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Larval dispersal and life cycle in deep-water hydrothermal vents : The case of *Rimicaris exoculata* and related species.

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Dedicatory

This work is dedicated to three generations of women that are always present in my past, present and future paths:

To my grandmothers Belén Ávila and Irma Ramos de Hernández,

to my mother Nelly D. Ávila de Hernández, and

to my daughter Laura V. Hernández Pirela.

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Introduction

1.1 Deep-water hydrothermal vents: functioning considerations

Deep-water hydrothermal vents are geological structures located on the ocean floor at depth greater than 200 m that bring hot fluids from the sub-seafloor to the deep sea environment. These structures are found in all oceans, but are mostly distributed along the major accretion zones of the tectonic plates. In areas where active rifting occurs, new ocean crust is generated by magma emission, and mainly composed of basalt. These geological processes create the conditions for the formation of hydrothermal vents. Today, vent emissions are mostly known along major ocean ridge systems: as in the Atlantic Ocean (Mid-Atlantic Ridge, MAR), Artic Ocean (Artic Mid-Ocean Ridge), Indian Ocean (South-West, South-East and Central Indian Ridge), Eastern Pacific (East Pacific Rise, North-east Pacific Ridge and Galapagos) and Southern Pacific (Australian-Antarctic Ridge). All these ridges are connected and form a continuous mid-ocean system. Moreover some vent systems occur outside of this global system, as for example in the Caribbean (Mid-Cayman Spreading Center, MCSC), Antarctic Ocean (East Scotia Ridge), Western Pacific (Back-Arc Basins, Mariana Basin, Izu-Bonin Arc and Okinawa Trough), linked to subduction processes and local spreading (Fig 1).

After the first vent field had been discovered in 1977 (Corliss et al. 1979), our knowledge on their number and distribution range kept increasing until now. For instance, only during the last five years, new vent fields have been described from the Mid-Cayman Spreading Center (Connelly et al. 2012), the western Pacific Back-Arc Basins (Fouquet et al. 2015), the Australian-Antarctic Ridge (Hahm et al. 2015), the East Scotia Ridge (Rogers et al. 2012) and the Central Bransfield Basin (Aquilina et al. 2013). Moreover, hydrothermal vents are found outside of the ridge axis (the Lost City site, Kelley et al. (2001)) increasing the potential area of distribution of these structures. Recent estimations of the predicted distribution of hydrothermal vents along ocean ridges underline the large number of potential hydrothermal vents that remain to be discovered (Beaulieu et al. 2015, Baker et al. 2016, German et al. 2016).

Vent activity is a result of the interaction between the cold and oxygenated seawater with hot seafloor layers within the oceanic crust, close to the magma chamber. Along the ridges, the new oceanic crust is formed by magma emission and become cooler and fractured as it moves away from the ridge

axis. During this process the cold oxygenated seawater penetrates the oceanic crust through fissures and is submitted to heat and pressure. This leads to supercritical fluids that interact with rock layers resulting in mineral dissolution and the discharge of those compounds through vent emission (Fig. 2). The composition of the vent fluids exhibits large variations depending on the mineral composition of the crust around the vent field and the geological activity. The undiluted fluids (so-called “end-member” fluids) beneath the crust in most of the vent systems share some common features: they are anoxic, highly reduced, and acidic (pH from 2–4). Moreover they are rich in silica, carbon dioxide, hydrogen sulfide, methane, hydrogen, iron, zinc, copper, and many other transition metals (Von Damm 1995) (Table 1). As it is expelled and enters the sea water, the end-member fluid interacts with the deep oxygenated seawater, generating different physical and chemical reactions leading to mineral precipitation. These precipitations lead to the formation of chimneys composed of mineral deposits rich in iron, cooper, zinc sulfides and many other elements. Around the vent emission, there are deposits of mineral particles formed by precipitations occurring within the vent plume, entrained within the water column and later redeposited around the vent fields.

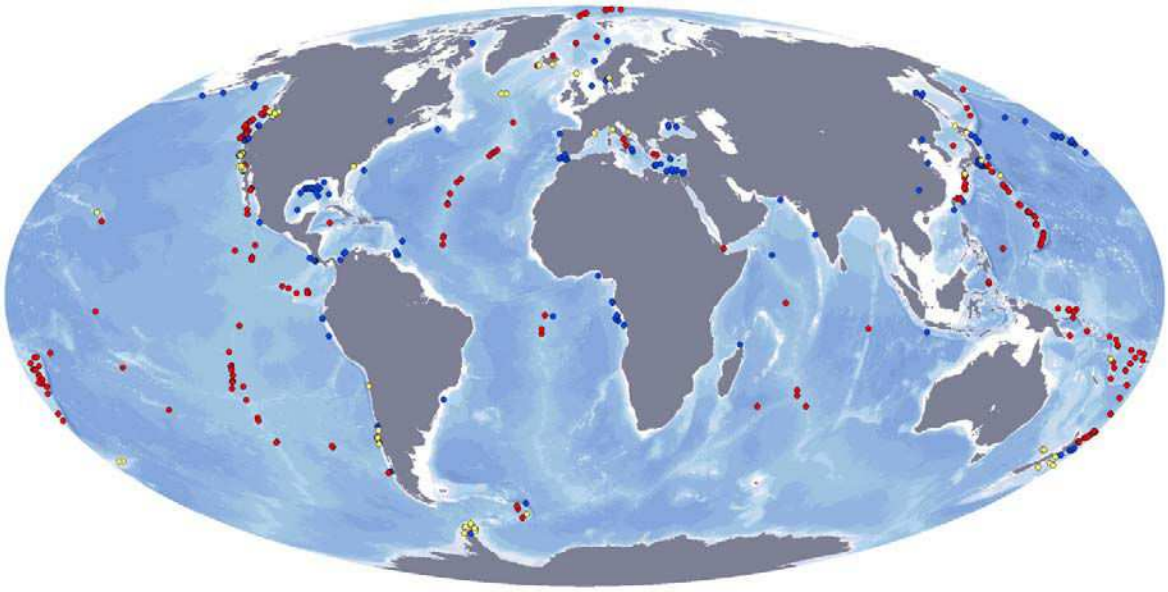

Figure 1. Global distribution of known hydrothermal vents and other chemosynthetic habitats. Red dots: hydrothermal vents, blue: cold seeps, yellow: whale fall. Image from German et al. (2011).

Four general types of venting processes are identified to date (German et al. 2010, Hodgkinson et al. 2015). The most common is associated to magma chambers under the ocean basaltic crust, associated with neovolcanism or tectonic plate activity, with hot emissions (up to 407 °C) with relatively low CH_4 and high metal concentrations. The second vent type is also a high temperature venting but involving serpentinization processes. In this case, the seawater interacts with mantel rocks which have been lifted up to the ocean floor during metamorphic processes. Since the composition of mantel rocks is different from that of basalts, different fluids are generated. This type of vent is characterized by high H_2, CH_4 and Fe concentrations in the vent fluids (eg. Rainbow vent field at the MAR). The third type of vent emission exhibits low temperature end-member fluids at 40-90 °C and high concentrations of H_2, CH_4 but low metal concentrations. This type of vent emission is only reported at the Lost City vent field, off of the ridge axis of the Mid-Atlantic Ridge (Kelley et al. 2001) and the Europe site of the Mid-Cayman Spreading Center (German et al. 2010). Recently, a new vent type was described at the Von Damn Vent Field of the Mid-Cayman Spreading Center, generated by the interaction of seawater with silica-rich basalt (mafic and ultramafic rocks) heated by magmatic interaction. It is characterized by warm end-member fluids (up to 215°C), high concentration of chloride and CH_4, moderate pH (5.8), low metal concentration and very high ratio (85-90 %) of silicate mineral (talc) in their precipitates (Hodgkinson et al. 2015).

Vent fluids can be separated according to their color and particles content. “Black Smokers” are generated by early deposition of anhydrite ($CaSO_4$) in the center of the vent emission, forming a pipe, followed by deposition of sulfide minerals in the inner layer of the pipe. Minerals found in the inner layer of the pipe include chalcopyrite ($CuFeS_2$) in high-temperature end-member emissions (≤ 330°C), and pyrite (FeS_2) or wurtzite ((Zn, Fe)S) at lower temperatures. Mineral structure restricts mixing between the seawater and the end-member fluid, and this “isolation” process is enhanced by the deposition of sulfide and sulfate minerals across the porous chimney wall (Tivey 2013). This results in the focalization of the emission of particles (including metal sulfides and oxides) that generates the ‘smoke’ of the chimneys (called ‘the plume’). However in vent emissions at lower temperatures (200-300 °C) the mixing process of the end-member fluids with seawater is different. Since the vent emission in this case is less active, more mineral particles precipitate inside of the pipe decreasing also the vent opening. The limited amount of sulfide particles in the fluids leads to a clearer fluid coloration, called “White Smoker Chimney”. In addition many white smokers lack anhydrite; instead zinc- and iron-sulfide minerals form the initial framework and internal structure. In other cases, barite and silica form the initial material, with internal deposits of barite, silica and sulfide minerals (Koski et al. 1994, Tivey 2013). Beside these compositions, black and white

smokers also exhibit differences in shape. Black smokers usually have few openings in a more or less vertical structure with a wide opening at the top (usually > 1 cm of diameter). White smokers usually have many narrow, connected conduits that can divert the flow horizontally and could be sealed by the mineral deposits (ie diffusing systems). Other structures found in white smoker chimneys are the beehive-shaped structures, which are bulbous, highly porous and with a hot surface (up to 70 °C), due to the fact that part of the fluid spread through the surface of the chimney (Fouquet et al. 1993, Koski et al. 1994).

A special case of vent chimney is found at the Lost City vent field. In this particular case, the temperature at the end-member fluid is much lower (< 100 °C), with a pH higher than seawater and low metal and sulfide concentrations. In this system, stalagmite-like structures are composed by calcite and/or aragonite ($CaCO_3$) and brucite ($MgOH_2$) which are saturated in the high pH of the fluid (Kelley et al. 2001, Kelley et al. 2005). Also the source of the hot fluids is not a transfer of the heat from the deeper crust layers. Instead, chemical reactions between sea-water and the mantle rocks (serpentinization) are highly exothermic and would be at the origin of heat in this particular system.

The hydrothermal vent fluid emissions also generate plumes during their ascension and mixing with seawater, especially the hot black smokers. The high temperature of the vent fluids decreases their density. As a consequence, the end-member fluid raises rapidly over the vent during the mixing processes. During the ascent, the fluid reduces its temperature and mix with the seawater resulting in an increase of the volume of the plume. Typically, vent fluids rise several 100s m above the seafloor and experience a 10^4-fold dilution up to the point where they reach a neutral buoyancy stage and start to spread horizontally. Neutrally buoyant plumes can generate heat and chemical anomalies that stretch 10s to 1000s of km down-current of their source (Baker 1990, 2013). Alsothe interaction between end-member fluids and sea water triggers a series of processes inside the plume including dilution, precipitation, chemical reactions and biological interactions.

Table 1. Composition of vent fluids in different hydrothermal vent systems

	Mid-Ocean Ridge	Back-Arc	Rainbow	Lost City	Von Damn	Seawater
T (°C)	≤ 405	278-334	365	≤ 91	215	2
pH (25°C)	2.8-4.5	< 1-5.0	2.8	10-11	5.8	8
Cl, mmol/kg	30.5-1245	255-790	750	548	667	545
Na, mmol/kg	10.6-983	210-590	553	479-485	589	464
Ca, mmol/kg	4.02-109	6.5-89	67	<30	16.4	10.2
K, mmol/kg	-1.17-58.7	10.5-79	20	-	17.5	10.1
Ba, µmol/kg	1.64-18.6	5.9-100	>67	-	8.4	-
H_2S, mmol/kg	0-19.5	1.3-13.1	1	<0.064	n.d.	-
H_2, mmol/kg	0.0005-38	0.035-0.5	13	<1-15	-	-
CO_2, mmol/kg	3.56-39.9	14.4-200	na	bdl	-	2.36
CH_4, mmol/kg	0.007-2.58	0.005-0.06	0.13-2.2	-	-	-
NH_3, mmol/kg	<0.65	-	-	-	-	-
Fe, µmol/kg	7-18700	13-2500	24000	-	n.d.	-
Mn, µmol/kg	59-3300	12-7100	2250	-	n.d.	-
Cu, µmol/kg	0-150	0.003-34	140	-	n.d.	-
Zn, µmol/kg	0-780	7.6-3000	160	-	-	-
Pb, µmol/kg	0.183-0.163	0.036-3.9	0.148	-	-	-
Co, µmol/kg	0.02-1.43	1	13	-	-	-
Cd, µmol/kg	0-0.910	-	0.130	-	-	-
Ni, µmol/kg	-	-	3	-	-	-
SO_4, mmol/kg	0	0	0	1-4	0	28
Mg, mmol/kg	0	0	0	<1	-	53

From Tivey (2007) (and references herein) and (Hodgkinson et al. 2015). N.d. not determined; -, not reported.

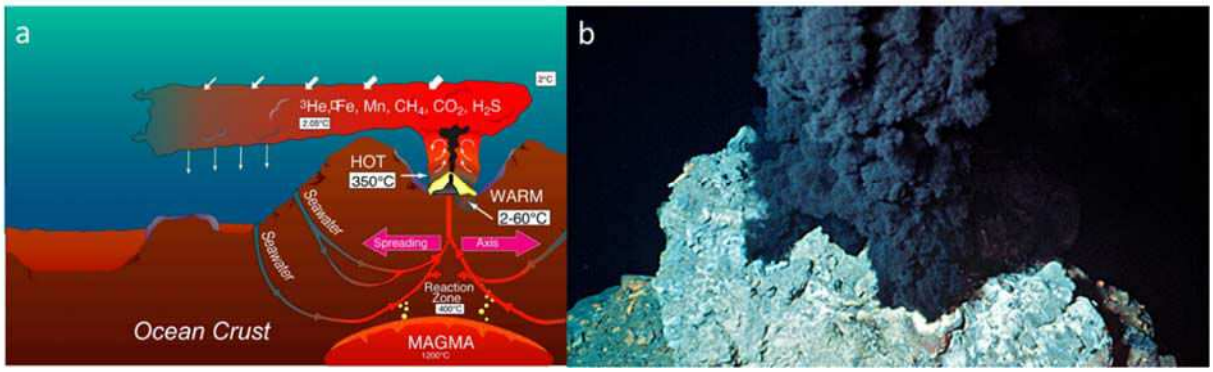

Figure 2. Geological settings and processes involved in hydrothermal vent activity. a, Schematic description of hydrothermal vent at a spreading center. b, Black smoker vent emission at the Snake Pit vent field on the Mid-Atlantic Ridge. Credits: a, NOAA Ocean Explorer; b, Woods Hole Oceanographic Institution

1.2 Deep sea hydrothermal ecosystems: a different way of life

Under these conditions of active fluid emission reaching toxic levels for most marine organisms, rich and abundant communities develop around the hydrothermal vents (Fig. 3). These communities are different from any other communities of the deep-sea benthos. They have characteristic forms of energy sources, assemblage structures, trophic relationships and environmental-biotic interactions. For these reasons, they are defined as 'deep-sea hydrothermal vent ecosystems' (Van Dover 2000). Although there are also marine shallow-water hydrothermal vents (<200 m depth), the physical and chemical features, the community structure and the level of habitat endemism of the species inhabiting shallow vents compared with deep-water ones, permit to separate these two environments as different ecosystems (Tarasov et al. 2005).

A remarkable feature of the deep-sea hydrothermal vent ecosystems is their source of energy and trophic structure. Unlike most marine ecosystems that are directly or indirectly sustained by the carbon sources generated by photosynthesis, deep sea vent systems rely mainly on microbial communities which are the main primary producers through chemoautotrophy (Fisher et al. 2007). Chemoautotrophy is the generation of organic matter from single carbon molecules coupled to reduction-oxidation reactions of others compounds (organic or inorganic molecules) as source of energy of the chemical reactions. In hydrothermal vents, the emission of fluids from the vents provides the ecosystem with reduced compounds required for chemosynthetic reactions driven by microorganisms. Although other deep-water marine ecosystems can be mostly sustained by chemosynthetic bacteria, as whale falls, wooden falls and minimum oxygen zones, they all depend on the deposition of organic matter originating from the surface

photosynthesis (Smith 2012). The only exceptions are the cold seep ecosystems and methane clathrates: here hydrocarbons originating either from biological (degradation of ancient organic matter deposited on the seafloor and buried into sediment layers) or geological processes are used by microorganism for primary production.

Cold seep ecosystems share ecological similarities with hydrothermal vents. Both show chemoautotrophy based trophic webs and a discrete distribution of sites usually dispersed at large scale. Athough many taxa appear to be shared at the family or even genus levels, species level community description indeed reveal a relatively low number of taxa shared betwwen these two types of "chemosynthesis-based" ecosystems. Hydrothermal vent are characterized by their high temperature and more focused release of reduced elements compared to fluid emissions at cold seeps. However, in terms of geological settings and environmental conditions, it is more and more clear that a continuum may exist between vents and seeps and that what separated vent and seep communities would be more the habitat characteristics (eg. depth, temperature or type of emission) than the vent or seep type itself (Levin et al. 2012, Plouviez et al. 2015, Portail et al. 2015). In most cases they are colonized by different taxa and are controlled by distinct environmental processes.

Despite the large diversity of microbial communities identified at hydrothermal vents (Sogin et al. 2006), few generalizations can be introduced for a broad description of the trophic processes. For instance, chemolithoautotrophic microorganisms can use the inorganic reduced compounds retrieved in the mix between vent fluids and seawater such as sulfide, elemental surfur, hydrogen or ferrous iron, as electron donors, and fix CO_2 in organic carbon. Such mechanisms are used by bacteria through different pathways, mostly related with the oxidation of reduced compounds. Primary production by chemolithoautotrophs sustains the heterotrophic compartment of both the microbial communities and, directly or indirectly, the rest of the hydrothermal community. Part of the trophic relationships between the primary producers (microorganisms) and the rest of the hydrothermal assemblages is through trophic networks of heterotrophic consumers at different levels. However metazoans, especially megafauna that largely dominates the vent biomass, host symbiotic bacteria. These symbiotic associations permit the direct transfer of the primary production generated by chemoautotrophy.

Hydrothermal vents show a high ratio of endemic species (Tunnicliffe 1992, Van Dover 2000) with different degrees of adaptation to the extreme environmental conditions and the particular trophic conditions, of an ecosystem based mostly on chemoautotrophy. For instance the vent fluids and the

surface of the chimney host thermoacidophilic microorganisms including the strain with the thermic record of life, the archaea 'strain 121' able to grow at 121 °C (Kashefi and Lovley 2003). Also some invertebrates can tolerate brief exposure to temperatures above 100 °C (Chevaldonne et al. 1992) and survive in culture conditions at 42°C (Ravaux et al. 2013). In terms of tolerance to toxic chemicals, different physiological mechanisms permit the tolerance and use of the elements released by the vent chimney. Detoxification of metals has been evidenced in microorganisms (Llanos et al. 2000, Edgcomb et al. 2004) and metazoans (McMullin et al. 2000, Kádár et al. 2006). Some bacteria exhibit metabolic pathways that permit the use of iron oxidation as a source of energy (Emerson et al. 2007). Although in microorganisms reduced compounds of vent fluids permit the chemosynthesis, for metazoans, the high concentrations of sulfide in the vent emission demands mechanisms of detoxification (McMullin et al. 2000). These mechanisms have been attributed to symbiotic bacteria in many species (Powell and Somero 1986, Vetter et al. 1987, McMullin et al. 2000).

Although vent communities are very diverse, showing variations at different spatial and temporal scales, they usually exhibit a reduced number of dominant taxa, some of which act as foundation species, that are associated with a suite of other, generally smaller, species. Distribution of the dominant taxa usually reflects biogeographical patterns of vent fauna at global scale. Hydrothermal vent communities exhibit major differences according to their biogeography, with distinction between those located on the mid-oceanic ridge system and those located at more isolated systems, such as back-arc ridges and smaller ridges (Bachraty et al. 2009). For instance, vent communities on the Mid-Atlantic Ridge are dominated by alvinocaridid shrimps and bathymodiolid mussels, whereas vestimentiferan tubeworms, alvinellid polychaetes and mussels are dominant at vent on the East-Pacific Rise. In the Western Pacific back-arc spreading centers, provannid gastropods, mussels and pedunculated barnacles are the most abundant species, with alvinocaridid shrimps present in some vents. Another characteristic assemblage found at the Scotia Sea vents includes *Kiwa* crabs, peltospirid gastropods and pedunculated barnacles. At global scale, the bathymodiolid mussels and the alvinocaridid shrimps are the most widespread taxa along the vents, both also colonizing cold seeps. However, they also show important changes in species composition between the biogeographical provinces (Table 2).

Table 2. Characteristic species of megafauna in the main vent biogeographical provinces

Biogeographical Provinces[1] (Vent fields or basins)	Dominant megafauna (taxa) [2]	References
North Iceland Mid-Ocean Ridge (Jan Mayen, Loki's Castle), 500-2400 m depth	*Sclerolinum* (pol), *Nicomache* (pol), *Skenea* (gas), *Exitomellita sigynae* (amp)	Pedersen et al. (2010), Schander et al. (2010)
Mid-Atlantic Ridge (Many vent fields from Menez Gwen-38°N- to Mephisto-5°S), 850-4200 m depth	*Bathymodiolus azoricus* and *B. puteoserpentis* (myt), *Rimicaris exoculata* and *Mirocaris fortunata* (alv), *Mariactis rimicarivora* (act)	Gebruk et al. (1997), Desbruyères et al. (2000)
Northeast Pacific, Juan de Fuca Ridge (Endeavour, Magic Mountain, Axial S.), 2200 m depth	*Paralvinella* spp. (pol), *Ridgeia picesae* (pol), *Lepetodrilus fucensis* and *Depressigyra globulus* (gas)	Sarrazin and Juniper (1999)
East Pacific Rise (Tamayo, Orozco, Clipperton, Siqueiros and others), 2500 depth	*Riftia pachyptila* (pol), *Bathymodiolus thermophilus* (myt), *Calyptogena magnifica* (ves)	Fornari et al. (2012) and references herein.
Central Indian Ridge (Kairei, Dodo, Solitaire, Edmond, Lonqi)	*Rimicaris kairei* (alv), *Bathymodiolus marisindicus* (myt), *Alviniconcha* (pro), *Chrysomallon squamiferum* (pel).	Nakamura et al. (2012), Watanabe and Beedessee (2015)
Western Pacific Back Arc (Mariana, Manus, North Fiji and Lau basins)	*Ifremeria nautilei, Alvinoconcha* spp. (pro), *Rimicaris* sp. (alv), *Vulcanolepas* and others barnacles, *Calyptogena* (ves)	Desbruyères et al. (2013)
Northwestern Pacific complex (Japan Ridge, Okinawa Trough and Arc, Izu-Ogasawara Arc), 300-1500 m depth	*Bathymodiolus septemdierum* and *B. platifrons* (myt), *Shinkaia crosnieri* (gal), *Shinkaicaris leurokolos, Alvinocaris longirostris* (alv), *Neoverruca* sp. (max)	Nakajima et al. (2014), Yahagi et al. (2015)
East Scotia Basin (Dog Head, Car Wash, Ivory Tower vent fields), 2400 m depth	*Kiwa tyleri* (kiw), *Vulcanolepas* (bal), *Lepetodrillus* sp., *Gigantopelta chessoia* (gas), Actinostolidae and Hormatiidae (ant)	Rogers et al. (2012)
Mid-Cayman Spreading Center (Von Damm and Beenbe vent fields), 2300-4960 m depth	*Rimicaris hybisae* (alv), *Maractis* sp. (act), *Iheyaspira bathycodon* (tur), *Provanna* (pro), *Escarpia* sp. and *Lamellibrachia* sp. (pol), *Bathymodiolus* sp. (myt)	Connelly et al. (2012), Plouviez et al. (2015)

[1]According to Bachraty et al. (2009), Rogers et al. (2012). [2]At different taxonomic levels: act, Actinaria; pol, Polychaeta; gas, Gastropoda; tur, Turridae; pro, Provannidae, pel, Peltospiridae; myt, Mytilidae; ves, Vesicomyidae; amp, Amphipoda; alv, Alvinocarididae; gal, Kiwaidae; max, Maxillopoda. References are a selection of papers on community structure for each area.

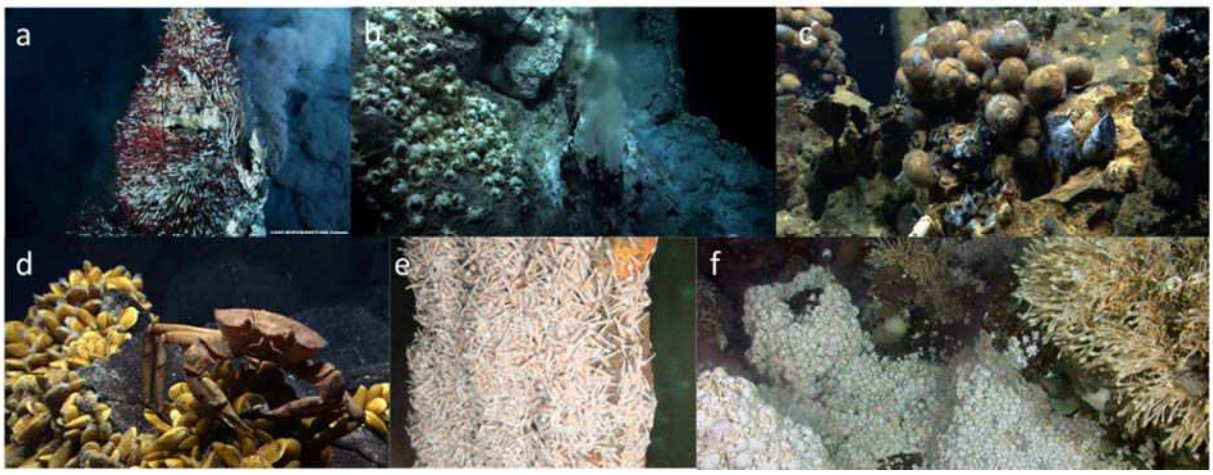

Figure 3. Examples of hydrothermal vent communities with different dominant taxa in different biogeographical provinces. a, Siboglinid tubeworms *Ridgeia piscesae* at Juan de Fuca Ridge; b, galatheid *Shinkaia crosnieri* in the Okinawa Trough; c, gastropod *Alvinoconcha* sp. in western Pacific Back Arc spreading centers; d, *Bathymodiolus* mussels and a crab on the Mid-Atlantic Ridge; e, swarms of the Alvinocaridid shrimp *Rimicaris hybisae* in the Mid Cayman Spreading Center; f, the chirostyloid *Kiwa tyleri* aggregated close to the emission and aggregations of barnacles (cf. *Vulcanolepas*), East Scotia Ridge. Credits: a, NEPTUNE Program, Canada; b-d, Bremen University; e, Connelly et al. (2012); f, Rogers et al. (2012).

