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Adapted to change: The rapid development of symbiosis in newly settled, fast-maturing chemosymbiotic mussels in the deep sea

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ABSTRACT

Symbioses between microbiota and marine metazoa occur globally at chemosynthetic habitats facing imminent threat from anthropogenic disturbance, yet little is known concerning the role of symbiosis during early development in chemosymbiotic metazoans: a critical period in any benthic species' life-cycle. The emerging symbiosis of *Idas* (*sensu lato*) *simpsoni* mussels undergoing development is assessed over a post-larval-to-adult size spectrum using histology and fluorescence *in situ* hybridisation (FISH). Post-larval development shows similarities to that of both heterotrophic and chemosymbiotic mussels. Data from newly settled specimens confirm aposymbiotic, planktotrophic larval development. Sulphur-oxidising (SOX) symbionts subsequently colonise multiple exposed, non-ciliated epithelia shortly after metamorphosis, but only become abundant on gills as these expand with greater host size. This widespread bathymodiolin recorded from sulphidic wood, bone and cold-seep habitats, displays a suite of adaptive traits that could buffer against anthropogenic disturbance.

1. Introduction

The growing scarcity and increasing extraction costs of consumable resources both on land and in shelf seas, has generated renewed commercial interest in the exploration and exploitation of deeper marine resources (Ramirez-Llodra et al., 2011). Communities identified to be under imminent threat include submarine benthos, which often include an organic-fall component. Organic falls provide localised influxes of labile organic matter and nascent substrata to the seabed that develop into highly ephemeral, ecologically complex habitats (e.g. megafaunal remains, vegetative debris, Smith, 2006; Bernardino et al., 2010; Laurent et al., 2013), however the conservation status of large-sized organic falls is one of the most poorly defined. In order to better understand the risks that human activities might pose to organic-fall habitats, there is an immediate need to first assess the degree of adaptability displayed by their associated communities.

Organic falls undergo physical and chemical transitions during degradation, culminating in a 'sulphidic stage', where the activity of sulphate-reducing bacteria drives the net efflux of sulphides (Treude et al., 2009; Laurent et al., 2013). In carcasses, the transitional process includes the breakdown of flesh by a combination of microbial degradation, microphages and mobile scavengers, the release of sulphides during the microbial decomposition of lipid-rich marrow, and the colonisation of exposed skeleton by sedentary symbiotic metazoans (reviewed in Smith, 2006). These can host heterotrophic symbionts, as in the posterior, root-like

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trophosome of bone-eating polychaete worms (genus *Osedax*, Katz et al., 2011), or chemosynthetic symbionts, such as those associated with the gill filaments of bone-colonising mussels (e.g. Southward, 2008; Fujiwara et al., 2010).

The emission of sulphides, H_2, CO_2, and – at times – alkanes, are features of many reducing habitats. Consequently, at higher taxonomic levels certain chemosymbiotic organisms are pervasive. The mussel subfamily Bathymodiolinae is almost ubiquitous across vents, seeps and organic falls globally (Duperron, 2010) and thus represents a keystone taxon. The majority of adult Bathymodiolinae host single or dual symbioses within (i.e. intracellular) or upon (i.e. extracellular) their gill filaments, typically involving chemoautotrophic bacteria, methylotrophic bacteria, or both (Dubilier et al., 2008), with some rare exceptions (no symbionts: *Idas argenteus* Ockelmann and Dinesen, 2011; Rodrigues et al., 2015, multiple symbioses: *Bathymodiolus heckerae* and *Idas modiolaeformis*, Duperron et al., 2007; Duperron et al., 2008). In at least some species, these symbioses have been demonstrated to provide some or all of the host's carbon and energy requirements (e.g. Riou et al., 2008). Bathymodiolins that colonise decomposing bones and plants tend to be smaller in size than other mytilid species (Lorion et al., 2013) and almost invariably possess symbiotic bacteria in their gill tissues (see Duperron et al., 2013 for Atlantic and Mediterranean species). Although most symbioses in these small-sized mussels are extracellular, some evidence for active host assimilation exists (e.g. *Idas washingtonius*, Deming et al., 1997).

One such small-sized species, "*Idas*" *simpsoni* (quotations indicate *sensu lato*), demonstrates a remarkable capacity to colonise diverse substrates under varying reducing conditions, having first been discovered on megafaunal skeletons (earliest: Marshall, 1900), on sunken wood (earliest: Marshall, 1901), and more recently on oil-drill cuttings (Hartley and Watson, 1993) and carbonate crust at a hydrocarbon seep (Ritt et al., 2012: identified as "*I.*" *simpsoni* post-publication by Génio et al., 2014; based on molecular data from Thubaut et al., 2013b). These habitats range from the North Atlantic to the Marmara Sea (Mediterranean) and from 73 to 1120 m in depth. "*Idas*" *simpsoni* therefore appears to be well-adapted to the varying conditions found at reducing habitats, though limited evidence has been accumulated to confirm why this is the case. This level of habitat flexibility is very rare in larger bathymodiolin mussels and uncommon in smaller species, excluding *I. washingtonius* and *I. modiolaeformis* (Rodrigues et al., 2015). In *I. modiolaeformis*, a small-sized bathymodiolin that colonises sunken wood and cold seeps, multiple habitat use almost certainly relates to this species metabolically diverse extracellular symbioses (with up to six distinct bacterial phylotypes identified on the gill filaments of one individual, Duperron et al., 2008). In contrast, despite colonising several habitat types where reduced fluid composition are expected to differ considerably, "*I.*" *simpsoni* is only known to harbour extracellular, gill-associated SOX symbionts (e.g. at a seep, Ritt et al., 2012; on mammal bones, Génio et al., 2014; on wood, SR Laming unpublished data), nestled between microvilli that border the gill bacteriocytes (Southward, 2008). A single record of Bacteroidetes (CFB) bacterium also exists, however its localisation remains unknown (molecular data only, Génio et al., 2014). Despite lower symbiont diversity, "*I.*" *simpsoni* has recently been recorded at greater densities than *I. modiolaeformis* where both species co-occur on sunken wood (SR Laming, unpublished data), suggesting that different competitive factors must exist other than the metabolic diversity of symbioses.

The acquisition of symbionts in many bathymodiolins appears to be horizontal (i.e. not inherited vertically from the parental germ-line, Bright and Bulgheresi, 2010), based on an overall lack of symbiont-host co-speciation (Won et al., 2003), the absence of symbionts in adult gonads of at least one species (*I. modiolaeformis*, Gaudron et al., 2012) and the ongoing incorporation of symbionts from external sources (Le Pennec et al., 1988; Wentrup et al., 2014). Accordingly, infection is believed to be environmental via 'free-living' bacteria, lateral from a proximal host (including conspecifics), or both. Identifying the moment at which symbionts first appear in the lifecycle remains challenging however (e.g. Salerno et al., 2005; Wentrup et al., 2013), though the initial symbiont-colonisation phase has been demonstrated in juvenile *I. modiolaeformis* (Laming et al., 2014). Even though early symbiont acquisition and development might provide a competitive angle for "*I.*" *simpsoni* over *I. modiolaeformis*, it is not known when this species' symbiotic association begins – be it before, during, or following settlement and metamorphosis (the latter being true of *I. modiolaeformis*, Laming et al., 2014) – and whether these associations develop further with host growth, as in most other bathymodiolins examined (Salerno et al., 2005; Wentrup et al., 2013; Laming et al., 2014), or remain unchanged (e.g. Streams et al., 1997). More generally, early life-history stages remain poorly understood in most chemosymbiotic bathymodiolin species save for a handful of studies (e.g. Berg, 1985; Arellano and Young, 2009; Génio et al., 2014; Laming et al., 2014). Limited data for "*I.*" *simpsoni* suggest a propensity for semi-continuous and abundant larval supply (Ritt et al., 2012; Génio et al., 2014) and sizes at first maturity equivalent to <5% of the species' maximum size (mature at shell length [SL] 1.8 mm, Génio et al., 2014; maximum SL 45 mm, Warén and Carrozza, 1990).

In light of current and future threats to reducing habitats and the general lack of biological data during the early life-history of bathymodiolins, the current study examined a size-spectrum of "*I.*" *simpsoni* specimens in order to 1) characterise the anatomical development of juvenile "*I.*" *simpsoni* up to maturity; 2) identify whether post-metamorphic "*I.*" *simpsoni* mussels are already symbiotic and if not, at what sizes bacteria first appear; 3) describe how bacterial distributions vary as the association develops with increasing host size, and ultimately; 4) establish whether features of early life-history and adult biology in "*I.*" *simpsoni* might influence its ongoing survival in increasingly disturbed environments.

