 ± 27.9 (38)
	Post-settlement growth	Time-averaged growth (mm d^{-1})	-	0.017–0.021	0.026♀ – 0 .028♂	...	Not calculable
	Rate of sexual maturation	SL at 1st maturity (mm)	< 3.6	2.35♂,	<5.6♂, <18.1♀	...	2.2–2.6 ♂
		Equivalent % SL_{max}	...	≈ 12% SL_{max}	<13%♂, <40%♀	...	5-6% SL_{max}
		Age 1st maturity, males/herm.	...	≈ 4 months ♂	<7 months ☿	...	Not calculable
		Age 1st maturity, females	...	...	<23 months ♀	...	Not calculable
	Development of Inner *descending* lamellae	# filaments: settlement	...	4–6	...	...	6
		1st acq. symbionts	...	6	...	...	6–7
		1st maturity	...	31	...	...	33
		Initiation of ascending lamellae	...	SL 0.77 mm	...	...	Could not be identified
	Initial acquisition of symbionts	Shell length (SL)	...	0.60 mm	...	...	0.43--0.51 mm
		% SL increase in after settling	...	49 %↑ PdII SL	...	...	8.3 - 10.6 %↑ PdII SL
	Specificity of association	Symbiont isolation in gills (SL)	...	≥ 4.37 mm	...	...	Not yet examined

* Includes oil drillings

Table 7.1 ***Continued from previous page***

			I. modiolaeformis		*"I." simpsoni*		
NUTRITION	**Symbiont densities** (see text for scaling)	Plantigrade	...	Aposymbiotic	...	...	Aposymbiotic
		Juvenile	...	Very low	...	...	Already Moderate
		Adult	Moderate–Very High	*Variable*: Low–Very High	Moderate	Moderate	High
	Symbiont diversity	# phylotypes	Typically ≈ 3–4, up to 6	**1–2**	Not confirmed	1	1
	Behavioural observations	Filter-feeding behaviour?	...	Yes	Yes (when < 22.5 mm)	...	...
	Alimentary system	Feeding grooves	...	Ventral (one/two gill^{-1})	Ventral (two gill^{-1})	...	Ventral (one gill^{-1})
		Labial palps	...	Ciliated, plicate	Ciliated, plicate	...	Ciliated, plicate
		Mouth	...	Present	Present	...	Present
		Oesophagus	...	Ciliated	Ciliated	...	Ciliated
	Digestive system	Digestive gland	...	Considerable diverticula	Considerable diverticula	...	Considerable diverticula
		Gut complexity	...	One intestinal loop	> one intestinal loop	...	> one intestinal loop
		Stomach	...	Style-sac present	Style-sac not observed	...	Style-sac present
		Gut contents	...	Yes in the hind-gut	Yes in the hind-gut	...	Yes in hind-gut/ stomach
	Endocrine system	Nephridia identified	...	Putatively	Definitively	...	...
	Trophic contributions	Stable isotopes $\delta^{14}C$	...	-27.4 – -20.7 ‰	-31.3 ± 0.4 ‰	-37.4 – -35.5 ‰	...
		Stable isotopes $\delta^{15}N$	...	1.9 – 4.8 ‰	8.7 ± 0.50 ‰	≤ 6 ‰	...
	Trophic mode during lifecycle (dominant processes in bold and larger font)	Larval (inferred)	...	Strict heterotrophy	...	...	Strict heterotrophy
		Plantigrade	...	Strict heterotrophy	...	...	Strict heterotrophy
		Juvenile	...	**Heterotrophy** Chemosymbiosis	...	...	Heterotrophy ≅ Chemosymbiosis
		Adult	Heterotrophy? **Chemosymbiosis**	*Variable*: however both are important	Heterotrophy ≅ Chemosymbiosis?	Heterotrophy? **Chemosymbiosis?**	Heterotrophy? **Chemosymbiosis**

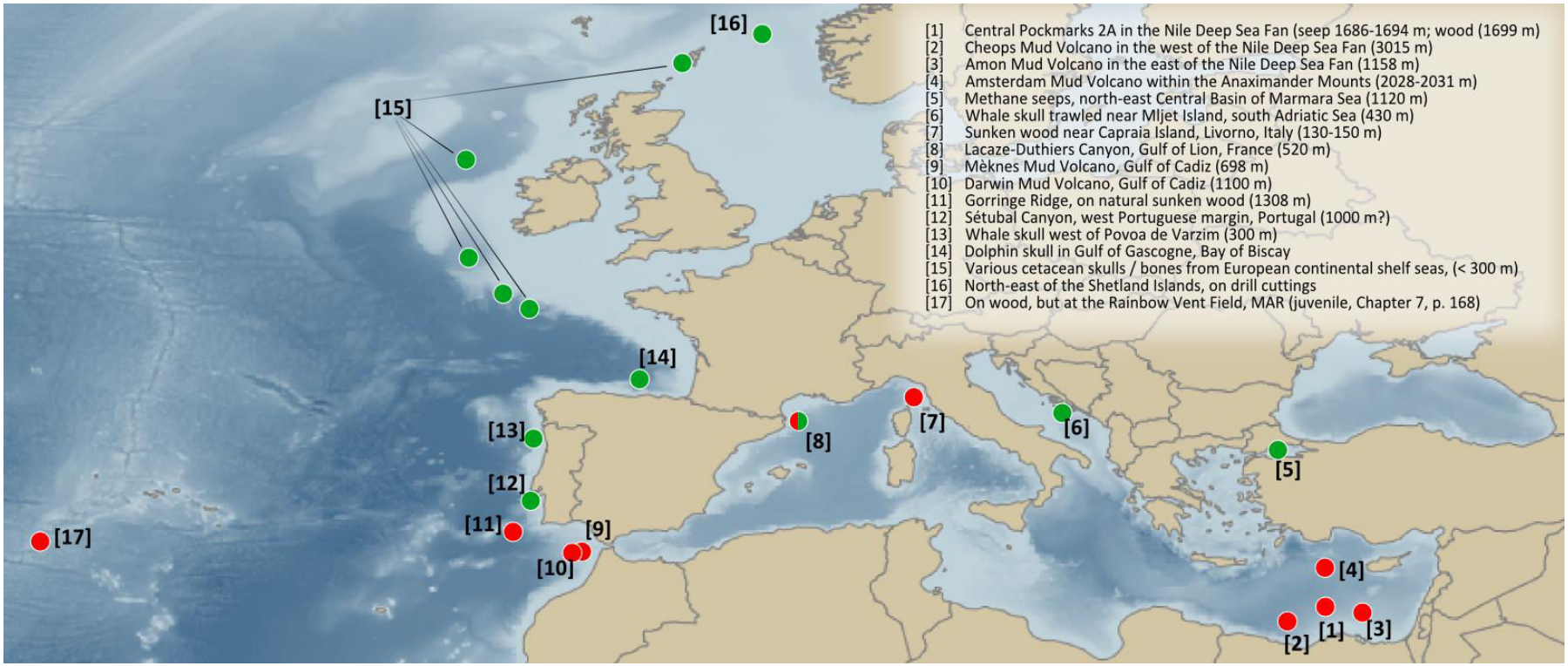


Figure 7.2 distribution of *Idas* (*s.s.*) *modiolaeformis* and *Idas* (*s.l.*) *simpsoni* based on literature and current research

particularly on sunken-wood accumulations – that additional incidences of co-habitation will be discovered. At methane seeps concentrated in the east Mediterranean, including the Marmara Sea, a lack of co-occurrence is surprising but may represent low sampling effort or, constraints on dispersal in *I.* (s*.l.*) *simpsoni* (e.g. across the Dardanelles strait, Ritt et al. 2012).