1.3 Bacterial symbiosis in hydrothermal vent species

Most of the dominant species at hydrothermal vents (Table 2) host microorganisms that contribute to their metabolism. The bacteria-host relationships are very diverse in term of identity and number of the partners involved, as well as in the degree of integration of the symbiotic relationship. In general, symbiosis can be distinguished according to the location of the symbiotic microorganisms. When the microorganisms are outside of the host cells, for example on external structures, the relation is called episymbiosis. In the case of bacterial colonization inside of the host (ie in organs, tissues or cells) the relation is called endosymbiosis. The episymbiotic relationships usually include farming-like behavior of the host, keeping and promoting the colonization of microorganisms for posterior harvesting and consumption (Thurber et al. 2011). This is the case in vent squat lobsters such as *Kiwa* sp. or *Shinkaia* sp. (Thurber et al. 2011, Watsuji et al. 2015, Zwirglmaier et al. 2015). However direct transfer of organic carbon from episymbiotic bacteria to their host trhough transcuticular exchanges is also demonstrated in the hydrothermal vent shrimp *Rimicaris exoculata* (Ponsard et al. 2013). In the endosymbiotic relationships, two main models can be recognized: i) the bacteria are found inside of specific cells (called bacteriocytes) within host organs such as the gills (Windoffer and Giere 1997, Duperron et al. 2005), ii)

the bacteria live inside of cells called bacteriocytes, occurring in a specialized internal organ called trophosome, sometimes representing a large proportion of the host (Dubilier et al. 2008). The degree of integration of the host-symbiotic relationship could involve more or less advanced morphological adaptations such as a the swelling of gills observed in *Bathymodiolus* or *Calyptogena* bivalves (Childress and Fisher 1992), a reduction of the digestive tract of the host as observed in *Rimicaris* shrimps (Segonzac et al. 1993), or even the complete lack of it as observed in *Siboglinid* tubeworms (Windoffer and Giere 1997, Childress and Girguis 2011). Bacterial symbionts may also undergo important modification when involved in a symbiosis such as genome reduction (Kuwahara et al. 2007).

Symbiotic bacteria have very diverse metabolic pathways, the most common ones at hydrothermal vents being sulfur-oxidation, followed by methane and hydrogen-oxidation (Dubilier et al. 2008, Petersen et al. 2011). Sulfur-oxidation can be achieved through different chemical pathways that use hydrogen sulfide or other reduced sulfur elements (such as thiosulfide, sulfite or elemental sulfur) in oxidative reactions to generate sulfate and obtain energy. Similarly, methane and hydrogen can be used as reduced compounds in oxidative reactions to obtain energy. In the case of methane oxidation, methane can serve as energy source or be directly incorporated into organic compounds. In sulfur and hydrogen oxidations, the energy obtained is used to produce organic compounds trhough CO_2 fixation, and more rarely trhough CO fixation. In addition, iron-oxidation has been suggested for episymbiotic bacteria as a new mechanism of energy production for CO_2 fixation (Zbinden et al. 2004, Jan et al. 2014), denoting the diversity of mechanisms involved in energetic production in symbiotic bacteria.

The mechanisms involved in the transfer of organic compounds from the symbiotic bacteria to the host show different degrees of complexity. The most simple mechanism is through harvesting and consumption of episymbiotic bacteria, as reported in the yeti crab *Kiwa puravida* (Thurber et al. 2011). In other cases of episymbiosis, the metabolites produced by the bacteria are translocated directly through the host surface. This mechanisms was initially suggested to occurs in pompei worms, *Alvinella pompejana* (Desbruyères et al. 1998), but was only demonstrated for the alvinocaridid shrimp *R. exoculata* (Ponsard et al. 2013). For species that host endosymbiotic bacteria, the digestion of the endosymbiotic bacteria themselves is the main mechanism of carbon transfer, instead of direct translocation of nutrients to the host (Riou et al. 2008, Childress and Girguis 2011) (Fig 4). However, experiments in tubeworms demonstrates that both mechanisms of nutrient translocation and bacterial digestion could be important for the host nutrition (Bright et al. 2000). The digestion of the endosymbionts requires a complex life cycle

organization for reproduction, growth and digestion of bacteria inside of the bacteriocyte in order to maintain an active symbiosis (Bright et al. 2000).

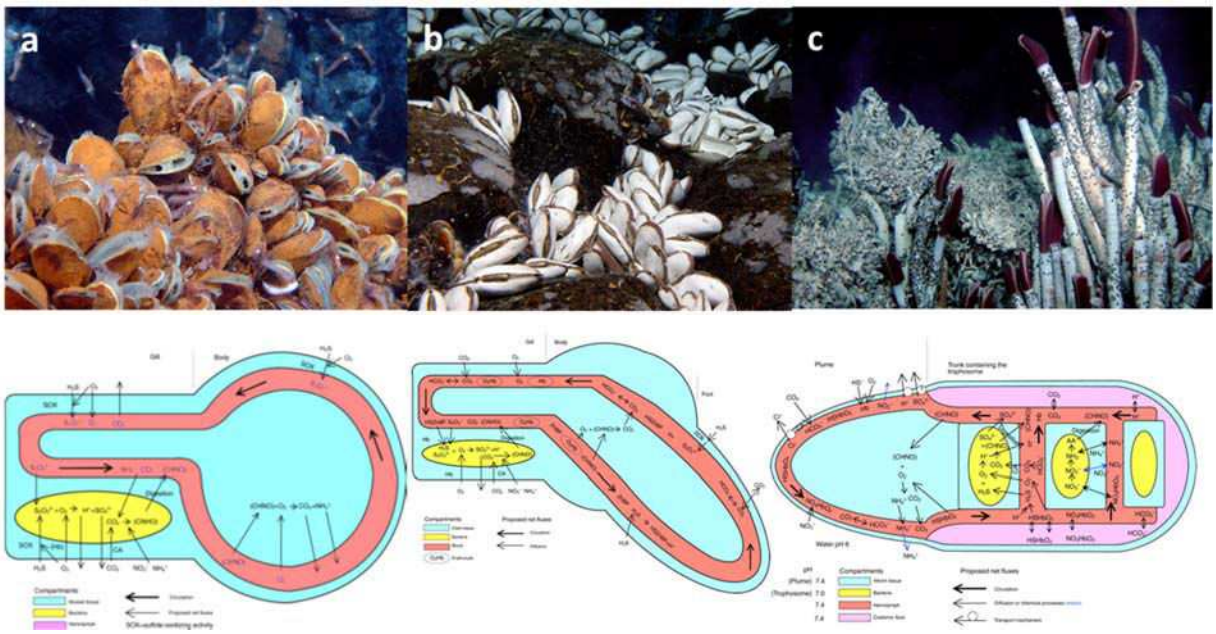

Figure 4. Three schematic diagrams of physiological interactions between endosymbiotic bacteria and host: (a) *Bathymodiolus* mussels, (b) *Calyptogena* clams and (c) *Riftia* tubeworms. Bacteria (yellow) are located in the gills (*Bathymodiolus* and *Calyptogena*) or in the trophosome (*Riftia*). The hemolymph (red) transports the products of the bacterial metabolism to the host tissue (blue) and in the tubeworms the hemolymph also transports the initial susbtrates (e.g. reduced compounds) and excretion products. Schemas obtained from Childress and Girguis (2011). Photo credits: a, c: Ifremer; b, WHOI.

The acquisition of bacterial symbionts at each host generation (transmission) follows two main mechanisms. Vertical transmission implies that the symbionts are passed from the parent to the egg or embryo during reproduction (Dubilier et al. 2008). In *Calyptogena* clams (*C. magnifica, C. phaseolifromis* and *C. pacifica*) endosymbotic bacteria are directly transmitted from the parental female to the eggs (Cary and Giovannoni 1993). However, vertical transmission of symbionts has rarely been demonstrated for vent species and horizontal transmission has been suggested in most of cases. In horizontal transmission, the symbionts are taken up from the environment or from co-occurring hosts during the development (Dubilier et al. 2008). Such transmission mechanism results in different evolutionary pathways between

the host and the symbionts, as reflected by their different phylogenies. This is the case, for instance, in *Bathymodiolus* mussels, where host and symbiont phylogenies reflect different evolutionary history (Won et al. 2003). This mechanism is also present in the acquisition of endosymbiots in vent tube worms, *Riftia pachyptila*, *Tevnia jerichonana* and *Oasisia alvinae* (Nussbaumer et al. 2006, Harmer et al. 2008) and seems to be the mechanism of acquisition of episymbiotic bacteria. Hypothesis of horizontal transmission for epibiotic bacteria is supported by the higher diversity in the bacterial assemblage of episymbionts and the affinities with bacteria collected in environmental samples of the host habitats (Dubilier et al. 2008). Even for species with vertical transmission, there is evidence that support a complementary horizontal transmission of bacteria (Stewart et al. 2008).

1.4 Connectivity in hydrothermal vents

The distribution of vent sites within each biogeographical province is discontinuous, and adjacent known vents are usually separated by distances from few kilometers to hundreds of kilometers, in some cases even thousands of km (Ramirez-Llodra et al. 2007, Bachraty et al. 2009). Moreover, vent sites can turn to be inactive or new vents can be generated according to the geological activity of the sea floor and to their position from the axis of the ridge. In some cases the hydrothermal vents can even show massive explosions, eliminating the communities settled previously and generating new substrates for *de novo* colonization (Shank et al. 1998, Mullineaux et al. 2010). In this context of discontinuous and ephemeral environments, the vent species manage to colonize almost all vent sites in different patterns depending on the biogeographical province and the species under study.

At community level the connectivity between vent ecosystems can be estimated based on biogeographical patterns in the taxa composition of hydrothermal vent (Bachraty et al. 2009, Rogers et al. 2012). Shared species between vent sites indicate that organisms are able to overcome the distance between vents in order to colonize them, at least during the evolutionary time of the species. Within biogeographic provinces, the discrete and ephemeral nature of the hydrothermal vent often brings an ecological scenario associated to an 'island pattern' of species distribution (Wilson and Robert 1987, Johnson et al. 2006b). For instance, there is a high proportion of shared species between vent sites of the Mid-Atlantic Ridge, despite distances between vent sites being over 1000s km, with differences reflecting mainly the depth of vent emission (Desbruyères et al. 2000). However, vent systems separated by similar

spatial ranges can show major differences in the species composition of the megafauna, as observed between the Mid-Atlantic Ridge and the Mid-Cayman Spreading Center (Plouviez et al. 2015).

At population level, the exchange of migrants between different vents has been estimated in some species through the analysis of gene flow between populations. The genetic structure of the population is usually linked to different levels of connectivity between populations. Some species show high rate of migrant exchange between closer vents (up to 100s km), but genetic flow is more restricted over 1000s of kms (Thaler et al. 2011), as occurs in tubeworm *Riftia pachyptila* (Vrijenhoek 1997). At large spatial scale, the restriction in genetic flow could be directly linked to the distance between the vents (Coykendall et al. 2011) or even associated with the position of the vents across the equator or between adjacent axes of the ocean ridge (Hurtado et al. 2004). For some taxa, as in vent limpets, complete isolation and speciation have been proposed at distance of few 100s km (Johnson et al. 2006a). However, there are many examples of species, particularly in vent shrimps, where gene flow restriction cannot be detected at scale of 1000s km, as reflected by the lack of differences in the population structure along the entire known distribution range (Teixeira et al. 2012, Beedessee et al. 2013, Yahagi et al. 2015).

Most of the benthic hydrothermal fauna is confined to the vent ecosystem due to their strict dependence to fluid emission and reduced compounds in order to maintain their symbiotic bacteria, or because of the use of sources of organic matter produced in the vent system by chemosynthesis. For these reasons, the connectivity of benthic megafauna in hydrothermal vents is probably driven by the dispersal and colonization abilities of the species early stages, mostly larval forms. Most of the species produce planktonic larval forms that inhabit the water column, and could be able to disperse and recruit at different hydrothermal vents. The combination of larval traits, larval physiological tolerance to the environmental conditions encountered during dispersal and the hydrodynamic conditions of the area determine the potential of the larvae for settling at different vents and promoting colonization and migrant exchange (Marsh et al. 2001, Mitarai et al. 2016).

There are four important features associated with the dispersal potential of larvae: the planktonic larval duration (PLD), the survivorship, the nutrition and the position in the water column during the larval period (Grantham et al. 2003). Other biological traits such as behavior, swimming and perception may also significantly influence larval dispersal. Unfortunately there are many knowledge gaps in these aspects of hydrothermal vent species biology that limit our understanding of the early stages of their life cycle. However, some findings denote the complex early life history of vent species and their dispersal. Most of

the vent species do not develop at atmospheric pressure, restricting rearing experimental approaches for direct observation of larval biology to the use of high pressure tanks (Tyler and Dixon 2000, Marsh et al. 2001, Pradillon et al. 2004, Brooke and Young 2009) with few exceptions (Koyama et al. 2005, Hamasaki et al. 2010). As a consequence, very few direct observation data are available, and only one species (*Gandalfus yunohana*) has been cultured through the complete planktonic phase (Hamasaki et al. 2010). Marsh et al. (2001) estimate the duration of the non-feeding larvae of the tubeworm *Riftia pachyptila* at 34 days, based on physiological modeling at 2°C and 250 atm. This estimation is similar for the duration of the complete development for the feeding larvae of the hydrothermal crab *Gandalfus yunohana* (34-60 days at 24-30°C and 1 atm, Hamasaki et al. (2010)). However, in other species the duration of the larval development could be much more extended. Arellano and Young (2009) estimate, in a cold-seep mussel species closely related with hydrothermal *Bathymodiolus* (*B. childressi*), a larval duration over a year based on settlement times and spawning seasons. The duration of the planktonic period could be also enhanced by developmental arrest and delay of metamorphosis that could permit the dispersal of the species over large distances and the detection of appropriated habitats for recruitment (Pradillon et al. 2001, Arellano and Young 2009, Adams et al. 2012).

For many of the hydrothermal vent species, the larval duration has been estimated indirectly from larval nutrition mode (Van Dover et al. 1985, Tyler and Young 1999). It is presumed that larvae able to feed on the plankton (planktotrophic) have greater dispersal potential than those with a nutrition based on the egg lipid reserves (lecitotrophic) which could constrain the energetic resources. Moreover, the nutrition mode of the larvae itself is usually estimated indirectly from the egg size and the adult fecundity of the species (Van Dover et al. 1985, Tyler and Young 1999). Different early life modes have been proposed for different phyla using these indirect approximations, the direct evidence and by extrapolations from shallow-water of non-vent relatives. For instance, in polychaetes, all modes of larval development, including lecitotrophic or planktotrophic trochophore larvae but also the complete lack of larval stages (direct development) have been proposed for *Paralvinella* spp. (Zal et al. 1995, Tyler and Young 1999). For bivalves, a lecitotrophic larva is suggested in vesicomyids and solemyids, but planktotrophic larvae is proposed for *Bathymodiolus* mussels. In crustaceans planktotrophic larvae are proposed in alvinocaridid shrimps and bythograeid crabs, but chirostyloid, as *Kiwa tyleri*, show lecitotrophic larvae (Tyler and Young 1999, Thatje et al. 2015). Although some of these inferences on larval modes have been corroborated by direct examination (Thatje et al. 2015), in other species, it seems that

more complex larval modes may exist (present work), and association of lecitotrophic early stages with limited dispersal is not always supported (van der Heijden et al. 2012).

Another important issue about the early life of deep sea hydrothermal species and their dispersal is the position of the larval stages in the water column during the dispersal period. Although the larval stages could be able to swim, their dispersal at medium or large scale is mostly driven by the hydrodynamic regime of the area. The variation in the vertical position of the larvae by active swimming or differential buoyancy can affect the velocity, the dispersal trajectory and even the food supply for the planktotrophic larvae (Young et al. 2012, McVeigh 2016). Different scenarios of dispersal and position of the larva in the water column have been proposed based on both direct and indirect evidences. In general, the dispersal scenarios are classified into four categories: i) lack of dispersal and recruitment at the vent origin of the larvae, ii) bottom current dispersal, iii) plume level dispersal and iv) surface water migration (Adams et al. 2012) (Fig. 5). For instance, a mechanism of larval retention in vent gastropods (including *Lepetrodrillus* spp., *Peltospira* spp. and *Cyathermia naticoides* as the most abundant) of the East Pacific Rise was proposed based on the increase of larval density close to the bottom and the vent source (Mullineaux et al. 2005), promoted by active positioning of the larvae close to bottom (Mullineaux et al. 2013). The transport of larvae using the vent plume could overcome the topographic limitations of near-bottom currents (Kim et al. 1994). This mechanism was proposed for *Lepetrodrilus* sp., peltospiroid gastropods and vent bivalves, supported by the higher concentration of larvae inside to the vent plume than in its surrounding (Mullineaux et al. 1995). Also, plume-mediated migration has been proposed to explain the dispersal of the tubeworm *Riftia pachyptila* (Marsh et al. 2001). Finally, vertical migration of the larvae to the photic zone has been proposed for some hydrothermal vent species based on the development of visual structures, as in the crab *Bytrograea thermydron* (Dittel et al. 2008), tolerance of larval cultures to shallow-water conditions, which is observed in *Gandalfus yunohana* (Hamasaki et al. 2010) or lipid isotopic signatures in alvinocaridid postlarvae (Dixon et al. 1998). Recently, Arellano et al. (2014) reported direct observation of vertical migration to the photic area, with collections of larvae of the deep-water cold-seep species *Bathynerita naticoidea* and *Bathymodiolus childressi* in shallow waters (0-100 m). These findings permit to suggest that *Bathymodiolus* species from hydrothermal vent could also use this mechanism for larval dispersal.

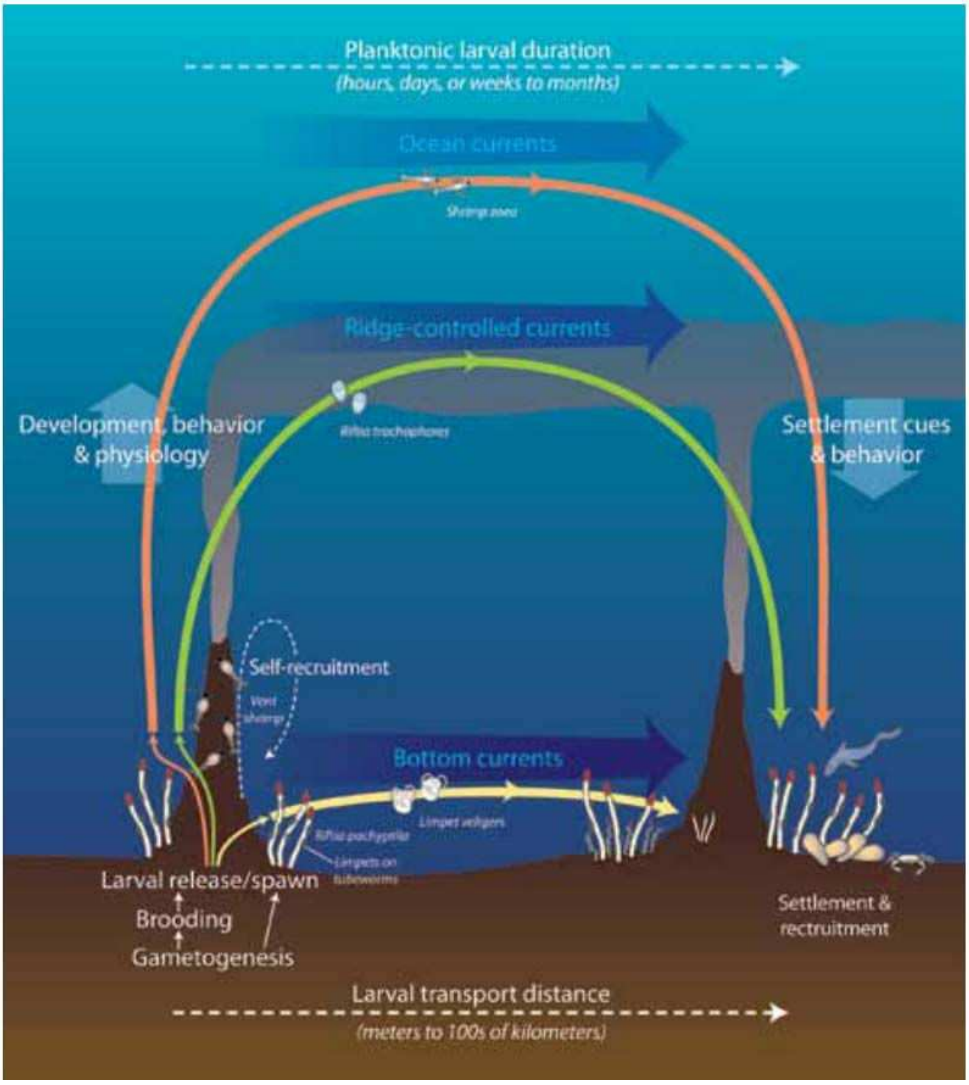

Figure 5. Simplified models of larval dispersal in hydrothermal vent species (Adams et al. 2012). The models illustrate the dispersal modes proposed for: *Riftia pachyptila*, following bottom currents (Brooke and Young 2009); gastropods with dominance of self-recruitment (Mullineaux et al. 2005); plume dispersal suggested in limpets (*Lepetodrilus* sp.), bivalves (*Calyptogena* sp.) and others (Mullineaux et al. 1995). Surface-water dispersal suggested in shrimps (alvinocaridids) and crabs (bythograeids) (Dixon et al. 1998, Dittel et al. 2008)

1.5 Reproductive patterns in vent species populations

At population level, the life cycle and ecological interactions generate patterns of reproduction and population structure. For the dominant species in vent systems, the population biology and reproduction is particularly important in terms of ecosystem functioning due their effect on the biomass

and energy flows (Van Dover 2002b, Govenar et al. 2004, Bergquist et al. 2007). In addition, patterns of colonization and succession are largely affected by the recruitment of dominant taxa at the early or intermediate successional stages of the community (Mullineaux et al. 2000, Sen et al. 2014). The population biology and resilience of the few dominant species have a large impact on the hydrothermal vent ecosystem structure and functioning. However, performing studies on dominant taxa represent a challenge due to dense aggregations found for these species (usually over 1000s ind m^{-2}), the different scales of distribution of the populations (from few meters to 1000 kms), the complex life cycles and the sampling limitations of hydrothermal vents ecosystems.

Analyses of gonad development indicate continuous or semi-continuous reproduction in many vent species. This is the case for the giant tubeworm *Riftia pachyptila* (Gardiner et al. 1992, Hilario 2005), the alvinellids worms *Alvinella pompejana* (Faure et al. 2007) and *Paralvinella pandorae* (McHugh 1989), peltospirid gastropods (*Nodopelta heminoda*, *N. subnoda* and *Peltospira operculata*) (Matabos and Thiebaut 2010), bythograeid crabs (*Bythograea laubieri and B. vrijenhoeki*) (Hilario et al. 2009) and alvinocarid shrimps (Ramirez-Llodra et al. 2000), among others (Tyler et al. 2008). The lack of major seasonal variation in the deep-sea has been proposed as the main cause of continuous gonadal production of these species (Tyler and Young 1999). However, other species show a seasonal maturation of the gonads. This is the case for *Bythograea thermydron,* that shows late oocyte maturation at the end of the autumn (Perovich et al. 2003). Seasonality has also been suggested in the vent mussel *Bathymodiolus azoricus* with observations of late maturation and spawning during late winter (Colaço et al. 2006, Dixon et al. 2006) and spring (Comtet et al. 1999). In the eastern Pacific vent polychaetes *Paralvinella plamiformis* and *P. grasslei* have been proposed to have several discrete breeding periods along the year (McHugh 1989, Zal et al. 1995). Late winter spawning in some hydrothermal vent species, as *Bathymodiolus azoricus*, and cold seep species, as *Alvinocaris stactophila,* has been associated with the increase of primary production in the euphotic zone during spring season which would enhance food availability for planktotrophic larvae (Copley and Young 2006, Dixon et al. 2006).

In addition to studies of gonadal maturation or observation of spawning events, reproductive patterns have also been inferred from recruitment patterns, discontinuous recruitment being associated with discontinuous spawning. In some species, gonadal development, spawning timing and patterns of recruitment seem consistent (McHugh 1989, Zal et al. 1995, Comtet and Desbruyeres 1998). However, for other species continuous or semicontinuous gonadal development has been observed, contrasting with the periodic brooding or recruitment. For instance in *Riftia pachyptila*, population size structure reflects discontinuous recruitment in time (Thiebaut et al. 2002), but gamete production appears to be a continuous process. In alvinocaridid shrimps, from the three species from the Mid-Atlantic Ridge with semi-continuous gonadal

maturation analyzed by Ramirez-Llodra et al. (2000), only one, *Mirocaris fortunata*, shows significant amount of brooding females during summer. The discrepancy between reproductive rhythms estimated from observations of gonad maturation and of recruitment patterns would be mostly explained by events occurring during the early life history of the species. For instance, larval duration and dispersal, as well as oceanographic processes could affect the larval migration and the time of recruitment (ref).

In motile species, post-recruitment processes could also affect the spatial distribution of the population according to life stage (ie juveniles and adults) or sex. For instance, in the alvinocarid shrimp *Rimicaris exoculata*, aggregations of juveniles have been reported inhabiting very close to adult populations (Vinogradrov and Vereshchaka 1995, Shank et al. 1998). Perovich et al. (2003) report a specific distribution pattern in brooding females of *Bythograea termidron* that suggests a migration at the vent periphery at the end of the brooding period. For the anomuran crab *Kiwa tyleri,* Marsh et al. (2015) described the distribution of the population along the gradient of hydrothermal vent conditions and proposed complex pattern of habitat utilization. This model includes the location of juveniles in the periphery of the vent emission and the colonization of the vent chimney exclusively by large males. Non-brooding females were found besides the large male assemblage but the brooding females migrated to the vent periphery during the egg development (Fig. 6).

Such diversity in biological traits and complex life cycles preclude the elaboration of inferences about the general functioning or dynamics of vent systems. Indeed, sampling design for population studies must take into account the potential level of spatial and temporal variation in the populations in order to elucidate the patterns of distribution.

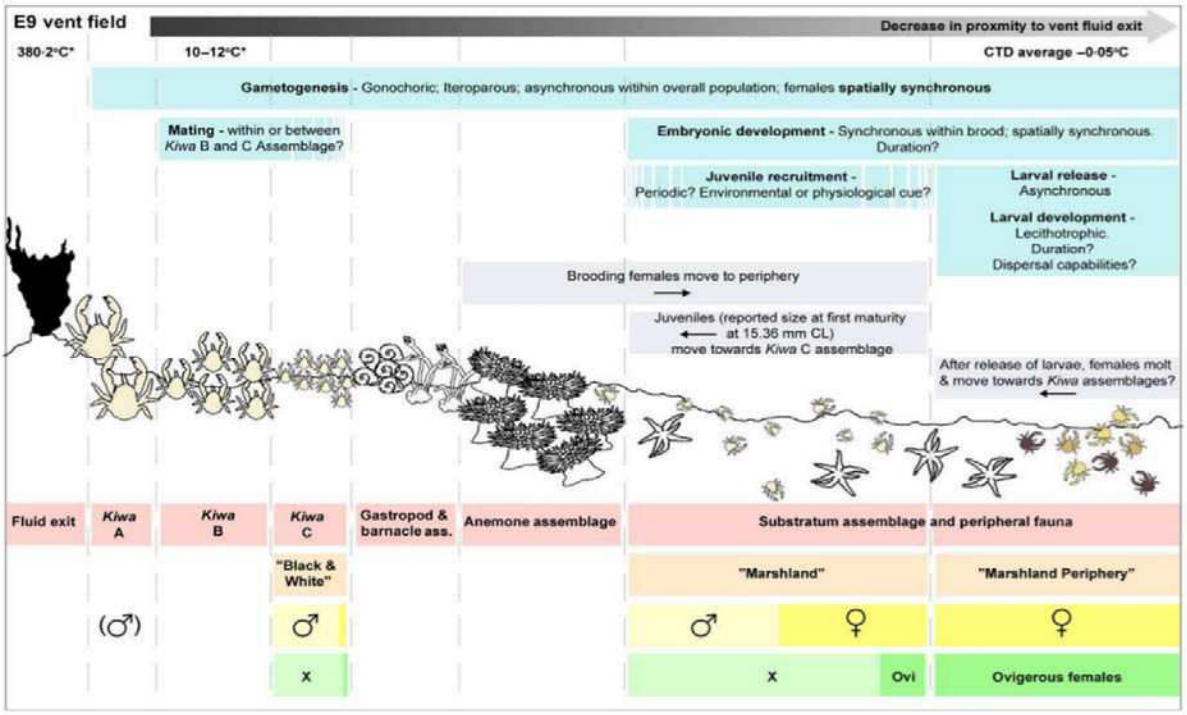

Figure 6. Schematic life history of *Kiwa tyleri* according to Marsh et al. (2015) based on spatial variation of population structure, at E9 vent field, East Scotia Ridge.