2. Methodology

2.1. Sampling and processing

In 2011, five bovine carcasses attached to concrete ballast were deployed at 1000 m depth in the Setúbal Canyon, located on the western Portuguese margin (38°16.85′N, 09°06.68′W). Details of the experiment may be found in Hilário et al. (in press). Some intact bone remains, colonised by a diverse array of fauna including numerous "*I.*" *simpsoni*, were recovered 18 months later (Nov. 2012 by ROV *Luso*, details in Génio et al., 2014; Hilário et al., in press). A random, size-stratified selection of these mussels from post-settlement to adult was used in the current study, fixed in 4% formaldehyde (in filtered seawater) for 2–4 h and preserved in 96% ethanol following serial transfer (70%, 80%, 96%, stored at room temperature).

2.2. Shell and preliminary soft tissue analysis

Shell dimensions recorded were for the prodissoconch I – the earliest-formed larval shell (PdI, n = 24), the prodissoconch II – the larval shell of the settling pediveliger (PdII, n = 38), and for entire specimens (i.e. including any deposition of juvenile 'dissoconch' shell, $n = 38$). Individuals were measured and photographed using a calibrated, camera-mounted dissection microscope (Nikon Elements, Japan). Shell heights (SHs) x lengths (SLs) of PdII and whole shells were measured according to Laming et al. (2014). PI SL was measured parallel to the vestigial provinculum (SH not taken). Shell

margin limits were distinguished by colour (see Fig. 1). Dissections were performed microscopically using custom-made tools (see Laming, 2014; Laming et al., 2014). General anatomy was recorded and tissue was extracted. 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. Counts were plotted against shell size where best-fit analysis was performed to assess gill proliferation as a function of SL. Analyses were performed using Sigmaplot (v. 11).

2.3. Embedding, sectioning and histology

Tissue was blotted dry and infiltrated (8 × 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 h). Orientations (sagittal, frontal and transverse) were equally represented across the size spectrum. Gelatine was removed with hand-hot water.

Resin blocks were wet-sectioned on a Leica EM Ultracut UC6 Ultramicrotome (Germany). Semi-thin 1-μm sections mounted 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. Periodic toluidine staining was performed to identify the cutting-axis and location (≈10-μm

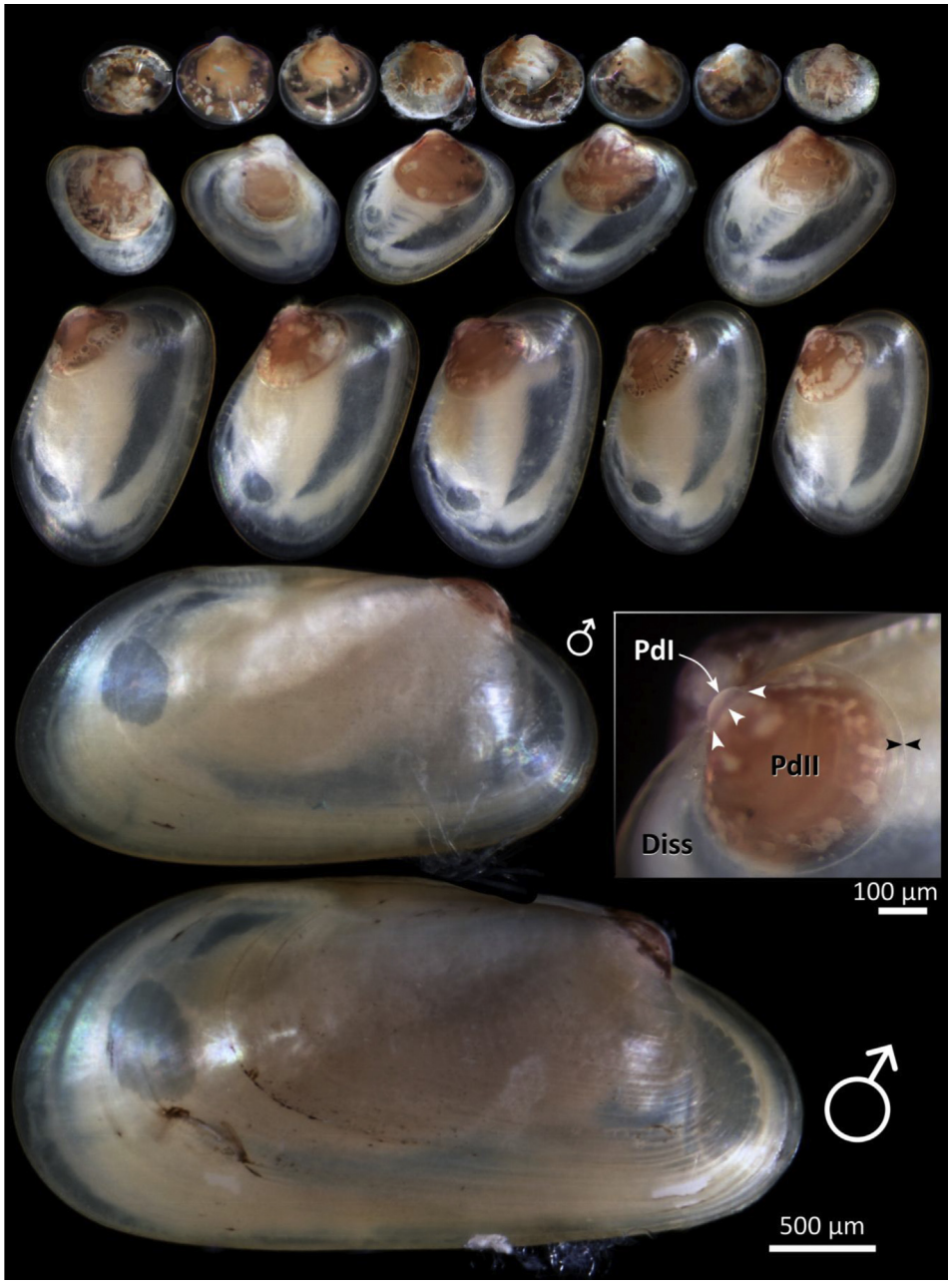

Fig. 1. Micrographs of some "*Idas*" *simpsoni* specimens examined. Micrographs are representative subsample of the total number of specimens examined for this study ($n = 38$). The top 8 specimens are the smallest individuals examined at <500 μm. The smallest mature individual and largest specimen examined are also pictured (small and large ♂ respectively). The inset image displays the regions of shell measured: larval prodissoconch I (PdI), margin indicated with dotted line; prodissoconch II (PdII) margin delineated between back arrowheads, and; the lighter-coloured post-metamorphic dissoconch shell (Diss).

intervals). Standard H & E staining procedures for thick sections were modified for semi-thin LR-white sections (see Laming et al., 2014). Once mounted, slides were viewed under a camera-mounted compound microscope (Evolution VF camera, Media Cybernetics, USA; Olympus BX61, Japan) and micrographs were processed and measured using Image-pro plus (v.5.1).

2.4. Examination of symbiont patterns

Select slide-mounted sections targeting the entire size-spectrum were equilibrated in phosphate buffered saline (PBS 1×), 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. To establish whether previously identified bacterial *Idas*-symbiont phylotypes occur 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 initially designed to target various symbiont 16S rRNA phylotypes identified in *I. modiolaeformis* and *B. heckerae*. These probes target M1 methanotrophs (probe ImedM-138), M2 methylotrophs (probe BhecM2-822), and the two phylotypes of sulphur oxidizers T1 and T2 (probes Bthio-193 and ImedT2-193, respectively, details in Duperron et al., 2008) following the post-permeabilisation steps only of the FISH protocol in Duperron et al. (2005). Slides were photographed as in the H & E analysis, but with a monochrome filter. Gill tissue from Lorion et al. (2012) provided the positive control.

3. Results

3.1. General specimen condition and classification

Larval Pds I and II were pearly white, and transparent red/orange, respectively. The dissoconch was glossy, colourless but iridescent and almost transparent in juveniles, or translucent and fleshy-pink in individuals >2 mm (examples in Fig. 1). Of the 38 mussels, 35 were immature. Five were post-larval 'plantigrades' (i.e. immature with no juvenile 'dissoconch' shell: SL 0.405–0.479 mm), three of which were used for soft-tissue analysis. Though rudimentary, most anatomy pertaining to later development was evident. Small (<1 mm) and larger (>1 mm) juveniles, in which dissoconch growth was observable 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 (e.g. Supplementary Fig. 1). Mean PdI lengths (±σ) were 95.9 ± 6.3 μm. Mean PdII heights and lengths (±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, $n = 38$).

3.2. Developmental patterns

3.2.1. Nervous and sensory system

Nerve ganglia were easily identified in all individuals, on account of their distinctive appearance and relatively conservative size (ganglia diameters ≈50 μm in plantigrades; ≈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 (Figs. 2–4). Prior to dissection, pigment spots were visible within the translucent tissue under the microscope as a pair of anterior black dots, 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 (e.g. Fig. 2B). In serial sections, 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. Fig. 2B).