Maximum recorded shell lengths (Table 7.1) for *I.* (*s.s.*) *modiolaeformis* now include a SL 19.4 mm (wood) which is larger than any previous maximum length documented (17 mm on seeps, Olu-Le Roy et al. 2004), while those for *I.* (s*.l.*) *simpsoni* include a SL 24.9 mm (wood), though it is possible that at least one individual exceeded this length on palmwood experiments (largest *I.* (s*.l.*) *simpsoni*-like specimen used by Justine Thubaut (Thubaut 2012). Assuming sunken wood can sustain *I.* (s*.l.*) *simpsoni* for a long enough period, maximum sizes will likely exceed this size, given the maximum size documented for this species overall (45 mm on a whale skull, observation during another species description, Warén and Carrozza 1990). As discussed above, the current research has established that *I*. (*s.s.*) *modiolaeformis* truly belongs in the *Idas* genus (based on the molecular characterisation of *I. argentus*). *Idas* (s*.l.*) *simpsoni* however, requires reassignment (Thubaut et al. 2013b; this research).

7-2.2. Theme 1: Reproductive biology

The *a priori* objectives of this Theme had been to "collate newly acquired data on gametogenic trends, sex ratios, fecundity and gamete sizes from live and preserved specimens". In this regard the research presented was generally successful (Table 7.1).

7-2.2.1 Sex-ratio patterns

The analysis of specimens of *I*. (*s.s.*) *modiolaeformis* from the Lacaze-Duthiers Canyon has provided new data, demonstrating that protandric sequential hermaphroditism is a persistent trait in this species, regardless of habitat type. However, patterns of sex-switching from small males to larger females differed somewhat depending on habitat (i.e. around the sizes at which the hermaphroditic state was found, Table 7.1). Sizes recorded at methane seeps and mud volcanoes from the eastern Mediterranean by Gaudron et al. (2012) displayed quite a complex pattern of sex-switching (functional males[29] SL 3.6–11.6; 'true' hermaphrodite SL 11.6 mm; functionally female only SL >7 mm). By comparison, sex-switching in adults of various sizes recovered from several oakwood deployments in 2012 in the Lacaze-Duthiers Canyon were more clear-cut (functionally males[30] SL <8.6 mm; 'true' hermaphrodite SL 9.3 mm, functionally female only > 9.2 mm). While the overall size at which switches in sex occurred at each site was roughly equivalent, the SL range over which sex-switching took place was narrower in samples from the Lacaze-Duthiers Canyon. This presumably reflects differences in habitat environments. Electron-donor supply is expected to be

[29] but with female acini undergoing follicular atresia
[30] single incidence of co-occurring female acini undergoing follicular atresia (SL indeterminate, cracked shell)

higher at seep sites though subject to microhabitat variability. The result is fluctuations in epigenetic factors that are presumed to determine sex alternations (Tyler et al. 2009; Gaudron et al. 2012). Although such variability is also known to occur in association with sunken wood (Laurent et al. 2013; Yücel et al. 2013), a large component of the mussels diet could be supported by particulate organic matter (POM), reflected in the complex stable isotopes results presented for mussels recovered from the Lacaze-Duthiers Canyon, particularly for nitrogen (Section 3-3.4.3, p. 135). The vertical flux of particulate organic matter in the Lacaze-Duthiers Canyon is likely to be relatively high, due to coastal proximity, cascading effects (Palanques et al. 2006) and shallower water depths in comparison to seeps in the eastern Mediterranean (525 m, in comparison to 1158–3015 m respectively). Thus, periods of high, less-refractory, POM input may trigger rather abrupt changes in sex within the submarine canyon, in order to take advantage of an episodic abundance in food. The remaining *I*. (*s.s.*) *modiolaeformis* mussels used in this research were those from the developmental size series collected from the eastern Mediterranean from wood and alfalfa substrates, deployed in an area known for methane seepage (Central Pockmarks 2A, NDSF; Chapter 4, p. 163). Conditions at this site effectively represent some sort of intermediate habitat state between the two other *I*. (*s.s.*) *modiolaeformis* sites. However, the largest individual collected from these experiments was only SL 6.54 mm, so data from this experiment can only support protandry generally (all mature individuals were male).

Analysis of sex-ratio patterns in *I.* (s.*l.*) *simpsoni* are somewhat more difficult to interpret. Contrasting patterns existed between the data available from wood associations in the Lacaze-Duthiers Canyon, and those from bone-colonising *I.* (s.*l.*) *simpsoni* in the Sétubal Canyon off the coast of Portugal (Table 7.1). In the former, only adults were collected, where the two smallest individuals were hermaphroditic, but functionally male (SLs 5.6 and 5.8 mm). No obvious size-dependent trend in sex could be identified statistically or even qualitatively (Section 3-4.3, p. 138), at least within the rather limited sample size available (*n* = 12). In contrast, trends in the developmental size series of *I.* (s.*l.*) *simpsoni* recovered from cow bones in the Sétubal Canyon, were comparable to patterns from the aforementioned developmental size series of *I*. (*s.s.*) *modiolaeformis*. All mature individuals (*n* = 3, of 38), analysed in the current research, were male. In addition, colleagues at the University of Aveiro working upon a larger subset of the total number of mussels (in the 100s) recovered from these experiments found that for the 18-month carcass deployment, only one (relatively giant) female was found (Ana Hilário, *personal communication*), putatively identified based on alcohol fixed histology (preservation was poor). The resulting sex ratio approached 1:1000 (♀:♂). However, considering the presence of two hermaphrodites of almost identical size at the Lacaze-Duthiers Canyons and the exclusively small males found at the Setúbal Canyon, sex-switching remains plausible. As adults however, instead of exclusively large females prevailing, it would seem that both sexes coexist. These contrasting trends in sex ratios evidently warrant further investigation, since the results herein are preliminary and derived from two quite different habitats with non-overlapping size ranges.

7-2.2.2 Gamete characteristics and gametogenesis

Due to the constraints of the live-mussel experiment undertaken at the Banyuls-sur-mer Marine Observatory (co-occurrence of two species, low fecundity, no sure means of species ID in real time), released oocytes from both species were inevitably pooled for fertilisation experiments. This, and a rather *ad-hoc* means of temperature control (use of tap-water baths), evidently presented some problems for maintaining larvae alive. Consequently much of the work that was envisioned, in relation to embryonic and larval development (see section 2-1, p. 107), was not realised fully. However, as only one oocyte type was obvious during oocyte collection and examination, this suggests that either the oocytes of both species are impossible to discriminate, or only one species spawned oocytes in significant numbers. The latter seems implausible since I was witness to several of the individuals spawning directly (although diameters of unreleased oocytes varied significantly between species). The net result is that gamete characteristics and estimates of fecundity had to be pooled across both species (Table 7.1). Gamete sizes were similar to those previously cited for *I. (s.s.) modiolaeformis* (Gaudron et al. 2012, having accounted for fixation effects) and are in keeping with recorded values for the prodissoconch I (representative of larval hatching size; see Table 7.1). The mean, released oocyte diameter of 59 µm, suggests that a minimal reserve of energy is invested in these oocytes (Young 2003), and goes some way to explain how relatively small specimens can spawn repeatedly and produce an average of ≈ 300 oocytes individual^{-1} (Table 7.1). Measurements of gametes were also made upon fixed gonad tissue in the current research, in both preserved mussels of each species from the live experiments and on the limited *I*. (*s.s.*) *modiolaeformis* adults available from the eastern Mediterranean and *I.* (s*.l.*) *simpsoni* adults from the Setúbal Canyon. Results from these measurements overlapped for oocytes and spermatids/spermatozoids within each species, consequently intraspecific gamete values for wood-associated mussels were pooled (regardless of site or embedding protocol, Table 7.1). As no *I.* (s*.l.*) *simpsoni* females from the Setúbal Canyon were analysed within the framework of this PhD, no comparable oocyte diameters exist at this location. Preserved gamete sizes for *I*. (*s.s.*) *modiolaeformis* measured within the current research consistently fell below those recorded for this species at seeps (Gaudron et al. 2012). However, this is suspected to be an artefact, either due to slight differences in sample processing or marginal variance in microscope calibrations. Spermatozoid acrosome lengths were basically identical in both species, while unreleased 'mature' oocyte diameters for each species differed significantly (Table 7.1). Given that this conflicts somewhat with the inferred homogeneity in released oocyte diameters across both species, it may indicate that the oocytes deemed mature in *I*. (*s.s.*) *modiolaeformis* specimens, may not have been developed fully (these were however, the largest oocytes retained subsequent to spawning).