1.6 Rimicaris exoculata and allies…

1.6.1 General biology and related species

R. exoculata is probably the most studied deep-water crustacean due to its importance in hydrothermal vent ecosystems at the Mid-Atlantic Ridge and its remarkable biology (Segonzac et al. 1993, Van Dover 2000, Schmidt et al. 2008a). However, there are still many gaps in our understanding of its life history, biology, reproduction and dispersal. This species has been discovered during the early exploration of the TAG vent field on the Mid-Atlantic Ridge (MAR) (Williams and Rona 1986), where it forms massive 'swarms' around the vent emission (Fig. 7). Currently known distribution of *R. exoculata* comprises the MAR vent fields deeper than 1700 m depth, between 45.5°N and 4.8°S. Vent fields included in its distribution are: Moytirra (45.48'N, 27.84°W, 2095 m), Lucky Strike vent field (37.28°N, 32.27°W, 1700), Rainbow (36.2°N, 33.9°W, 2285 m), Broken Spur (29.16°N, 43.17°W, 3050 m), TAG (26.1°N, 44.8°W, 3600 m), Snake Pit (23.4°N, 23.95°W, 3500 m), Logatchev (14.75°N, 44.98°W, 3028 m), Ashadze 1 (12.97°N,

44.9°'W, 4088 m) and Red Lion (4.8°S, 12.4°W, 3047 m) (Desbruyères et al. 2000, Komai and Segonzac 2008, Fabri et al. 2011, Wheeler et al. 2013).

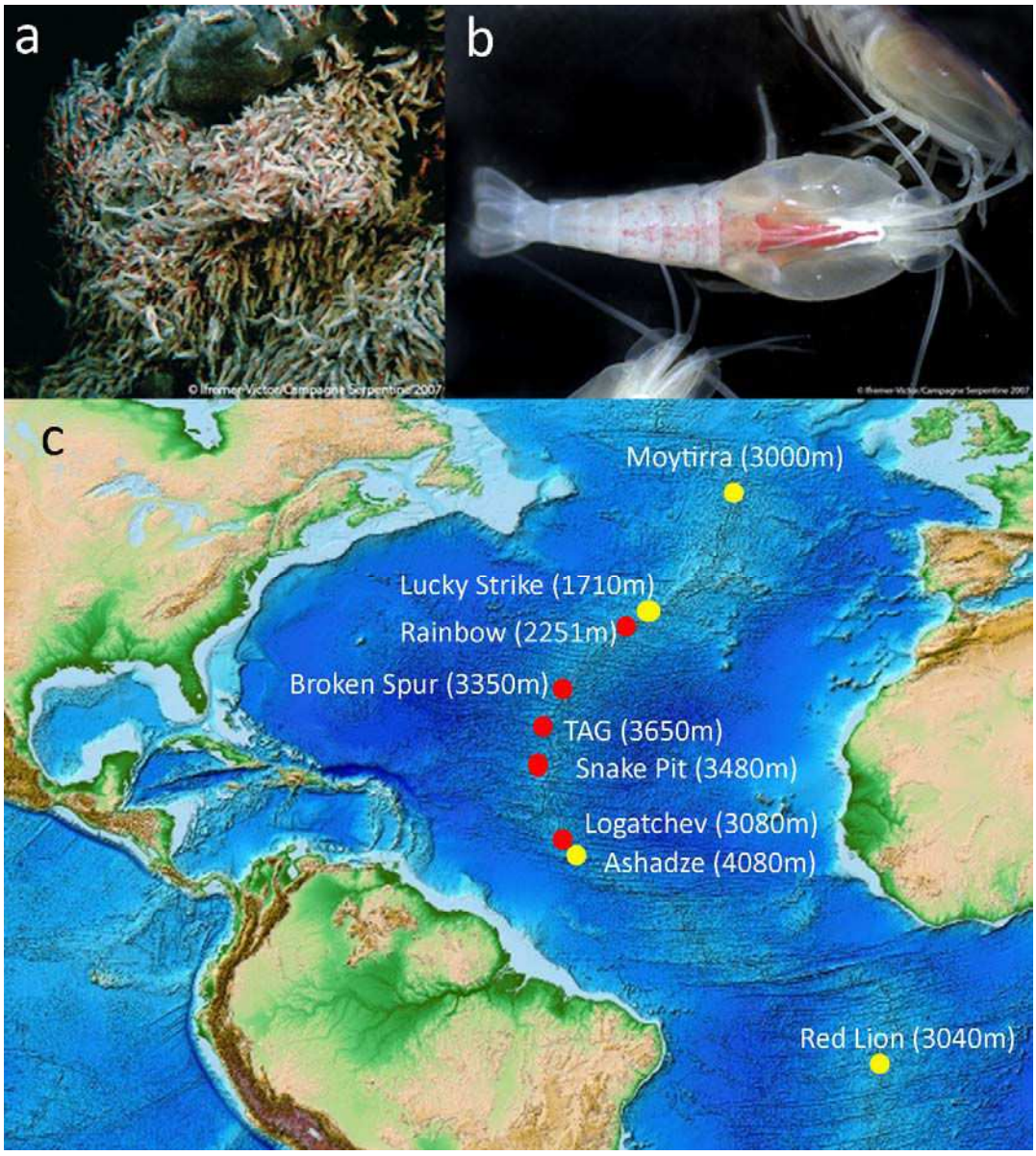

Figure 7. *Rimicaris exoculata* habitat, habitus and distribution. a, chimney of the Logatchev vent field colonized by a swarm of *R. exoculata*. b, adult specimens. c, distribution along the Mid-Atlantic Ridge, location in yellow are vent fields with scattered populations of *R. exoculata* or variable occurrence. Top pictures ®Ifremer, distribution base on Desbruyères et al. (2000), Desbruyères et al. (2001), Copley et al. (2007), Komai and Segonzac (2008) and others (see above).

A distinctive morphological feature of this species is the modification of the cephalothorax region with a laterally expanded branchial chamber. The branchiostegites (the lateral sections of the cephalothorax) are hypertrophied, which results in the increase of the space in the branchial chamber (Fig. 7). In addition, the scaphognathites and the exopods of the first maxillipeds (appendices found in the branchial chamber) are both enlarged and ornamented with thick setae on their surface. Other mouth part structures such as the second and third maxillipeds, also show thick setae on the surfaces near to the branchial chamber (Williams and Rona 1986). These morphological modifications are related with the development of an episymbiotic bacterial community hosted within this enlarged branchial chamber. Bacteria colonize the scaphognathites, the first maxilliped exopods and the inner surface of the branchial chamber (Segonzac et al. 1993, Zbinden et al. 2004, Corbari et al. 2008b). The scaphognathite has a mechanical function of ventilation of the branchial chamber and participate in gill cleaning (Brusca and Brusca 2003). The modification of the exopod of the first maxilliped into an additional scaphognathite-like structure suggests an increased ventilation of the branchial chamber.

Another major morphological peculiarity of *R. exoculata* is the lack of pedunculated eyes commonly found in decapod crustaceans. Instead, adult specimens develop a special photoreceptor dorsally, under the carapace, which appears as two wing-shaped white spots, connected in the frontal section. The retinal structures of this photoreceptor show adaptations to the perception of dim light (Chamberlain 2000). Pelli and Chamberlain (1989) estimated that such vision structure could permit the perception of the Black-Body radiations emitted by the hot vent fluids, allowing a visual orientation towards the areas close to the vent emission. This shrimp is also able of chemical perception of the sulfide emitted by the vent chimney using its first pair of antennae, which could permit additional orientation in the vent ecosystem (Renninger et al. 1995).

After the initial description of this unsual shrimp, related species have been identified forming large aggregates in other vent systems, such as *Rimicaris kairei* on the Central Indian Ridge (Van Dover et al. 2001, Watanabe and Beedessee 2015) and *Rimicaris hybisae* on the Mid-Cayman Spreading Center (Connelly et al. 2012, Nye et al. 2012). These species share biological and ecological features with *R. exoculata,* including the adaptation of the branchial chamber and mouth parts for hosting episymbiotic bacteria and the modified dorsal vision structures (Watabe and Hashimoto 2002, Nye et al. 2012, Streit et al. 2015).

1.6.2 Symbiosis in R. exoculata

As other dominant species living at hydrothermal vents*, R. exoculata* host symbiotic bacteria that contribute to its nutrition. The bacteria are hosted in both the hypertrophied branchial chamber and in the digestive tract of the shrimp with a clear partition in the assemblage composition between the two compartments (Zbinden and Cambon-Bonavita 2003, Zbinden et al. 2008, Durand et al. 2010, Petersen et al. 2010, Durand et al. 2015) (Fig. 8). The bacterial community in the branchial chamber is diverse in terms of composition and metabolic activity, but is generally dominated by Epsilon and Gammaproteobateria. The bacterial metabolic activities associated with this branchial chamber assemblage include oxidation of sulfur, iron, methane and even hydrogen (Zbinden et al. 2008, Hügler et al. 2011, Jan et al. 2014). The bacterial metabolic activity and the environmental conditions promote the deposition of mineral oxides (mainly iron oxides) on the surface of the branchial chamber and associated structures (scaphognathites)(Schmidt et al. 2008b). With time, these deposits accumulate and form a crust of minerals embedding the bacteria (Zbinden et al. 2004, Corbari et al. 2008a). As other crustaceans, the shrimps must molt in order to grow, and the mineral-bacteria crust is removed together with the exoskeleton during the molting process. During the adult life of the shrimp, a series of successive cycles of colonization by episymbiotic bacteria, mineral deposition and molt occur (Corbari et al. 2008b), leading to a regular renewal of this symbiotic community.

In *R. exoculata* the stomach is strongly reduced (Segonzac et al. 1993), and the epibiotic bacteria is found mostly in the hindgut. The shrimp gut microbiota includes Deferribacteria, Mollicutes, Epsilon- and Gammaproteobacteria, forming a resident bacterial community (Zbinden and Cambon-Bonavita 2003, Durand et al. 2010, Durand et al. 2015) (Table 3). Although the lumen of the anterior section of the gut up to the stomach is removed at each molt, the midgut is devoid of cuticule and host most of the bacteria, representing an internal episymbiotic compartment of the shrimp.

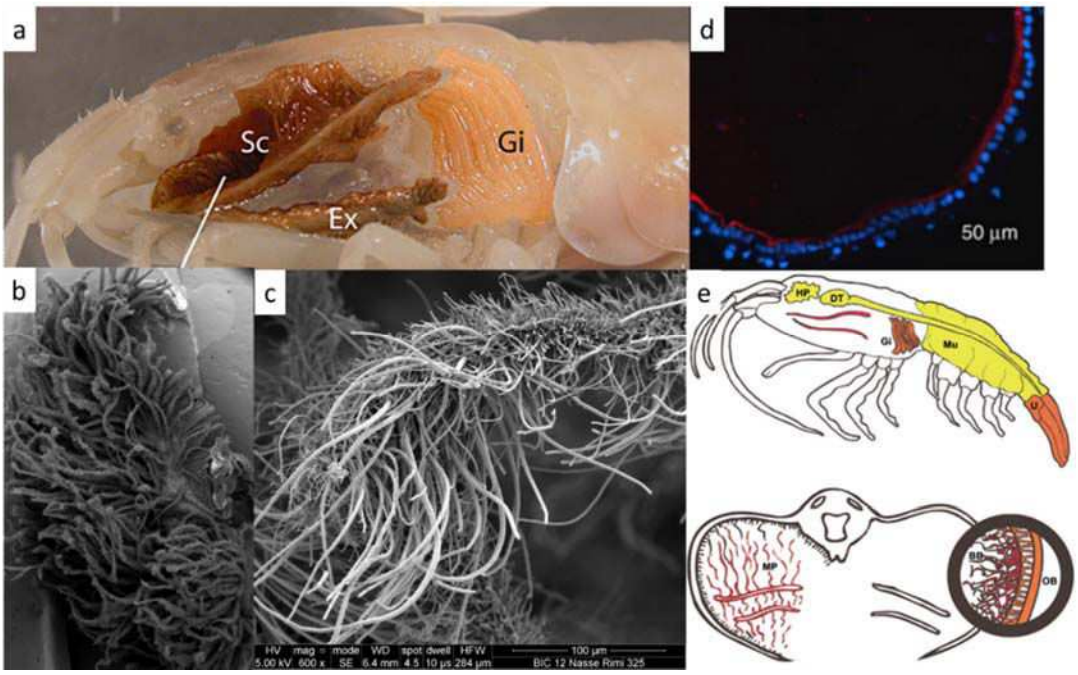

Figure 8. *Rimicaris exoculata* and its symbiotic bacterial communities. a, dissection of the branchial chamber, showing the gills (Gi), the scaphognathite (Sc) and the exopodite (Ex) (Petersen et al. 2010). b, SEM image of a scaphognathite with multiple projected setae. c, SEM image of a seta of the scaphognathite colonized by bacteria. d, FISH analysis of the transversal section of the midgut showing bacterial layer in the lumen (red) surrounded by cellular layers of the gut (blue) (Durand et al. 2010). e, absortion of the organic carbon produced by the episymbiotic bacteria, highest levels in red, lowest levels in yellow (Ponsard et al. 2013).

Experiments with labeled compounds (isotope-labeled inorganic carbon and radiolabeled organic compounds) demonstrated the direct nutritional transfer of organic carbon from the bacteria to the shrimp, especially from those living in the branchial chamber, that could represent the major source of nutrition for the shrimp (Ponsard et al. 2013). Additional nutritional sources could include ingestion of the bacteria obtained from shrimps exuviae, gut microflora activities and grazing bacteria of the chimney walls (Van Dover et al. 1988, Segonzac et al. 1993, Zbinden et al. 2004, Corbari et al. 2008b, Durand et al. 2010).

Table 3. Bacterial phylotypes identified in *R. exoculata*. Data compiled from branchial chamber (mainly branchiostergite and scaphognagtite), gut (stomach and midgut) and eggs (external surface).

	Branchial chamber						Gut			Eggs
Phylogenetic groups	Polz & Cava-naugh 1995[1]	Zbinden et al 2008[1]	Petersen et al. 2010[1]	Hügler et al 2011[1]	Guri et al 2012[1]	Jan et al 2014[2]	Zbinden & Cambon-Bonavita 2003[1]	Durand et al. 2010[1]	Durand et al. 2015[1]	Guri et al 2012[1]
Archaea						●				
α-proteobacteria		●			●	●			●	●
β-proteobacteria					●	●		●	●	
γ-proteobacteria		●	●	●	●	●	●	●	●	●
δ-proteobacteria		●		●	●	●		●	●	●
ε-proteobacteria	●	●	●	●	●	●	●	●	●	●
ζ-proteobacteria						●			●	
CFB*			●	●	●	●		●	●	●
Firmicutes						●		●	●	
Deferribacteres							●	●	●	
Entomoplasmatales							●			
Mollicutes								●	●	
Verrucomicrobiae								●	●	
Aquificae						●			●	
Actinobacteriae									●	
Thermodesulfobacteria									●	
Choroflexi									●	

*Cytophaga, Flavobacteria and Bacteroidetes. [1]Identified by cloning analyses, [2]Identified by metagenomics analysis.

Guri et al. (2012) found that eggs collected on brooding females located close to the vent emission at the Logatchev site were colonized by episymbiotic bacteria. The bacterial assemblages found on the eggs were widely dominated by Gammaproteobacteria, but also include Epsilonproteobateria, Bacteroidetes, Alphaproteobacteria and Deltaproteobacteria (Fig. 9). Some of the bacteria detected on eggs were closely related to the episymbiotic bacteria found in the shrimp branchial chamber, but an

important shift in composition was found between eggs, juveniles and adult shrimps (Guri et al. 2012). While the epibiotic bacterial community on eggs was dominated by Gammaproteobacteria, including methanotrophs, the branchial chamber of juveniles and adults was mostly colonized by Epsilonproteobacteria (Guri 2011, Guri et al. 2012). However, many questions remain about the bacteria associated with the eggs. For instance, it is not known how the bacterial colonization on the eggs starts and evolves through the embryonic development until hatching of the larva. The specificity of the egg-bacteria association has also not been tested.

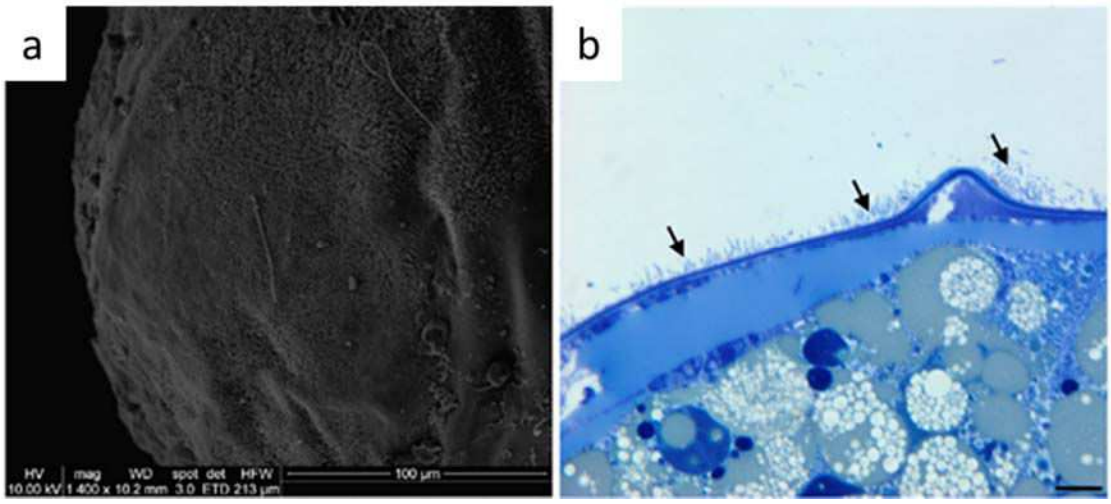

Figure 9. Bacterial colonization on the surface of *R. exoculata* eggs. a, SEM of an egg surface colonized by bacteria. b. transversal thin section of an egg showing the attached bacteria (arrows). From Guri et al. (2012).

The surface of the eggs and the mucus coat associated with the eggs brood represent a new surface available in the environment during the brooding period. The associated bacterial community could then be generated by an unspecific colonization process instead of a specific symbiotic process, implying bacterial lineages selection. Time of colonization and environmental conditions of each vent could control the eggs bacterial assemblages, instead of a symbiotic relationship. Then the bacterial assemblages on eggs would be similar to those found in similar structures exposed to the same environmental conditions during the same time. For instance, the endosymbiotic bacteria living in the gut of *R. exoculata* could be considered as specific since the composition of these gut communities were very similar in shrimps collected at four vent fields with contrasting environmental conditions and presenting also different free-living microbial communities (Durand et al. 2010, Durand et al. 2015). Determining the

eggs bacterial composition at different embryonic stages and at different vent sites and comparing them to structures exposed to the same conditions could bring clues about the relationship between eggs and bacteria, their specific nature and the variations through the embryonic development.

1.6.3 Early life and dispersal

Direct observations of larval dispersal and early life history of *R. exoculata* are not available which precludes a clear understanding of the species life-cycle. Most of the current information and hypotheses about the early stages of alvinocaridid are based on indirect evidences. For instance, the nutrition of the larvae of *Alvinocaris lusca* was inferred to be planktotrophic in early studies, based on the comparison of the egg size of this species with the egg size in decapods with planktotrophic and lecitotrophic development (Van Dover et al. 1985). This hypothesis of planktotrophic larval development has then been extended to other alvinocaridid shrimp, including *R. exoculata* (Ramirez-Llodra et al. 2000, Copley and Young 2006, Ramirez-Llodra and Segonzac 2006, Miyake et al. 2010). The planktotrophic larvae in decapods is associated with a non-abbreviated larval phase sustained by external food sources, allowing for extended dispersal of the larva that reach the post-larval phase after a series of larval stages (Anger 2001). The extended dispersal of planktotrophic alvinocaridid larvae was supported by a report of Herring and Dixon (1998) that mentioned the occurrence of postlarvae more than 100 kms away from vent sites. Moreover, several population genetic studies in alvinocaridid species suggest levels of connectivity consistent with high dispersal potential between populations separated by 100s or 1000s km (Teixeira et al. 2012, Beedessee et al. 2013, Yahagi et al. 2015).

Although the egg size has been used as a first approximation to infer the feeding mode in decapod crustacean larvae (Van Dover et al. 1985), accurate estimations require direct examination of the larvae (Anger 2001). The main characteristic to identify if a larvae is planktotrophic or lecitotrophic in decapods is the development of the mouthparts in the larval stages. In lecitotrophic larvae, the feeding structures are lacking or show a lower development than in planktotrophic larvae. In addition the lipid reserves of lecitotrophic larvae are usually larger because metabolic requirements are not sustained by external food sources. Similarly, the larval period could be estimated as abbreviated or extended based on the degree of development of early larval stages. In larvae with abbreviated development, morphological structures observed in the first larval stage exhibit characteristics that are expected for advanced stages. For instance, abdominal pleopods or eyestalks are not observed in larvae just after hatching, except in species

with abbreviate development (Anger 2001). Detailed larval description could thus bring useful information on the larval biology (Thatje et al. 2015).

According to Pond et al (Pond et al. 1997c), there is an important shift in the lipid composition and isotope signature between the eggs and early juveniles of *Mirocaris fortunata*, with a lipid composition showing a chemosysnthesis signature in eggs (as in the adult specimens) to a lipid composition that includes phototrophic microplankton markers in juveniles. Similar changes in lipid composition were found between adults and juveniles of *R. exoculata* (Pond et al. 2000a). Lipid composition with photosynthetic origin were also reported for post-larval stages identified as *R. chacei*, *R. exoculata* and *Alvinocaris markensis* (Pond et al. 1997a). Based on the variation of lipid composition in alvinocaridids, Dixon et al. (1998) and Copley et al. (1998) postulated that the early stage would display a vertical migration and would inhabit upper ocean layers consuming planktonic material from the photic zone. This would enhance the dispersal of larvae that would be transported within stronger surface currents, with a posterior recruitment to the deep-water hydrothermal vents at postlarval stage. Although this model represents the current hypothesis of dispersal of alvinocaridid shrimps (Adams et al. 2012), it is still not clear in which depth range inhabit the planktonic stage of this taxa. All known plankton records of alvinocaridid early stages are post-larval stages restricted to the bathypelagic environment (Herring 1998, Herring and Dixon 1998, Herring 2006). In addition experiments performed with the first larval stages of *Mirocaris fortunata* show that they can tolerate low pressure but did not survive at temperatures corresponding to shallow waters (Tyler and Dixon 2000). In addition, a lipid composition with photosynthetic signatures is found also in bathypelagic shrimps (Pond et al. 2000b) and even in adult benthic stage of *Alvinocaris markensis* at the hydrothermal vent (Pond et al. 1997b). Therefore, without direct observations of larval stages within surfaces waters, it will not be possible to validate the hypothesis of a vertical migration of alvinocaridid larvae up to the surface.

The lack of consistency between observations related to early life history of *R. exoculata* and other alvinocaridids is also affected by the gaps in information about the larval morphology. Since most of the information currently available concerns either the eggs, or the recruited post-larval stages, juveniles and adults, a large gap remains between the egg hatching and the post-larval stage found at vents. This could be pivotal to understand the dispersal mechanisms of the species. Information about the larval morphology in alvinocaridids is limited to brief comments and pictures of specimens obtained after hatching of brooding females posterior to their collection *in situ* (Miyake et al. 2010, Guri et al. 2012, Nye et al. 2012). Field samples of early-stage larvae are very limited, only larvae (presumably first Zoea) of

Opaepele loihi had been reported from deep-waters at the Nikko Seamount (Miyake et al. 2010). Experimental cultures of larvae obtained from on board hatching are also rare (Tyler and Dixon 2000, Koyama et al. 2005). In particular, for *R. exoculata* the very few collections of brooding females and their low tolerance at atmospheric pressure (Shillito et al. 2008) limit their potential for biological experiments with hatched larvae. These limitations affect our ability to obtain direct information about the early life history, such as the larval nutrition or modes of development based on larval morphology (Anger 2001), the larval tolerance to environmental parameters or the habitats occupied during the larval phase.

1.6.4 Population biology and reproduction

R. exoculata and related species represent the only actively moving hydrothermal vent taxa that colonize habitats close to the vent emission and form dense aggregations. The ability of the shrimp for rapid response to the environmental conditions and the potential for short term migration between different vent habitats is a very distinctive trait. In most other vents, the habitats close to the vent emission are dominated by sedentary (tubeworms or bivalves beside the vent emission) or slow-moving species (gastropods, galatheids, chirostyloids).

The large aggregations found at the top of the vent emission are very heterogeneous in composition due to the dynamic activity of the shrimp and the configuration of the vent chimney. However, in general, the populations living closer to the emission did not show major changes in density. Between consecutive decades no-variation in density is reported at the vent fields of TAG (Copley et al. 2007) and Logatchev (Gebruk et al. 2010). At short temporal scale, observations performed at the TAG site show a rapid redistribution in response to major perturbations (Copley et al. 1999). Also short-term changes in the distribution of the specimens around a minidiffuser chimney were observed coupled with tidal variations (Copley et al. 1999). The *R. exoculata* populations at the Broken Spur vent field appear to be rather peculiar since they do not form large aggregations in most of the vent emissions (Copley et al. 1997). However in this case the chimney areas with high, medium or low densities (at scales of 1000s, 100s or 10s ind m^{-2}) did not show significant variation in density during consecutive years (1993-1994) (Copley et al. 1997), and dense aggregations were observed after a decade (in 2005, no quantitative estimation available)(Galkin and Demina 2016). These patterns suggest that the *R. exoculata* populations have a dynamic equilibrium within its range of distribution without drastic temporal changes in the population size, at least up to decadal scale.

However, near the latitudinal and bathymetric limits of the known distribution of *R. exoculata* the populations did not form dense aggregations and do not show drastic temporal variations. For instance, at Lucky Strike vent field, dense aggregations of *R. exoculata* were reported at the site Y3 (1700 m depth) during the DIVA 2 cruise in summer 1994 (Desbruyères et al. 2001), and some shrimps were collected during the MAR97 cruise in summer 1997 (Shank et al. 1998), but they did not appear in the observations from the MORMARSAT 2010-2015 cruises (MOMARSAT cruise reports). Similarly, at the southern limit of the known distribution, at the Shrimp Farm site of the Red Lion vent field (4.8°S) the dense aggregations of *R. exoculata* observed in April 2005 were absent in May 2006 (Haase et al. 2007). Recent reports of *R. exoculata* populations in the northern section of the MAR (Nye et al. 2012, Wheeler et al. 2013) or below 4000 m depth (Fabri et al. 2011) did not include large aggregations but only scattered specimens.