3.2.2. Alimentary system

Already well-developed in plantigrades, the alimentary system underwent roughly proportional increases in volume and complexity with increasing shell size (Fig. 2A versus 2C). In plantigrades examined histologically ($n = 3$), ciliated labial palps were already well-developed (Supplementary Fig. 2), with a ciliated oesophagus, stomach and digestive gland. The post-larval 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 Fig. 2). It was not clear whether palps were coupled directly with gill bars at this size. The entire alimentary system occupied about a third of the area of the soft tissue in mid-sagittal section (Fig. 2A).

In juveniles, the alimentary system increased in size relative to shell length. Sequential HE sections with detailed annotation from two similarly sized juvenile individuals are presented, cut in the transverse (Fig. 3) and sagittal (Fig. 4) planes. These individuals were representative of general trends observed (summarised using colour schematics: full-page versions can be found in Supplementary Figs. 3 and 4). Labial palps were more elongate than in plantigrades, coupled to the most anterior gill filaments (e.g. Fig. 4A). The longer oesophagus was orientated at a more acute angle to the hinge-line than in plantigrades (Fig. 4A and B). Digestive diverticula flanked the oesophagus and stomach on all sides, but particularly laterally and dorsally (Fig. 3A and B; Fig. 4A). The mid-gut looped back anteriorly almost immediately after its posterior origin from the stomach, with at least one additional intestinal loop in the upper hindgut (Fig. 3A and B). Styles in the stomach could not be identified. The stomach and digestive gland together occupied the majority of the dorsal region (Figs. 3–4).

Alimentary organs and tissues in the three adults identified were the most complex structurally (not shown) and were greatest in size, in proportion with larger SLs. Though dorso-ventrally compressed to accommodate the rapidly expanding gill chamber, the stomach and digestive diverticula still represented a notable portion of the visceral mass in the dorsal region.

3.2.3. Attaining maturity

The first individual to be identified as mature (male) had a SL of 2.61 mm, based on clear evidence of spermatogenesis (Supplementary Fig. 1). However gonad tissue was already highly developed, so this individual may have been mature for some time prior to fixation. Several preceding mussel sizes were examined in detail but no evidence of gametogenesis was visible. The two larger specimens were also identified to be mature males.

3.3. Development of the gills in the context of symbiosis

3.3.1. Gill development

Gill filaments in plantigrades and the smallest juveniles were densely ciliated with only a minimal non-ciliated abfrontal region on the interior face of filaments (Fig. 2B; Supplementary Fig. 2). At first, "*I.*" *simpsoni* plantigrades had two sets of 5–6 rudimentary gill bars, forming a 'gill basket'. These ultimately would have become the first (inner) pair of descending lamellae. Filaments differentiated in the posterior-most parts of the gill axes within a 'budding' zone, and then elongated ventrally as neighbouring posterior

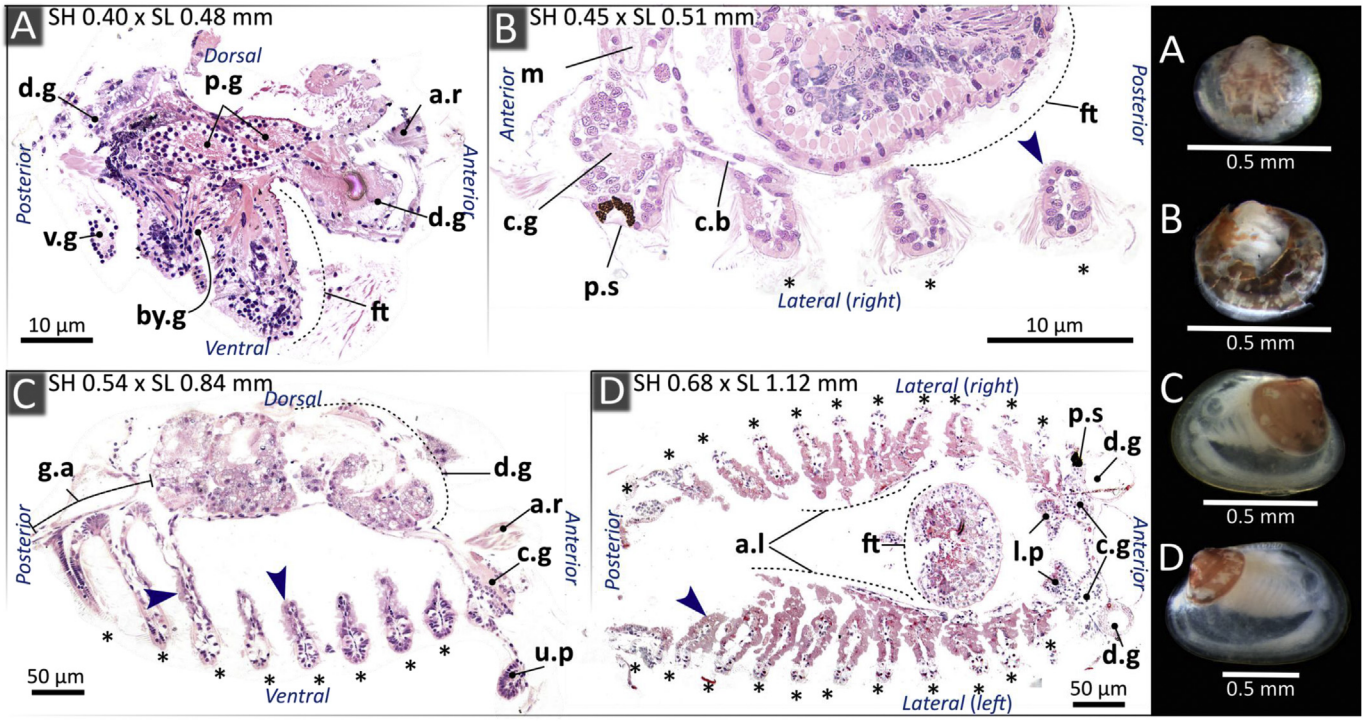

Fig. 2. Overview of Haematoxylin and Eosin-Y developmental histology in *"Idas" simpsoni*. A) Post-larval plantigrade in mid-sagittal section. B) Right lateral view of ventral region of small juvenile, in frontal section. C) Lateral region of a larger juvenile in sagittal section. D) Ventral region of a larger juvenile in frontal section. Arrowheads indicate bacteria identified during HE staining. Shell micrographs are prior to dissection of specimens [A–D]. a.l *ascending lamella*, 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.

filament buds continued to form and differentiate. Filaments became more numerous (see Fig. 5), more elongate dorso-ventrally and deeper fronto-abfrontally as SL increased. The increase in filament depth related to the expansion of the non-ciliated regions of each filament abfrontally (detail in Fig. 3D). Bacteria populated these regions in all but the smallest individuals (Fig. 2A, see next section). At any size, the shortest filaments were located posteriorly, suggesting that these regions are the site of new filament formation (e.g. Fig. 2D). An unusual morphological trait in this species was the discovery of a thin connecting band of tissue along the antero-posterior length of the gill filaments (*connecting band* in Fig. 2B and D). The secondary (inner) ascending lamellae, which extended dorsally from the ventral margin of the inner descending lamellae, were first identified in a mussel of SL 1.05 mm. Gill filament numbers increased linearly in "*I.*" *simpsoni* with increasing SL (Fig. 5; Linear regression, $r^2 = 0.98$, $F_{1,\ 31} = 1236$, $p < 0.001$) from 5 and 6 simple, highly ciliated gill bars in plantigrades to 46 filaments in one inner descending lamella of the largest specimen examined (SL 3.74 mm).

3.3.2. Patterns in symbiont acquisition, proliferation and diversity

No evidence existed for the acquisition of bacteria prior to settlement and metamorphosis. Signals were entirely absent in post-larval plantigrades during DAPI analysis (Fig. 6B), HE staining (not shown) and toluidine-blue staining (Supplementary Fig. 2). In one of the smallest developing juveniles (SL 0.50 mm, PdII settlement size 0.44 mm, equating to a 13.8% increase in SL following settlement), there remained no evidence for bacteria based on the same analyses (Fig. 6B). 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). Excluding the aforementioned aposymbiotic juvenile at SL 0.50 mm, all larger specimens analysed using DAPI and FISH ($n = 15$ in total) were host to abundant aggregations of symbionts on the non-ciliated regions of their gill filaments (examples in Figs. 6–7) and much lower abundances on dorsal non-ciliated visceral epithelia (Figs. 3B and 4B: regions indicated with arrowheads, Supplementary Fig. 5), on the epithelial sheaths of retractor muscles, and rarely, on the dorsal surface of the foot. Being particularly delicate, little or no mantle epithelia survived sample processing, so bacterial colonisation of these epithelia could not be confirmed.

