Population connectivity of hydrothermal-vent limpets along the northern Mid-Atlantic Ridge (Gastropoda: Neritimorpha: Phenacolepadidae)

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The red-blooded limpet 'Shinkailepas' briandi (Neritimorpha: Phenacolepadidae) is one of the commonest gastropod species at deep-sea hydrothermal vents on the Mid-Atlantic Ridge (MAR). We investigated its population connectivity along MAR as the first such study for gastropods and explored the importance of larval migration for the distribution of vent-endemic animals. Our analyses, based on 1.3-kbp DNA sequences from the mitochondrial COI gene, showed a panmictic population throughout its geographic and bathymetric ranges that span from the northernmost and shallowest Menez Gwen vent field (38°N; 814–831 m depth) to the southernmost and deepest Ashadze field (13°N; 4090 m). Early development of this species is presumed to have a long pelagic duration as a planktotrophic larva; the hatchling with a shell diameter of 170–180 μm attains a constant settlement size of 706 ± 8 μm (mean ± SD). Retention of eye pigmentation in newly settled juveniles, along with the genetic panmixia, suggests that the hatched larva of 'S.' briandi migrates vertically to the surface water, presumably to take advantage of richer food supplies and stronger currents for dispersal, as has been shown for confamilial species at hydrothermal vents and cold methane seeps.

Keywords: biogeography, chemosynthetic environment, deep sea, larval ecology, limpet, panmixia, population genetics, protoconch, *Shinkailepas briandi*, vertical migration

INTRODUCTION

Hydrothermal vent fields in the deep sea are typically distributed along the mid-ocean ridges generated by seafloor spreading, the volcanic arcs and back-arcs created by subduction, and also on ridge flanks and hot-spot volcanoes (Mullineaux, 2014). Since the discovery on the Galapagos Rift in 1977, at least 521 active vent fields have been documented around the world (Beaulieu *et al.*, 2013). Their invertebrate communities – predominantly composed of molluscs, crustaceans and annelids – depend on the primary production of chemoautotrophic bacteria (Van Dover, 2000) and occur with high levels of endemism and biomass (Desbruyères *et al.*, 2006).

The Mid-Atlantic Ridge (MAR) forms one of the five biogeographic provinces for the global hydrothermal vent communities (Moalic *et al.*, 2012). At least nine vent fields exist on MAR to host faunal assemblages at the depth range of 810–4200 m (Kelley & Shank, 2010; Wheeler *et al.*, 2013). More than 225 endemic species have been reported from this province with richness ranging from less than 30 to over 100 at each field (Kelley & Shank, 2010). Individual