Gametogenic and spawning periodicity were both thought to be semi-continuous based on the minimal amounts of connective acinal or inter-acinal tissue, which suggests active gametogenesis as opposed to the storage of gametes (Le Pennec and Beninger 2000), and on the capacity to spawn multiple times (live experiments, possibly indicative of dribble spawning). Certainly mussels were ready to spawn in

June of 2012 and May 2013 in the Gulf of Lion. However, this assessment remains speculative in the absence of time series sampling that might identify relative changes in gametogenic patterns (e.g. such as in Tyler et al. 2007a), or changes in the amount of inter-acinal tissue or the thickness of the acinal walls, indicative of seasonal gametogenesis (Le Pennec and Beninger 2000).

7-2.3. Theme 2a: Larval developmental biology subtheme

The objectives of both the larval and post-larval development subthemes were the most ambitious of the current research. Of the two, the latter was met most fully by the research presented herein. The objectives of the larval developmental work were well met at the design stage but fell short in terms of useable results. Preparations for live observations were considerable. As a part of this, aquaria were designed and constructed based on several previously validated systems both academically and in commercial hatcheries. The resulting tanks included holding aquaria, which were used successfully during the observational experiments (Section 3-3.1.1, p. 130) and larval post-hatching tanks, which though built and tested, were ultimately not employed. Unfortunately due to the constraints imposed by the facilities, the species under examination and of working alone when employing the fertilisation protocol, larval development observations remained unattainable. During this process however, a great deal was learnt about other behavioural aspects of these species which proved highly informative, principally in relation to nutrition (section 7-2.5.3, p.264). This first attempt though not wholly successful, had provided insight and impetus to try to repeat the fertilisation protocol based on what had been learnt. This was scheduled to be repeated in dedicated, temperature-controlled, filtered, closed-aquarium systems in the University of Aveiro and employing a monospeciate assemblage (together removing several of the constraints previously encountered). However, the necessary coincidence of suitable facilities and test organisms stipulated in the objectives of this research was not realised (see Methodology section 5-3, p. 207). However, a limited amount of data concerning adult reproduction (see previous section), fertilisation and early embryonic development were gathering during the course of the live spawning experiments performed in Banyuls-sur-mer (Table 7.1).

7-2.3.1 Fertilisation rates and embryogenesis

Fertilisation rates were low, despite a 1:1000 oocyte-to-sperm mixing ratio. A combination of working alone (gametes remained on ice for an overly long period of time) and the pooling of oocytes from two species are likely to be the principal causes for this, rather than any natural biological phenomenon. In addition, a large proportion of fertilisation incidences will have been hybridisations, and thus doomed from the start. However, the formation of a fertilisation membrane, a vitellin jelly coat, and subsequently the first polar body were identified following to gamete mixing. As far as it went, embryogenesis was relatively slow, with the 4-cell stage being first identified at around 11 hours (about double the time taken in shallow-water *Mytilus* spp.), but typical of deep-sea Bathymodiolins (Arellano and Young 2009). Cleavage patterns

appeared to be spiral based on Hoechst staining. Embryonic development beyond the 4-cell stage did not occur, as the embryos died following these observations.

7-2.4. Theme 2b: Post-larval developmental biology

The objectives set out for this subtheme were met almost entirely in full. In only two instances were objectives not met. The first was in attempts to use immuno-labelling to track the migration of the primordial germ-line on its course to forming gonads in mature individuals. This was trialled at the University of Caen, in collaboration with Anne-Sophie Martinez. However issues concerning either the methacrylate embedding medium or a *vasa*-like gene mismatch (the oyster vasa-like gene marker was employed, based on its availability at that lab), could not be overcome. This was instead inferred from cell-clumps which could not be assigned to another tissue type (see Chapter 4, p. 163). The second, which was to use larval shells for possible chemical analysis (e.g. trace-elements or stable-isotopes analysis) was limited technically and by sample availability (insufficient volumes of shell). However, a considerable quantity of new data is now available regarding the developmental biology of both *I*. (*s.s.*) *modiolaeformis* and *I.* (s*.l.*) *simpsoni* (Table 7.1). These were based on size-frequency distributions (*I*. (*s.s.*) *modiolaeformis*), larval-shell measurements (prodissoconch I and II) and descriptions of developmental patterns in key anatomical features with increasing size, considered prudent to the survival of these mussels in reducing habitats (Table 7.1).

7-2.4.1 Larval biology, inferred from larval shell characteristics

The examination of vestigial larval features in post-larval, juvenile and adult shells revealed a consistent trend in both *I*. (*s.s.*) *modiolaeformis* and *I.* (s*.l.*) *simpsoni*, regarding the relative dimensions of the prodissoconch I (a proxy for larval hatching size, Lutz et al. 1980) and the prodissoconch II (a proxy for size at settlement and metamorphosis, Lutz et al. 1980). In both species, the prodissoconch I was easily identifiable as a result of its pearly white appearance, while the prodissoconch II could be discerned due to its red/orange coloration. Thus their measurement was possible under a high magnification using a dissection microscope only. Despite this potential technical limitation, values recorded were in close agreement with those found for these species in the literature (Gaudron et al. 2012; Ritt et al. 2012), in individuals from quite different habitats (Table 7.1) although the ratios of prodissoconch II SL and SH in *I*. (*s.s.*) *modiolaeformis* collected from wood (those cited in table two are from the eastern Mediterranean only), appeared to be greater than from equivalent measures in specimens from seeps (Gaudron et al. 2012), resulting in a slightly more elliptical larval shell shape. The overall approximate area (and thus volume) of the shells were comparable however. Assuming similar growth rates in the plankton, this suggests similar transport times for *I*. (*s.s.*) *modiolaeformis* larvae settling in the east Mediterranean, regardless of substrate 'choice'. Larval shell dimensions in *I.* (s*.l.*) *simpsoni* were similarly consistent across

habitat types (seeps and bones), though admittedly this is in comparison to a single shell measured by Ritt et al. (2012). In both cases, the small prodissoconch I (and of course, gamete size, Table 7.1) along with the large disparity between hatching and settling size, suggests low maternal energy investment oocyte^{-1} and a long dispersal period in the plankton. This has previously been cited as evidence for a planktotrophic larval phase (Lutz et al. 1980; Arellano and Young 2009). Having the flexibility of feeding in the plankton has both positive and negative consequences, given the energy that must be spent searching and catching prey, and the elevated risks of predation, associated with an extended planktonic duration. However, a precedence for planktotrophic dispersal, within the top 100 m of the water column has been demonstrated rather conclusively (both in terms of physiological feasibly and by direct capture for the larger seep species *Bathymodiolus* (*s.l.*) *childressi*, (Arellano and Young 2009; Arellano and Young 2011; Arellano et al. 2014). In fact the arguments used by Lutz et al. (1980), have since been applied to multiple examples (see 1-9.3.2, p. 70 for details) from the Bathymodiolinae (*s.l.*), suggesting that this trait is a ubiquitous one in the subfamily.