In the smallest symbiotic mussels, bacteria could be individually discerned when viewed with 100× objective lens in contrast to larger individuals, where bacteria were often too numerous to identify individually (e.g. Fig. 6, two inset magnified regions). This indicates that bacterial abundances on a given area of filament increased with increasing SL, beyond the multiplying effect of expanding gill surface-area with increasing host size. Symbionts increasingly occluded the inter-filamentary spaces in gills of larger sized host individuals (SL > 1 mm, Figs. 2, 4, 6 and 7), even though the abundance of bacteria on other non-ciliated epithelia remained comparatively unchanged based on microscopic observations (Supplementary Fig. 5). No evidence for hypertrophy was found (i.e. tissue engorgement); the latero-abfrontal regions of gill filaments, heavily colonised by bacteria, were delicate and narrow in sagittal cross-section (e.g. Fig. 2C). Despite employing a wide range of specific oligonucleotidic probes during FISH analysis, only the probe Bthio-193 targeting sulphur-oxidizing bacteria yielded signals on the filaments of *"I." simpsoni*. Bacteria that hybridised with the general Eubacterial probe EUB338 simultaneously hybridised with the specific probe Bthio-193 during FISH (both probes signals matched closely when overlaid, Fig. 7) indicating that SOX bacteria were the single abundant bacterial type. Exclusively EUB338-

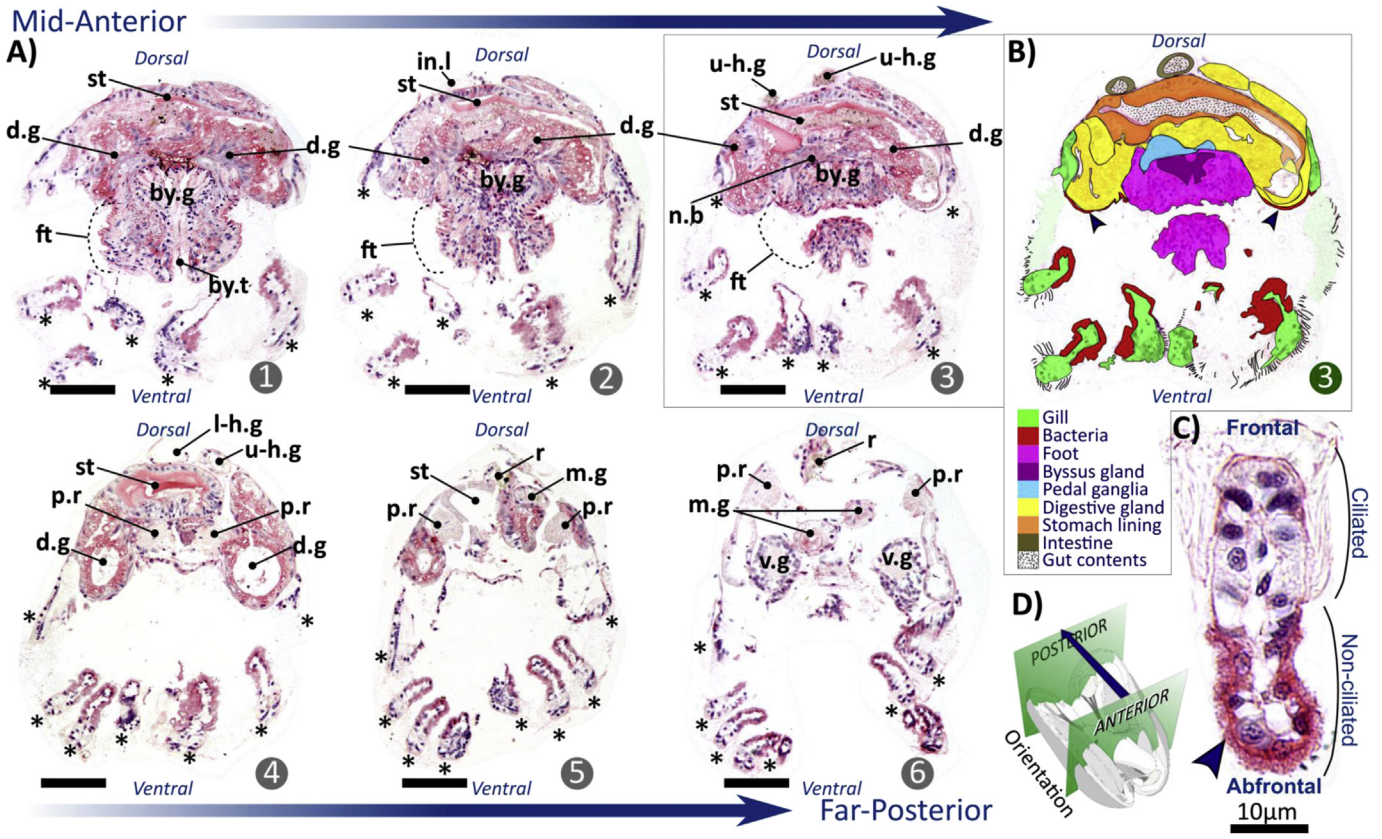

Fig. 3. Serial Haematoxylin and Eosin-Y stained transverse sections of a juvenile *"Idas" simpsoni* (SH 0.59, SL 0.92 mm). A) Series of sections taken at sequential locations along the antero-posterior axis [❶ = Mid-anterior, ❻ = Far-posterior]. B) A schematic that segregates the various organs and tissues identified in ❸ (non-gill bacteria indicated with arrowheads). An enlarged version is available in Supplementary Fig. 3. C) High magnification view of gill filament in frontal section. Arrowhead indicates bacteria (strong Eosin-Y staining). D) A generic *Idas*-like mussel drawing, with region of sections displayed (approximate). by.g *byssus gland*, by.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.r *posterior retractor*, r *rectum*, st *stomach*, u-h.g *upper hindgut*, v.g *visceral ganglion*. * = gill filament. Scales in [A] are 50 µm.

hybridised bacteria were only identified very rarely, in the midst of dense masses of double-hybridised bacteria. DAPI and the specific probes revealed complete overlap in putative bacterial signal, indicating that bacteria identified using DAPI signal alone could be trusted (Fig. 6A; Fig. 7). Though signals from the FISH probes in smaller mussels were less brilliant due to lower bacterial abundances (e.g. Fig. 7B: Bthio-193), signal remained distinguishable from background fluorescence. Incidences of poor signal-to-noise ratio were rare (e.g. specimens of SL 0.85 and 0.97 mm in Fig. 6B) but were specimen-specific.

4. Discussion

The current study advances our knowledge concerning the developmental biology of organic-fall bathymodiolins from post-settlement up to sexual maturation. Both the developmental anatomy and the initial colonisation of tissues by symbiotic bacteria are documented in "*I.*" *simpsoni* for the first time. The data suggest that "*I.*" *simpsoni* may be a highly adaptable species, both in terms of the rate at which an abundant symbiosis is established, and the possible nutritional flexibility that a well-developed alimentary system may provide.

4.1. Anatomical development in "I." simpsoni following metamorphosis

Shell morphometrics of the bone-, wood- and seep-colonising mussel "*I.*" *simpsoni* (collected from bone remnants in current study) were similar to those described for other bathymodiolin mussels at early life stages (e.g. Arellano and Young, 2009; Laming et al., 2014; Rodrigues et al., 2015). Striking similarities also existed between the developmental anatomy in juvenile "*I.*" *simpsoni* from the current study and equivalent specimens of the chemosymbiotic wood- and seep-colonising mussel *I. modiolaeformis* (collected from sunken plant remains, Laming et al., 2014; see Fig. 8). As noted in that study, some developmental traits also appear to be common to asymbiotic mytilids (e.g. *Mytilus edulis*, Bayne, 1976; Cannuel et al., 2009), most likely relating to conservative aspects of the entire family's ancestry.