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fields along MAR are separated not only by geographic distances of several dozens to thousands of kilometres but also large topographic discontinuities, bathymetric differences and shearing oceanic currents. These potential barriers to dispersal might favour divergence among sites (Van Dover, 1995; Desbruyères *et al.*, 2000).
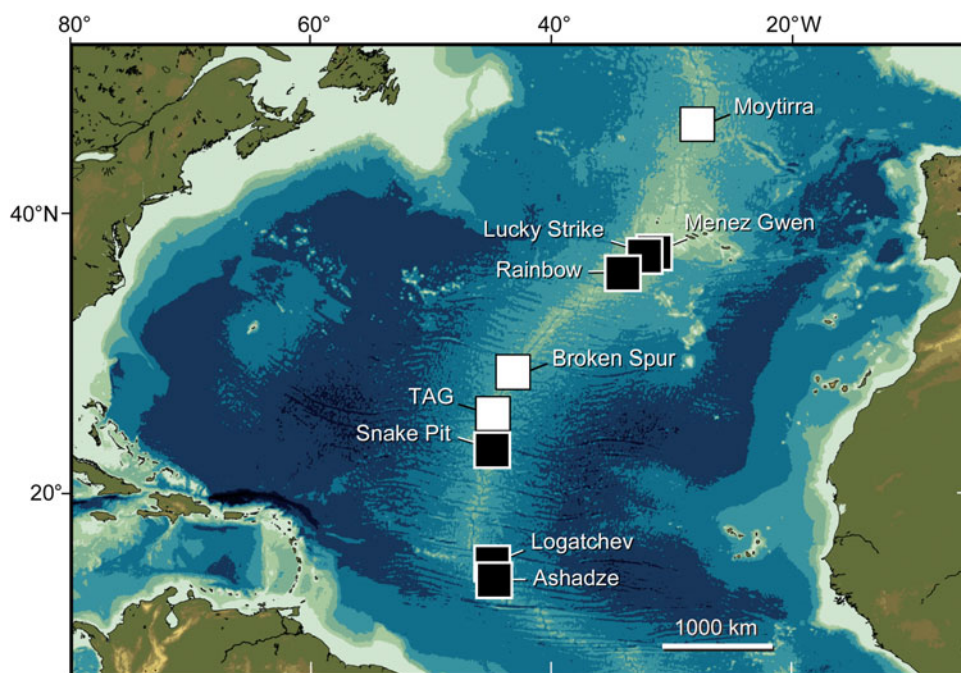
Previous studies on the genetic connectivity of vent animals along MAR suggest different levels and patterns of variation, presumably depending on their larval ecology. Among five animal lineages so far investigated in this context, two lineages are each divided by a dispersal barrier into multiple species or populations, while three others exhibit genetic panmixia. The former includes the case of *Bathymodiolus azoricus* and *B. puteoserpentis*, reciprocal sister species in the bivalve family Mytilidae. These mussel species have largely parapatric distributions in the north and south, respectively, with a narrow overlapping zone at the Broken Spur vent field at 29°N (O'Mullan *et al.*, 2001; Breusing *et al.*, 2016; see Figure 1 for the location of vent sites). Genetic discontinuity has also been shown for the commensal annelid *Branchipolynoe seepensis* that lives inside the mantle cavity of the same mussels, but in this case between the Snake Pit (23°N) and Logatchev (15°N) sites to the south (Daguin & Jollivet, 2005).

Three shrimp species of the family Alvinocarididae instead show population connectivity along the entire ridge. *Rimicaris*

Fig. 1. Location of hydrothermal vent fields along northern Mid-Atlantic Ridge. Solid and open squares represent vents with and without 'Shinkailepas' briandi, respectively. DNA sequences were determined for specimens from Menez Gwen, Lucky Strike, Logatchev and Ashadze.

exoculata, among the most abundant animal species at vents on MAR, has a panmictic population that stretches 7100 km from the Rainbow field at 36°N to the South MAR at 5°S (Teixeira *et al.*, 2011, 2012). Another species in the same genus similarly shows genetic homogeneity between the Lucky Strike (37°N) and Logatchev (15°N) vent fields (Teixeira *et al.*, 2013: ESU2). The third, confamilial species in the genus *Alvinocaris* demonstrates population connectivity not only within MAR but also with cold methane seeps in the Gulf of Mexico and off the west coast of Africa (Teixeira *et al.*, 2013: ESU1). Larvae of alvinocaridid shrimps are thought to feed and disperse in mid-water, far above the influence of a hydrothermal plume, so that such barriers as depth, seafloor topography and transform faults would not constrain the connectivity of its local populations (Teixeira *et al.*, 2011; see also Adams *et al.*, 2012). Additional information on the population connectivity and early-life traits of various animal taxa on MAR would contribute towards a more inclusive understanding of formation of the hydrothermal vent fauna.

Here we focus on a gastropod species with wide geographic and bathymetric distributions to explore the larval dispersal and connectivity between vent fields along MAR. The red-blooded limpet 'Shinkailepas' briandi Warén & Bouchet, 2001 (Neritimorpha: Phenacolepadidae; a new genus is now being proposed elsewhere) represents one of the commonest gastropods that occur at a depth range of 810–4090 m in the Menez Gwen, Lucky Strike, Rainbow, Snake Pit, Logatchev and Ashadze fields (Warén & Bouchet, 2001; Kelley & Shank, 2010; Fabri *et al.*, 2011; Figures 1 & 2). This species is found in clusters on rocks and shells of *Bathymodiolus* in volcanically active zones near venting orifices (Cuvelier *et al.*, 2011). Its digestive system, including the radula, shares the same characteristics with other phenacolepadids (Fretter, 1984; Warén & Bouchet, 2001), suggesting grazing on bacterial mats on hard substrates (Beck, 1992; Kano *et al.*, 2002). The aim of this paper is to investigate the

population connectivity of 'S.' briandi along MAR as the first such study for gastropods and to explore the importance of larval duration and vertical migration for the biogeography of vent-endemic animal species.