In vent fields where shrimps are observed in large aggregations close to the vent emission, scattered adults are also found in surrounding habitats lacking focused fluids exits, together with others alvinocaridid species (Copley et al. 1997, Gebruk et al. 2000). However, it is not clear if the variations in adult shrimp density between habitats is linked to a variation in population structure, as occur in the vent crustacean *Kiwa tyleri* (Marsh et al. 2015). Shank et al. (1998) found more females in samples collected at different vent fields (especially Broken Spur, TAG, Snake Pit and Logatchev), however the authors did not conclude on the processes underlying this apparently biased sex-ratio: variation in population structure with local aggregation of females or overall biased sex-ratio.

For the juveniles, there is evidence that early stages collected in this habitat belong to both *R. exoculata* and *R. chacei* (Komai and Segonzac 2008), but partition of species at juvenile stage in the nurseries and their spatial distribution are still unknown. In addition juveniles have been reported inhabiting with adult aggregations (Shank et al. 1998, Copley et al. 2007), but stages of these juveniles and their proportion in the populations remain unclear. Vereshchaka (1997) proposed a size of transition from juvenile to adult stage (12-13 mm carapace length-CL) based on the morphology and depletion of lipid reserves. In addition the later author found a lack of morphological variation according to sex, except for the primary sexual structures. Other aspects of population structure, as size structure, are based on pooled samples, thus limiting the estimation of small scale spatial variations (Gebruk et al. 1997, Vereshchaka 1997).

As other carideans, *R. exoculata* has separated sexes (gonochorism) and multiple reproductive periods during its life cycle (Ramirez-Llodra et al. 2000, Copley et al. 2007). Although there is no description of the mating behavior, it is assumed that the fecundation occurs by spermatophore (sperm

packets) transfer from the male to the female ventral surface of the abdomen (Bauer 1976). Following this process, the egg spawning occurs. In shrimps, the eggs are extruded from gonopores placed at the base of the third pair of walking legs. During spawning, the eggs are placed under the abdomen, allowing the contact with the male sperm. The latter process could be promoted by the action of the abdominal appendices (pleopods)(Bauer 2013). In addition, the pleopods of caridean shrimps produce a coat secretion that covers the fertilized eggs and facilitate their attachment to the setae of the pleopods and between the eggs themselves, forming a brood mass (Fisher and Clark 1983). The eggs are incubated on the abdomen of the female during the complete embryonic development. During this period, the embryo develops into a planktonic larval stage, called Zoea, which is released during hatching (Van Dover et al. 1985, Anger 2001).

Although reproduction in *R. exoculata* seems to follow general traits observed in carideans, it also has many intriguing gaps. Oocyte size frequency distribution in gonads suggests a lack of reproductive seasonality (Ramirez-Llodra et al. 2000, Copley et al. 2007). However, very few brooding specimens have been collected since the description of the species (Williams and Rona 1986, Ramirez-Llodra et al. 2000, Gebruk et al. 2010, Guri et al. 2012). As a consequence, the realized fecundity (number of eggs per brood) has been estimated from observation on only one specimen (Ramirez-Llodra et al. 2000). The lack of brooding females also contrasts with the large density of the populations and the strong genetic connectivity among vent fields along the MAR (Teixeira et al. 2012), which should be supported by a large larval pool.

To explain the contradiction between an apparently non-seasonal reproduction, which suggest that brooding females may be encountered more or less continuously, and a striking lack of observation of brooding females, it has been proposed that brooding females may migrate to the periphery of the vents during brooding and hatching (Tyler and Young 1999, Ramirez-Llodra et al. 2000, Copley et al. 2007). This hypothesis argues that migration of brooding females at the vent periphery could avoid the exposure of embryos to the toxic emissions of the hydrothermal vent fluids. The brooding-migration hypothesis was proposed to explain the population distribution of the hydrothermal vent crab *Bythograea thermodron* (Perovich et al. 2003) and the chirostyloid *Kiwa tyleri* (Marsh et al. 2015). For both species, higher proportion of brooding or hatching females are found in areas at the periphery of the vent emission (Perovich et al. 2003, Marsh et al. 2015). In *R. exoculata*, despite the large amount of collections of this shrimp since the discovery of the MAR vent ecosystems, no evidence of such migration behavior has been reported so far. However, *R. exoculata* adults from the periphery of the vents were never sampled in

sufficient number to test the occurrence of brooding females in these habitats (Copley et al. 2007). Moreover, recent findings of few brooding females in samples collected close to the vent emissions seem to reject the brooding-migration hypothesis (Gebruk et al. 2010, Guri et al. 2012). However, the lack of quantitative approach in these reports and the lack of samples collected at the periphery of the vent preclude to draw any conclusion on whether these findings represent an isolated event or a consistent evidence to reject the hypothesis of brooding female migration.

An alternative hypothesis that could also explain the lack of brooding females is the occurrence of reproductive seasonality in *R. exoculata.* Indeed, sampling dates during previous cruises are strongly biased to the summer season (due to favorable meterological conditions), thus possibly missing brooding females if reproduction is periodic and occurs during seasons not sampled so far. For instance the brooding period of the cold-seep alvinocaridid *Alvinocaris stactophila* at the Louisiana Slope occurs during winter (Copley and Young 2006). Similarly, the MAR vent mussel, *Bathymodiolus azoricus,* exhibits seasonal reproduction with spawning in January (Colaço et al. 2006). However, in *R. exoculata,* the occurrence of a similar pattern of spawning restricted to winter seems inconsistent with the presence of ripe gonads in samples collected during summer and autumn (Ramirez-Llodra et al. 2000, Copley et al. 2007). In addition, the lack of systematic sampling did not allow obtaining so far any direct evidence of winter reproduction. In order to determine if the apparent lack of brooding females is explained by seasonal reproduction or by migration of brooding females, it is necessary to conduct systematic sampling in the different habitats where the species is encountered (close to the vent emission and at the periphery), and to increase the sampling effort to cover different seasons. Determining the location and environmental conditions experienced by brooding females is also important because it may impact very early life stages, ie developing embryos, as well as the bacteria that are associated with them.

1.6.5 Species delimitation debate in Rimicaris and implications in life cycle interpretation

In general, the family Alvinocarididae has a long history of problems dealing with species delimitation. The identification of species based only on morphological traits has been problematic in this group. In some cases, morphological variations between adult and juvenile stages and the phenotypic variation in adult stage have been confounded with species level variation (Shank et al. 1998, Shank et al. 1999, Komai and Segonzac 2003). In other cases, nearly cryptic phenotypes with allopatric distributions are recognized as different species (Watabe and Hashimoto 2002). Although many cases of species

delimitation problems have been resolved with the implementation of phylogenetic reconstructions based on genetic sequences, some complicated clades remain with species complexes (Teixeira et al. 2013, Vereshchaka et al. 2015). The species delimitation in the family have a direct effect on the interpretation of postlarvae and recruitment (Herring 1998, Herring and Dixon 1998), lipid profiles (Pond et al. 1997a), variation of symbiosis along maturation stages (Guri et al. 2012) and connectivity between deep-water chemosynthetic ecosystems (Teixeira et al. 2013).

Currently, there is a debate about the species delimitation in some *Rimicaris* that could affect the interpretation of the life cycle and ecology for the species than inhabit the top of the vent. The recent exploration of the vent field at the Mid Cayman Spreading Center in the Caribbean Basin brought the discovery of *R. hybisae* (Nye et al. 2012) which have a similar ecological niche to *R. exoculata* on the Mid-Atlantic Ridge and *R. kairei* on the Central Indian Ridge. In addition to be closely related phylogenetically, the three *Rimicaris* species could be considered as being ecologically homologous since they represent the dominant megafauna close to the vent emission in their respective vent systems, forming dense aggregations and exhibiting bacterial symbiotic communities in their branchial chamber reflecting a specialized nutrition mode relying on chemoautotrophy (Van Dover 2002a, Streit et al. 2015). Moreover all of them exhibit evidence of high genetic flow between vents within their respective geographic distribution range (Teixeira et al. 2012, Beedessee et al. 2013, Plouviez et al. 2015).

According to the morphology and the partial sequences of some mitochondrial and nuclear genes, *R. hybisae* is closely related to *R. chacei* that inhabits the Mid Atlantic Ridge vents (Nye et al. 2012). Teixeira et al. (2013) proposed that *R. chacei* and *R. hybisae* would be the same species, based on the genetic distances of several genes between the two taxa. This would also denote that the Mid-Atlantic Ridge and the Mid Cayman Spreading Center would be connected by the exchange of migrants of the *R. chacei/R. hybisae* species complex. The hypothesis presented by the former authors was also supported by analyses performed by Vereshchaka et al. (2015), who recommended additional analysis. In this particular case, the resolution of species delimitation could bring clues about the life cycle of dominant taxa in hydrothermal vents and the connectivity between vent systems.

1.7 Objectives of the study

My objectives were to gain insights into the population, reproductive and larval biology of *Rimicaris* shrimps in order to decipher their life cycle and attempt to resolve several paradoxes that emerged from the very partial data accumulated so far (species with high biomass populations extending over broad geographic ranges in patchy habitats, but with extremely few records of reproductive individuals). Specifically, I had three objectives focusing on aspects of the reproduction and early development of Alvinocaridid shrimps, using more specifically *Rimicaris exoculata* from MAR vent sites as a model to:

i. Understand the early larval life of Alvinocaridid shrimps in terms of nutrition capability and developmental duration using detailed morphological observations of the first planktonic larval stage in order to infer the dispersal mode of these deep-sea shrimps.
ii. Analyse reproductive patterns in populations of *R. exoculata* on the MAR in order to understand how it may influence population structure and dynamics as well as recruitment patterns.
iii. Examine interactions of early developping embryos with bacterial symbiotic communities in order to investigate potential symbiosis acquisition mechanisms in *R. exoculata* which specialized on symbiotic chemosynthetic nutrition.

A fourth more global objective was to assess a recent hypothesis on species delimitation in *Rimicaris* shrimps in the Atlantic Ocean, suggesting that two species with contrasting ecological niches related to their degree of specialization on symbiotic chemoautotrophic nutrition were indeed one species. The close examination of this species complex brought evolutionary perspectives on colonisation patterns in *Rimicaris* shrimps, and a more global context to our understanding of their life-cycle.

The objectives of this work are addressed in four chapters:

Chapter 1: The first larval stage of *R. exoculata* and three other species of alvinocaridid shrimps (*Mirocaris fortunata, Alvinocaris muricola* and *Nautilocaris saintlaurentae*) is described. Morphological features are analyzed in order to estimate the larval biology. A new combination of larval traits is proposed for decapod crustaceans. Inferences on larval nutrition and extension of development permit a contrast to the previous hypothesis of larval dispersal for these species. In addition, the morphology of the larvae is compared with that of related caridean families (mostly deep-water taxa) in order to make inferences about the phylogeny and the occurrence of larval traits.

Chapter 2: An analysis of the population structure and the reproduction of *R. exoculata* is performed using samples collected at the Snake Pit and TAG vent fields during January-February 2014. The objectives are to i) test the variation in sex and stage among shrimps collected in different microhabitats of the vent edifice, ii) determine the occurrence of brooding females and iii) describe the reproductive output. The study includes systematic sampling for the same habitat (aggregation of shrimps close to the vent emission) in different vent field and between different habitats at the TAG site. An important variation of the sex ratio between habitats is described for first time. The reproductive output is also described and discussed in the context of previous hypothesis (brooding female migration and seasonal reproduction). The reproductive output (egg number per brood and egg size) is compared between vent fields.

Chapter 3: The bacterial community associated with the eggs of *R. exoculata* is analyzed at different embryonic stage and between different vent fields. In addition, the bacterial assemblage of the egg is compared to that found on the pleopods of the parental females. The objective is to determine if the bacterial community that develops on the egg surface exhibit a pattern suggesting a specific bacteria-egg relationship and describe its change through the embryonic development. Diverse bacterial assemblages are found in both eggs and pleopods, with a community structure consistent with a specific bacteria-host relationship in eggs. Changes in the bacterial assemblages associated with embryonic development are also detected. Hypotheses related with geographical variation in the egg bacterial assemblages and the functional relationship with the host are proposed.

Chapter 4: The objective of this chapter is to contribute to the current debate about the species status of two *Rimicaris* shrimps living in the Atlantic Ocean, *R. hybisae* and R. *chacei* (Teixeira et al. 2013). The problem of species delimitation within these 2 putative species is addressed in the context of the life cycle of the species and their colonization patterns on Atlantic vent sites. We used population genetics and phylogenetic approaches to test hypotheses of single or two separated lineages, and discuss several aspects of the biology and ecology of the shrimps in an integrative approach of the species delimitation problems.

Chapter 1

Morphology of First Zoeal Stage of Four Genera of Alvinocaridid Shrimps from Hydrothermal Vents and Cold Seeps: Implications for Ecology, Larval Biology and Phylogeny

Sur la morphologie du premier stade de Zoea chez quatre genres de crevettes Alvinocarididés des écosystèmes hydrothermaux et des émissions de fluides froids : implications pour l'écologie, la biologie larvaire et la phylogénie

Synthèse

Les Alvinocarididés sont une famille de crevettes endémiques des écosystèmes des sources hydrothermales et/ou d'émissions froides, qui n'ont été observées qu'une seule fois en dehors de ces environnements (*Alvinocaris* sp., 'Chinchorro bank', Golfe du Mexique). En outre, c'est un groupe très diversifié, avec beaucoup d'espèces décrites et présentant d'importantes différences morphologiques et biologiques. Ces crevettes sont distribuées tout autour du globe, entre 200 et 5000 m de profondeur, à l'exception des océans polaires. La distribution de certaines espèces d'Alvinocarididés s'étend sur plusieurs milliers de kilomètres et se caractérise par des habitats ponctuels et dispersés. Beaucoup de ces crevettes sont des organismes dominants de leurs habitats et peuvent former des agrégations de milliers d'individus, en particulier les espèces des sources hydrothermales. Les études de génétique des populations chez les Alvinocarididés indiquent des flux génétiques importants entre les populations, en dépit de la distance. Ces résultats tendent à montrer une grande connectivité génétique, sans doute favorisée par une vaste dispersion des stades larvaires.

Cependant, la biologie des larves d'Alvinocarididés et les mécanismes associés à leur dispersion sont inconnus. La petite taille des œufs suggère que les larves d'Alvinocarididés sont planctotrophes (qui se nourrit du phytoplancton). De plus, la variation de la composition lipidique entre les crevettes juvéniles venant de recruter et les adultes suggère que les larves se nourrissent de matériel d'origine photosynthétique provenant des eaux de surface pendant leur dispersion. Cette observation a été à l'origine d'une hypothèse de migration verticale des larves après l'éclosion, consommant du phytoplancton pendant leur dispersion dans les eaux de surface, et migrant à nouveaux vers les sources hydrothermales ou les zones d'émissions de fluides froids, où elles recrutent après avoir fini leur développement larvaire. Bien que ce scénario soit accepté comme le modèle de biologie et dispersion larvaire des Alvinocarididés, il n'est pas supporté par des preuves directes et les preuves proposées sont contestables.

Le développement larvaire des crevettes Caridés est composé de plusieurs stades de larve Zoés, suivi par plusieurs stades de larve 'Decapodid'. Le nombre et la durée des stades larvaires peuvent varier entre les espèces et au sein de la même espèce, pour moduler la durée de la vie larvaire. L'étude du premier stade larvaire peut donner des indices pour comprendre la biologie de la larve et la durée de la période larvaire. Ce travail fournit la première description détaillée du stade de Zoé 1 de quatre espèces d'Alvinocarididés : *Rimicaris exoculata* et *Mirocaris fortunata*, des sources hydrothermales de la dorsale médio-Atlantique (MAR), *Alvinocaris muricula*, des émissions de fluides froids de la marge du Congo, et *Nautilocaris saintlaurentae,* des sources hydrothermales des îles de Wallis et Futuna (Pacifique ouest). Les objectifs sont de i) Fournir des descriptions détaillées pouvant contribuer à l'identification de larves dans les échantillons de plancton, ii) Faire une estimation du régime alimentaire et de la durée de l'étape pélagique, en utilisant la morphologie de la larve, iii) Comparer la morphologie de la larve, les régimes alimentaires et les modes de vie larvaire entre les Alvinocarididés et des familles associées, composées principalement de crevettes abyssales, iv) Reconsidérer les hypothèses de dispersion larvaire des Alvinocaridídées en prenant en compte les résultats obtenus.

Les larves des quatre espèces ont été obtenues à partir de l'éclosion d'œufs de femelles gravides récoltées au fond par un submersible, puis remontées sur le bateau sans compensation de la pression hydrostatique. Les femelles gravides ont été récoltées durant les missions Biozaïre 2 (Golfe de Guinée, site Regab, 3150 m profondeur, Novembre 2001), Serpentine (MAR, site Logatchev, 3037 m profondeur, Mars 2007), Futuna 3 (îles de Wallis et Futuna, Fatu Kapa, 1554 m profondeur, Juin 2012), Momarsat (MAR, site Lucky Strike, 1739 m profondeur, Septembre 2013) et BICOSE (MAR, site TAG, 3625 m profondeur, Janvier 2014). De plus, un groupe de femelles gravides de *R. exoculata* ont été récoltées et ramenées à bord lors de la mission BICOSE en utilisant la chambre de récupération isobare PERISCOP. Ces femelles ont été maintenues dans la chambre BALIST (300 bars, 5°C) durant 24 h. L'éclosion a été détectée dans la chambre BALIST et les larves actives ont été transférées à la chambre pressurisée PICCEL. Après une brève décompression durant le transfert, les larves ont été maintenues vivantes à 300 bars de pression et 8°C durant 96h. Les larves récoltées en conditions pressurisées et les larves écloses à pression atmosphérique ont été comparées pour estimer l'effet de la pression à l'éclosion sur la morphologie larvaire.

De plus, nous avons examiné les larves récupérées d'échantillonnages de plancton de l'environnement profond. Les analyses portent sur six larves d'Alvinocarididés récupérées sur la marge du Congo grâce à des pièges à particules déployés à 3150 m profondeur au niveau des émissions de fluides

froids du site Regab (mission Congolobe, Janvier 2012). Nous avons également analysé des larves récoltées sur la dorsale Atlantique avec la pompe à larves SALSA. La pompe SALSA a été déployée sur le site TAG à 3637 m profondeur durant la mission BICOSE (février 2014) à la périphérie de la source hydrothermale. Des six larves d'Alvinocarididés récupérées par SALSA, trois ont été analysées pour l'identification jusqu'au niveau de l'espèce. Les larves récoltées par le piège à particules au niveau du site Regab et une larve récoltée avec la pompe SALSA sur TAG ont été identifiées morphologiquement. Pour deux des larves de la dorsale Atlantique, l'identification a également été effectuée par extraction d'ADN et amplification du gène cytochrome oxydase I (COI). Une reconstruction phylogénétique du COI parmi les Alvinocarididés a été réalisée pour parvenir à l'identification de ces larves.

Lors de la description morphologique des larves zoés des quatre espèces, certaines structures ont permis d'estimer le régime alimentaire et la durée du stade larvaire. Le développement des appendices de la bouche de la larve (la mandibule, la maxillule et la maxille) a été analysé pour déterminer si le régime est planctotrophe (les dents et les épines développées, les soies internes abondantes) ou lécitotrophe (pas de développement des dents et épines, les soies internes réduites). La présence des pléopodes, péréiopodes et le développement des maxillipèdes ont été comparés pour déterminer si la durée de la vie larvaire est abrégée (les pléopodes et/ou les péréiopodes présents, endopodes des maxillipèdes développés) ou si elle est prolongée (absence des pléopodes et des péréiopodes, développement faible de l'endopode dans les maxillipèdes). Une recherche bibliographique sur les différents types de larves a été effectuée dans les familles associées aux Alvinocarididés. Aussi une reconstruction phylogénétique à partir du gène 18S ribosomal a été réalisée afin de mettre en relation les traits d'histoire de vie des larves avec la phylogénie des familles associées.

Les larves de *R. exoculata* récoltées et maintenues sous pression et les larves écloses à pression atmosphérique sont de morphologie similaire. Ce dernier résultat permet valider les descriptions des larves obtenues à partir des éclosions à pression atmosphérique. La morphologie des larves est très similaire entre les quatre espèces, bien que les adultes soient morphologiquement très différents et que les espèces appartiennent aux trois sous-familles d'Alvinocarididés. De plus, les larves des Alvinocarididés sont très distinctes de celles des autres Caridés (Fig S1). La morphologie des larves et adultes et la génétique moléculaire supportent le statut monophylétique des Alvinocarididés. Les structures morphologiques des larves sont des outils fiables pour l'identification de la famille, mais permettent plus difficilement d'identifier l'espèce. Cependant, l'identification morphologique des larves de *R. exoculata* récupérées avec la pompe SALSA sur la MAR a été confirmée par les analyses génétiques du gène COI (Fig

S2). De la même façon, les larves de *A. muricola* récupérées dans le piège à particules sur le site Regab de la marge du Congo ont été identifiées grâce aux critères morphologiques. Ce travail représente la première identification fiable de larves d'Alvinocarididés en environnement profond, sur les sources hydrothermales et sur les sites d'émissions de fluides froids. La description de la larve permet une identification rapide au niveau de la famille, mais la faible variabilité inter-espèces et le nombre limité de descriptions disponibles compliquent l'identification au niveau de l'espèce. En revanche les protocoles d'extraction d'ADN et les analyses du gène COI sont plus pratiques et fiables pour l'identification de larves au niveau de l'espèce.

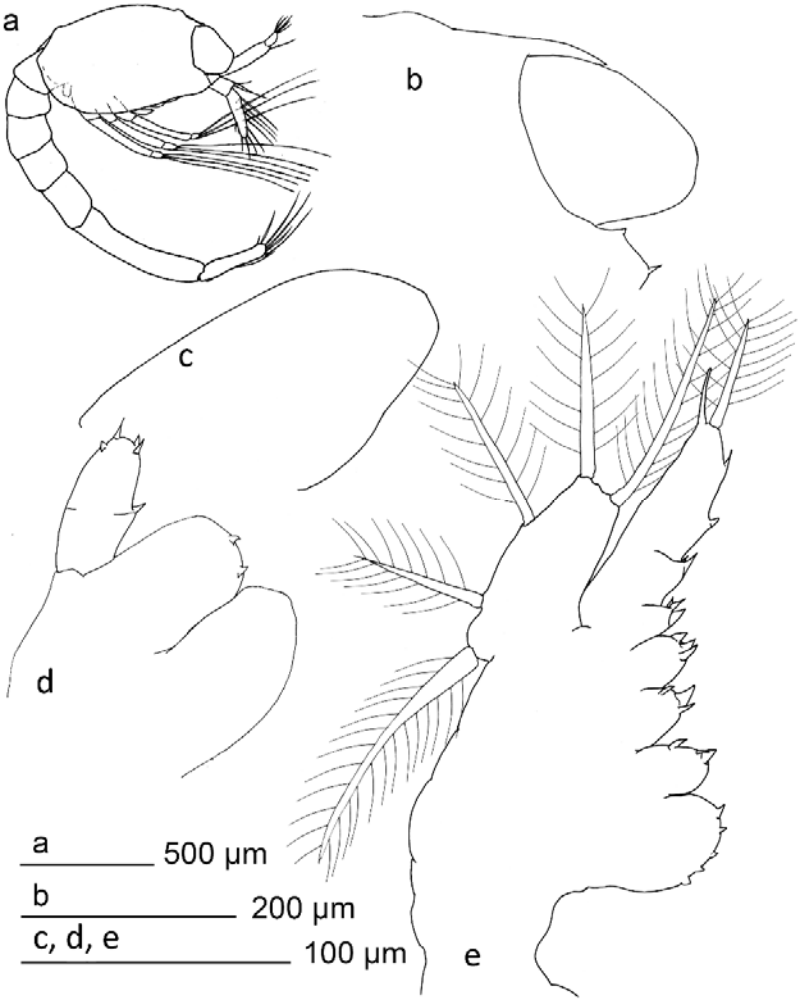

Figure S1. Zoé I de *Rimicaris exoculata*, a) habitus, b) région frontale, c) mandibule, d) maxillule, e) maxille.

Les larves zoé I d'Alvinocarididés n'ont pas de mandibules développées (Fig. S1c). Certaines petites épines dispersées sont trouvées sur les marges internes des mandibules, maxillules, maxilles et endopodes des maxilipèdes (Fig S1c-e). Les soies des marges internes sont absentes sur les appendices de la bouche et très réduites sur les endopodes des maxilipèdes. En plus du faible développement des appendices de la bouche, les importantes réserves lipidiques de la larve suggèrent un régime larvaire de type lécitotrophe. Ces résultats permettent de réfuter l'hypothèse d'un mode de vie larvaire planctotrophe, du moins dans la phase larvaire initiale, postulée à cause de la petite taille des œufs. Un régime lécitotrophe implique par contre que la larve n'a pas besoin d'effectuer une migration verticale pour consommer du plancton des eaux de surface durant le premier stade larvaire. Les larves récupérées sur les sources hydrothermales et les sites d'émission de fluies froids favorisent aussi une hypothèse de dispersion dans l'environnement profond.