7-2.4.2 Post-settlement growth

Although derived estimates for time-averaged minimum growth rates cited herein make some assumptions about each species' growth, they appear to be in gross agreement with the closest related species available for comparison within the bivalves (see section 4-5.1.2, p. 177). The assumptions of the methodology in Chapter 4 (p. 163 onwards; Laming et al. 2014), rendered estimates of growth in *I.* (s.*l.*) *simpsoni* on cow bones from the Setúbal canyon impossible however, since this represented a stratified random subsample of mussels only (i.e. largest individuals recorded were not available). However, following the approach employed for eastern Mediterranean *I*. (*s.s.*) *modiolaeformis* specimens (section 4-5.1.2, p. 177; Laming et al. 2014), rough estimates of growth could be inferred for *I*. (*s.s.*) *modiolaeformis* and *I.* (s.*l.*) *simpsoni* from the Lacaze-Duthiers Canyon wood deployments as well. These were based on SL in the relatively large male and female adults, but over longer deployment periods. Despite the fact that the largest specimens of *I.* (s.*l.*) *simpsoni* in the current research was not the very largest recovered (Thubaut 2012), comparable growth rates for both species were identified, with those in *I.* (s.*l.*) *simpsoni* being marginally greater and separated by sex (Table 7.1); estimates for each sex used the largest male and female respectively. By way of validation, the growth rates calculated independently for *I*. (*s.s.*) *modiolaeformis* from wood deployments in the NDSF and Lacaze-Duthiers Canyon, were found to be in close agreement. The relatively similar averaged growth rates across quite divergent shell sizes suggests that, much like *I. argenteus* (Dean 1993), growth in these species is unusually consistent with increasing size, a trait that is probably either a function of their lineage, habitat or both. Discerning the contribution from habitat effects however, would require growth data from these environments, which are currently unavailable.

7-2.4.3 Rate of sexual maturation

Identifying the rate of maturation in juvenile mussels represented the central aim as far as developmental biology was concerned. Size at first maturity was assessed based on the smallest size at which all stages of

gametogenesis were identifiable. Despite their markedly different maximum sizes, both *I*. (*s.s.*) *modiolaeformis* and *I.* (s.*l.*) *simpsoni* matured as males at SL a little over 2 mm (NDSF on wood, and Setúbal Canyon on bones, respectively). In the case of *I*. (*s.s.*) *modiolaeformis*, the smallest mature individual identified was 2.35 mm long (approximately 12% of its SL_{max}, Table 7.1), where the gonad tissue was barely discernible and poorly structured, i.e. in its earliest, least-developed incarnation. At this size reproductive output is likely to be low, however as a male this presents less of an obstacle, given the investment per spermatozoid is also minimised. All individuals above this size were both mature and male (Laming et al. 2014; section 4-4.4, p. 171). For *I.* (*s.l.*) *simpsoni,* although a size at first maturity was identified at 2.6 mm (bone-associated Setúbal specimens, approximately 6% of its SL_{max}, Table 7.1), the gonad was considerably more developed, which suggests that a notable period of time has passed since the initial establishment of functional gonad tissue in this individual. As no evidence for gametogenesis was observed in the next smallest individual (2.2 mm), size at first maturity was constrained simply to the size difference between these specimens. By combining growth rate estimates and the size at first maturity, estimates for age at first maturity in *I*. (*s.s.*) *modiolaeformis* were around 4 months following settlement. In the absence of growth estimates for the Setúbal Canyon *I.* (*s.l.*) *simpsoni* mussels, no such value could be estimated for this site. However, given all individuals of this species were mature at the Lacaze-Duthiers Canyon, this suggests a maturation of less than 6 months based on growth rate estimates and the smallest individual found for this species (5.6 mm functional male; Table 7.1), which was already in the early stages of a switch in sex (likely male to female). Collectively these rates of maturation are well within the longevity of larger wood and bone accumulations, based on the literature available (Smith et al. 2002; Smith 2006; Bernardino et al. 2010; Cunha et al. 2013; Yücel et al. 2013), making these mussels well adapted to these temporally finite habitats. However, the estimate for size and age at first maturity in females, and thus first reproduction in the population (i.e. both gametes are available for fertilisation), was not possible for *I*. (*s.s.*) *modiolaeformis*. In *I.* (*s.l.*) *simpsoni*, since the smallest female was already quite large (SL 18.1 mm), size and age at first maturity as a female are both large and late at <18.1 mm (by definition) and <23 months respectively. Given that these mussels represented a subset of the total mussels found on the palmwood deployment, it is quite likely that smaller females not used in the current research existed. This fact, and the likely underestimate of growth (for similar sub-sampling reasons), suggest that these values are likely to be conservative. In effect, this fact holds true for all growth (and thus age at first maturity) estimates cited.

Given the dearth of data available for maturation rates of wood-associated reproductive development in the literature, such rough estimates though based on certain assumptions can still provide direct evidence to support what has otherwise been well-understood in theory, but assumed in practice: like the wood-boring bivalves before them (Tyler et al. 2007b), which establish the apparently necessary preconditions for sulphophilic species to settle, chemosymbiotic mussels reach sexual maturity in an impressively short passage of time.

7-2.4.4 Gill development and growth

Symbionts play a vital role in the lifecycle of *Idas* spp. Of particular interest for this research was the initiation of this symbiosis, in terms of acquisition and localisation. Thus, it was of prime importance that the structures within which these bacteria are housed were investigated. The development and proliferation of gills in *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* was thus studied in detail. In some respects the observed patterns of development echoed those identified in the shallow-water mussel *M. edulis* (Cannuel et al. 2009), particularly the emergence of new gill filaments from the distally located budding zone, and the order of development; the descending lamellae of the inner demibranchs developed first, and grew in length and filament number (Table 7.1), before the initial development of the ascending lamella of the inner demibranch. The onset of ascending-lamella development was at 0.77 mm in *I. modiolaeformis*, similar to that of the same lamellae in *M. edulis* which appear "at < 1mm" (Cannuel et al. 2009). Although the outer demibranch is known to be fully developed in larger *I*. (*s.s.*) *modiolaeformis* adults (5 mm +), the moment at which this begins developing appears to exceed SL 4.37 mm[31], in the mussels examined from the eastern Mediterranean. In *I.* (*s.l.*) *simpsoni*, the initiation of ascending lamellae was not identified at all in the samples from bone deployments in the Setúbal canyon; that said, these observations were based on histology alone, as specimen soft tissues were not examined prior to embedding. By the time the mussels had matured, 31 – 33 filaments in each inner descending lamella had developed, where filament number and SL were directly proportional (4-4.5, p. 173).

7-2.4.5 Acquisition of (or infection by) symbionts

Part and partite to measuring gill development was the examination of associated symbiont densities and if possible, the moment that symbionts were first acquired or first infected the host: the relative degree of mediation of each partner remains unknown. A principal result common to both species was the initial absence of symbionts in post-larval plantigrades. The most parsimonious conclusion is therefore that larvae arriving at settlement sites as pediveligers were probably also aposymbiotic. This suggests strict heterotrophy during larval dispersal, which further supports the likelihood of planktotrophic development, since this remains the only means by which nutritional needs could be met (in the absence of both a yolk reserve and symbionts). Despite being the larger-sized species, *I.* (*s.l.*) *simpsoni* actually acquired symbionts at a slightly smaller size both in absolute and relative terms: SLs were 0.43–0.51 mm as opposed to 0.60 mm in *I*. (*s.s.*) *modiolaeformis*, representing quite different proportional changes in shell sizes following settlement (i.e. % increase in SL when compared with the Pd II size; Table 7.1).

[31] the largest individual at 6.54 mm was not entirely sectioned due to time constraints, and may have possessed the beginnings of outer demibranchs at its most posterior region

7-2.5. Theme 3: Dynamics of nutrition

In terms of the initial objectives, much of what was decided at the outset was achieved, with the exception of some of the more refined aspects of FISH analyses. Using basic histological and targeted *in situ* hybridisation techniques symbionts were localised and patterns in the symbiotic association with size were documented.