MATERIALS AND METHODS

Population genetic structure of 'Shinkailepas' briandi was analysed using a total of 34 specimens deposited in the Swedish Museum of Natural History (SMNH). These specimens originated from four hydrothermal-vent fields, including Menez Gwen (2 specimens; SMNH-89175 and 89169), Lucky Strike (11; SMNH-84610), Logatchev (6; SMNH-92814) and Ashadze (15; SMNH-103209 and 103211), which collectively cover the geographic and bathymetric ranges of the species (Table 1; Figure 1).

Total DNA was extracted from foot tissue using the DNeasy Tissue Extraction Kit (Qiagen). 1327-bp fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene were amplified by the polymerase chain reaction (PCR) using the primer pair LCO1490 and COIa-NER (Folmer *et al.*, 1994; Kano & Kase, 2004). Amplification was performed in 25 μl reaction mixtures of 17.5 μl DDW, 2.5 μl EX Taq buffer (10×), 2.0 μl dNTP mixture (2.5 μM each), 0.3 μl of each forward and reverse primer (20 μM), 0.13 μl Ex Taq Hot Start Version (TaKaRa) and 2.5 μl genomic DNA. PCR reactions were carried out with an initial denaturation step at 94°C for 40 s, followed by 35 cycles consisting of a denaturation step at 94°C (40 s), an annealing step at 42°C (60 s) and an extension step at 72°C (90 s). Amplicons were purified by ExoSAP-IT (USB) following the described protocol.

Sequencing reactions were prepared using a Big Dye Terminator Cycle Sequence Kit 3.1 (Applied Biosystems) and either of the newly designed internal primers COIf2-SHL (forward; 5′-ATTTTRATTATTCCTGGRTT-3′) and COIr2-
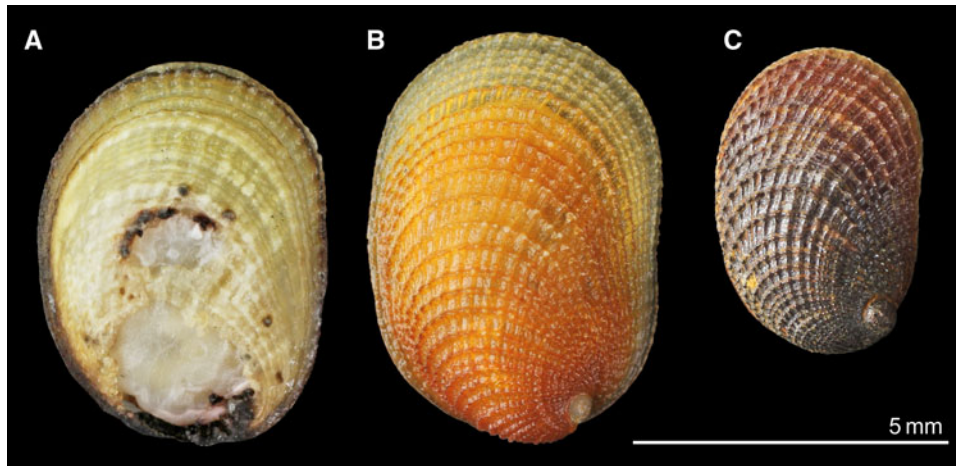
Fig. 2. '*Shinkailepas*' *briandi* from Menez Gwen (A; SMNH-89175; DNA: AORI_YK#866), Logatchev (B; SMNH-92814; AORI_YK#867) and Ashadze (C; SMNH-103209; AORI_YK#2872). All specimens were used for DNA sequencing.