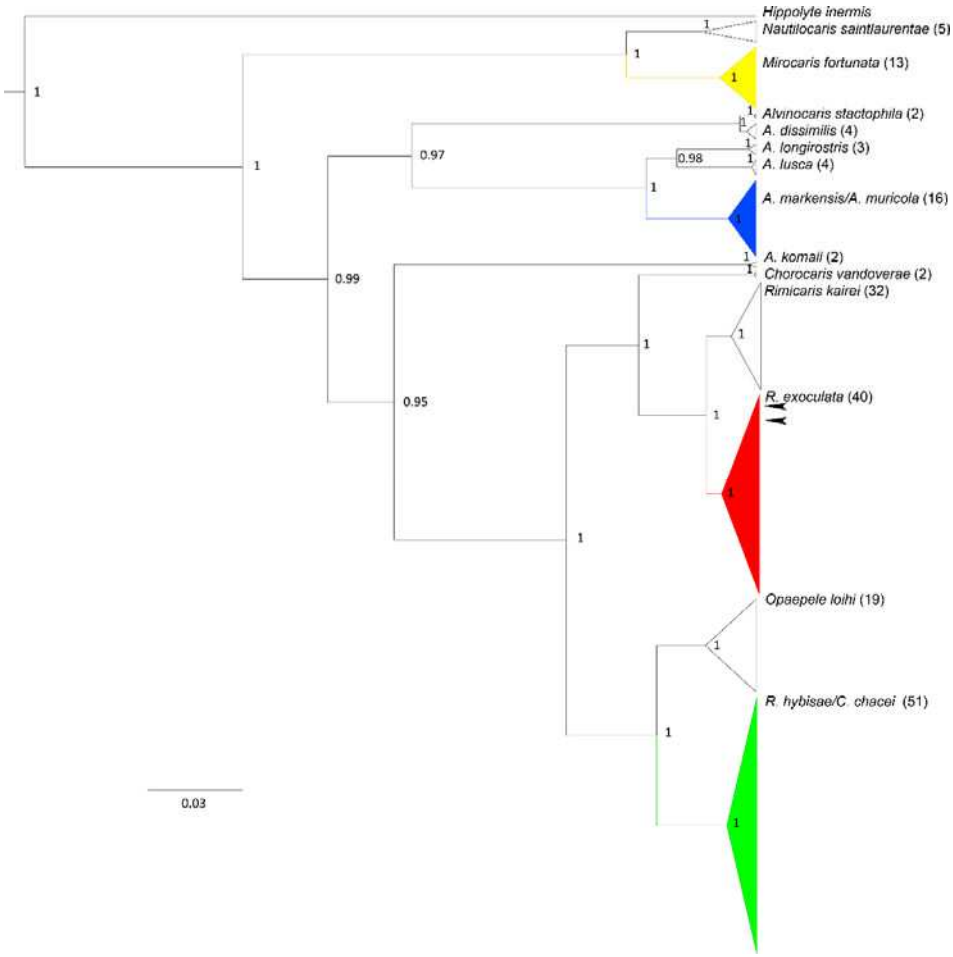

Figure S2. Reconstruction phylogénétique du gène COI chez les Alvinocarididés. Les flèches indiquent les sequences de larves récupérés avec la pompe SALSA

La lécitotrophie larvaire des crustacés décapodes est généralement associée à un développement larvaire écourté, un mécanisme associé à une stratégie de rétention larvaire. Mais la morphologie de la larve des Alvinocarididés est une combinaison particulière de caractéristiques de lécitotrophie et de développement prolongé. Comme les autres larves au développement prolongé, la Zoea I des Alvinocarididés ne montre pas de pléopodes ou de péréiopodes, et la morphologie des maxilipèdes correspondent à des structures adaptées à la natation (ie exopodes développés avec des soies longues). Les expériences de culture de larves d'Alvinocarididés n'en sont encore qu'aux étapes préliminaires, mais elles montrent que la larve peut rester plus de deux mois sans muer vers le stade suivant. Si ces estimations sont exactes, les larves d'Alvinocarididés pourraient présenter la plus longue période en Zoea I des crustacés décapodes, et la durée totale du stade larvaire pourrait être hyper prolongée si les stades

larvaires suivant ont des échelles de temps similaires. Ces caractéristiques, et en particulier l'autosuffisance alimentaire du premier stade larvaire, permettraient la colonisation d'habitats dispersés sur de grandes distances, comme les sources hydrothermales et les sites d'émissions de fluides froids. A cause de la quantité de réserves lipidiques, qui semblent insuffisantes pour un développement larvaire prolongé complètement lécitotrophe, nous émmettons l'hypothèse d'un stade larvaire planctotrophe pendant le 2eme stade de Zoé.

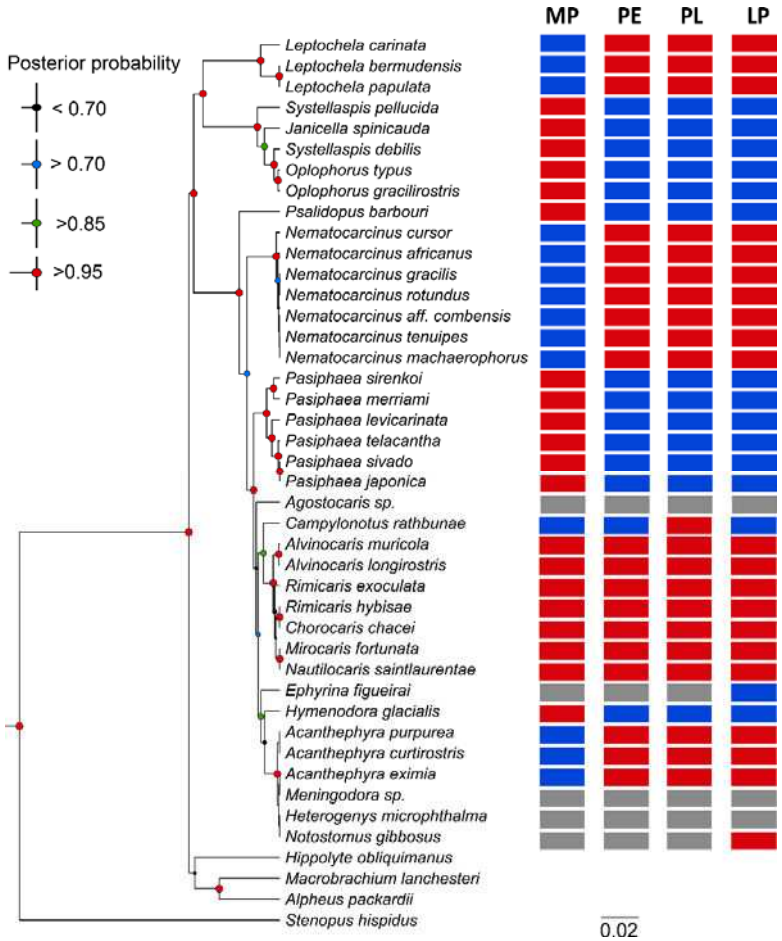

Figure S3. Reconstruction phylogénétique du gène 18S chez les Alvinocarididés et familles associées, et traits larvaires de la Zoé I. MP : appendices de la bouche (bleu, développés ; rouge, non développés), PE et PL : péréiopodes et pléopoes (bleu, présents; rouge : absents), LP : durée de la phase planctonique (bleu, abrégée ; rouge, prolongée).

La distribution des caractères larvaires dans la reconstruction phylogénétique des Alvinocarididés et des familles associées montre que le régime alimentaire (lécitotrophe/planctotrophe) et la durée de vie larvaire (abrégée/prolongéé) sont présentes sous toutes les combinaisons possibles entre les familles (Fig S3). A l'exception de certains cas, les combinaisons régime alimentaire/ durée de vie larvaire sont constants au sein des familles. De plus, les groupes estimés comme paraphylétiques (ie Oplophoridae *sensu lato*, Pasiphaeidae *sensu lato*) montrent une séparation claire dans les types de larves et dans la reconstruction phylogénétique. Il est possible que le type de larve ancestral (planctotrophe et avec développement prolongé) ait évolué plusieurs fois selon différentes combinaisons dans les familles analysées. Les différentes combinaisons régime alimentaire/ durée de vie larvaire peuvent représenter une caractéristique évolutive clé au niveau de la famille. Les Alvinocarididés sont la seule famille connue de crustacés décapodes ayant une larve lécitotrophe et une durée larvaire prolongée. Cette configuration peut permettre la colonisation efficace des habitats chimiosynthétiques dispersés et favoriser une longue dispersion dans l'environnement profond.

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RESEARCH ARTICLE

Morphology of First Zoeal Stage of Four Genera of Alvinocaridid Shrimps from Hydrothermal Vents and Cold Seeps: Implications for Ecology, Larval Biology and Phylogeny

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Abstract

Alvinocaridid shrimps are endemic species inhabiting hydrothermal vents and/or cold seeps. Although indirect evidences (genetic and lipid markers) suggest that their larval stages disperse widely and support large scale connectivity, larval life and mechanisms underlying dispersal are unknown in alvinocaridids. Here we provide for the first time detailed descriptions of the first larval stage (zoea I) of four alvinocaridid species: *Rimicaris exoculata* and *Mirocaris fortunata* from the Mid-Atlantic Ridge, *Alvinocaris muricola* from the Congo Basin and *Nautilocaris saintlaurentae* from the Western Pacific. The larvae were obtained from onboard hatching of brooding females (either at atmospheric pressure or at habitat pressure in hyperbaric chambers) and from the water column near adult habitats, sampled with plankton pumps or sediment traps. Major characteristics of the alvinocaridid larvae include undeveloped mandible and almost complete absence of setation in the inner margin of the mouth parts and maxillipeds. Although the larvae are very similar between the four species studied, some morphological features could be used for species identification. In addition, undeveloped mouthparts and the large amount of lipid reserves strongly support the occurrence of primary lecithotrophy in the early stage of alvinocaridids. Although lecithotrophy in decapod crustaceans is usually associated with abbreviated larval development, as a mechanism of larval retention, morphological and physiological evidences suggest the occurrence of an extended and lecithotrophic larval stage in the Alvinocarididae. These traits permit the colonization of widely dispersed and fragmented environments of hydrothermal vents and cold seeps. Distribution of larval traits along the phylogenetic reconstruction of the Alvinocarididae and related families suggest that lecithotrophy/planktotrophy and extended/abbreviated development have evolved independently along related families in all

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potential combinations. However, the Alvinocarididae is the only taxa with a combination of lecithotrophy and extended larval development.

Introduction

Shrimps of the family Alvinocarididae inhabit deep-waters in the Atlantic, Pacific and Indian Oceans, usually at depths greater than 1000 m [1]. They occur at hydrothermal vents and/or cold-seeps, and could represent the dominant species in these ecosystems, even in some cases forming large aggregations of thousands of individuals per m^2 [2,3]. Some species such as *Rimicaris exoculata* harbour symbiotic bacteria in their gill chambers and within their guts, which supply nutrients to the shrimp in a complex mutualistic association [4], whereas other species depend on grazing on chemoautotrophic bacteria and detritus in their adult stage [2].

However, populations of these shrimps exhibit high genetic connectivity along the Mid-Atlantic Ridge [5], between different vent and cold seeps systems in the Atlantic [6] and in vents systems of the Western Pacific and Indian Oceans [7,8]. This suggests a notable ability for dispersion and migration in widely spaced habitats. Juveniles and adult stages of alvinocaridids inhabit close to the vents and cold seeps due to their dependence on bacterial-detrital grazing or to their need to supply their symbiotic bacteria with reduced compounds, limiting adult migration [4,6]. Colonization of new habitats and connectivity along their geographic range must be promoted by larval forms. However, information about larval stages is scarce, limited to reports of post-larvae in plankton samples [9,10], estimations of larval development mode based on egg size [11], and on board observations of larvae hatched from brooding females [10, 12–17]. In general, published data on larval forms of alvinocaridid shrimps include only brief descriptions or some illustrations, but without clues about important larval traits, differences between species or comparison with other caridean shrimps.

As other carideans, brooding females of alvinocaridids maintain their eggs below the abdomen during the embryonic development until the hatching, which is followed by a planktonic larval stage [15,17,18]. Larval history appears to occur in the water column, where, after a series of molts, post-larval stages recruit in the benthic system of hydrothermal vents and cold seeps. However no information is available about the duration, dispersion or development of larvae, and no field samples of larval stages have been reported at the present (except briefly by Miyake [19]). The absence of larval descriptions in this group is a gap to be resolved, because it could help to identify larvae in the plankton and elucidate mechanisms of dispersion and larval history. Also larval morphology can bring important cues about the early life history of the species [20] and provide useful morphological features to help interpreting both phylogenies [21,22] and ecology [23,24]. In this paper we describe the first larval stage of four genera of Alvinocarididae obtained from hatching of gravid females and some larvae identified from field plankton samples.

Material and Methods

Collection and preparation of larval stages

Brooding females of *Rimicaris exoculata*, *Mirocaris fortunata*, *Nautilocaris saintlaurentae* and *Alvinocaris muricola* were collected from hydrothermal vents on the Mid-Atlantic Ridge (*R. exoculata* and *M. fortunata*), in the Western Pacific (*N. saintlaurentae*), and at cold seeps of the Congo Basin (*A. muricola*) (Table 1). Shrimps were collected with a suction sampler connected to the submersible (Nautile or ROV VICTOR 6000), and in most of cases, were brought

Table 1. Cruise and sample information for the larvae herein studied.

Species	Sample	Location	Site	Coordinates	Cruise	Depth (m)	Date	Sampling gear
R. exoculata	Lab. hatched	MAR	Logatchev	14°45'N; 44° 57'W	Serpentine	3037	March 2007	Suction sampler
R. exoculata	Lab. hatched	MAR	TAG	26°08' N; 44° 49' W	BICOSE	3635	January 2014	Suction sampler
R. exoculata	Lab. hatched, with habitat pressure	MAR	TAG	26°08' N; 44° 49' W	BICOSE	3635	February 2014	Suction sampler, pressure compensation
R. exoculata	Plankton samples	MAR	TAG	26°08' N; 44° 49' W	BICOSE	3637	February 2014	Plankton pump
A. muricola	Lab. hatched	Gulf of Guinea	pockmark Regab	05°48'S; 09° 42' W	Biozaïre 2	3150	November 2001	Suction sampler
A. muricola	Plankton samples	Gulf of Guinea	pockmark Regab	5°48' N; 9°42' W	Congolobe	3150	January 2012	Sediment trap
M. fortunata	Lab. hatched	MAR	Lucky Strike	37°17' N; 32° 17' W	Momarsat	1739	September 2013	Suction sampler
N. saintlaurentae	Lab. hatched	Wallis and Futuna	Fatu Kapa [30]	14°N; 177°°W	Futuna 3	1554	June 2012	Suction sampler

For the laboratory reared larvae, sampling information refers to the brooding female.

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on board the research vessel (N/O *L'Atalante* or *Pourquoi Pas*?) without pressure compensation. Adult females with eggs in advanced stage of development were examined, and in some of them, eggs had hatched (maybe due to recovery stress). After hatching, active larvae, rests of eggs and no-motile larvae were fixed in 4% buffered formalin, 10% glutaraldehyde, or 96% ethanol. Adult females were preserved and identified by external morphology [25,26] and by DNA mitochondrial COI sequence (for *N. saintlaurentae*).

Brooding females of *R. exoculata* collected during the BICOSE cruise in January 2014 at the TAG site of the MAR were brought on board with pressure compensation in the PERISCOP chamber [27] and were maintained in the BALIST chamber [28] at 300 bars and 5°C during 24 hours. Hatching occurred in the pressure chamber and active larvae were separated after a short period of decompression. Larvae were maintained in the PICCEL chamber [29] at 300 bars and 8°C during 96 hours. Larvae hatched at habitat pressure were compared with the larvae hatched at atmospheric pressure in order to assess the potential effects of hatching at low pressure on larval morphology.

Additionally, larvae collected in deep-water plankton samples from different locations were studied. Larvae of alvinocaridid shrimps (6 specimens) from the Congo Basin were collected using sediment traps deployed at 3150 m depth at the cold seep site REGAB in the Congo Basin. On the MAR, a larval pump SALSA (Serial Autonomous Larval Sampler- Ifremer) deployed at the TAG site at 3637 m captured 6 specimens of alvinocaridid larvae, 3 of which are included in the present study (Table 1). The morphology of these larvae was compared with the larvae obtained from hatching on board for species identification.

For larvae obtained from on board hatching, from 8 to 15 larvae of each species were fully dissected, and mounted on slides for observations and drawings with a Leica-Leitz microscope with camera lucida. Measurement of total length (TL) and carapace length (CL) were performed for each specimen and variation in the number of setae and spines were noted, including variation between the appendices of the left and right side in the same specimen. For the larvae obtained from field samples, some specimens were dissected for observation and

drawings. For general considerations during dissection, mounting, description and illustration we followed Clark *et al.* [31].

Some specimens from both laboratory hatching and field samples were prepared for Scanning Electron Microscopy (SEM) analyses. Specimens previously fixed in 2.5% glutaraldehyde (seawater) or in 4% formalin were washed (3 times, 5 min each) in distilled water and post-fixed in 0.8% osmium tetroxide (OsO_4) for 1 h. Later the samples were washed 3 times in water and fixed again in OsO_4 for 1h, rinsed (6 times, 5 min each) in distillated water and dehydrated in graded ethanol series (10, 25, 35, 50, 65, 70, 80, 90, 96, 100%, 5 min each, 3 times for the last 5 grades). Samples were submitted to a critical point (Leica EM CPD300), sputter in gold, and observed in SEM.

DNA extraction, amplification, sequencing and phylogenetic reconstruction

Extractions were performed from whole larvae, or 1–2 pleopods for the adult specimens collected at MAR and Western Pacific hydrothermal vents, using the CTAB method (cetyl trimethyl ammonium bromide [32]). Amplifications of the COI gene were performed in a 50 μL solution of 1X reaction buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 0.6 mM of each primer (LCOI1490 and HCOI2198 [33]), 1.25 U *Taq* polymerase and 1–4 μL extracted DNA (depending on the DNA concentration). We performed 35 cycles of amplification with an annealing temperature of 52°C. Similarly, amplifications of the 18S rRNA gene were performed in a 50 μL solution of 1X reaction buffer, 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 mM of each primer (18S1 Forward and 1498 Reverse [34]), 1.25 U *Taq* polymerase and 1 μL extracted DNA. We performed 30 cycles of amplification with an annealing temperature of 51°C. All PCR amplifications were conducted on a GeneAmp PCR system 9700 (Applied Biosystems). PCR products were purified and sequenced at Macrogen, Inc. (Netherlands) using the amplification primers for COI and for the 18S gene the following primers: 18S-3F, 18S-bi, 18S-1F, 18S-5r, 18S-1373F, 18S-505r [34–36].

Other sequences included in the phylogenetic reconstructions were obtained from different studies, mostly related with phylogeny and population genetics on alvinocaridids [5–7,37–41], and available in genbank (S1 Table and S2 Table). For the phylogenetic reconstructions, the sequences were aligned using MUSCLE [42]. Phylogenetic trees were constructed using Bayesian Inference (BI) in BEAST version 1.8 [43]. Configurations of the evolutionary model for each data set were selected according to the best-fit obtained from Model Generator [44]. The selected model of nucleotide substitution for the COI was HKY + I + G, considering a speciation Yule process. Whereas for the 18S, the best-fit model was HKY + G, with a speciation Yule process. 20^6 generations trees with sampling each 1000 generations were performed, the first 25% of the trees were discarded, the rest of the trees were summarized using Treeannotator. Robustness of the inferred trees was evaluated using posterior probabilities. For the COI phylogenetic reconstruction, few sequences (8 of the 201 COI sequences analyzed) obtained from Genbank were discarded due inconsistences (outgroup sorting, and non-monophyletic distribution of the same species) observed in early analyses.

Relationship of the DWCC phylogenetic reconstruction and larval traits

In order to interpret larval morphology traits in an ecological and evolutionary context, literature data on larval traits in related families were also examined. 18S rRNA nuclear gene was used to reconstruct a phylogeny of alvinocaridid shrimps and related families. According to Braken *et al.* [39], Li *et al.* [40] and Aznar-Cormano *et al.* [41] Alvinocarididae are closely related to 7 other families of mainly deep-water Caridea (Oplophoridae, Nematocarcinidae,

Agostocarididae, Campylonotidae, Pasiphaeidae, and Psalidopodidae, with some differences between authors), herein mentioned as deep-water caridean clade (DWCC, although these are not the only deep-water families and include a few shallow-water taxa). Sequences of these major taxa were obtained from GenBank, including those used in the phylogenetic analyses of the former studies [39–41] and others as well (S2 Table). Moreover we obtained new sequences of the 18S gene for *R. chacei*, *M. fortunata* and *N. saintlaurentae*. Rather than bringing a new phylogenetic proposal for the group, our intent was to set an evolutionary framework to analyze the occurrence of larval traits in the DWCC.

In order to compare the phylogenetic relationships along the DWCC and the occurrence of larval traits, we sorted the larval information related to the species considered in the phylogenetic reconstruction. We considered general traits of the larval morphology as indicators of some aspects of the early larval biology. Undeveloped mouth parts (absence of incisive and molar processes in the mandible, and lack or poor setation and spination in the maxillule and maxilla) was considered as indicator of primary lecithotrophy, because the larvae is not able to take external source of food and so relies on its lipid reserves [45]. On the contrary, larvae with developed mouth parts were categorized as planktotrophic, because the larvae have the ability to capture and ingest food when it becomes available. However some planktotrophic larvae could also hatch with large amount of lipid reserves (usually accomplished by large eggs > 1 mm), which could enhance their nutritional flexibility [20]. Unfortunately the descriptions of lipid reserves in the group are scarce and range between just "oil drops" to isotopic analysis, and a more accurate classification distinguishing facultative lecithotrophic from planktotrophic larvae was limited in some cases.

Moreover the presence of pereiopods and/or pleopods in hatching larvae was considered as a trait related with abbreviated development [20,45]. In general, decapod crustacean larvae hatch without pereiopods or pleopods. The development of these structures occurs usually as the larvae molt into more advanced stages. The occurrence of these structures in the first stage suggests advanced development and reduction of the larval instars. For some species considered in our phylogenetic analysis, no information is available regarding the larval development. In these cases, traits were inferred from other species of the same genus with existing knowledge about larval traits (but without genetic data that prevented their inclusion in our dataset) (S2 Table). Although larvae of the same genus usually show interspecific differences in spines and setae, there is no evidence of variation within genera for the major larval traits herein considered (i.e. mouth parts development, occurrence of pereiopods and pleopods) in the DWCC.

Ethics statement

No specific permissions were required to collect the samples used here during the Serpentine and BICOSE cruises (international waters). During the cruises Biozaïre 2 and Congolobe, samples were collected with permission from the Gabon Government. Samples were collected during the cruise MOMARSAT with permission of the Azores Regional Government, Portugal, and during the Futuna cruise with permission of the French Government. The study did not involve endangered or protected species.

Results

Description of first larval stage in alvinocaridids shrimps

***Rimicaris exoculata* Williams and Rona, 1986. Mid-Atlantic Ridge (Figs 1, 2, 3A–3D, 3G and 3H).** Dimensions: LC = 0.61±0.06 mm; LT = 2.41±0.12 mm.

Carapace with tiny rostrum, sharp but hidden between the eyes, reaching around a half of the eyes length. Eyes sessile. A small anterior-dorsal hump present. Pterisgostomial spine

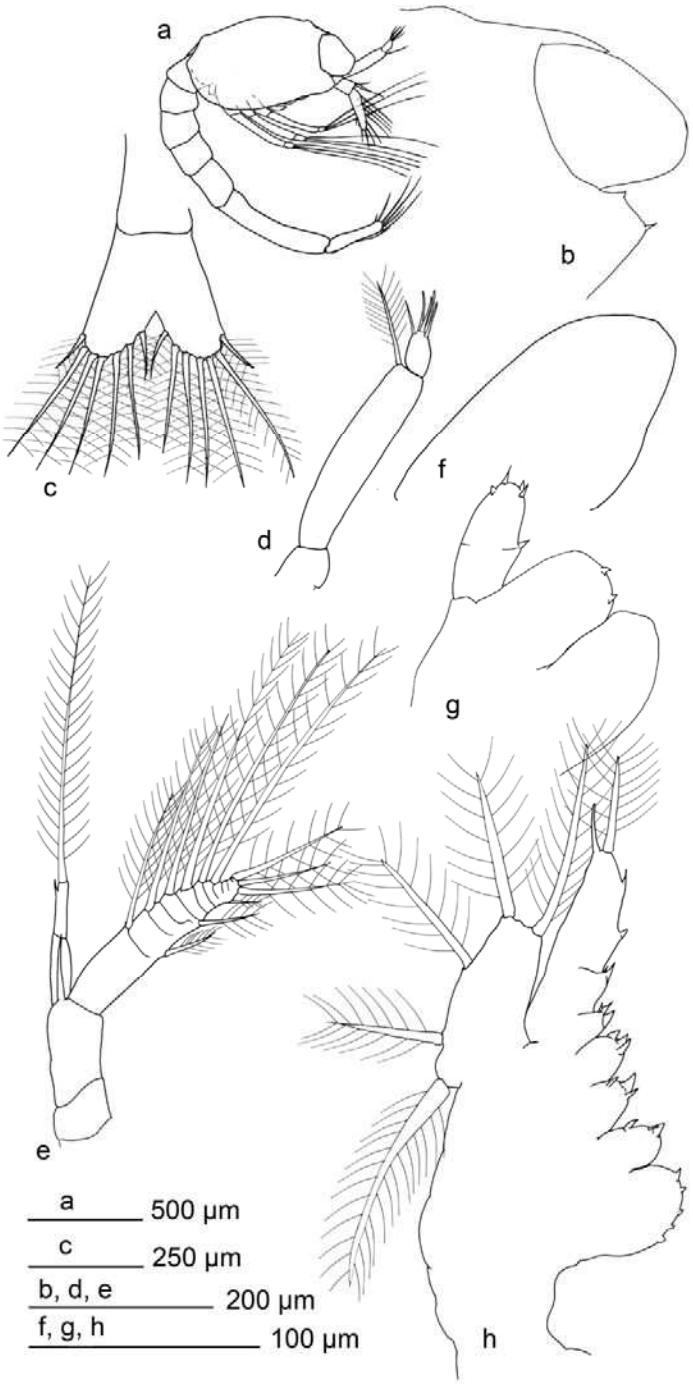

Fig 1. *Rimicaris exoculata* zoea I. a) habitus, b) distal section of carapace, c) telson, d) antennule, e) antennae, f) mandible, g) maxillule, h) maxilla.

doi:10.1371/journal.pone.0144657.g001

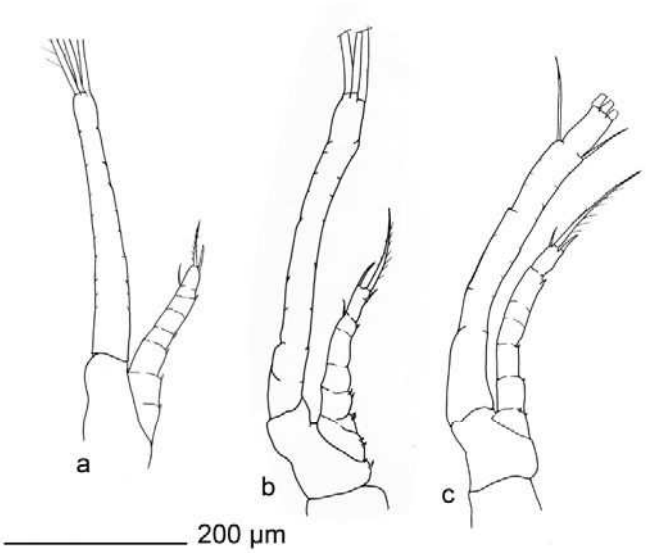

Fig 2. *Rimicaris exoculata* zoea I. maxilipeds, a) first, b) second, c) third.

doi:10.1371/journal.pone.0144657.g002

present, followed by small sub-ocular spine. Posterior margin bilobulated in dorsal view. Antennulae uniramous, peduncle unsegmented with 1 distal setulose setae. Distal segment (outer flagellum) with 0–1 setae simple and 3–5 aesthetascs. Basal segment of the antennae (peduncle) unsegmented, armed with a large spine inserted near to the endopod, and approximately 0.6 times the length of the endopod. Endopod bi-articulated, second article with subdistal spine, absent in some specimens, and large setulose setae. Exopod distally segmented and armed with large setae (setulosae), 7 in the inner margin, 3 distally inserted and 2 smaller in the outer margin.

Labrum and paragnathes present, thumb-like. Mandible thumb-like, unsegmented. Incisive and molar processes absents, 0–3 small distal spines. Palp absent. Maxillule with 0–4 spines in coxal endite. Basal endite with 2–3 spines. Endopod composed by two segments, with mesial spine in the inner margin and 2–4 distal spines. Maxilla with endite coxal and basal bilobulated, coxal endite with 3–8 spines in the first lobule and 1–3 spine in the second. Basal endite with 2–4 spines in the first lobule and 1–3 spines in the second. Endopod unsegmented, with two proximal small lobes armed by 1–3 spines and 1–2 spines respectively. Followed by 2 subdistal spines, one distal spine in the outer margin and distal setulose setae 2–5 times the size of the distal spine. Margin of the scaphognathite with 5 marginal setulose setae.