7-2.5.1 Qualitative symbiont densities with size

Following symbiont acquisition which was in very small juveniles (Table 7.1), the number of bacteria to be found on the abfrontal and lateral non-ciliated regions of gill filaments depended strongly on species and/or habitat (Table 7.1). The terminology may be considered as follows. *Very low* indicates a sporadic distribution of individual bacteria; *low* represents a layer on filament that averages ≈ 1-2 bacteria deep; *moderate* implies a layer consistently >2 bacteria deep but where individual bacteria remain visible and; *high* assemblages are so dense that bacteria cannot be individually discerned, instead forming a fluorescent 'cloud'. The rare instances of *Very* High, concerned the situation where no inter-filamentary voids were visible, i.e. the bacteria located on adjacent filaments were in direct contact, effectively forming a continuum. In *I.* (*s.s.*) *modiolaeformis* in the eastern Mediterranean specimens displayed very low numbers of symbionts at first acquisition and increases in densities though witnessed, were quite slight, attaining low densities in young adults only. In contrast, once they appeared densities of symbionts in *I.* (*s.l.*) *simpsoni* recovered from bone quickly increased with increasing SL, reaching moderate levels long before maturation, with mature mussels displaying borderline-high symbiont densities. This is all the more extraordinary when taking the relative sizes of *I.* (*s.l.*) *simpsoni* collected from bones versus those collected from wood (bone: 0.51–3.74 mm; oak and palmwood: 3.75 – 24.2 mm). On these substrates, *I.* (*s.l.*) *simpsoni* were exclusively adult, and yet general densities appeared to be moderate based on DAPI analysis (FISH proved problematic when investigating *I.* [*s.l.*] *simpsoni* from wood). Without equivalent sizes of each mussel species on alternative reducing habitats, it is not possible to disentangle the various factors which have led to the widely different densities witnessed in both species. However, such factors probably relate to the availability and reliability of electron-donor and free-living bacterial supply at each habitat, with some species-specific confounding factors. The low number of symbionts in the eastern Mediterranean mussels suggests that despite methane seepage occurring in the area of wood deployment, reducing fluids (or free-living symbionts) were in low supply or, they had dissipated before reaching the CHEMECOLI. From a nutritional point of view it's thus hard to know how such densities alone could possibly sustain the mussel's metabolic needs. Unfortunately stable isotopes analyses of pooled post-larval and adult soft tissue were of insufficient mass to be analysed, despite trying. So no direct evidence exists regarding possible nutritional sources.

7-2.5.2 Symbiont diversity

Using oligonucleotide probes previously designed to target the six known phylotypes for *I*. (*s.s.*) *modiolaeformis* (Duperron et al. 2008) as well as an additional *Bathymodiolus* spp. thiotroph phylotype, BangT-642 (Duperron et al. 2005), symbiont diversity was assessed in a subset of the mussels collected from the NDSF (wood and alfalfa-filled CHEMECOLI), Lacaze-Duthiers Canyon (oakwood, 2012; palmwood and wood-filled CHEMECOLI, 2013) and Setúbal Canyon (on cow bones). The level of diversity documented in *I*. (*s.s.*) *modiolaeformis* associated directly with methane-seeping carbonate crusts in Pockmarks Central 2A region of the NDSF, eastern Mediterranean (up to 6 symbiont phylotypes, Duperron et al. 2008) was never identified in *I*. (*s.s.*) *modiolaeformis* mussels in this research, including those from the same region, but colonising wood. Despite the fact that these individuals occurred in a similar region, only a single T1-thiotroph phylotype was identified (not presented, signals very low). In the western Mediterranean, *I*. (*s.s.*) *modiolaeformis* recovered from oakwood deployments in the Lacaze-Duthiers canyon in 2012[32] were found to house at least two phylotypes, T1-thiotrophs and M1-methanotrophs. Of the two the T1-thitrophs were present at very high densities, raising some questions about how efficiently water can flow through filaments under bacterial saturation. Methanotrophs were at low to moderate densities located at interior regions of the gill, thought to be close to the dorsal gill axis (orientation of dissected gill tissue was difficult to confirm). The presence of such high symbiont levels suggests that the supply of sulphides (and presumably significant traces of biogenic methane) were considerable under the specific conditions experienced by those mussels tested for bacteria (3 of 30 *I*. (*s.s.*) *modiolaeformis* collected that year). Oakwood samples were contained with netting and placed on the seafloor, so it is thought that sulphides and methane production were through sulphate-reducing bacteria and methanogens internal to the wood (Fagervold et al. 2013; Yücel et al. 2013) and possibly in the underlying sediment and faecal matter of *Xylophaga* spp. (Fagervold et al. 2014).

Curiously, the two specimens of *I.* (*s.l.*) *simpsoni* collected from the same oakwood substrata experiments (though not on identical pieces of wood) were found to contain moderate levels of putative bacteria, however the phylotypes could not be confirmed with FISH, despite identical fixation, probes and hybridisation conditions. Since the general eubacterial probe employed only weakly hybridised with bacteria, this suggests that issues persisted with the specificity of hybridisation rather than the presence of novel bacterial phylotypes. FISH and DAPI were inconclusive (but suggested a surprisingly low number of symbionts) for the larger palmwood specimens used in the live fertilisation experiments. These specimens had been kept alive in aquaria for several weeks during spawning induction trials (Section 3-4.4.3, p. 144) with access to unfiltered seawater (due to the constraints of the facilities). Access to particulate organic matter from shelf seas (inlet supply), rather than more refractory material within the canyon, may have

[32] the year prior to samples collected for live experiments

contributed to a certain degree of symbiont loss and a switch in trophic dominance from chemosymbiosis to heterotrophic filter-feeding. Issues were also initially encountered when attempting to identify the phylotypes of highly abundant putative bacteria in *I.* (*s.l.*) *simpsoni* from cow bones in the Setúbal Canyon. The level of auto-fluorescence in gill sections suggested that formalin fixation had been overly long (performed remotely by a colleague). However, following an extended stint in the freezer, slides responded better during examination. Generic eubacterial-probe signal was high enough to overcome backgrounds levels, demonstrating that at the very least, the observed accumulations on non-ciliated regions of the gill filaments noted in histological sections, were eubacterial in origin. In some larger individuals TI-thiotrophs hybridisation also took place and to a lesser extent in juveniles too, though signals were of lower intensity, which may reflect a reduced number of or activity in bacteria present.

Consequently, while it was possible to establish some idea of symbiont diversity in *I*. (*s.s.*) *modiolaeformis* (though hampered by low densities in juvenile East Mediterranean mussels), the question of diversity in *I.* (*s.l.*) *simpsoni* remains unresolved. Diversity in *I.* (*s.l.*) *simpsoni* does appear to be lower than in *I*. (*s.s.*) *modiolaeformis* based on the limited data available (Ritt et al. 2012; this research), however as the results of the current research and previous work demonstrate (e.g. Duperron et al. 2008 *versus* Rodrigues et al. 2013), a larger picture of general levels of diversity can only be established with sampling at multiple sites. It's also worth noting that none of the FISH analyses herein (and in other studies) accommodated the possible role played by archea in mussel symbioses, though such archeal-eukaryotic associations are not known in bivalves, being (currently) described in the Porifera, Ciliophora and Protista only (Wrede et al. 2012).