SHL (reverse; 5′-AAYCCYAAAATACCAATTGA-3′). The reaction mixtures were analysed on ABI PRISM 3130xl sequencers after purification with a Big Dye XTerminator Purification Kit (Applied Biosystems). Obtained sequences were aligned and trimmed to exclude the amplification primers in MEGA 6.06 (Tamura *et al.*, 2013). The final sequences (1276 bp each) have been deposited in DDBJ/ENA/GenBank databases with accession numbers LC215293–LC215326.

The population genetic structure of the species was analysed using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). We estimated haplotype diversity h (Nei, 1987), mean number of pairwise difference π_1 (Tajima, 1983) and nucleotide diversity π_2 (Nei, 1987) for each local population. A parsimonious haplotype network was reconstructed using TCS 1.21 (Clement *et al.*, 2000). Estimates of pairwise F_{st} and exact test of differentiation (Hudson *et al.*, 1992; Raymond & Rousset, 1995) were also conducted among the populations of the Lucky Strike, Logatchev and Ashadze vent fields, from which sufficient numbers of individuals could be sequenced. Moreover, the demographic history of the species was inferred by a mismatch distribution analysis (Rogers & Harpending, 1992) and by computing Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997) in ARLEQUIN.

The following material was also investigated based on morphological criteria to verify previous occurrence records of the study species: ∼300 specimens from Menez Gwen (SMNH-50960, 89169 and 89175 and Muséum national d'histoire naturelle, MNHN), Lucky Strike (SMNH-21173, 43246,

45411, 50925, 81666, 84610, 89167, 89168, 89182 and 89188 and MNHN), Rainbow (SMNH-50407 and 50409), Snake Pit (MNHN), Logatchev (SMNH-92814, 92830 and 103249) and Ashadze (SMNH-103194–103199, 103204 and 103208–103211). These specimens cover all known localities and populations of the Shinkailepadinae on MAR.

Some specimen lots included post-settlement juveniles with intact protoconchs or larval shells. The diameter of the protoconch, which corresponds to the shell size at settlement, was measured for 12 specimens from Lucky Strike (7 specimens; SMNH-21173 and 84610 and MNHN), Logatchev (2; SMNH-92830) and Ashadze (3; SMNH-103199). The measurement was made with 5 μm precision by tracing the outline of the protoconch using a Nikon SMZ1500 stereomicroscope equipped with a drawing tube (see Fukumori & Kano, 2014 for details). More than a hundred pre-hatched larvae were also obtained and measured by opening 23 egg capsules on conspecific adult shells from the Lucky Strike field (SMNH-21173; see Warén & Bouchet, 2001: figure 32).

RESULTS

Thirty-four nucleotide sequences of the partial COI gene (1276 bp) were determined for '*Shinkailepas*' *briandi*. Each population of the Menez Gwen, Lucky Strike, Logatchev and Ashadze vent fields showed a high haplotype diversity ($h =$ 1.00 ± 0.50) with high numbers of polymorphic sites (35 ≥ K ≥ 2) and mean pairwise differences (π_1 ≥ 2.00), as well as

Table 1. Locality data for '*Shinkailepas*' *briandi* used in population genetic analysis.

Locality	Coordinates	Depth (m)	Expedition and dive numbers
Menez Gwen	37°30′N 31°31′W	814–831	MoMARETO 2006, st. PL299
Lucky Strike (Crystal Vent)	37°17′N 32°16′W	1721	Alvin Dive 3119
Logatchev	14°43′N 44°56′W	2700	MoMARETO 2007, st. PL316
Ashadze	12°58′N 44°51′W	4090	Serpentine 2007, st. 311

Table 2. Genetic diversity of mitochondrial COI sequences in '*Shinkailepas*' *briandi* (1276 bp).