Maxillipeds 1–3 similar in shape. Endopod with irregular segmentation, between 5–6 articles. Distal article with 1–3 plumodenticulate setae, occasionally with additional spine. Inner margin of endopod with 2–6 disperse, small spinules, sometimes with small subdistal setae. Exopod with superficial and incomplete segmentation, with a shallow furrow-like surface in the inner face and flat outer surface. Distally the exopod is armed with 3 large setulose setae. Pereiopod only as rudimentary bud, without segmentation, unarmed.

Abdomen with 6 somites, first segment overlap with the carapace. Last segment larger than others, thinner and dorsally compressed. Setae and spines absent. Ventral humps present in

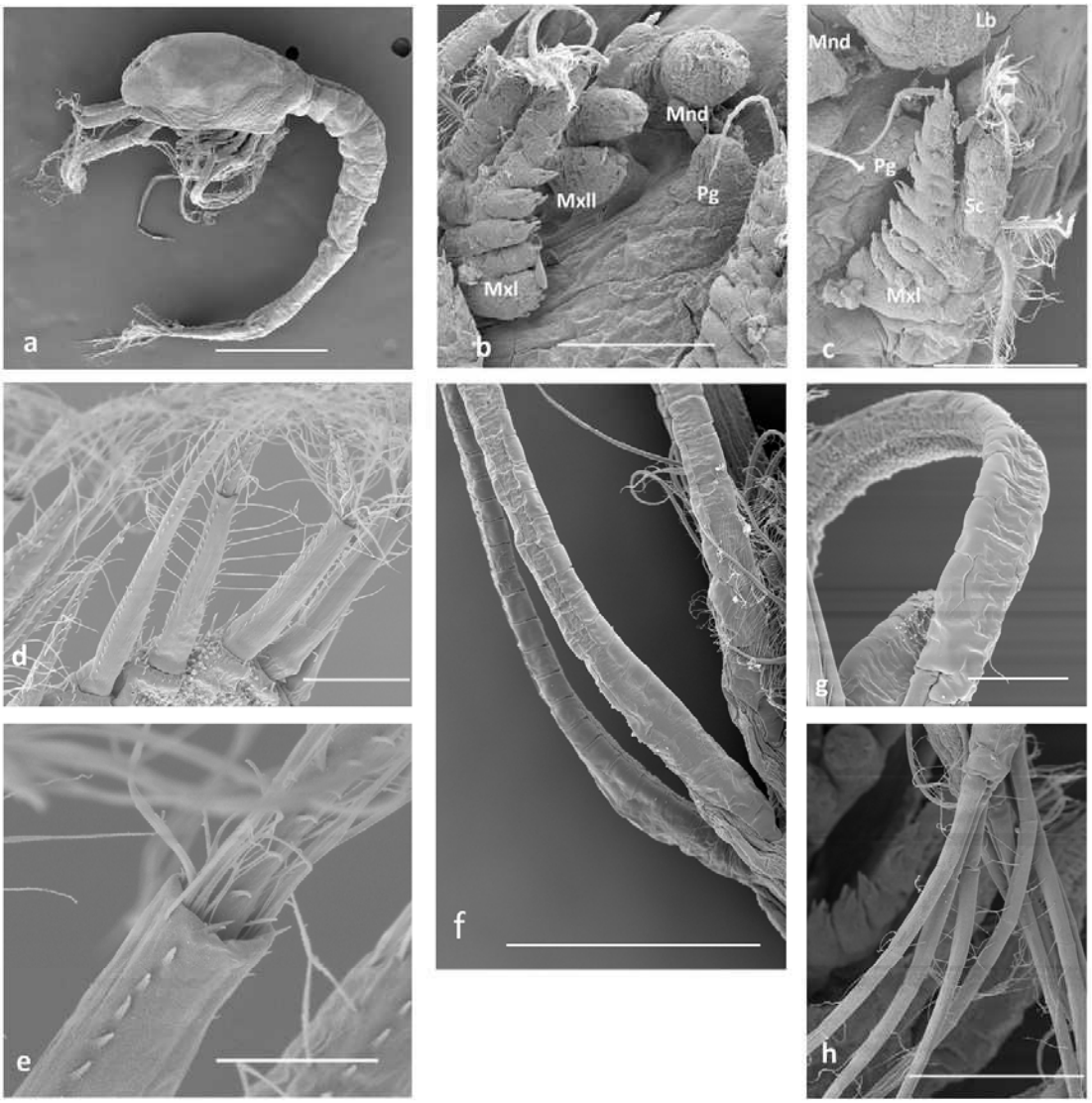

Fig 3. SEM details in some alvinocaridid zoea I larvae. A.- *Rimicaris exoculata*, lateral view; b, c.- *R. exoculata*, mouth parts; d, e.- *R. exoculata*, plumodenticulate seta of telson; f.- *Mirocaris fortunata*, exopods of 1–3 maxillipeds; g, h.- *R. exoculata*, exopod of 2nd maxilliped and distal setae. Mnd, mandibule; MxII, maxillule; MxI, maxilla; Pg, paragnathe; Lb, labrum.

doi:10.1371/journal.pone.0144657.g003

the 1–5 somites, pleopods and uropods absents. Telson bilobulated, distal margin with 7 plumodenticulate setae in each lobe.

Remarks: The larvae hatched in the BALIST chamber and maintained at 300 bars during 96 h did not show differences with the specimens hatched at atmospheric pressure. Although some variation occurs between specimens in the number of spines on the mouth parts and on the maxillipeds, their range completely overlap between the larvae hatched at atmospheric

pressure or at habitat pressure. Hatching at atmospheric pressure does not appear to have an effect on the morphology of the larvae of *R. exoculata* and we assume that larvae of other species obtained after hatching at atmospheric pressure reflect then the morphology of those at their habitats.

***Mirocaris fortunata* (Martin and Christiansen, 1995). Mid-Atlantic Ridge (Figs 3F and 4).** Dimentions: LC = 0.56±0.027 mm; LT = 2.32±0.08 mm.

Carapace with tiny rostrum, sharp but hidden between the eyes, reaching around a half of the eyes length. Eyes sessile. A small anterior-dorsal hump present. Pterisgostomial spine present. Posterior lateral margin bilobulated in dorsal view. Antennulae uniramous, peduncle unsegmented, setulose setae inserted distally. Distal joint (outer flagellum) with 3–5 aesthetascs and, eventually, 1 setae. Antennae with basal segment (peduncle) unsegmented, armed with a distal spine 0.6 times the size of the endopod. Endopod bi-articulated, second article with tiny subdistal spine, sometimes absent, and large setulose setae. Exopod distally segmented and armed with large setae (setulosae), 7 in the inner margin, 2–3 distally inserted and 2 smaller in the outer margin.

Mandible thumb-like, unsegmented. Incisive and molar processes absents, unarmed or with 1–2 small spines. Palp absent. Labrum and paragnathes present, thumb-like, unarmed. Maxillule with coxal endite with 2–4 distal spines. Basal endite with 3–6 spines, some minute. Endopod composed by two segments, with mesial spine in the inner margin and 2 distal spines. Maxilla with two lobules in coxal and basal endites. Coxal endite with 5–6 spines in the first lobule and 2–3 spines in the second. Basal endite with 3 and 4 spines in each lobule. Endopod unsegmented, with two proximal small lobes armed with 3 and 2 spines respectively, followed by 2 subdistal spines and two distal spines. Margin of the scaphognathite with 5 marginal setulose setae.

Maxillipeds 1–3 similar in shape. Endopod with segmentation irregular, uncomplete. Subdistal setae present followed by 2–3 distal setae, irregular in size, setulose. Inner margin of endopod with 5–6 disperse, small spinules. The first maxilliped shows up to 13 spines between the margin of the endopod and coxal-basal margin. Exopod with superficial incomplete segmentation, with 3 large setulose setae distally inserted. Inner and outer faces of exopod show a flat surface, inner also with small furrow. Some specimens also with 1–2 thin sub-distal setae. Pereiopods only as rudimentary bud, without segmentation, unarmed.

Abdomen with 6 somites, first segment overlap with the carapace. Last somite larger and laterally thinner. Setae and spines absent. Ventral humps present in the 1–5 somites, pleopods and uropods absent. Telson bilobulated, distal margin with 7 plumodenticulate setae in each lobe.

***Nautilocaris saintlaurentae* Komai & Segonzac, 2004. Western Pacific (Figs 5 and 6).** Dimensions: LC = 0.58±0.004 mm; LT = 2.20±0.01 mm.

Carapace armed with tiny rostrum, sharp but hidden between the eyes, reaching around a half of the eyes length. Eyes sessile. A small anterior-dorsal hump present. Pterisgostomial spine present. Posterior margin laterally bilobulated. Antennulae uniramous, peduncle unsegmented, setulose setae inserted close to distal joint. Distal segment (outer flagellum) with 3–5 aesthetascs. Basal segment of the antennae (peduncle) unsegmented, with large spine inserted near to the endopod (0.6–1.1 times the size of the endopod). Endopod bi-articulated, second article usually with small subdistal spine and large setulose setae. Exodop distally segmented and armed with large setae (setulosae), 6–7 in the inner margin, 2–3 distally inserted, and 2 smaller in the outer margin.

Mandible thumb-like, unsegmented. Incisive and molar processes absents, unarmed or with 1–2 small spines. Palp absent. Labrum and paragnathes present, thumb-like, unarmed. Maxillule with coxal endite with 0–5 small spines. Basal endite with 1–4 spines, some minute.

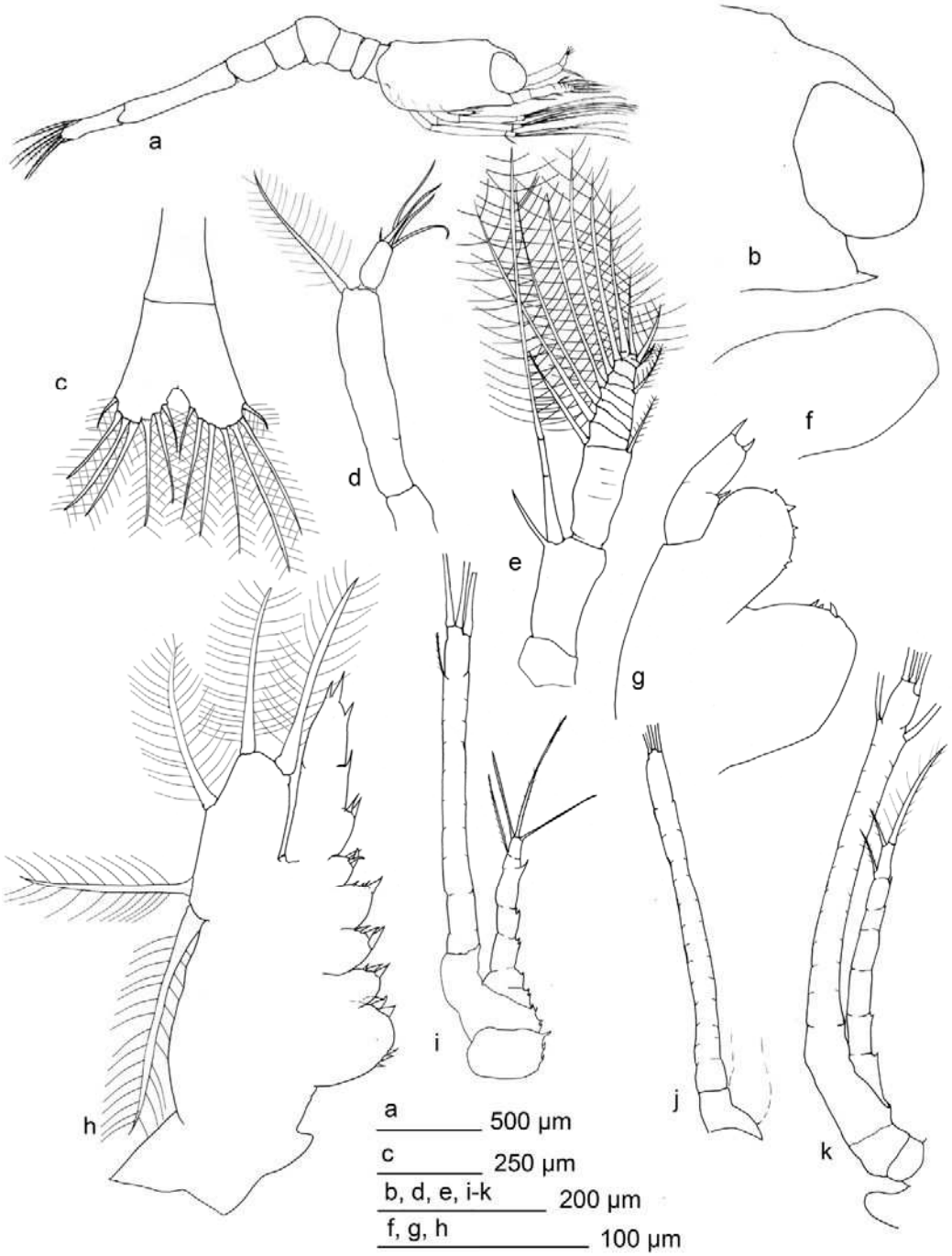

Fig 4. *Mirocaris fortunata* zoea I. a) habitus, b) distal section of carapace, c) telson, d) antennule, e) antennae, f) mandible, g) maxillule, h) maxilla, i-k) first to third maxillipeds respectively.

doi:10.1371/journal.pone.0144657.g004

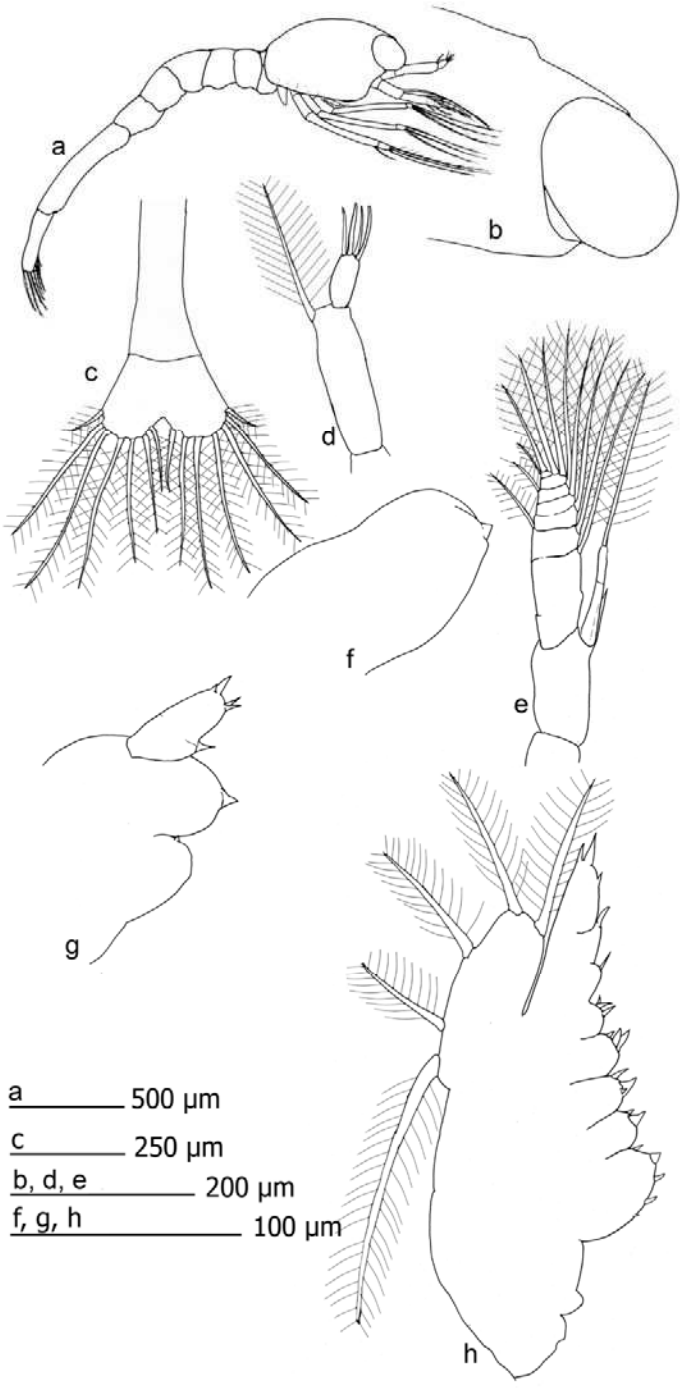
a
b
c
d
e
f
g
h
a 500 µm
c 250 µm
b, d, e 200 µm
f, g, h 100 µm

Fig 5. *Nautilocaris saintlaurentae* zoea I. a) habitus, b) distal section of carapace, c) telson, d) antennule, e) antenna, f) mandible, g) maxillule, h) maxilla.

doi:10.1371/journal.pone.0144657.g005

Endopod composed by two segments, with 1–2 mesial spines in the inner margin and 2–4 distal spines. Maxilla with endite coxal and basal bilobulated, coxal endite with 4–8 spines in the first lobule and 2–3 spines in the second. Basal endite with 2–3 spines in the first lobule and 2–4 in the second. Endopod unsegmented, with two proximal small lobes armed with 1–3 and 1–2 spines respectively, followed by 2 subdistal spines. Distal margin with two spines, a single spine in some specimens. Margin of the scaphognathite with 5 setulose setae.

Maxillipeds 1–3 similar in shape. Endopod with segmentation irregular, 3–6 joints. Distal joint with 2–3 setae, and 1–2 small subdistal setae, all setulose. Inner margin of endopod with 0–8 disperse, small spinules. Exopod show superficial segmentation at lateral sides and flat inner and outer faces. Small furrow also on the inner face of expodod. Three large setulose setae inserted distally. Some specimens also with 1–2 sub-distal setae. Pereiopods only as rudimentary bud, unarmed.

Abdomen with 6 somites, first segment overlap with the carapace. Last somite larger and laterally thinner. Setae and spines absent. Ventral humps present in the 1–5 somites, pleopods and uropods absent. Telson bilobulated, distal margin with 7 plumodenticulate setae in each lobe.

***Alvinocaris muricola* Williams, 1988. Eastern Atlantic, Congo Basin** (Fig 7). Dimensions: LC = 0.57±0.02 mm; LT = 2.13±0.07 mm.

Carapace with tiny rostrum, sharp but hidden between the eyes, reaching around a half of the eyes length. Eyes sessile. A small anterior-dorsal hump present. Pterisgostomial spine present. Posterior margin laterally bilobulated. Antennulae uniramous, peduncle unsegmented, large setulose setae inserted close to distal joint. Distal segment (outer flagellum) with 5 aesthetascs. Antennae with basal segment (peduncle) unsegmented, armed by a large spine inserted

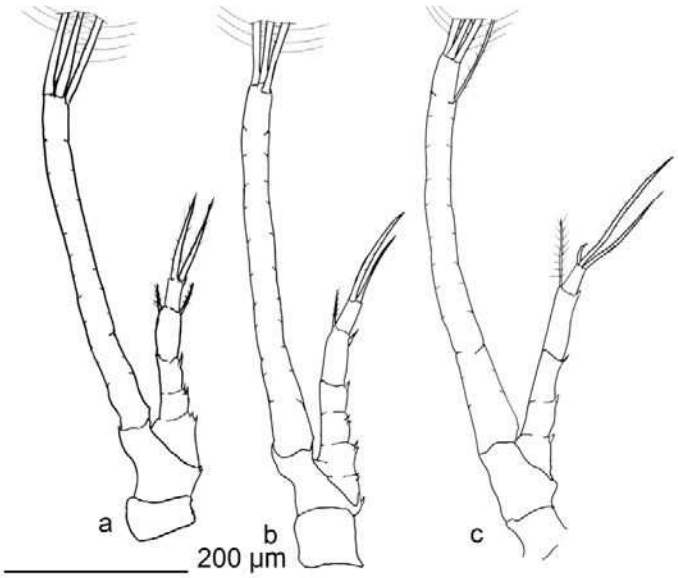

Fig 6. *Nautilocaris saintlaurentae* zoea I. maxillipeds, a-c) first to third respectively.

doi:10.1371/journal.pone.0144657.g006

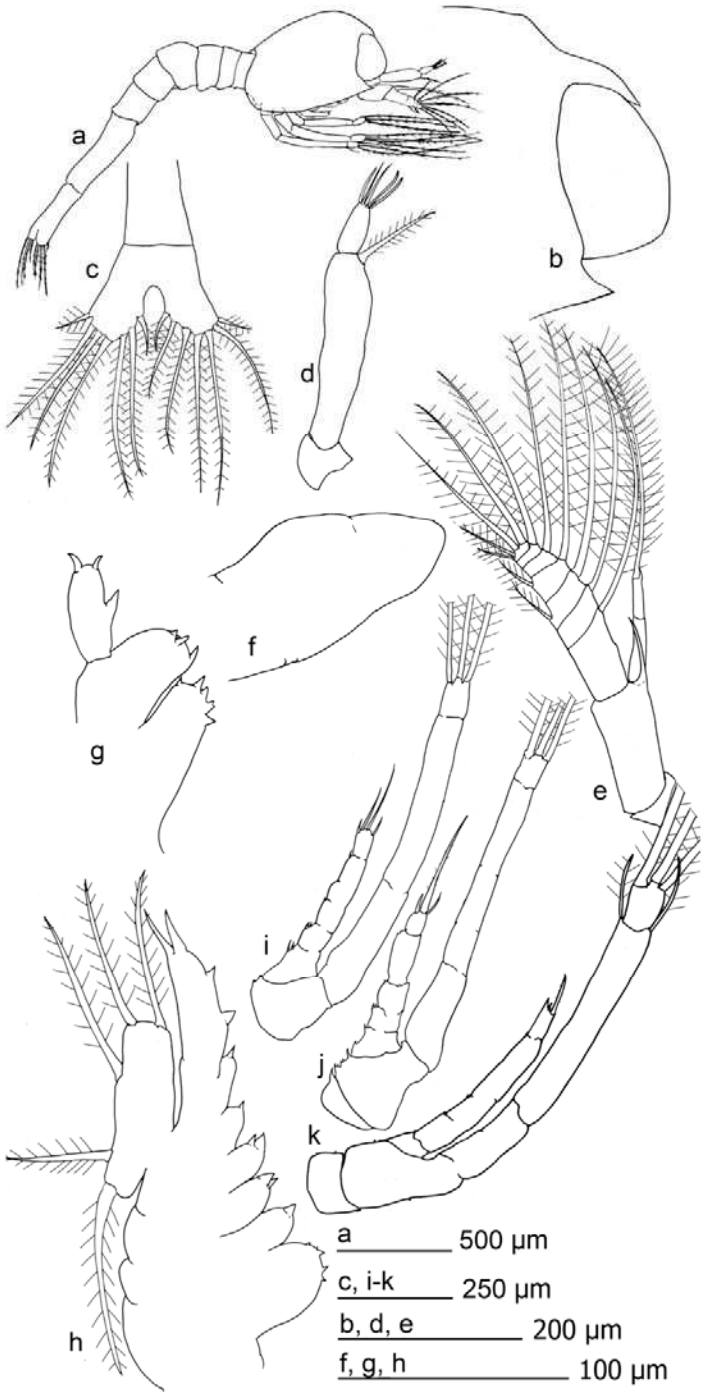
a
b
c
d
e
f
g
h
i
j
k
a 500 µm
c, i-k 250 µm
b, d, e 200 µm
f, g, h 100 µm

Fig 7. *Alvinocaris muricola* zoea I. a) habitus, b) distal section of carapace, c) telson, d) antennule, e) antenna, f) mandible, g) maxillule, h) maxilla, i-k) first to third maxillipeds respectively.

doi:10.1371/journal.pone.0144657.g007

near to the endopod. Endopod bi-articulated, second article with distal spine and large setulose setae. Exopod distally segmented and armed with large setae (setulosae), 6 in the inner margin, 2 large and 2 small setae distally inserted, 2 small setae inserted in the outer margin.

Mandible thumb-like, unsegmented. Incisive and molar processes absents, unarmed. Palp absent. Labrum and paragnathes present, thumb-like, unarmed. Maxillule with coxal endite with 5 strong spines, one bifid. Basal endite with 3 spines. Endopod composed by single segment, with mesial spine in the inner margin and 2 curved spines distally. Maxilla with coxal and basal endites bilobulated, coxal endite with 5 small and 1 large spines in each lobule, and basal endite with 2 spines in each lobule. Endopod unsegmented, with two proximal lobes armed with 1 spine each followed by 2 subdistal spines, one distal small and thick setae (setulose) and distal spine. Margin of the scaphognathite with 5 setulose setae.

Maxillipeds 1–3 similar in shape. Endopod with segmentation irregular, composed by 4–8 joints. Distal joint with 1 large and 2–3 small setae. Inner margin of endopod with 6–8 disperse, small spinules and 4–5 spinules in the basis. Exopod superficially segmented in lateral sides, distally armed 3 large setulose setae and occasionally with 1–2 small sub-distal setae. Inner and outer faces of the endopod flat, with small furrow in the inner surface. Pereiopods only as rudimentary bud, without segmentation, unarmed.

Abdomen with 6 somites, first segment overlaps with the carapace. Last somite larger and laterally thinner. Setae and spines absent. Ventral humps present in the 1–5 somites, pleopods and uropods absent. Telson bilobulated, distal margin with 7 plumodenticulate setae in each lobe.

Morphological identification of alvinocaridid larvae collected by plankton samplers

The larvae collected with the plankton pump on the MAR show the same morphological features as the larvae hatched from *R. exoculata* females onboard. These characters include the setae at the tip of the endopod of the maxilla and the small subocular spine, which are distinct from other species herein studied. No other species except *R. exoculata* exhibit a subocular spine in the carapace, although this small spine was, rarely, absent in some *R. exoculata* specimens. Moreover the setae at the tip of the endopod of the maxilla is present in *R. exoculata* and *A. muricola*, but in the latter species, it is variable in shape from a spine to a small and thick setae with few setulae. Other species, *M. fortunata* and *N. saintlaurentae* only show two spines at the tip of the endopod.

The shrimp larvae collected with the sediment traps in the Congo Basin shows similar characters as those of specimens of *A. muricola* that hatched onboard. Although all alvinocaridid species herein studied are very similar to each other and the knowledge of alvinocaridid larval morphological variation is still low, a thick short setae at the tip of the endopod of the maxilla was observed only in *A. muricola* and in the specimens collected with sediment traps. Moreover *A. muricola* is the only alvinocaridid species in the Congo cold seeps. The other species with a distal setae in the maxilla endopod, *R. exoculata*, shows a long setae and a large spine besides, and also usually a small subocular spine, which is absent in *A. muricola*.