4.1.1. Nervous and alimentary system

Like many smaller-sized bathymodiolins (e.g. *Idas macdonaldi*, Gustafson et al., 1998; *Idas iwaotakii*, Thubaut et al., 2013a; *I. modiolaeformis*, Laming et al., 2014), "*I.*" *simpsoni* retains anatomical vestiges that echo a hypothesised, heterotrophic, evolutionary past (Lorion et al., 2013, possibly from shallow-water habitats). Post-larval nervous systems are well-developed in plantigrades of "*I.*" *simpsoni*, reflecting the complex sensory requirements of settling mytilid pediveligers generally (Bayne, 1971). Juveniles of "*I.*" *simpsoni* differ from *I. modiolaeformis* in having simple eye spots (Laming et al., 2014). The presence of extensive digestive diverticula and the ongoing development of recurrent intestinal loops first identified in "*I.*" *simpsoni* at SL 0.94 mm, are features now lost from the rudimentary gut of many *Bathymodiolus* spp. (Kenk and Wilson, 1985; Gustafson et al., 1998). Preliminary findings from adults of "*I.*" *simpsoni* found on sunken wood, suggest

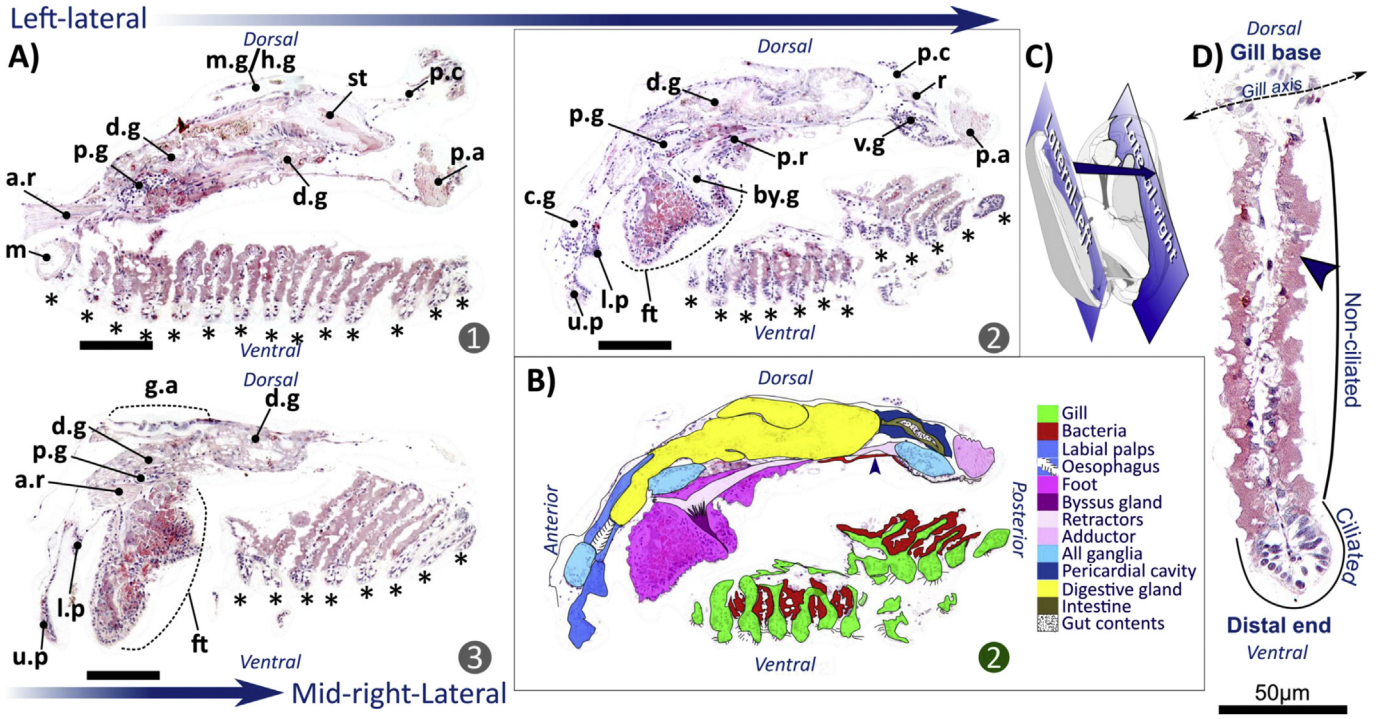

Fig. 4. Serial Haematoxylin and Eosin-Y stained sagittal sections of a juvenile "*Idas*" *simpsoni* (SH 0.59, SL 1.05 mm). A) Series of sections taken at sequential locations along the latero–lateral axis [❶ = Left-lateral side, ❸ = Right-lateral side]. B) A schematic that segregates the various organs and tissues identified in ❷, highlighting the location of non-gill bacteria (arrowhead). An enlarged version is available in Supplementary Fig. 4. C) A generic *Idas*-like mussel drawing, with region of sections displayed (approximate). D) High magnification view of gill filament in sagittal section (bacteria indicated with arrowhead). 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. Scales in A) are 100 µm.

that at least one additional intestinal loop develops at larger sizes (Laming, 2014). The current study did not identify a style-sac: an organ responsible for enzymatic and mechanical digestion in coastal mytilids (Giusti, 1970). This is believed to be present in both *I. modiolaeformis* (Laming et al., 2014) and the giant seep mussel "*B.*" *boomerang*, where it is conjoined with the mid-gut (Von Cosel and Olu, 1998). Since the use of almost identical preservation protocols in both '*Idas*' studies rules out problems relating to preservation, its absence may be symptomatic of the distinct evolutionary history of "*I.*" *simpsoni* (based on its phylogeny, Thubaut et al., 2013b).

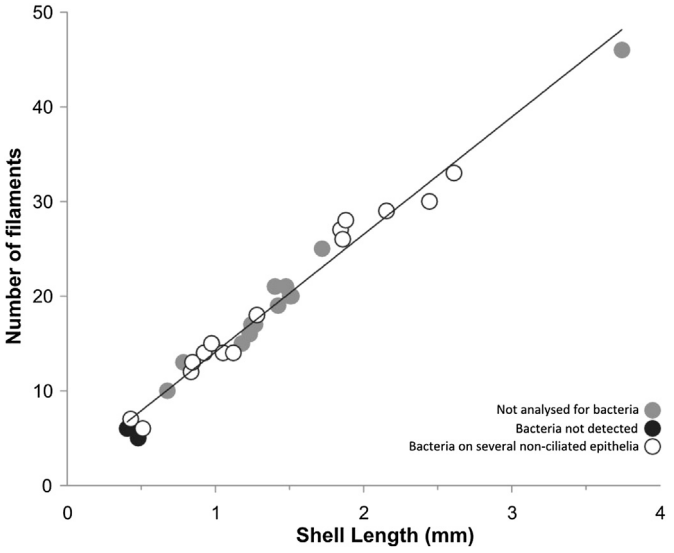

Fig. 5. Gill-filament counts in the inner demibranch's descending lamella of "*Idas*" *simpsoni*. Filament counts in one inner-demibranch descending lamella (n = 33) as function of SL. Bacteria appeared most abundant on abfrontal and lateral non-ciliated gill filament surfaces, when identified (white dots), (DAPI and FISH, n = 15).

4.1.2. Gill filaments

The processes that underlie early gill development and proliferation in "*I.*" *simpsoni* appear to follow those of small- and large-sized chemosymbiotic mussels alike (e.g. *I. modiolaeformis*, *Bathymodiolus puteoserpentis* and *Bathymodiolus azoricus*, Wentrup et al., 2013, 2014; Laming et al., 2014) and other mussel species with filibranch gills (e.g. Leibson and Movchan, 1975; Cannuel et al., 2009), at least across the size ranges of the current study. Thus the initial number of gill bars either side of the post-larval gill basket in "*I.*" *simpsoni* plantigrades (5–6, current study) is similar to both *I. modiolaeformis* (4–5, Laming et al., 2014) and *M. edulis* (≈6, Cannuel et al., 2009), where posterior-most gill bars are no more than ciliated buds. However, the initial rate of filament formation in "*I.*" *simpsoni* is lower than that of *I. modiolaeformis* (Laming et al., 2014; the only mytilid for which such detail is available), so that at greater SLs, relatively fewer filaments are found in "*I.*" *simpsoni* by comparison (Fig. 8).

These filaments normally form the first of four pairs of gill lamellae in filibranch bivalves (Cannuel et al., 2009). Due to the limited size range examined here (and in Laming et al., 2014), only the development of the first and second pairs were recorded: the descending and ascending lamellae of the left and right inner demibranchs respectively. In the current study, ascending lamellae were first identified in "*I.*" *simpsoni* at SLs similar to *M. edulis* (<1.05 mm in current study; and <1 mm in Cannuel et al., 2009; respectively). However this pair of lamellae appears at smaller SLs