Locality	N	Nh	K	h	π_1	π_2 (×10^{-2})
Menez Gwen	2	2	2	1.00 ± 0.50	2.00 ± 1.73	0.16 ± 0.19
Lucky Strike	11	11	31	1.00 ± 0.04	8.33 ± 4.18	0.65 ± 0.37
Logatchev	6	6	17	1.00 ± 0.10	7.47 ± 4.07	0.59 ± 0.37
Ashadze	15	15	35	1.00 ± 0.02	6.69 ± 3.34	0.52 ± 0.29

N, number of specimens; *Nh*, number of haplotypes; *K*, number of polymorphic sites; *h*, haplotype diversity; π_1, mean number of pairwise differences; π_2, nucleotide diversity.

a low nucleotide diversity ($\pi_2 \leq 0.0065$; Table 2). The reconstructed haplotype network consisted of 32 (including two shared and 30 singleton) haplotypes, which differed by one to 13 substitutions from each other (Figure 3). One of the two shared haplotypes originated from the Menez Gwen (38°N; 814–831 m depth) and Ashadze (13°N; 4090 m) vent fields, and the other from Ashadze and Lucky Strike (37°N; 1721 m). Neither the pairwise F_{st} nor the exact test showed significant genetic differentiation among the local populations of Lucky Strike, Logatchev and Ashadze (Table 3).

The mismatch distribution analysis suggested that the panmictic population of '*S.*' *briandi* along MAR has experienced a sudden demographic expansion. The observed unimodal distribution with a mean of 7.12 substitutions (Figure 4) was not significantly different from the theoretical distribution under the sudden expansion model ($\tau = 8.43$) with a sum of squared deviations of 0.008 ($P = 0.24$). The sudden expansion was also suggested by significantly negative ($P < 0.05$) values of Tajima's D (-1.98) and Fu's Fs (-25.05). By applying the evolutionary rates of COI gene for invertebrates (pairwise distance of 1.4–2.4% per million years; see Wilke, 2003), the population expansion of the species could be calculated to have occurred 0.28–0.48 million years ago (8.43 substitutions in 1276 base pairs).

All specimens treated in this study had conchological and anatomical characteristics of the species, as shown for the type specimens in the original description (Warén & Bouchet, 2001), although the shell size and colour of mineral deposits on the shell tend to differ among sites (Figure 2). The occurrence of '*S.*' *briandi* was thus verified for not only the above four localities but also for the Rainbow and Snake Pit vent sites. No other species was found in our material of the Shinkailepadinae from MAR.
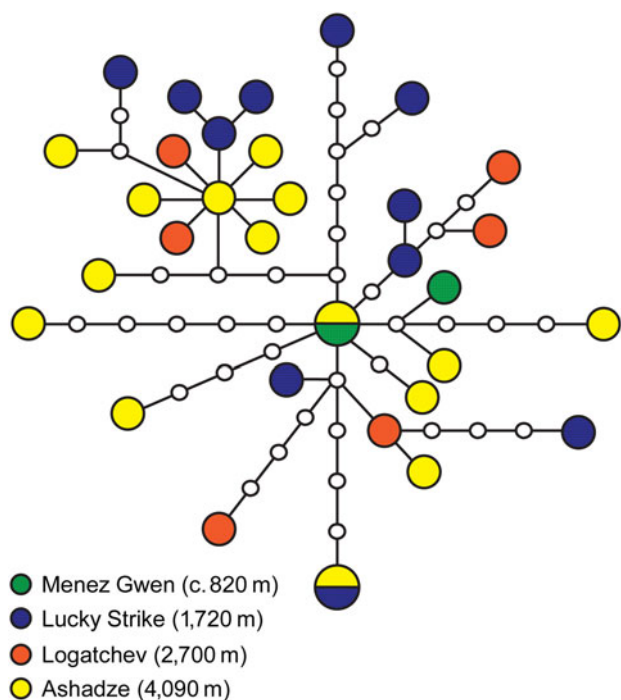
Table 3. Genetic differentiation tests among three local populations of '*Shinkailepas*' *briandi* based on 1276-bp sequences of mitochondrial COI gene (upper right: results of exact test; lower left: pairwise F_{st} values).

Locality	Lucky Strike	Logatchev	Ashadze
Lucky Strike		*	*
Logatchev	-0.0381*		*
Ashadze	0.0007*	0.0078*	

None of the values were significant (*$P > 0.05$).