Molecular identification of alvinocaridid larvae

The phylogenetic tree obtained from Bayesian inferences with the COI gene shows three very divergent clades (Fig 8). The first clade consists of the genera *Mirocaris* and *Nautilocaris*, and

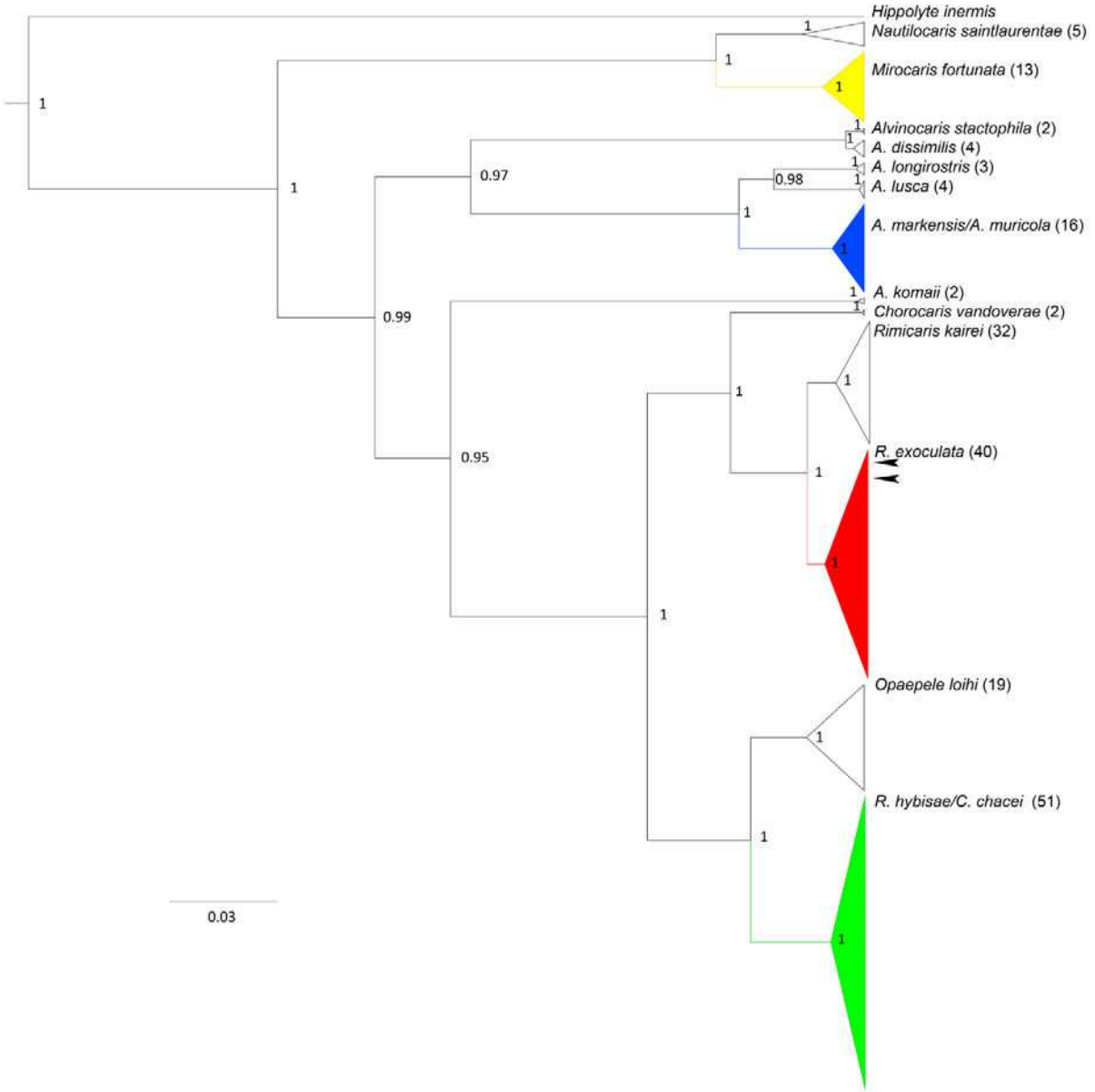

Fig 8. Phylogenetic relationships of Alvinocarididae shrimps based on the Bayesian Inference of COI gene using HYK + I + G evolutionary model. Species or monophyletic species-complex are cartooned and MAR species are in colors. Arrows shown the position of the larvae collected in the plankton samples of the MAR. Number of sequences in parentheses.

doi:10.1371/journal.pone.0144657.g008

includes the *N. saintlaurentae* hatching female considered in the present study. It is a sister group of the rest of Alvinocarididae. The second clade includes all species of *Alvinocaris*

(6 spp.) except *A. komaii*. This group includes the sequence of Alvinocaridid larvae studied by Koyama *et al.* [10] which is affiliated with sequences of *Alvinocaris longirostris*. The third clade includes the genera *Rimicaris*, *Chorocaris* and *Opaepele*, in addition to one species of *Alvinocaris* (*A. komaii*). This 3-clade configuration is consistent with previous phylogenetic reconstruction based in COI gene [37], including the position of *A. komaii* [46]. The two species complexes formed by *Alvinocaris muricola/markensis* and by *Rimicaris chacei/hybisae*, both proposed by Teixeira *et al.* [6] and confirmed by Vereshchaka et al. [1] also appear in our phylogenetic analysis. This tree topology was expected since we used the same data as those presented by former authors, and it is further supported in our analysis which also includes a new sequences of *R. hybisae* obtained by Plouviez *et al.* [38]. All the species and species-complexes in our analysis are well supported by the posterior probabilities of the Bayesian Inference. The larvae collected with the larval pump at the TAG site on the MAR and identified from their morphology as *R. exoculata*, fall in our tree with the other *R. exoculata* samples. The large divergence with other alvinocaridid species from the MAR brings no doubt about the genetic confirmation of the morphological identification of these larvae.

Larval traits along the DWCC

Strong phylogenetic reconstructions of the DWCC based on several nuclear and mitochondrial loci are already published, but they do not necessarily include species with published information on larval traits. Here we selected species from different DWCC families with such knowledge as well as genetic information (most of the time 18S rRNA sequence) available. Our phylogenetic reconstruction of the DWCC based on the 18S gene (Fig 9) shows some differences with previous reconstructions [39–41], particularly in the position of Acanthephyridae and some families represented by single species (Campylonotidae, Psalidopodidae and Agostocarididae). These differences are attributed to the taxa coverage and locus selection. However the general topology of the tree is in agreement with some of the important phylogenetic features in the group previously suggested. Particularly, our tree also reflects the separation of Acanthephyridae *sensu stricto* from Ophoploridae [39,41,47], the polyphyletic status of Pasiphaeidae [39], and retains the monophyletic status of Nematocarcinidae [39,40] (although our analysis includes a single genus) and Alvinocarididae [1, 37,39–41,46–48]. Moreover in all genera with more than one species (except *Acanthephyra* and *Systellaspis*) the genetic relationship was closer within genus. Since we recover here the main trends previously proposed in broader analyses, we consider our tree suitable to make inferences about larval morphology and ecology.

Based on general traits of the first larval stage (mouth parts development and pereiopod or pleopod development), DWCC considered here could be separated in four main groups: 1: lack of pereiopod and pleopods, and developed mouth parts, 2: presence of pereiopod or pleopods and developed mouth parts, 3: presence of pereiopod or pleopods and undeveloped mouth parts, 4: lack of pereiopod and pleopods, and undeveloped mouth parts. The first group includes species from several families (Pasiphaeidae: *Leptochela*, Nematocarcinidae: *Nematocarcinus*, Acanthephyridae: *Acanthephyra*). Larval traits associated with planktotrophic and extended development are considered plesiomorphic due to their general distribution in decapod crustaceans in both shallow and deep-water habitats and their occurrence in the basal taxa of Caridea (Disciadidae and Rhynchocinetidae) according Bracken *et al.* [39] and Aznar-Cormano *et al.* [41]. The second group includes Campylonotidae. Facultative primary lecithotrophy is also present in this group [49]. The third group, with abbreviated larval development (hatching in advanced stage) and undeveloped mouth parts, typically exhibits lecithotrophy, which is found in several families (Ophophoridae, Pasiphaeidae: *Pasiphaea*, Acanthephyridae:

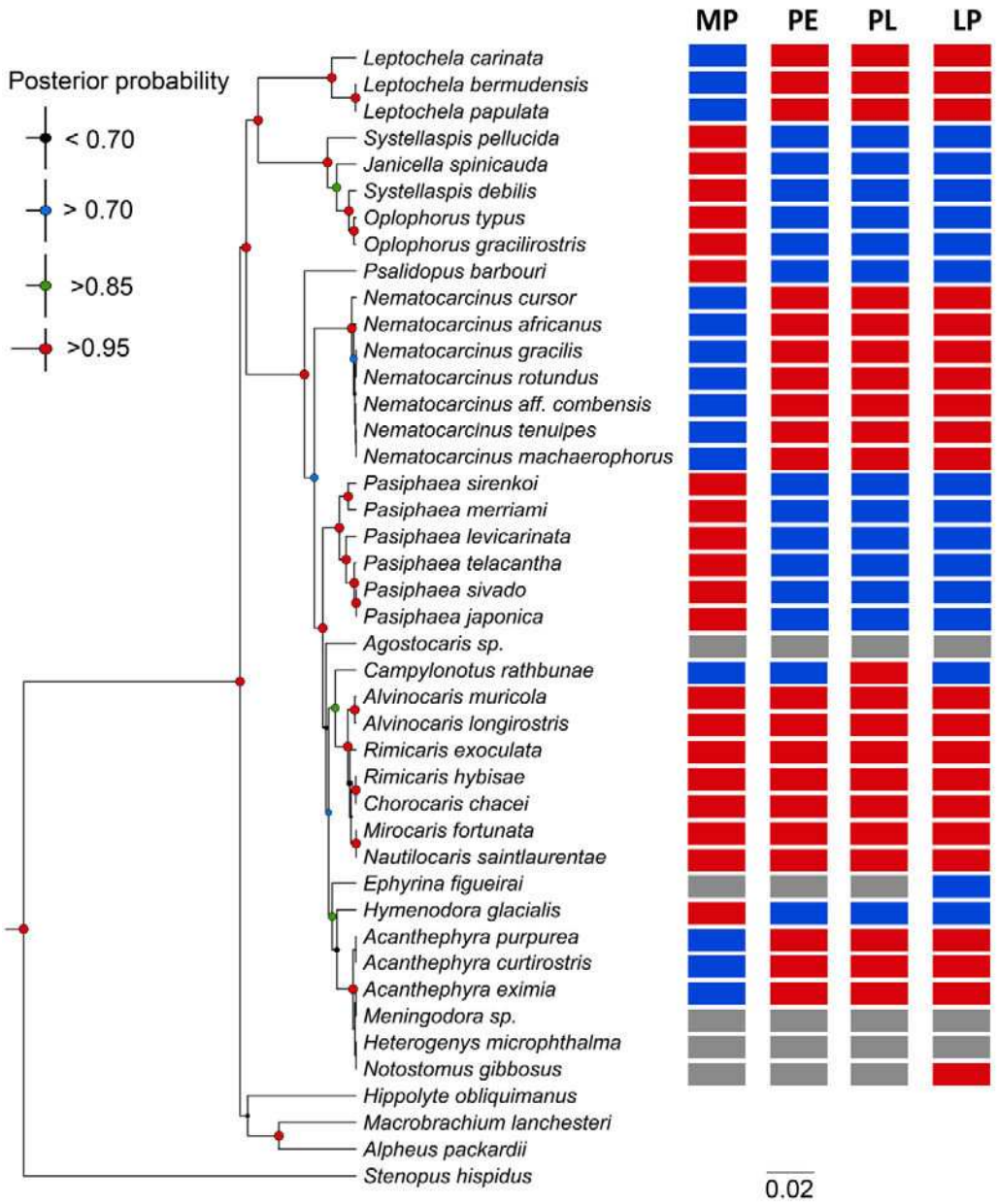

Fig 9. Phylogenetic relationships between Alvinocarididae and related families and distribution of larval traits along the tree. Phylogenetic reconstruction is based on the Bayesian Inference of 18S gene using HYK + G evolutionary model. Larval traits of first zoeal stage: MP, mouth parts developed (blue), non-developed (red); PE, pereiopods present (blue), absent (red); PL, pleopods present (blue), absent (red); LP, larval development abbreviated (blue), extended (red). Gray squares, non-information available.

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Hymenodora and Psalidopodidae). The forth combination of traits is found only in Alvinocarididae.

Discussion

Comparison of larvae between alvinocaridid species

Early stages of Alvinocarididae exhibit particular features such as a notorious lack of development in mouth parts and few setation in the inner margin of the maxillipeds. This observation is not an artifact due to abnormally precocious hatching caused by recovery stress. The absence of a cover layer over the appendices and telson confirms that larvae analyzed here are at first zoeal stage instead of a prezoea stage [50,51] that could occur just after hatching. Moreover, the similarity between the larvae of *R. exoculata* obtained on board from hatching at atmospheric pressure or in pressurized chambers and in the plankton pump also demonstrates that the low degree of development of mouth parts is neither the product of an ontogenetic anomaly caused by the pre-hatching decompression nor abnormal early hatching of undeveloped larvae.

The absence of setation on the mouth parts (except for the scaphonagtite, and the tip of the endopod of the maxilla) and on the endopod margin of the maxillipeds, with the almost complete absence of spines on the carapace and abdomen bring a configuration very similar between the larvae herein studied. Spinulation of mouth parts shows intraspecific variation that overlaps interspecific variation. However *R. exoculata* can be separated from the other species by the presence of a spine and setulose setae on the distal section of the endopod of the maxilla and a small sub-ocular spine on the carapace, additional to the pterigostomian spine. The other species studied exhibit two spines at the tip of the endopod of the maxilla, or one spine and one thick setae (with few or no setulae), but only the pterigostomian spine on the carapace. In addition, the distal spine projected on the external margin of the endopod of the maxilla is usually larger in *R. exoculata* than in other species. *A. muricola* can be distinguished by the occurrence of a thick setae at the tip of the endopod of the maxilla, although this character showed some variation from small simple spine-like setae to large setae occasionally with few setulae (resembling *R. exoculata*). The intraspecific variation in spinulation completely overlaps between *M. fortunata* and *N. saintlaurentae*, limiting the distinction of larvae of the 2 species based on morphological characters (Table 2).

The larvae collected in plankton samples from Regab in the Gulf of Guinea are identified as first larval stage of *A. muricola*. These larvae, as well as those collected from a gravid female from the same site, showed a higher degree of morphological variations than zoea from the three other species herein studied. These variations were not related to growth and molting to the next larval stages since we did not observe changes in size or new structures (i.e. more setation or changes in size and shape in the appendices) as it would be expected after molting, as compared with the larvae obtained from onboard hatching. Adults and juveniles of *A. muricola* exhibit a wide range of morphological variations in some characters such as rostrum and carapace width [25]. *A. muricola* is the only alvinocaridid species known to inhabit the Congo basin cold seeps so far. However, this species is also found in the Barbados Prism, the Gulf of Mexico and the Blake Diapir [52]. Moreover, phylogenetic studies based on both mitochondrial (COI) and nuclear (18S rRNA) genes suggest that *A. muricola* and *A. markensis* from hydrothermal vents on the MAR are a single species [6]. *A. muricola* thus seems to be a morphologically plastic species, with plasticity also occurring in larvae as observed here, widely distributed and able to colonize different habitats, although morphological variability has not been explicitly related to specific locations or habitats so far.

Larvae collected with the plankton pump at the TAG vent field on the MAR were identified as first zoea stage of *R. exoculata* based on their external morphology. Both specimens

Table 2. Variation in larval structures in Alvinocaridid larvae.

	R. exoculata		*M. fortunata*		*N. saintlaurentae*		*A. muricola*	
	MF	Range	MF	Range	MF	Range	MF	Range
Carapace								
Subocular spine	p	ra-p	a		a		a	
Antennule								
Aesthetascs	4	3–5	4	3–5	4	3–5	4	4–5
Other spines or setae	0	0–1	1	0–1				
Antenna								
Basal spine ratio/endopod	0.6	0.4-.06	0.6	0.6–0.7	0.8	0.6–1.1	0.6	0.4–0.6
Endopod, Nbr of joints	2	1–2	2	1–2	2		2	
Spine in the last joint	l, sd	a-l, sd-d	s, d	a-s	s, sd	s-l	d, l	s-l
Mandible								
Nbr of spines	1	0-3s	0	0–2	0	0–2	0	0–1
Maxillule								
Nbr spines coxal endite	2	0–4	3	2–4	4	0–5	5	3–7
Nbr spines basal endite	2	2–3	3	3–6	2	1–4	3	1–3
Subdistal spine endopod	1	1–2	1		1	1–2	1	1–3
Nbr of distal spines	2	2–4	2		3	2–4	2	
Maxilla								
Spines lobe 1 coxal	5	3–8	6	5–6	5	4–8	5	4–8
Spines lobe 2 coxal	3	1–3	3	2–3	3	2–3	2	2–3
Spines lobe 1 basal	3	2–4	3	2–3	3	2–3	3	2–3
Spines lobe 2 basal	3	2–4	3	2–4	3	2–4	3	2–4
Spines lobe 1 endopod	3	1–3	3		3	1–3	3	1–3
Spines lobe 2 endopod	2	1–2	1	1–2	2	1–2	1	1–2
Inner distal projection	st		sp		sp		st	1–2
Size of inner distal projection	l	m-l	S		s		s	1–2, s-l
Outer distal spine	m	m-l	S		s	a-s	s	a-l
Maxilliped 1								
Endopod segmentation	5	5–6	4	4–6	6	i5-6	5	
Distal setae	2pd	1-2pd+ 0–1, s+ 1-3sp	2pd	2-3pd +1sp	3pd	2-3pd +1-2sp	1pd+1sp	1-2sp
Spinules inner margin	6	2–8	2	2–8	5	3–11	4	2–5
Spinules in the basis	2	0–8	4	0–4	3	0–5	3	0–4
Exopod segmentation	3		3		3	2–3	3	
Distal setae	3		3	1–3	3	2–3	3	2–3
Subdistal Setae	0	0–1	1	0–1	0	0–1	0–1	0–2
Maxilliped 2								
Endopod segmentation	6	5–6	i4	3–6	i6	3–6	6	5–6
Distal setae	2	1–3+0-1s+ 0-1sp	2	2–3+1sd, s	2	2–3+1sd, s	1	1–2+0-2s+1-2sp
Spinules inner margin	5	0–5	4	4–10	3	3–8	3	3–4
Spinules in the basis	0	0–3	0	0–3	1	0–4	3	2–3
Exopod segmentation	3		3	2–4	3	2–3	3	
Distal setae	3		3	3–4	3	2–3	0	
Subdistal Setae	0		0	0–3	1	0–1		
Maxilliped 3								
Endopod segmentation	5	5–6	i4	4–6	i6	3–6	6	5–6
Distal setae	2	1–2+ 0-1s+ 0-1sp	2	2–3+1sd	2	2–3+1sd	2 sp	0–2+0-1sp
Spinules inner margin	3	0–5	5	4–11	0	0–4	2	2–4

(*Continued*)

Table 2. (*Continued*)

	R. exoculata		*M. fortunata*		*N. saintlaurentae*		*A. muricola*	
	MF	Range	MF	Range	MF	Range	MF	Range
Spinules in the basis	0	0–2	0	0–2	0		3	2–3
Exopod segmentation	3		3		3		3	
Distal setae	3		3		3	2–4	3	
Subdistal Setae	0	0–2	0	0–2	1	1–2	0	

Columns show the mean or most frequent number or character (MF) and range. For each larval feature (only features with variation is considered). Keynote: a, absent; p, present; ra, rarely absent; s, small; m, medium; l, large; st, setae; sp, spine; d, distal; sd, subdistal; i, irregular. X-X denote a range and X+X denote additional structure (e.g. Distal setae: 0–2+0-1s = from 0 to 2 setae and from 0 to 1 small setae).

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examined show a tiny subocular spine and large setae at the tip of the endopod of the maxilla, which is consistent with the description in *R. exoculata*. This identification is also confirmed by COI barcode. The occurrence of ovigerous females bearing eggs close to hatching (pers. observation) explain the presence of early larval stages in the water column near the vent, which probably originates from TAG and have not yet dispersed away.

The close similarities between the larvae of *Rimicaris*, *Mirocaris*, *Nautilocaris* and *Alvinocaris* and their differences with other carideans (see next section), support the monophyletic status of Alvinocarididae previously suggested based in molecular phylogeny [37,39–41] and morphology, although genetic evidence suggests that the generic relationships within the family require a revision [1, 6,37]. Unfortunately, our taxa coverage and the overlapping of larval characters between the species herein studied preclude any approximation of within family phylogenetic relationship based on larval morphology.

Morphology and larval biology of alvinocaridids

The absence of both masticatory processes in the mandible and setation in mouthparts that could participate in the process of capture and manipulation of food suggests that the alvinocaridid first larval stage is a non-feeding larva. This contrasts with previous hypothesis of planktotrophic larval nutrition in Alvinocarididae [9,11,18,53] based on indirect (egg size) evidence [11]. Although egg size in alvinocaridids is relatively smaller than in other species with lecithotrophic larvae [54], the accumulation of triacylgycerols, wax esters and monounsaturated fatty acids in eggs of alvinocaridid species [55] supports the hypothesis of primary lecithotrophy in this family. The occurrence of lipid storage in early stages is common in decapod larvae for standing eventual starvation or even lecithotrophy [20], but the suppression of feeding structures is rare. Lipid reserves in a lecithotrophic larvae or lacking of developed feeding structures has been documented previously in land crabs [56,57], symbiotic crabs [58], symmetric hermit crabs [59–61], galatheids [62,63], alpheid shrimps [64,65], freshwater shrimps (Atydae, [66]) and in some deep water Oplophoridae, Acanthephyridae and Pasiphaeidae [66, 67]. Larvae of these species also exhibit developed pleopods or pereiopods, which are common in late larvae, suggesting abbreviated development. These features are associated with restricted larval dispersal due to the short development period and larval or postlarval retention [56,58,64,68]. In alvinocaridids, although the early larval stage is lecithotrophic, the absence of pleopod and pereiopod does not support abbreviated development and genetic connectivity also suggest high dispersal [5,6,51]. Moreover, Koyama *et al.* [13] report that the early stage (zoea I) larvae of *Alvinocaris* sp. (which belong to *A. longirostris* according to our COI

analysis) could survive for at least 74 days under laboratory conditions (atmospheric pressure, 4.5°C).

Related taxa (Pasiphaeidae) do not develop mouthparts during the larval stage and could survive without food source until the post-larvae [69], however these larvae also have abbreviated development that could be completed in 12 days (at 13°C). In alvinocaridids, the large amount of lipid reserve could be not enough to support the survivorship and growth in a complete extended larval development. According to Pond *et al.* [55] adults and eggs of *M. fortunata* have a similar lipid composition, dominated by monounsaturated and saturated fatty acids, lipids being transferred from the parent during vitellogenesis. However, between adults of both *R. exoculata* and *M. fortunata* and their respective juveniles, a shift in lipid composition occurs, with lipids in the juveniles dominated by polyunsaturated fatty acids and with different isotopic signature [55,70]. This suggests the accumulation of new lipid reserves before the recruitment to the vents, which could supply, at least partially the period between the recruitment, maturity and the acquisition of symbiotic bacteria [16]. Since the generation of new lipid reserves usually requires feeding, according to Pond *et al.* from a photosynthetic carbon origin [55,70], it is expected that the early lecithotrophic period would be followed by a feeding period during the larval development.

Although lipid composition of *R. exoculata*, *R. chacei*, *A. markensis* and *M. fortunata* at juvenile stage suggest feeding from photosynthetic source [55,70], similar to bathypelagic shrimps living close to hydrothermal vents in the MAR [71], it is still unclear which habitat the larval stages use. Post-larval stages of Alvinocarididae have been collected at a long distance from their potential origin (> 100 km) [9,70,72] in bathypelagic habitat between 1990–3060 m. Although Alvinocarididae early stages seem to tolerate large pressure variation, with larvae that can, in some cases, hatch and survive at atmospheric pressure ([12,13], present study), temperature tolerance may constrain the upper limit of the bathypelagic habitat [12]. An alternative hypothesis to explain the presence of lipids with photosynthetic isotopic signature in alvinocaridid shrimp is the feeding on particles descending to the aphotic zone, which are found near to the hydrothermal plumes [73] or in the open sea. At the present there is no direct evidence to support the occurrence of alvinocaridid larvae in the photic zone.

Differences in larval morphology of alvinocaridid larvae with others caridean shrimps

General characters in the Alvinocarididae first larvae include tiny rostrum hidden between the eyes, pterisgostomial spine present, setation absent in mouth parts (except for scaphognathite and occasionally the tip of the endopod of the maxilla), mandible thump-like unarmed or with only 1–2 small spines, incisive and molar processes absent, maxilliped 1–3 similar in form and size, three large setae in the distal join of the exopod and 1–3 distal setae in the tip of the endopod, inner margin of endopod only with spinules. Since the present study is the first detailed description of early stage of alvinocaridid shrimps larvae, no other information is available yet for comparisons within the family.

In order to compare the larval morphology of Alvinocarididae with other carideans, it is important to consider their phylogenetic relationships. Although this family had been included in the Superfamily Bresilioidea [74], molecular evidence did not support monophyly at this level [39,41] or relationship between Alvinocarididae and Bresiliidae [41]. Phylogenetic relationships proposed by Tokuda *et al.* [75] suggest monophyly of Alvinocarididae with Palaemonidae, however general phylogenetic reconstruction of carideans using both mitochondrial and nuclear genes support the phylogenetic relationship of Alvinocarididae with Oplophoridae, Acanthephyridae, Nematocarcinidae, Pasiphaeidae [39–41], and also Agostocarididae,

Psalidopodidae [39] and Campylonotidae [40]. Although there are evidences of polyphyly within Pasiphaeidae, all members tested of this family belong to the same general clade [39–41].

Related with a close morphological comparison of the larvae, Thatje *et al.* [76] highlighted the larval similarities between Campylonotidae and Oplophoridae *sensu lato* (Oplophoridae+-Acantephyridae) based on the absence of external setae in maxillule, occurrence of four well developed endites on the maxilla and presence of exopods in all pereiopods. These structures of the maxillule and maxilla are described in the first larval stage of Oplophoridae, Acanthephyridae, Nematocarcinidae, Psalidopodidae and Campylonotidae [49,76–79], except for the occurrence of external setae in some Nematocarcinidae. Although setal interpretation is not possible for Psalidopodidae, due to description of late embryonic stage [79], both pairs of coxal and basal endites of the maxilla are present in this larva. Additionally, in three genera of Oplophoridae (*Systellaspis*, *Janicella* and *Oplophorus*), two Acanthephyridae (*Hymenodora* and *Acanthephyra*) and two Pasiphaeidae (*Pasiphaea* and *Parapasiphae*) a first larval stage with undeveloped mouth parts has been described [67,69,77]. Although the occurrence of pleopods at this stage for all previous taxa suggest also abbreviated larval development [66,77], which also had been suggested for Psalidopodidae [79,80]. The combination of lack of development of mandible and maxillule, the almost complete absence of setation at the inner margin of maxillule, maxilla and maxillipeds and the absence of pleopods all advocate for a new larval configuration in marine caridean shrimps.

Scattered distribution of the genera with lecithotrophic larvae or abbreviated development in the present phylogenetic reconstruction suggests that reduction of mouth parts development and abbreviated development evolved independently along major taxa. No pattern is observed comparing the distribution of the larval traits in the phylogenetic reconstructions proposed previously [39–41,47], although differences in the position of the taxa occurs between studies due to gene and taxa coverture. However, combinations of larval traits are consistent within families or monophyletic units (for Pasiphaeidae), except for *Hymenodora glacialis* and *Ephyrina figuerai*, which show different traits compared to other Acanthephyridae. Polyphyletic taxa, such as Oplophoridae (sensu lato) (Ophophoridae + Acanthephyridae) [39,41,47] and Pasiphaeidae [39], exhibit distinct larval trait combinations, which further support the genetic evidence that split them into monophyletic groups. Concerning Acanthephyridae, the species with abbreviated development are in a sister position of other Acanthephyridae with extended development, although the information about the larval traits in this group is incomplete. Larval traits seem to evolve through different events to the acquisition of the larval form, however these events are not observed at genera or species level of diversification, at least for most of the DWCC. The sequence of acquisition of different larval traits cannot be determined because the relationships between the monophyletic units are still not fully resolved, and there are discrepancies between the previous studies.