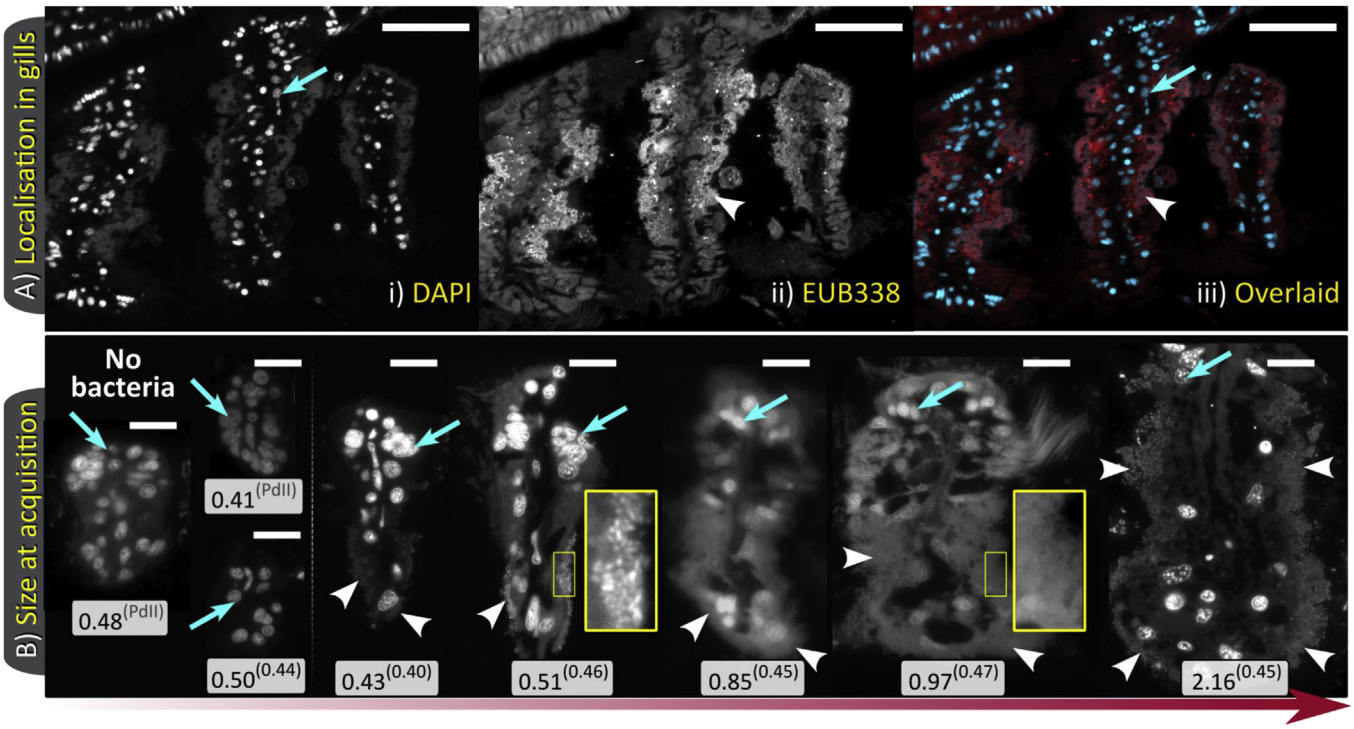

Fig. 6. Analysis of symbiont localisation and the period of acquisition using fluorescence microscopy. A) A sequence of images taken in succession using multiple epifluorescence source frequencies, with nucleic acid-labelling [i] DAPI and generic eubacterial 16S RNA-labelling [ii] EUB338. These are overlaid 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 specimens. Whole shell lengths (mm) indicated in the grey boxes, the prodissoconch II (≡settling size) is superscripted in parentheses if it differed (PdII = plantigrades). Blue arrows indicate epithelial nuclei, arrowheads identify bacterial signals. The largest specimen in [B] is the same as that of [A]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in *I. modiolaeformis* (<0.71 mm, Laming et al., 2014), perhaps as a result of the higher rates of filament differentiation. These differences could also be a consequence of scale since of the three species, *I. modiolaeformis* attains the smallest maximum SLs (19.5 mm, Laming, 2014) in comparison with "*I.*" *simpsoni* and *M. edulis* (45 mm, Warén and Carrozza, 1990; and 60–70 mm, Bayne, 1976 respectively).

Although common in *Bathymodiolus* spp. from vents and seeps, no evidence could be found for hypertrophied gill filaments in the current study, suggesting that this condition is not a feature of "*I.*" *simpsoni*. This is supported by the first ever study to describe the presence of extracellular, gill-associated bacteria in "*I.*" *simpsoni* (Southward, 2008), wherein gill filaments were reportedly more typical of coastal mytilids than related, *Bathymodiolus* spp.

4.2. Development of symbiosis following settlement

4.2.1. The distribution and abundance of symbionts

Once settled and metamorphosed on bone material, *"I." simpsoni* appears to undergo minimal growth before detectable symbionts first appear (e.g. 8% increase in SL). These symbionts are predominantly SOX Gammaproteobacteria, based on the degree of signal overlap between bacteria hybridised with generic eubacterial and specific SOX probes and the fact that no other phylotype-specific probe elicited a FISH signal. Previous studies that have examined symbioses in "*I.*" *simpsoni* have determined SOX symbionts to be the dominant bacterial type present on gill tissues (Ritt et al., 2012; Génio et al., 2014), arranged between small microvilli (Southward, 2008). Thus, unlike some species for whom symbiont diversity can be high (especially *B. heckerae*, with four distinct symbiont phylotypes, Duperron et al., 2007; and *I. modiolaeformis*, with one to six symbionts in varying combinations, Duperron et al., 2008; Lorion et al., 2012; Rodrigues et al., 2013; Laming et al., 2014), "*I.*" *simpsoni* displays little diversity in its symbiosis. Within a narrow range of SLs following their initial appearance in very small juveniles, symbionts in the current study appear to increase rapidly in abundance on the external, non-ciliated surfaces of gill epithelia reaching high abundances based on microscope observations (e.g. Fig. 4; Fig. 6B even at SLs 846 and 974 μm). The abundance of SOX bacteria on other exposed epithelia remained comparatively low, however they did not disappear altogether to leave an exclusively gill-associated symbiosis, as has been observed in other bathymodiolins (Wentrup et al., 2013; Laming et al., 2014). Considering that such tissue-specificity was first identified in *I. modiolaeformis* of SL of 4.37 mm (Fig. 8), the current study's specimen size-range may not have been sufficiently large to witness this process.

The only other chemosymbiotic mussel available for direct comparison is *I. modiolaeformis*, colonising wood and alfalfa substrata (Laming et al., 2014). Comparing specimens of equivalent SLs for each species (Fig. 8), SOX symbionts first appear in *"I." simpsoni* at smaller SLs following only a marginal investment in growth (e.g. SL 0.43 and 0.50 mm), according to barely perceptible dissoconch deposition and limited changes in anatomy. This in spite of the fact that larger maximum sizes are documented in "*I.*" *simpsoni* (SL_{max} = 45 mm, cited within the species description for *Idas ghisottii*, Warén and Carrozza, 1990). In *I. modiolaeformis*, size at first acquisition is larger at SL 0.60 mm (Laming et al., 2014) and represents a greater increase in SL following settlement (≈50%, in contrast to 8% in *"I." simpsoni*). Furthermore, based on fluorescence microscopy (e.g. Fig. 6 in the current study, versus Fig. 5 of Laming et al., 2014), symbiont abundances are unequivocally higher in "*I.*" *simpsoni*, to such an extent that even the largest *I. modiolaeformis*