7-2.5.3 Observations related to heterotrophy, assimilation, digestion and excretion

In adults of both *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni*, filter feeding behaviour was observed, where particles were actively transported to the buccal region by the gills, along the ventral particle groove. Mucous entrainment was not evident in the frontal regions of the gill, suggesting that particle entrainment relied on fluid dynamics, as in shallow-water mytilids (Jørgensen 1981; 1990). At the mouth, particles were ingested in advance of mucous accumulation which was seemingly not ingested (the degree of mucous is likely to have been stress related, Jørgensen 1990). Observations made of the extent of the digestive system in both species, but particularly *I.* (*s.l.*) *simpsoni* in which the intestinal tract appeared to loop more than once, indicate that the capacity to assimilate food, within the stomach (style-sac was putatively identified but the style was not preserved) and in the digestive diverticula. Equally in most cases, contents in the hind-gut were identified either histologically as heavily stained, optically fractal material, or as dark coloured regions during dissection (material compaction made identification unambiguous). Nephridia were also putatively identified in some instances, and certainly in the larger *I.* (*s.l.*) *simpsoni* where their organisation rendered them conspicuous among other tissues. No gastric shields were seen in any specimens. Thus anatomically and – crucially – behaviourally, both species are capable of, and engage in

facultative heterotrophy (Table 7.1). This is supported (or at least not contradicted) by the $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures identified for each species. In *I*. (*s.s.*) *modiolaeformis* from oakwood (2012), signatures were in agreement with the dual symbiotic state identified during FISH (though nitrogen may be photosynthetically-derived), while the pooled sample from wood-filled CHEMECOLIs (2013) indicated a heterotrophy-dominated mixotrophic state. In *I.* (*s.l.*) *simpsoni*, signatures identified for specimens on palmwood suggested mixotrophy (or an unidentified dual symbiosis), in comparison to the thiotrophic nutrition of this species at seeps (Ritt et al. 2012; Section 3-3.4.3, p. 135; Table 7.1). The $\delta^{15}N$ signatures were usually high for *I.* (*s.l.*) *simpsoni* on palmwood, but akin to their substrate, suggesting locally derived nitrogen, perhaps from free-living heterotrophic microbes somehow feeding on the palmwood directly.

7-2.5.4 Trophic mode during lifecycle

The changing patterns of symbiont densities in the gills of mussels during development and the heterotrophy-related data generally, allow some overall inferences to be made concerning nutrition across ontogenetic shifts. As larvae, both species are likely to be heterotrophic, based on the absence of symbionts in post-larval plantigrades. Depending on the species, but more probably on the habitat conditions, symbiont densities appear to increase with growth. At some point during late juvenile or early adult life, densities are believed to be sufficiently high to represent the dominant source of nutrition, particularly in *I.* (*s.l.*) *simpsoni* bone-colonisers. The evidence together suggests that symbiont diversity and densities are highly variable, and that habitat conditions may ultimately determine what the relative contributions are from chemosymbiosis and heterotrophy. Consider that, with moderate to high symbiont densities as adults, *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* colonising seeps are likely to rely almost entirely on chemosymbiotic nutrition (Table 7.1). Using this as a benchmark, symbiont densities in adult *I*. (*s.s.*) *modiolaeformis* on wood are highly variable, from the dense thiotroph associations found in specimens recovered from oakwood in the Lacaze-Duthiers Canyon, to the low densities on pinewood and alfalfa from the NDSF, eastern Mediterranean and in small adult *I*. (*s.s.*) *modiolaeformis* found in the Lacaze-Duthiers Canyon, the latter being known to engage in filter-feeding. In *I.* (*s.l.*) *simpsoni* found on wood (Lacaze-Duthiers canyon), stable isotopes results, moderate densities of putatively identified symbionts in adults (excluding those used in the live experiments), and the filter-feeding behaviour observed in this species below a certain size, all point towards a mixotrophic diet. Finally, while there is likely to be a heterotrophic component to the diet of *I.* (*s.l.*) *simpsoni* on bone falls, the evident abundance of symbiotic bacteria even at early stages of development, suggest that chemosymbiosis forms the primary source of nutrition within this habitat (Table 7.1)

7-3 The flexibility of mixotrophy

Prior to the research undertaken in this thesis, little or nothing was known of several aspects of the lifecycle biology of small-sized mussel species, which colonise a variety of reducing habitats. Through this research several key attributes can now be described for the first time (though some have been inferred in literature

previously). The adaptability of *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* to their various environments is astounding and unquestionable. It helps to explain their collectively wide geographical and bathyal distribution, their capacity to take advantage of multiple reducing habitat types, and their ability to adapt to the heterogeneity of these environments. In the latter case, this seems to be in part, through a retained level of motility (a trait common to loosely analogous shallow-water counterparts) which is thought to facilitate access to reduced compounds for chemosymbiosis. In particular however, it is their capacity to supplement chemosymbiotic nutrition with facultative filter feeding that permits such extraordinary flexibility.

7-3.1.1 The lifecycle of *Idas* (*s.s.*) *modiolaeformis*

In terms of what we can now state definitively or infer with confidence, the post-larval to adult period of these mussels lifecycle has benefited the most form this research. To help visualise what has been established for *Idas* (*s.s.*) *modiolaeformis* (as an example), I return to the lifecycle of the bathymodiolins presented in the introduction (Figure 7.3). In this case the main details summarised in Table 7.1 are included as annotations and as two anatomical schematics, based on multiple dissections. In effect, within a short period of time after settling and metamorphosing on wood (≈ 17 days), mussels have already acquired their symbionts. Mounting symbiont densities are compounded by an exponential increase in filament surface area as the gills develop allometrically (Chapter 4, p. 163), and the ascending lamellae begin to develop (≈ 27 days). With the energy provided by chemosymbiosis and filter feeding the race towards maturity as males is at a relatively fast rate of growth, in comparison to analogous species. So it is that within 4 months, mussels have matured as males. By 8.5 months, bacteria have become isolated to gill filaments only, presumably mediated by the host. As a protandric species, a period of time passes as the mussels grow, before an eventual switch in sex occurs (seemingly after at least 12 months), which requires a brief period as a hermaphrodite. As yet, the exact timing of this switch in sex is not known for either species on wood, however at seeps all functional *I*. (*s.s.*) *modiolaeformis* females were above SL 7 mm while the largest hermaphrodite had a SL 11.6 mm. If such sizes are typical of the species generally (i.e. independent of growth, then the first females could likely have appeared after 13.5 months (using the growth rate 0.017 mm d^{-1}), while the most delayed switches in sex would be expected to occur at around 22 months (employing a SL 11.6 mm), assuming immediate settlement in both cases.