The colourless, multispiral protoconchs of '*S.*' *briandi* were fairly constant in size (mean and standard deviation of the diameter: 706 ± 8 μm; range: 695–720 μm). No significant difference was observed among the populations of Lucky Strike (704 ± 7 μm), Logatchev (710 ± 5 μm) and Ashadze (710 ± 9 μm) by one-way ANOVA ($P = 0.43$). The shell diameter at hatching was estimated to be 170–180 μm, according to the sizes of 24 ready-to-hatch veligers in five egg capsules (two with 180-μm diameter and 22 others with 170 μm).

DISCUSSION

The present study first revealed the genetic population structure of a gastropod species endemic to deep-sea hydrothermal vents along the Mid-Atlantic Ridge. Our analyses based on 1.3-kbp mitochondrial DNA sequences from a total of 34 specimens of '*Shinkailepas*' *briandi* showed a panmictic population in the Menez Gwen, Lucky Strike, Logatchev and Ashadze vent fields. These vent fields are separated by up to 3150-km distances and distributed in a depth range of 814–4090 m, collectively covering the geographic and bathymetric distributions of the species (Figure 1). The mismatch distribution analysis and the values of Tajima's D and Fu's Fs suggested that the panmictic population of '*S.*' *briandi* has experienced a sudden demographic expansion, which apparently followed a bottleneck event in the last half a million
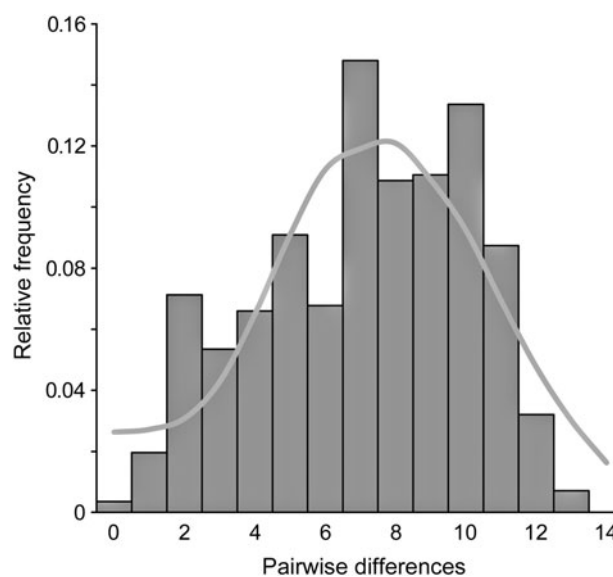

● Menez Gwen (c. 820 m)
● Lucky Strike (1,720 m)
● Logatchev (2,700 m)
● Ashadze (4,090 m)

Fig. 3. Haplotype network for '*Shinkailepas*' *briandi* based on 1276-bp sequences of mitochondrial COI gene from 34 individuals. Colour and circle size reflect locality and haplotype frequency, respectively; undiscovered haplotypes are shown as small open circles. Each line represents one mutational step.

Fig. 4. Mismatch distribution of '*Shinkailepas*' *briandi* based on 1276-bp sequences of mitochondrial COI gene. Bars show observed distribution; line denotes expected distribution under sudden expansion model.

years. A similar demographic history along MAR has previously been suggested for the alvinocaridid shrimp *Rimicaris exoculata*, with an estimate time of population expansion of 250,000 years ago (Teixeira *et al.*, 2012). Such bottlenecks could have resulted from unstable and ephemeral hydrothermal activities (Holm & Hennet, 1992), although no significant geological record exists for a plausible explanation for either of the limpet or shrimp species (see Teixeira *et al.*, 2012). Two more species of alvinocaridid shrimps exhibit genetic panmixia along MAR (Teixeira *et al.*, 2013).

The present limpet species develops as a long-lived planktotrophic larva (Warén & Bouchet, 2001; Fukumori & Kano, 2014), as alvinocaridid shrimps do (Ramirez Llodra *et al.*, 2000; Young, 2003). Whereas *in-situ* sampling has never been conducted to elucidate the larval behaviour of the limpet, an unidentified, vent-endemic alvinocaridid shrimp has been collected as a pelagic larva at a depth of 800–1000 m, which is ~1000 m above nearby vent sites (Herring, 2006). Stable isotopic analyses of post-settlement larvae of confamilial shrimps likewise suggest dependence on photosynthetic nutrition in their pelagic life (Stevens *et al.*, 2008). The alvinocaridid larvae are thus thought to feed and disperse in mid-water, far above the influence of a hydrothermal plume, which then might have resulted in their high population connectivity and wide geographic distributions (Teixeira *et al.*, 2011; see also Beedessee *et al.*, 2013).