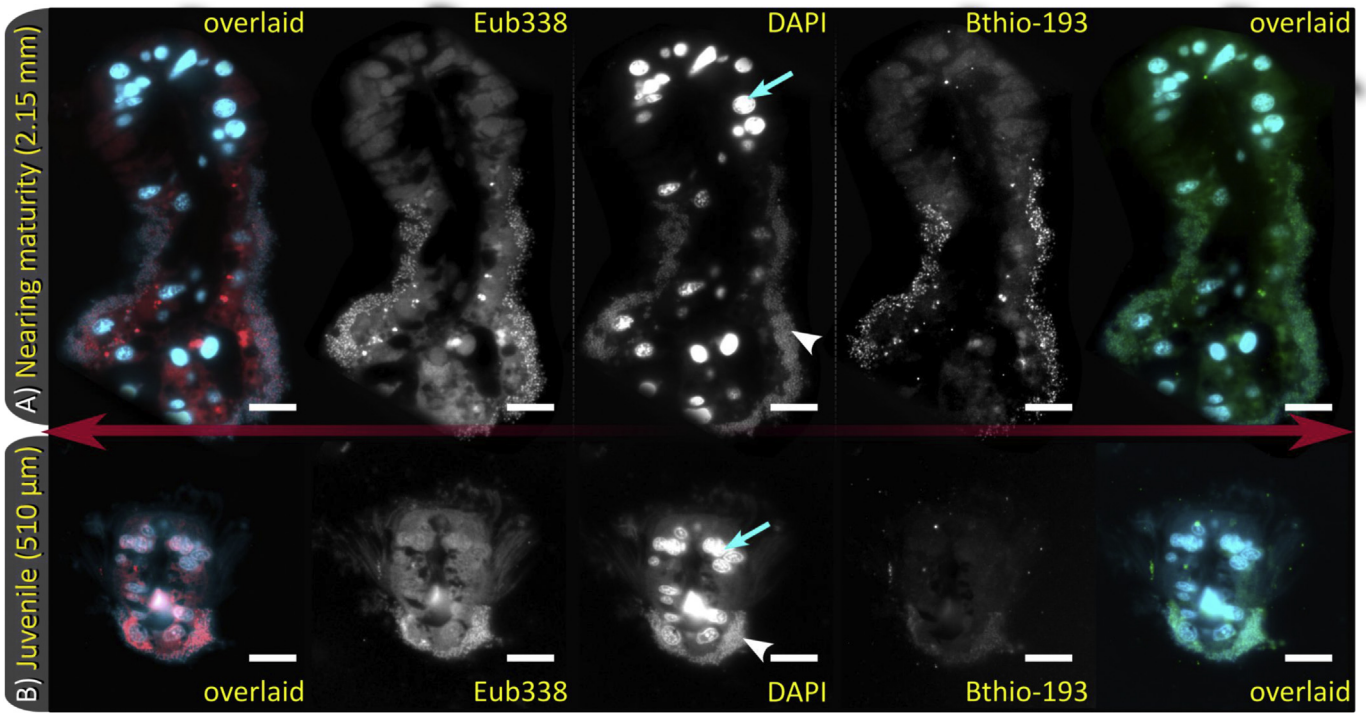

Fig. 7. Fluorescence *in situ* hybridisations of one of the largest specimens not confirmed as an adult, and a small juvenile. Dual hybridisations of fluorochrome-labelled bacterial probes and DAPI in sections of host gill. In both specimens (A, B), DAPI staining (centre) targets nucleic acids indiscriminately, Cy3-labelled EUB338 signal targets most Eubacteria, and Cy5-labelled Bthio-193 targets T1-thiotrophs. Superimposed signals of different wavelengths: DAPI and EUB338 to the left, DAPI and Bthio-193 to the right. A) Signals from a frontally sectioned filament in a mussel approaching maturity. B) Frontal filament section in one of the smallest mussels to have any bacterial symbionts, displaying similar results, though probe emission intensities displayed lower signal to noise ratios. Blue arrows indicate epithelial nuclei, arrowheads indicate bacterial signals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specimen examined (SL 6.54 mm, Fig. 5k in Laming et al., 2014) had lower apparent in-section abundances than "*I.*" *simpsoni* individuals a fraction of this size (e.g. Fig. 6 of the current study, SLs 0.97 mm and 2.16 mm). Similar comparisons of FISH analyses in the current study with equivalent FISH-based studies examining intracellular symbioses in larger vent and seep species indicate that although gills are not hypertrophied in "*I.*" *simpsoni*, its extracellular SOX symbionts occur at similar abundances, qualitatively, to those of *Bathymodiolus* spp (e.g. Duperron et al., 2007; Wentrup et al., 2013, 2014). Thus while the acquisition of symbionts is a gradual process in *I. modiolaeformis* living on wood substrata, it appears to be rapid in *"I." simpsoni* occurring on bone substrata, with the association proliferating over a relatively small host size-range at a rate far beyond that seen in *I. modiolaeformis* (Laming et al., 2014).

4.2.2. The potential origins of symbiont patterns

The almost immediate appearance of relatively dense aggregations of symbiotic bacteria in the gills of *"I." simpsoni* indicates the presence of a readily available, locally abundant, supply of symbiont candidates. The same could not be argued for the *I. modiolaeformis* settling on vegetative debris in the study by Laming et al. (2014), where poor symbiont supply may have hindered bacterial acquisition. Regardless of the substrate to which "*I.*" *simpsoni* is attached, host populations often form tightly packed clumps (Ritt et al., 2012; Génio et al., 2014; Laming, 2014) typical of highly gregarious mytilid species. *Idas modiolaeformis* on the other hand occurs more evenly dispersed over a substrate, with individuals only rarely in direct contact with other conspecifics (S. Duperron, personal observation). These distinct distributions may be critical in the acquisition and transmission of symbionts, as gregarious behaviour could provide a supplementary pool of proximal symbionts, readily available via lateral transmission (Bright and Bulgheresi, 2010). Habitat specific differences in sulphide supply may also affect the availability (and growth rates) of symbionts. However, since the occurrence of free-living forms of mussel symbionts has yet to be demonstrated and it is almost impossible to entirely exclude the possibility that larvae arrive pre-equipped with a small 'seed' population of bacteria, ascertaining the exact origins of mussel symbionts requires further study.

4.3. Possible nutritional modes throughout the life-history of "I." simpsoni

4.3.1. Larvae are obligate planktotrophs

SLs recorded for larval shells PdI (current study) and PdII (Génio et al., 2014; current study) of "*I.*" *simpsoni* agree with Ritt et al. (2012) describing a then-unidentified, seep-associated species "*Idas* nov sp." ($n = 1$, PdI SL ≈ 95 μm; PdII SL ≈ 450 μm), identified post-publication as *"I." simpsoni* (Génio et al., 2014). SLs for the PdI in other, related species are roughly similar (74–137 μm), though PdII dimensions vary considerably more (Table 1). Even accounting for this, like many other bathymodiolins the mean PdII SL of 459 μm in "*I.*" *simpsoni* is considerably greater than those of shallow water species (e.g. *Mytilus* spp. reach PdII SLs of 120–300 μm in 4 to 8 weeks, additional examples in Fuller and Lutz, 1989). Since the PdII SL approximates that of the settling pediveliger then, assuming similar growth rates, this indicates a more extended period of transport before settlement– longer still if lower temperatures suppressed growth rates. Given that the PdI SL of 96 μm relates to the newly formed straight-hinged veliger SL of "*I.*" *simpsoni* (Lutz et al., 1980), the corresponding relatively small oocyte diameter

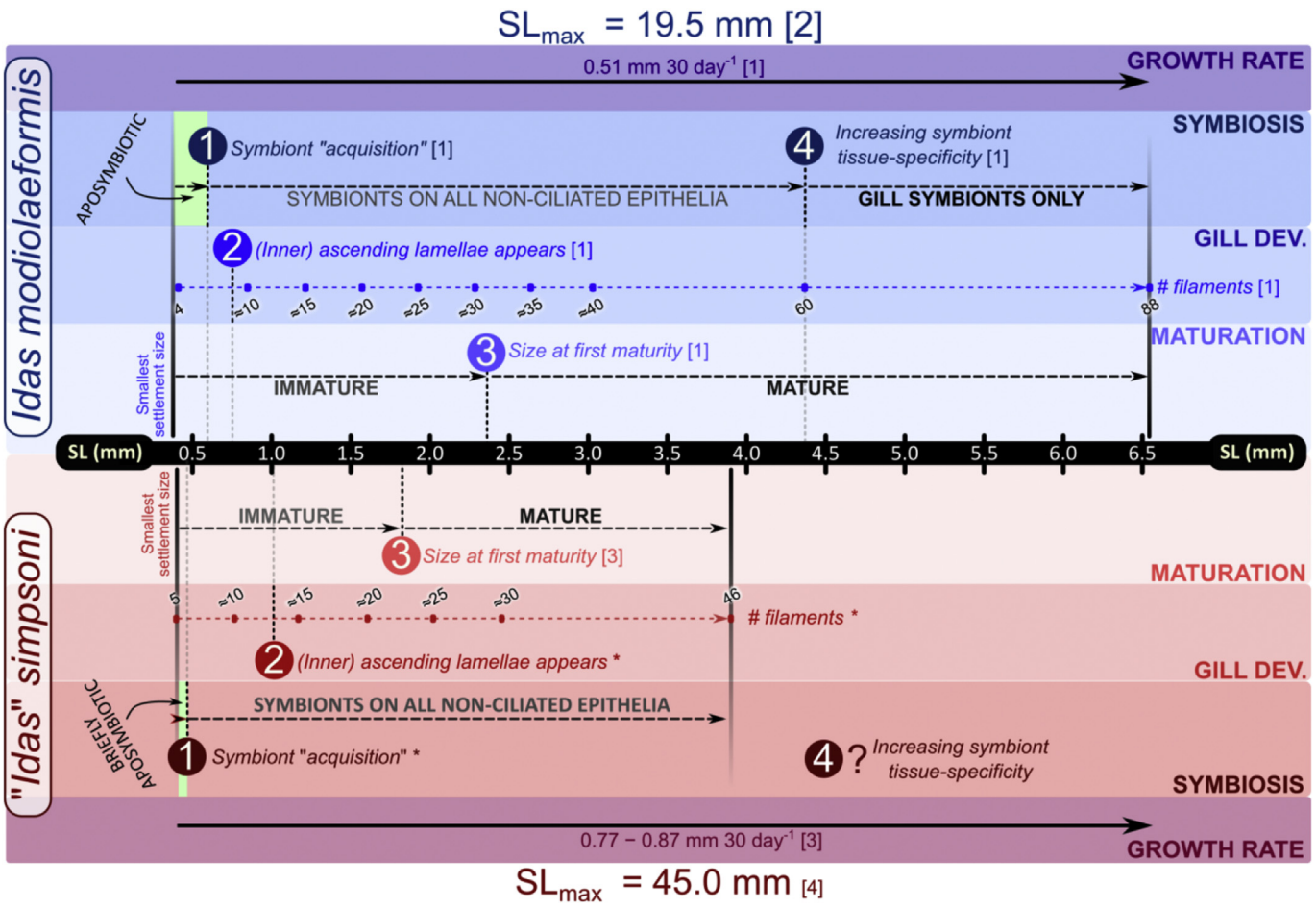

Fig. 8. Comparative summary of anatomical development in "*I.*"*simpsoni* and *I. modiolaeformis*. Schematic summarises developmental data available for two chemosymbiotic mussels, "*I.*"*simpsoni* (red regions) and a comparator species, *I. modiolaeformis* (blue regions). Points ❶, ❷, ❸ and ❹ indicate critical junctures during development, in order of shell length (SL, scale in middle). [1] – Laming et al. (2014), [2] – Laming (2014), [3] – Génio et al. (2014), [4] – Warén and Carrozza (1990), Asterisks indicate the current study's data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of <96 μm would confer limited yolk reserves to the developing embryo. With minimal yolk, these larvae require an alternative source of energy to realise their extended PLD (e.g. Lutz et al., 1980). Considering bacterial signals were entirely absent from all epithelia in plantigrades of "*I.*" *simpsoni*, chemosymbiotic larval nutrition seems unlikely, though the pre-acquisition of a very low seeding population of bacteria cannot be entirely ruled out. Thus the larvae of "*I.*" *simpsoni* are very likely to be obligate heterotrophs.