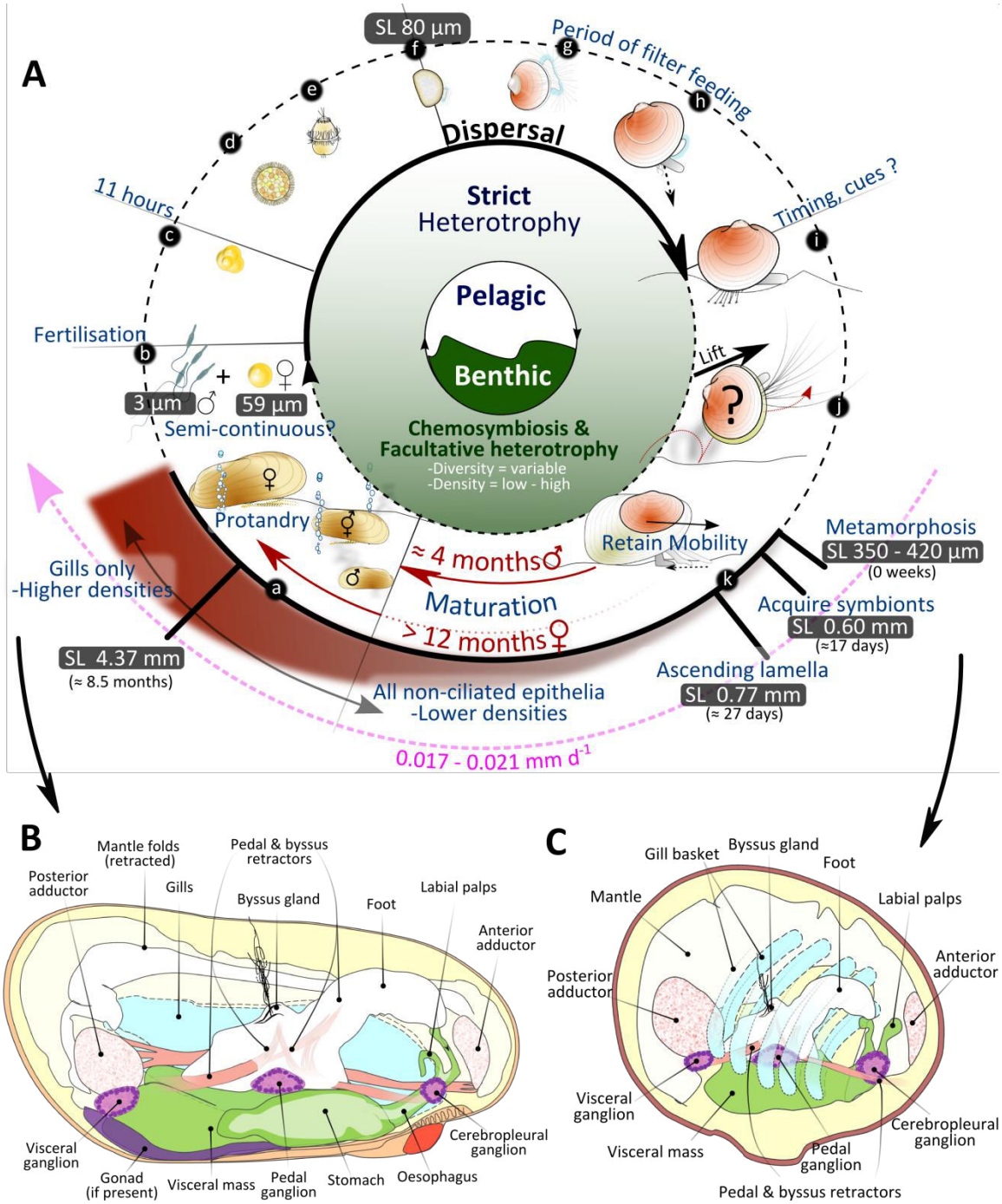


Figure 7.3 The lifecycle of *Idas* (*s.s.*) *modiolaeformis*

Having reached size or age at first reproduction (i.e. a combination of males and females present at once), adults are thought to produce and spawn gametes semi-continuously throughout the year. Presumably intraspecific larviphagi is prevented somehow. Following fertilisation, if embryonic development is anything to go by (i.e. 11 hours for 4 d-cell formation), larval growth is probably slow (Arellano et al. 2014).

7-3.2. Gigantism, Miniaturisation, nutrition and maturation

The geological age of habitats is probably vents→seeps→wood→whale. However in terms of the evolution of the Bathymodiolinae, fossil records suggest that the mussels appear on seeps first, then wood habitats very closely followed by whale associations when whales became large enough or lipid-rich enough to support sulphophilic organisms, and finally, at vents. It is not hard to imagine an *Idas*-like ancestor with a single bacterial association taking one of two paths in terms of evolution from the Early to Middle Miocene onwards (Lorion et al. 2013); either down the avenue of gigantism with a dependence on reducing fluids and a reduction in gut functionality, or down the avenue of paedomorphism, retention of full filter feeding capacity and the evolution of superior symbiont flexibility in order to cope with more unpredictable highly ephemeral organic fall conditions. These trends are supported by remarkably consistent patterns of size within the fossil record. These show that seep-colonising mytilids were less than 50 mm in length until the Early Miocene (Amano and Little 2005; Kiel et al. 2010; Saether et al. 2010; Amano and Jenkins 2011), and then their dimensions abruptly increased from SL 10 cm in the Mid-Miocene to more than 30 cm in extant species. This flexibility in the subfamily (*s.l.*) as a whole has resulted in members being represented at nearly all types of reducing habitat. Even in the larger-sized bathymodiolins that have sacrificed some complexity in their gut for a greater reliance on symbionts, filter feeding still persists (Page et al. 1990; Page et al. 1991). This behaviour may be a widespread trait in the subfamily, but one which has gone out of regular use in some species in the absence of a strict need for it.

Vents and seeps appear to provide higher energy environments with a greater amount of colonisable space, in comparison to typically spatially limited and ephemeral organic falls. These factors may have dictated the extremes that exist in terms of size at these habitats (Saether et al. 2010). Mussels inhabiting relatively sparse organic debris might have benefited from heightened paedomorphism which would constrict the growth of the species, reduce spatial competition and permit the allocation of energy resources towards reaching maturity sooner (Lorion et al. 2013). The current research (and other studies) demonstrate that mussels from organic falls reach sexual maturity quickly and at much reduced sizes (Tyler et al. 2009; Ockelmann and Dinesen 2011; Gaudron et al. 2012; Laming et al. 2014).

7-3.3. Convergence versus divergence

Developmental patterns of germ-line cell migration, size-at-first-maturity, gill growth and symbiont acquisition and localisation are presented for the post-larva to adult transition. Evidence for protandry supports previous findings (Gaudron et al. 2012) while data on gametes, larval shells and the likelihood of feeding, being aposymbiotic, fit with the assumptions made for many of the members of the subfamily. These aspects are what have made them such an intriguing group to study. Biological traits aside from symbiosis appear to reflect conserved ancestral phylogenetic traits, particularly in aspects of development

(e.g. cleavage patterns, embryology, gross adult anatomy). Equally, some traits have probably evolved subsequently, such as the disappearance of abfrontal cilia and the hypertrophy of gills, both of which are likely to have facilitated an increase in bacterial symbiont densities historically, permitting host organisms to place greater dependence upon their symbionts. The former may be a by-product of neoteny, thought to be particularly prevalent in small-sized Bathymodiolinae (*s.l.*, Génio et al. 2012).

7-3.4. Concluding remarks

Based on the absence of bacterial signals, plantigrades were aposymbiotic, indicating strict heterotrophy in larvae and early post-larvae. This information is of particular utility in dispersal modelling, though currently it is not certain where in the water column larvae principally disperse. Symbiont acquisition may be a critical juncture in the lifecycle of small-sized multi-habitat mussels, but the retention of a heterotrophic condition releases them from an obligation to 'acquire, or die'. As a consequence of their trophic flexibility, the distribution of these mussels is astoundingly widespread. The only apparent constraint is the need for a hard substratum to attach to (a trait that select bathymodiolin species have even dispensed with (e.g. *B.* [*s.l.*] *boomerang*). These observations on early development in *I.* (*s.s.*) *modiolaeformis* represent evolutionary adaptations to their ephemeral, reducing habitats. Extraordinary rates of adaptive radiation in the last 100 Ma suggest some event(s) took place which accelerated speciation, hypothesised to be the initial (repeated and independent) incorporation of free-living bacteria as symbionts in host species. Host speciation is suspected to have been facilitated by the incorporation of symbionts with diverse metabolic capabilities (e.g sulphur-and methane-oxidisers). The flexibility of symbiont associations and retention of filter-feeding and assimilation capabilities certainly furnish both *I*. (*s.s.*) *modiolaeformis* and *I.* (*s.l.*) *simpsoni* with an unrivalled level of flexibility within the Bathymodiolinae studied to date.

7-3.5. Future considerations and perspectives

7-3.5.1 Phylogeny

Family-to-subfamily hierarchy and generic cladistics within the Bathymodiolinae remain contradictory, as a consequence of conflict between classical constraints of taxonomic nomenclature for species described prior to the discovery of reducing environments, and data emerging from state of the art molecular techniques and analyses. Accordingly, the finer details concerning the systematics of the Bathymodiolinae must continue to be elucidated as part of an overhaul to reconcile polyphyletic (e.g. *Idas*, *Adipicola*) and paraphyletic taxa (i.e. the Bathymodiolinae *sensu stricto*) that contain many of the most thoroughly studied species in terms of deep-sea biology and ecology (Jones et al. 2006; Miyazaki et al. 2010; Fontanez and Cavanaugh 2013; Lorion et al. 2013; Thubaut et al. 2013b).