The high genetic connectivity of '*S.*' *briandi* among the horizontally and vertically scattered vent habitats most probably resulted from a similar large-scale dispersal of larvae in a water column extraneous to hydrothermal vents. Most interestingly, their newly settled juveniles bear a pair of black eyespots, which are lost in larger individuals (Warén & Bouchet, 2001). The retention of eyes in the juveniles of deep-sea gastropods might suggest their vertical migration as swimming larvae to the photic zone (Bouchet & Warén, 1994). Such vertical migration has actually been demonstrated – by collecting larvae from the surface water – for the confamilial *Thalassonerita naticoidea* (= *Bathynerita naticoidea*; Fukumori *et al.*, in preparation) that inhabits cold methane seeps at depths from 400 to 1700 m in the Caribbean region (Arellano *et al.*, 2014). Converging evidence from larval behaviour, temperature optimum and population genetics indicates that this is also the case for a vent-endemic species of the family. *Shinkailepas myojinensis* in the north-west Pacific (442–1227 m depth) arguably takes advantage of richer nutrition and stronger currents in the surface water (Yahagi *et al.*, 2017).

The planktotrophic larvae of *T. naticoidea* and *S. myojinensis* are thought to spend at least 7–12 months before settlement (Arellano *et al.*, 2014; Yahagi *et al.*, 2017). The settlement size of planktotrophic neritimorphs is remarkably stable within a species (Kano, 2006) and it is probably not a coincidence that these phenacolepadids with such a long larval phase are larger at settlement than most other members of the subclass (Fukumori & Kano, 2014: table 1). The diameter of their shells increases significantly from 170 μm at hatching to 670–680 μm at settlement in *T. naticoidea* (Arellano *et al.*, 2014: figure 1) or from 140–160 μm to 720 μm in *S. myojinensis* (Fukumori & Kano, 2014; Yahagi *et al.*, 2017). '*Shinkailepas*' *briandi* has comparable sizes at both hatching (170–180 μm) and settlement (695–720 μm), implying a similarly long pelagic period for this species. It is also crucial to note that neritimorph larvae have an ability to remain pelagic for a few or

several months after attaining the settlement size, if a suitable settlement cue is not found (Scheltema, 1971; Lesoway & Page, 2008; Fukumori & Kano, 2014). The panmictic population of '*S.*' *briandi* along MAR has resulted from effective dispersal by their long-lived planktotrophic larvae, which likely migrate to and grow in the surface water, regardless of the deeper occurrences of their adults, hence a longer distance and duration of vertical migration than *T. naticoidea* and *S. myojinensis*.

Growing evidence suggests that other vent endemic animals, including at least some alvinocaridid shrimps and bythograeid crabs, also migrate not just to mid-water column but to the euphotic layer as planktotrophic larvae (Yahagi *et al.*, 2017). On the other hand, not all planktotrophs experience the warm, phytoplankton-rich surface water (see Young, 2003 for review). The planktotrophic mussels of the genus *Bathymodilus* on MAR, and their commensal annelid with lecithotrophic development, are each composed of multiple lineages that are divided by a dispersal barrier (Jollivet *et al.*, 2000; O'Mullan *et al.*, 2001; Breusing *et al.*, 2016). The vertical migration to the surface might perhaps be hampered by the physiological preference and/or tolerance of larvae in *Bathymodilus*. Planktotrophic larvae of another bathymodioline species in the Gulf of Mexico have been experimentally shown to have a lower thermal tolerance than those of the phenacolepadid *T. naticoidea* (Arellano *et al.*, 2014). Future studies on larval behaviour and temperature optimum for more numerous species would enable to evaluate this hypothesis and shed further light on the biogeography of the vent endemic fauna.

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