4.3.2. A propensity for mixotrophy?

As young juveniles, the presence of a developing gut and a growing number of bacteria associated with the gill suggests a potential to ingest and assimilate particulate or dissolved organic matter as well as utilise possible symbiont-based nutrition. The

Table 1
Larval shell-length measurements from this study and from related species.

Species	Habitat	PdI μm ± σ (*n*)	Pd II μm ± σ (*n*)	From
"*I.*" *simpsoni*	Bone	**96 ± 6.3 (24)**	457–478	This study, [1]
	Seep	≈95 (1)	≈450 (1)	[2]
I. modiolaeformis	Seep	79 ± 3.4 (6)	379 ± 11.7 (14)	[3]
	Wood	80 ± 5.1 (20)	398 ± 17.5 (35)	[4]
I. argenteus	Wood	≈98 (1)	≈459 (1)	[5]
I. iwaotakii	Wood	74 ± 9.4 (15)	544 ± 37.6 (15)	[6]
T. fisheri	Seep	–	460 (1)	[7]
"*B.*" *heckerae*	Seep	137 (1)	120–600	[8]
"*B.*" *childressi*	Seep	113 ± 2.0 (5)	443 ± 8.8 (5)	[9]
B. thermophilus	Vent	95–110	400–470	[10]
B. azoricus	Vent	–	524 ± 20.0 (93)	[11]
M. edulis	Rocky shore	94–120	120–252	[12]

Genera: *I.* – *Idas*, *T.* – *Tamu*, *B.* – *Bathymodiolus*, *M.* – *Mytilus* (inverted commas = *sensu lato*).
Data from this study in bold. Other sources: [1] – Génio et al. (2014), [2] – Ritt et al. (2012), [3] – Gaudron et al. (2010), [4] – Laming (2014); Laming et al. (2014), [5] – Ockelmann and Dinesen (2011), [6] – Thubaut et al. (2013a), [7] – Berg (1985); Gustafson and Lutz (1994); Craddock et al. (1995), [8] – Turner and Lutz (1984); Salerno et al. (2005), [9] – Arellano and Young (2009), [10] – Comtet and Desbruyeres (1998), [11] – Gustafson et al. (1998), [12] – Sprung (1984); Fuller and Lutz (1989).

latter would require a means to assimilate the energy and carbon generated by symbionts. This might be achieved by the lysis of bacteria after being engulfed by phagocyte-like cells in developing juveniles (e.g. as identified in Southward, 2008), evolved from an ancestral immunological response to the presence of bacteria perhaps. As the ingestion of symbionts in "*I.*" *simpsoni* remains hypothetical, this would represent an intriguing avenue for further study and would be best supported by stable isotopes data, not possible in the current study due to organism size and numbers.

The persistence of a well-developed gut in tandem with SOX symbionts in adults, which are presumed to provide a nutritional role, is not a trait exclusive to "*I.*" *simpsoni*. The retention of a fully-formed digestive system appears to be the norm in smaller-sized chemosymbiotic bathymodiolins regardless of habitat (Gustafson et al., 1998; Thubaut et al., 2013a; Laming et al., 2014). Support for mixotrophy also exists in the vent-, bone- and wood-colonising species, *I. washingtonius*, which displays quite variable $\delta^{13}C$ and $\delta^{15}N$ isotope ratios, depending on the habitat from which it is collected. Values range from those indicative of chemosymbiosis (at vents on the Juan de Fuca Ridge and larger whale falls in Monterey Bay, Smith and Baco, 2003; Mckiness et al., 2005; Bergquist et al., 2007), to ratios equivalent to benthic primary consumers (e.g. shallower, smaller whale carcasses, Smith and Baco, 2003), with specimens of *I. washingtonius* on wood falling between the two extremes (Smith and Baco, 2003). Even certain *Bathymodiolus* spp. display a capacity for filter-feeding upon, and assimilating, organic material (e.g. Page et al., 1990; Page et al., 1991). If the SOX symbionts found in specimens of "*I.*" *simpsoni* in the current study are indeed assimilated as a source of carbon and energy, then in highly sulphidic conditions the importance of an anatomically functional gut may be reduced. However, the release of reduced chemical compounds at reducing habitats is often highly heterogeneous (e.g. Le Bris et al., 2008), wherein mixotrophy involving filter-feeding and chemosynthetic symbiont nutrition might provide a means to ensure that a continuous energy resource remains available. Indeed, active engagement in filter-feeding has been documented in both "*I.*" *simpsoni* and *I. modiolaeformis* (SR Laming unpublished data), and probably remains necessary for many bathymodiolins species, even at vents (Martins et al., 2008).

4.4. Habitat adaptability and coping with change

Many Bathymodiolinae specialise in colonising either vents, seeps or organic falls exclusively (Duperron, 2010). "*Bathymodiolus*" spp. are sometimes described as an evolutionary 'dead-end' in terms of adaptation to their habitat (e.g. reduced digestive systems, internally integrated symbionts, a higher dependency on chemosynthetically derived nutrition based on stable isotopes data, Cavanaugh et al. 1992; Dubilier et al., 2008). Applying this logic, smaller bathymodiolins might be described as being intermediary by comparison. However, current evidence for active selection towards a greater dependency on symbiosis over successive generations, is speculative at best (e.g. based on phylogenetics Distel et al., 2000; Jones et al., 2006; Kyuno et al., 2009; Fontanez and Cavanaugh, 2013). There are no arguments to suspect that bathymodiolins are not already suitably adapted to their various reducing habitats. "*Idas*" *simpsoni* is documented to occur at multiple habitat types (wood, bones, seeps) and over large geographical, thermal and barometric ranges (Thubaut, 2012). This species is one in a small selection of smaller-sized chemosymbiotic mussels that demonstrate a high level of flexibility and tolerance to environmental variability (e.g. Fujiwara et al., 2007; Lorion et al., 2009). "*Idas*" *simpsoni* also displays several specific adaptations identified in the current study: rapid establishment of a dense host–symbiont association and the retention of an anatomically functional alimentary system. Considering both these life-history traits and the reproductive biology of "*I.*" *simpsoni* (e.g. early size at first maturity, possible protandry, semi-continuous – and apparently abundant – larval supply, Ritt et al., 2012; Génio et al., 2014), it appears that populations of "*I.*" *simpsoni* may prove relatively robust to predicted increases in anthropogenic disturbance, assuming an ongoing supply of chemically reduced substrate.

The rapid appearance and relatively immediate abundances of SOX symbionts in "*I.*" *simpsoni*, contrast the high symbiont diversity but slower rates of symbiont acquisition seen in *I. modiolaeformis* living at seeps and on wood, respectively. These may reflect differences between the two species symbiont compatibility, the effect of habitat conditions, and differing life habits in each species. Being derived from similar coastal processes, organic falls are likely to occur in regions which see a greater overall flux of detritus, such as submarine canyons. Thus although chemosymbiosis is prevalent in the adults of most mussel species colonising organic falls, the retention of an anatomically functional gut could reflect active, ongoing selective pressures (i.e. a gut remains a requirement for survival because mussels are mixotrophic), particularly if symbiont productivity is subject to intermittent or limited supplies of reduced-fluid emissions (Laming et al., 2014). Such attributes in "*Idas*" *simpsoni* could prove vital as a means of adaptation, if pending threats predicted to affect organic-fall hotspots are realised in the future (e.g. submarine canyon benthos, Ramirez-Llodra et al., 2011). Indeed, the ability to colonise a wide array of habitats in tandem with the adaptive traits of "*I.*" *simpsoni*, may have already buffered against probable declines in sunken carcass availability in the last century, due to historical whaling activity and current fisheries activity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2015.07.014.

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