7-3.5.2 The larval/post-larval black box

The bathymodiolins are probably one of the most widespread chemosymbiotic bivalve groups in reducing environments worldwide, and yet they have never been recorded in background abyssal faunal

communities (Duperron 2010; Duperron et al. 2013). This suggests that for these mussels, interspersed soft-sediment environments do not provide refuges and must be traversed in order to populate new reducing habitat sites. This either reinforces the hypothesis that larval dispersal must be considerable in order to maintain the apparent levels of connectivity between distant sites (e.g. *Bathymodiolus* sp., Schultz et al. 2011) or of course, it suggests that reducing-habitat frequency may be greatly underestimated.

Larval development takes many forms, with many species adopting a planktonic phase (over 70% in the Atlantic: Thorson, 1950; Connell, 1961; Milekovsky, 1971; Vance, 1973). Of the larval phases that are planktonic, planktotrophy is the most prevalent mode in marine invertebrates, particularly in the tropics (Thorson, 1950; Pechenik, 1999), but also in deep sea environments (Young 2003). This larval mode requires minimal oocyte investment, as the oocytes hatch quickly into heterotrophic, planktonic larvae with a minimal or absent yolk reserve. Large numbers of young may be produced per unit of energy expended (high fecundity), and the capacity to feed permits an extended period of larval transport, which may facilitate dispersal (Cowen and Sponaugle 2009). However their planktonic prey is dependent on fluctuating environmental conditions, so successful larval development is constrained to periods when food availability is high (Pechenik, 1999). Moreover, the cost of prolonged planktotrophy is in increased mortality from predation and abiotic pressures (e.g. over-dispersal or dispersal into unsuitable environment). Mortality is typically very high at up to 99% before settling (Thorson, 1950; Milekovsky, 1971).

Evidently, with data being accumulated on the distribution and phylogeny of deep-sea metazoan species in chemosynthetic habitats from many of these underrepresented regions, there is an immediate need to resolve how dispersal is acting to influence connectivity within and perhaps between these environments to better understand their resilience to disturbance, particularly in light of the gathering interest in the deep-sea for mining minerals (Van Dover 2011a).

7-3.5.3 Resilience to and recovery from disturbances

Despite the fragmented knowledge available for habitat connectivity and stability in the deep sea, some indications of resistance to disturbance can be surmised based on habitat characteristics and some opportunistic studies of recolonisation of 'nascent' habitat. The resistance of a community to disturbances imposed by human activities will depend on its predisposition to similar levels of natural disturbance. Accordingly, much emphasis has been placed on the need to better describe the patterns of distribution and connectivity, which exist courtesy of larval dispersal in the deep-sea, particularly in spatially isolated reducing habitats. This is an expanding and evolving field, evident for example, from the changeable nature of accepted biogeographic provinces described for vent species globally (Bachraty et al. 2009; Rogers et al. 2012). Clearly, as more sites are discovered and explored, the resolution and thus reliability of the global biogeography for vent species will likely increase. Contrastingly, in hydrocarbon seeps, or at least those located upon the Atlantic Equatorial Belt, isobathyal distance appears to be of less importance than that of depth in determining species composition and similarity between regions, particularly between 1000m and

2000m (Olu et al. 2010), suggesting a degree of connectivity between seep habitats within a given depth range. The level of connectivity across organic falls remains difficult to assess since predicting organic fall distributions and locating them is not as straight-forward as for vents – using known and predicted regions of geothermal activity and then water-chemistry anomalies locally, and seeps – identifying regions with a geologically stable history for organic sedimentation, that are now subjected to some form of sediment destabilisation.

However by layering global remote sensing data depicting the present-day distribution of flood-plain located forests worldwide and the main (i.e. navigable) global network of rivers, it is possible to identify which regions of the planet should see the greatest proportion of coastal efflux of wood material (refer to *Introduction* Figure 1.7).

7-3.5.4 Moving forward: facing the challenges head-on

Current initiatives (German et al. 2011) that have largely arisen in the wake of the Census of Marine Life (CoML) and particularly the Chemosynthetic Ecosystem Science field project (or ChEss), are focused on the expansion of our understanding of basic larval biology, including the supply of larvae to newly available substrate for colonisation (e.g. Serpent project through INDEEP), ecosystem function and environmental characterisation (e.g. Interridge- specifically examining vent systems in the Pacific Ocean) and the biodiversity and distribution of deep-sea species in these habitats (e.g. several European projects such as the recently finished Hotspot Ecosystem Research and Man's Impact On European Seas (HERMIONE project), and DOSMARES, a Portuguese-Spanish collaborative research project examining deep-water submarine canyons and slopes in the Mediterranean and Cantabrian seas). In the framework of these initiatives, the expansion of our understanding of larval biology in deep-water environments ought to be possible and indeed encouraged. Greatest gain in this area would include characterisation of dispersal potential (e.g. the pelagic larval duration coupled with oceanographic models) in conjunction with realised dispersal of keystone species (derived from genetic connectivity and elemental fingerprinting). This would not only provide two independent measures of connectivity, which can be co-validated against one another, but it also would help determine the degree of export or retention at natal sites. In addition, through the application of elemental marker techniques it may be possible to begin mapping population connectivity within the deep-ocean and help inform networks for marine protected areas (Christie et al. 2010; Van Dover 2011a). Finally the characterisation of larval nutrition during its pelagic phase, using isotopic and nutritional analysis may yield exactly the information required to better define the conditions needed for successful *in-vitro* larval rearing to characterise development. Once this is possible, many of the other larval traits for which empirical data are now emerging in shallow-water species, may eventually be categorised in deep-water descendents, such as swimming behaviour, behavioural responses to environmental stimuli in terms of orientation both during dispersal and at competency and the

consequences of stress, tolerance and parental effects on larval development. Indeed the next decades of deep-sea larval biology can only be improved by such advances.

Deep-sea marine science is now at a critical juncture in its history. The advent of technology for scientific exploration was only made possible by the investment of telecommunications companies and those interested in offshore hydrocarbon fuel extraction. Ironically, it is through these avenues of development that ROVs have become so accessible to scientific ocean exploration. Deep-sea environments are subject to multiple levels of environmental impact, including deep-sea fisheries and imminent mineral-extraction exploration using huge underwater drilling apparatus. This comes at a time when we still have so much to learn and gain from describing these habitats more definitively (Van Dover 2011b).

At present, a niche exists for new researchers to fill: that of the deep-sea larval developmental biologist. Such a research niche is rare in biological studies and whilst the challenges it represents are not insignificant, the importance of the potential results of such research cannot be overstated. Studies examining coastal systems, which have acted to shape the way in which we approach the discipline of larval biology, reproductive kinetics, supply, recruitment into the adult population and thus the sustainability of community compositions remain seminal pieces of marine scientific literature (e.g. Hatton 1938; Coe 1953; Connell 1961; Thorson 1966; Woodin 1974; Gaines and Roughgarden 1985; Roughgarden et al. 1988; Connolly et al. 2001). Prof. Craig Young, one of the most experienced and influential researchers to have invested several decades of his academic career in the study of larval and reproductive biology, alongside many up and coming deep-sea researchers whose field of expertise resides in reproductive biology, have recently reiterated the need for research into the larval biology of deep-sea organisms. The dearth of such researchers was palpable at the last Deep-sea Biology Symposium in December 2012, where only one researcher, Prof. Craig Young himself, presented any data which actually incorporated empirical larval biology (within the context of a modelling approach, Young et al. 2012). Addressing the lack of investment in this research discipline in deep-sea organisms, could significantly contribute to our understanding of the dynamics of deep sea benthic environments, as they has in shallow water systems. Prof. Craig Young put it quite succinctly, when he was heard to say:

"... Genetics cannot answer *every* question"

7-4 References for Chapter 7

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