Although low variation in the larval traits is observed within the families (or monophyletic units) in the DWCC, other carideans show differences at this level. For instance, three distant and diverse monophyletic families as Alpheidae, Pandalidae and Atyidae [39–41] have species with planktotrophic and extended development but also species with lecithotrophic and abbreviated development [65,81,82]. This variation is associated with the distribution of the species in different habitats and the acquisition of different mechanisms for dispersal or for retention of the offspring. In alvinocaridids the restriction of the species to hydrothermal vents and cold seeps habitats and the common characteristics of these systems (deep waters, fragmented and widely distributed and dominance of bacterial chemosynthesis) could be related to the lack of diversification of larval traits.

Conclusion

Alvinocaridid first larval stage is very distinctive from other decapod crustaceans because of the combination of undeveloped mouth parts and lack of pereiopods and pleopods, which suggest lecithotrophy but extended larval development. The larvae are very similar between the four genera and species studied, but minor morphological structures could be used for species identification. The larval traits observed in Alvinocarididae contrast with other larval models in decapod crustaceans, where lecithotrophy is associated to abbreviated development and some degree of larval retention. This model is consistent with a wide dispersal in the oligotrophic bathypelagic environment to colonize fragmented habitats such as hydrothermal vents and cold seeps. In the DWCC the scattered distribution of traits associated with lecithotrophic/planktotrophic and abbreviated/extended development suggests that they evolved independently in different combinations.

Supporting Information

S1 Table. Genebank references of COI sequences used in the present study.
(PDF)

S2 Table. Sequences (18S gene) included in the phylogenetic reconstruction and the larval traits of the species. The list includes information on larval traits of species not considered in the phylogenetic reconstruction, but used to make inferences on closely related species included in our phylogenetic reconstruction but without available data on larval traits (see methods).
(PDF)

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Author Contributions

Conceived and designed the experiments: IHA FP MACB. Performed the experiments: IHA FP. Analyzed the data: IHA FP. Contributed reagents/materials/analysis tools: FP MACB. Wrote the paper: IHA FP MACB.

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S1 Table. **Genebank references of COI sequences used in the present study.**

Species	Genebank number	Reference
Hippolyte inermis (outgroup)	JF794740.1	[1]
Mirocaris fortunata	KT210460	Present study
	KT210450	Present study
	KT210451	Present study
	KT210452	Present study
	KT210453	Present study
	KT210454	Present study
	KT210455	Present study
	FJ769225.1	[2]
	FJ769226.1	[2]
	AF125430.1	[2]
	AF125431.1	[2]
	AF125432.1	[2]
	AF125433.1	[2]
Alvinocaris dissimilis	AB779491.1	Hiraoka,R. et al. (unpublished)
	AB779492.1	Hiraoka,R. et al. (unpublished)
	AB779493.1	Hiraoka,R. et al. (unpublished)
	AB779494.1	Hiraoka,R. et al. (unpublished)
Alvinocaris komaii	EU031816.1	[3]
	KP759373.1	[4]
Alvinocaris longirostris	AB222051.2	[5]
	AB222050.2	[5]
Alvinocaris lusca	AF125407.1	[2]
	AF125406.1	[2]
	AF125405.1	[2]
	AF125404.1	[2]
Alvinocaris markensis	KC840880.1	[6]
	KC840879.1	[6]
	KC840893.1	[6]
	KC840886.1	[6]
	KC840881.1	[6]
	KC840882.1	[6]
	KC840883.1	[6]
	KC840884.1	[6]
	KC840885.1	[6]
Alvinocaris muricola	KC840887.1	[6]
	KC840888.1	[6]
	KC840889.1	[6]
	KC840894.1	[6]
	KC840891.1	[6]
	KC840892.1	[6]
	KC840890.1	[6]
Alvinocaris stactophila	AF125410.1	[2]
	AF125411.1	[2]
Alvinocaris sp.	AB128829.1	[7]
Rimicaris chacei	KT210443	Present study

	KT210444	Present study
	KT210445	Present study
	KC840932.1	[6]
	KC840933.1	[6]
	KC840939.1	[6]
	KC840938.1	[6]
	KC840930.1	[6]
	KC840929.1	[6]
	KC840940.1	[6]
	KC840935.1	[6]
	KC840931.1	[6]
	KC840934.1	[6]
	KC840936.1	[6]
	KC840928.1	[6]
Rimicaris vandorvae	AF125417.1	[8]
	AF125418.1	[8]
Opaepele loihi	DQ328838.1	Jones et al. (unpublished)
	AF125437.1	[8]
	AF125436.1	[8]
	DQ328825.1	Jones et al. (unpublished)
	DQ328824.1	Jones et al. (unpublished)
	DQ328837.1	Jones et al. (unpublished)
	DQ328826.1	Jones et al. (unpublished)
	DQ328823.1	Jones et al. (unpublished)
	DQ328835.1	Jones et al. (unpublished)
	DQ328832.1	Jones et al. (unpublished)
	DQ328820.1	Jones et al. (unpublished)
	DQ328830.1	Jones et al. (unpublished)
	DQ328834.1	Jones et al. (unpublished)
	DQ328821.1	Jones et al. (unpublished)
	DQ328827.1	Jones et al. (unpublished)
	DQ328833.1	Jones et al. (unpublished)
	DQ328831.1	Jones et al. (unpublished)
	DQ328819.1	Jones et al. (unpublished)
Rimicaris exoculata	HM125956.1	[9]
	HM125918.1	[9]
	HM125927.1	[9]
	HM125935.1	[9]
	HM125950.1	[9]
	HM125949.1	[9]
	HM125937.1	[9]
	HM125926.1	[9]
	FN392999.1	[10]
	HM125911.1	[9]
	HM125910.1	[9]
	HM125921.1	[9]
	HM125943.1	[9]
	KT210447	Present study
	KT210446	Present study
	FN393004.1	[10]

	KT210448	Present study
	KT210449	Present study
	AF044057.1	[8]
	AF125419.1	[8]
	AF125403.1	[8]
	AF125402.1	[8]
	AF125401.1	[8]
	AF125398.1	[8]
	AF125399.1	[8]
	AF125400.1	[8]
	AF125420.1	[8]
	AF125440.1	[8]
	FN393000.1	[10]
	HM125925.1	[9]
	FN393005.1	[10]
	FN393007.1	[10]
	FN393006.1	[10]
	HM125922.1	[9]
	FN393001.1	[10]
	FN393003.1	[10]
	FN392996.1	[10]
	FN393002.1	[10]
	FN392998.1	[10]
	FN392997.1	[10]
Rimicaris hybisae	JN850607.1	[11]
	KJ566979.1	[12]
	KJ566987.1	[12]
	KJ566988.1	[12]
	KJ566974.1	[12]
	KJ566990.1	[12]
	KJ566971.1	[12]
	KJ566970.1	[12]
	KJ566992.1	[12]
	KJ566969.1	[12]
	KJ566975.1	[12]
	KJ566995.1	[12]
	KJ566980.1	[12]
	KJ566991.1	[12]
	KJ566981.1	[12]
	KJ567001.1	[12]
	KJ566997.1	[12]
	KJ566986.1	[12]
	KJ566976.1	[12]
	KJ566996.1	[12]
	KJ566989.1	[12]
	KJ566985.1	[12]
	KJ566968.1	[12]
	KJ566982.1	[12]
	KJ566977.1	[12]
	KJ566973.1	[12]

	KJ566978.1	[12]
	KJ566983.1	[12]
	KJ567003.1	[12]
	KJ567002.1	[12]
	KJ567000.1	[12]
	KJ566999.1	[12]
	KJ566998.1	[12]
	KJ566984.1	[12]
	KJ566972.1	[12]
Rimicaris kairei	AB813089.1	[13]
	AB813107.1	[13]
	AB813101.1	[13]
	AB813105.1	[13]
	AB813090.1	[13]
	AB813099.1	[13]
	AB813088.1	[13]
	AB813097.1	[13]
	AB813096.1	[13]
	AB813095.1	[13]
	AB813094.1	[13]
	AB813093.1	[13]
	AB813092.1	[13]
	AB813091.1	[13]
	AB813108.1	[13]
	AB813106.1	[13]
	AB813104.1	[13]
	AB813103.1	[13]
	AB813102.1	[13]
	AB813100.1	[13]
	AB813098.1	[13]
	AB813087.1	[13]
Nautilocaris sainlaurentae	KT223499	Present study
	KT223500	Present study
	KT223501	Present study
	NC_021971.1	[14]
	KF226726.1	[14]

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S2 Table. **Sequences (18S gene) included in the phylogenetic reconstruction and the larval traits of the species.** The list includes information on larval traits of species not considered in the phylogenetic reconstruction, but used to make inferences on closely related species included in our phylogenetic reconstruction but without available data on larval traits (see methods).

	18S		Larval Morphology	General larval traits (first zoea)				
SPECIES	Source	Genbank	Source	Mouth parts development	Trophic status	Pereiopods	Pleopods	Type of development
PASIAPHAEIDAE								
Leptochela bermudensis	[1]	EU868785	[2,3]	Developed	Planktotrophic	Absent	Absent	Extended
L. papulata	[1]	EU868784						
L. carinata	[1]	EU868786						
L. gracilis			[4]	Developed	Planktotrophic	Absent	Absent	Extended
Pasiphaea japonica	[5]	JF346260	[6]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
P. merriami	[1]	EU868796						
P. levicarinata	[5]	JF346261						
P. sirenkoi	[7]	KP725823						
P. sivado	[7]	KP725826	[8]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
P. tarda			[3,8]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
P. telacantha	[7]	KP725828						
Parapasiphae sulcatifrons			[3,8]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
OPLOPHOIDAE								
Janicella spinicauda	[7]	KP075856.1	[3]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
Oplophorus typus	[9]	GQ131929.1	[3]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
O. gracilirostris	[9]	GQ131928	[3]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
O. spinosus			[10]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
Systellapis debilis	[1]	EU868775	[10]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
S. pellucida	[5]	JF346250						
ACANTHEPHYRIDAE								
A. cutirostris	[1]	EU868769						

A. brevirostris			[11]	Developed	Planktotrophic	Absent	Absent	Extended
A. purpurea	[1]	EU868770	[10,12]	Developed	Planktotrophic	Absent	Absent	Extended
A. eximia	[9]	GQ131916						
A. acutifrons			[12]	Developed	Planktotrophic	Absent	Absent	Extended
A. stylorostrata			[12]	Developed	Planktotrophic	Absent	Absent	Extended
Hymenodora glacialis	[9]	GQ131915	[3,13]	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
Ephyrina figuerai	[14]	AM087654	[3]					Abbreviated
Meningodora sp.	[9]	GQ131931						
Notostomus gibbosus	[9]	GQ131917	[10]					Extended
Heterogenys microphthalma	[9]	GQ131932						
NEMATOCARCINIDAE								
Nematocarcinus africanus	[7]	KP725769						
N. lanceopes			[15]	Developed	Planktotrophic	Absent	Absent	Extended
N. cursor	[1]	U868766						
N. rotundus	[1]	EU868765						
N. gracilis	[5]	JF346240						
N. tenuipes	[5]	JF346248						
N. aff combensis	[5]	JF346238						
N. longirostris			[15]	Developed	Planktotrophic	Absent	Absent	Extended
AGOSTOCARIDAE								
Agostocaris sp.	[1]	EU868716						
PSALIDOPODIDAE								
Psalidopus barbouri	[1]	EU868804	[16]*	Undevelodep	Primary lecitotrophy	Present	Present	Abbreviated
CAMPYLONOTIDAE								
Campylonotus rathbunae	[5]	JF346246	[17]	Developed	Facultative lecitotrophy	Present	Absent	Abbreviated
C. vagans			[18,19]	Developed	Facultative lecitotrophy	Present	Absent	Abbreviated

C. capensis			[18]	Developed	Facultative lecitotrophy	Present	Absent	Abbreviated
C. semistriatus			[18]	Developed	Facultative lecitotrophy	Present	Absent	Abbreviated
ALVINOCARIDIDAE								
Alvinocaris longirostris	[5]	JF346247	[20]**			Absent	Absent	Extended
A. muricola	[1]	EU868717	this study	Undevelodep	Primary lecitotrophy	Absent	Absent	Extended
Rimicaris exoculata	[14]	AM087652	this study	Undevelodep	Primary lecitotrophy	Absent	Absent	Extended
R. hybisae	this study	KT210459	[21]***	Undevelodep	Primary lecitotrophy	Absent	Absent	Extended
R. chacei	this study	KT210456						
Mirocaris fortunata	this study	KT210458	this study	Undevelodep	Primary lecitotrophy	Absent	Absent	Extended
Nautilocaris saintlaurentae	this study	KT210457	this study	Undevelodep	Primary lecitotrophy	Absent	Absent	Extended
Outgroups								
Stenopodidae								
Stenopus hispidus	[5]	JF346253						
Hippolytidae								
Hippolyte obliquimanus	[1]	EU868752						
Alpheidae								
Alpheus packardii	[1]	EU868720.1						
Palaemonidae								
Macrobrachium lanchesteri	[7]	KP725756.1						

*Late embryo

**Analysis of COI sequence of *Alvinocaris* sp. submitted by Koyama *et al.* [20] suggest that the species belong to *A. longirostris* (see COI analysis in the present study). The mouth parts of the larvae are not shown in [20], we assume that they are similar than *A. muricola.* Other traits are observed from the illustration

***The mouth parts of the larvae are not shown in [21], we assume that they are similar than *R. exoculata.* Other traits are observed from the illustration.

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Chapter 2

Population structure and reproduction of the alvinocaridid shrimp *Rimicaris exoculata* on the Mid-Atlantic Ridge: variations between habitats and vents fields

Structure populationnelle et reproduction chez la crevette Alvinocarididae Rimicaris exoculata sur la dorsale médio-Atlantique : Variation entre les habitats et les sites hydrothermaux

Synthèse

La crevette *R. exoculata* est l'espèce dominante de la plupart des sources hydrothermales de la dorsale médio-Atlantique (Mid-Atlantic Ridge : MAR), en particulier de celles situées à plus de 2000m de fond. Cette espèce forme généralement des agrégations de milliers d'individus, proches de la source hydrothermale. Les éléments réduits libérés par l'émission hydrothermale sont utilisés par les bactéries symbiotiques que les crevettes abritent dans la chambre branchiale et le tube digestif. Les bactéries symbiotiques sont impliquées dans la nutrition de la crevette, permettant à la population de se maintenir en forte densité et de dominer la biomasse de ces habitats. Cette espèce est l'un des organismes les plus étudiés de la dorsale médio-Atlantique, mais des questions persistent concernant son cycle de vie et sa biologie. En dépit des nombreux échantillons de *R. exoculata* qui ont été récoltés pendant les trente années qui ont suivi la description de l'espèce, peu d'informations sont disponibles sur la structure en âge et en sexe de l'espèce.

En plus de l'habitat principal de la crevette, à proximité de la source hydrothermale, d'autres zones sont aussi identifiées comme des habitats potentiels de *R. exoculata*. Par exemple, des juvéniles ont été identifiés dans les agrégations avec les adultes, mais aussi hors des agrégations et dans des groupes trouvés à la périphérie de la cheminée. Des crevettes adultes dispersées ont également été observées à la périphérie de la source hydrothermale. Ces observations suggèrent une répartition de la population dans différents habitats, mais l'absence d'échantillonnage systématique ne permet pas de déterminer cette répartition de façon claire, ni de comprendre la structure de la population au sein des différents habitats. De plus, la co-existence avec d'autres Alvinocarididae au seinde certains de ces habitats, en particulier pour les stades juvéniles, reste à déterminer. L'analyse de la proportion relative des individus en termes de sexe ou de stade de développement, ainsi que l'étude de la structure en taille peuvent permettre de mieux connaitre les profils de colonisation du système hydrothermal et l'utilisation des différents habitats durant le cycle de vie de la crevette.

Concernant la reproduction de *R. exoculata,* les travaux précédents sur le développement de la gonade chez *R. exoculata* suggèrent que sa reproduction est semi-continue durant l'année. Cependant, peu de femelles gravides ont pu être échantillonnées depuis la découverte de l'espèce. Ces résultats laissent à penser que les femelles gravides réalisent une migration hors de la source hydrothermale pour limiter l'exposition des stades embryonnaires à l'émission toxique. Bien que ce type de comportement ait été identifié chez d'autres crustacés hydrothermaux, chez *R. exoculata,* les données disponibles ne permettent pas de valider cette hypothèse. Par exemple, aucune crevette gravide n'a été trouvée à la périphérie des sources hydrothermales, ce qui aurait pu indiquer un habitat nourricier. De plus, les dates d'échantillonnage sont fortement biaisées entre été et automne, empêchant toute hypothèse alternative de reproduction saisonnière chez la crevette. Des échantillons de femelles gravides ont récemment été récoltés sur une source active du champ hydrothermal Logatchev à la fin de l'hiver (Mission Serpentine, Mars 2007). Bien que ces derniers résultats présentent une preuve préliminaire contre l'hypothèse de migration de femelles gravides, il est nécessaire de récolter des informations plus précises (proportion de gravides, stade de développement, quantité d'œufs, etc.) et de répéter l'échantillonnage pour tester la présence d'un rythme saisonnier.

Ce travail fournit la description de la structure populationnelle et de la reproduction chez *R. exoculata* de deux sources hydrothermales (les champs hydrothermaux de Snake Pit et TAG) à environ 3500 m de fond (Fig S1). Les échantillons ont été récoltés entre Janvier et Février 2014 pendant la mission BICOSE. Le protocole expérimental inclut plusieurs prélèvements dans les agrégations proches de la source hydrothermale (site actif) de deux champs. En plus, sur le champ TAG, des prélèvements ont été effectués à la périphérie de la source hydrothermale, à proximité des émissions diffuses (site diffus) et dans des zones sans émissions détectées mais avec crevettes (site inactif). Ce protocole de prélèvement prend ainsi en compte la variation entre habitats (actif, diffus et inactif sur le site TAG), la variation à grande échelle (comparaison entre deux sites actifs séparés par environ 300 km, sur TAG et Snake Pit) et la variation à petite échelle entre les prélèvements pris dans la même source et le même habitat. Les prélèvements ont été effectués par le robot sous-marin téléopéré (ROV) Victor 6000, avec un aspirateur.

Tous les spécimens récoltés (N= 3 445) ont été mesurés (longueur de carapace, CL) et séparés par stade (juvéniles A, B et C, sub-adultes et adultes) et sexe. L'identification des juvéniles de type 'A' a été vérifiée par le séquençage du gène cytochrome oxydase I et la reconstruction phylogénétique des Alvinocarididés. Les œufs ont été séparés des femelles gravides et comptés. Ils ont ensuite été classifiés selon le développement de l'embryon en trois stades (début, milieu et avancé) et le volume de l'œuf a

été mesuré. Pour chaque échantillon la proportion de juvéniles, mâles et femelles a été enregistrée, ainsi que la structure en taille. De plus, la reconstruction des cohortes a été estimée avec l'analyse de Bhattacharya. Différentes analyses statistiques ont été effectuées pour décrire les profils de distribution de la population entre les différents habitats et la reproduction.

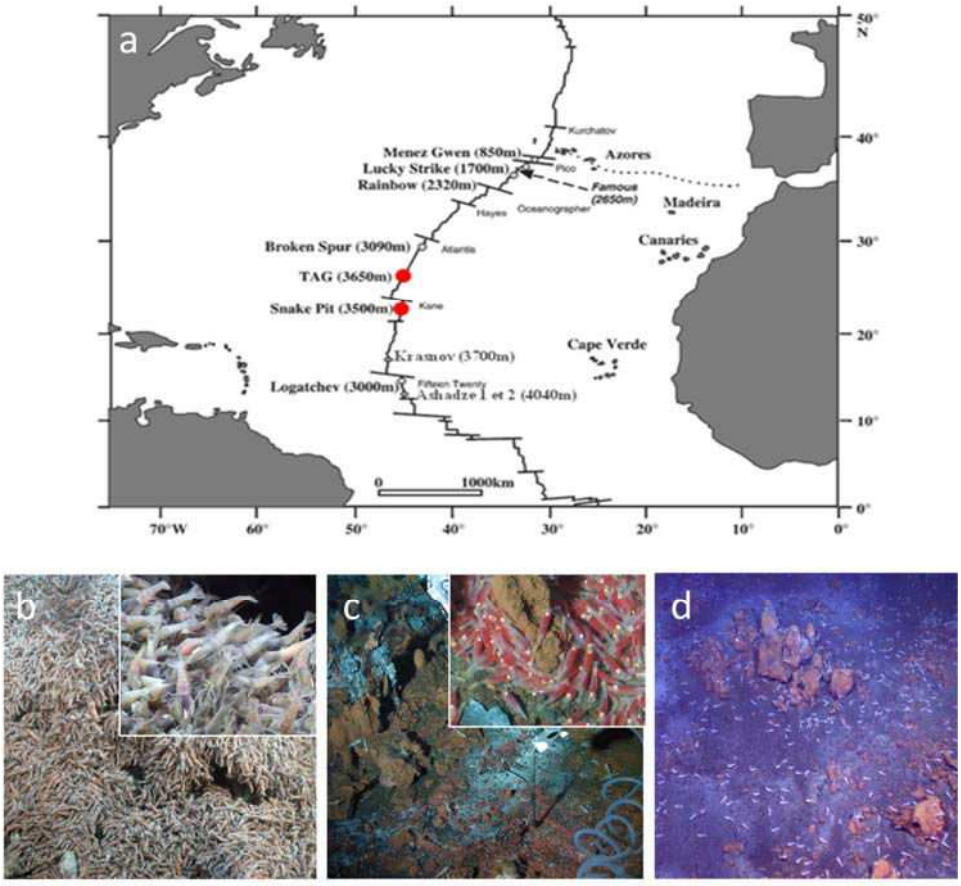

Figure S1. Sources hydrothermales et habitats explorés. a) Section de la dorsale medio-Atlantique avec la position des champs Snake Pit et TAG, b) Agrégations de crevettes sur le site active, c) Agrégations de juvéniles dans le site diffuse, c) Adultes dispersés dans le site inactive.

Des différences importantes ont été trouvées dans la structure populationnelle entre les habitats (Fig S2). Les femelles dominent les agrégations du site actif des deux champs hydrothermaux, avec une proportion générale de 61-97 % de la population, suivi par les subadultes et les stades juveniles. Les mâles

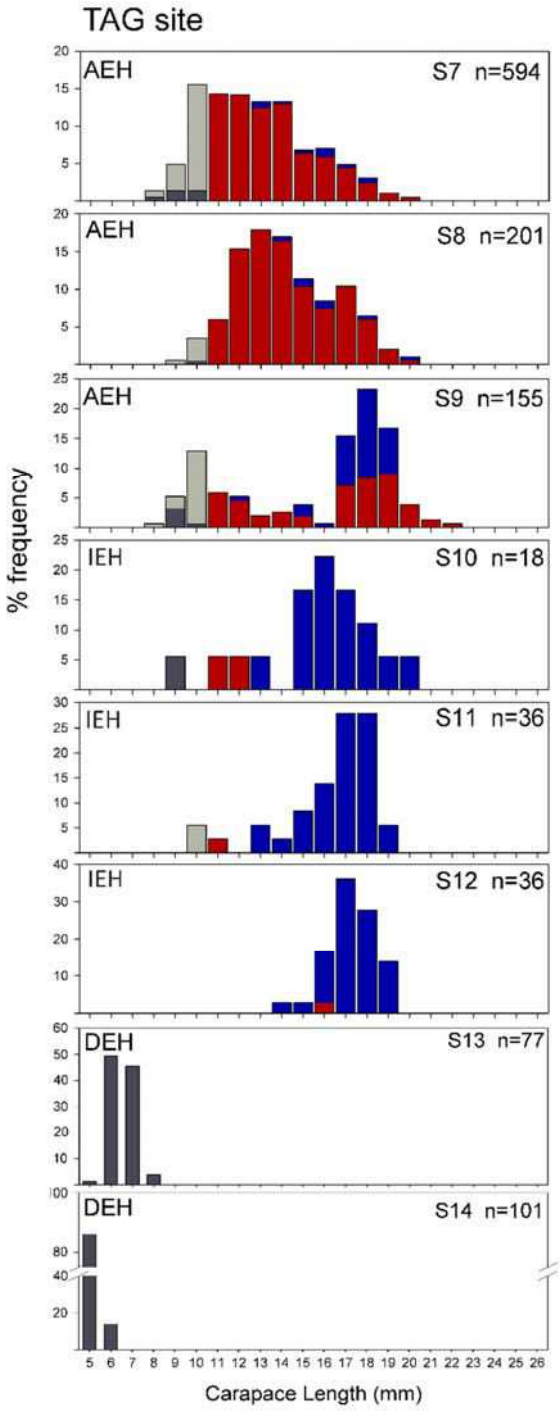

Figure S2. Distribution de fréquence par taille de la crevette. Prélèvements dans le champ TAG, AEH : site actif, IEH : site inactif, DEH : site diffus. Bleu : mâles, rouge : femelles, gris foncé : juvénile, gris clair : subadultes asexués. La plupart de juvéniles dans le site diffus sont *R. chacei*.

du site actif représentent généralement moins de 15% de la population, avec un seul prélèvement où ils constituaient plus de 30% de la population (31.7%). En revanche, le site inactif de la source hydrothermale de TAG est colonisé presque uniquement par des mâles adultes dispersés, qui composent 88.9-97.3 % des crevettes, suivis des femelles adultes. Les agrégations de juvéniles observées dans la zone de faible diffusion étaient composées presque uniquement par le premier stade juvénile de *R. chacei* avec peu de juvéniles de *R. exoculata.*

La structure en taille de la population du site actif est similaire entre les champs hydrothermaux, en dépit de la variation à petite échelle. La structure de taille est polymodale dans les deux champs, impliquant un recrutement discret. Cinq cohortes ont été identifiées entre les différents échantillons, avec des variations détectées dans différents échantillons au sein de la même source hydrothermale. Deux cohortes sont associées aux stades juvéniles, subadultes et petits adultes, qui constituaient plus de 60% de la population, tandis que les autres trois cohortes sont composées d'adultes en proportions différentes.

La variabilité des spécimens en termes de stade de maturité, de sexe et de taille permet de proposer une reconstruction du profil d'utilisation des différents habitats durant le cycle de vie (Fig S3). Le recrutement commence au stade juvénile 'A' (5-6 mm CL) à côté et dans les agrégations des sites actifs, ou (en moindre proportion) dans la périphérie. Après

recrutement, les juvéniles muent en deux stades plus avancés et chevauchant (7.3 – 11.38 mm CL pour les stades B et C). Ces juvéniles muent vers le stade subadulte, avec une faible variation de la taille (7.3 – 13 mm CL) et la différentiation sexuelle externe commence à 10.5 mm de CL, durant la transition vers le stade adulte. Bien que les mâles et les femelles aient un intervalle de taille similaire (mâles : 10.4-24.9, femelles : 10.5-25.5 mm CL), la taille moyenne des mâles est supérieure à celle des femelles. Les deux sexes habitent dans le site actif durant la phase juvénile et le stade subadulte, mais après la différentiation sexuelle, la plupart des mâles adultes effectuent une migration vers la périphérie de la source hydrothermale, dans le site inactif. Les crevettes adultes abritent des bactéries symbiotiques dans leur chambre branchiale qui dépendent des émissions de fluides riches en composés réduits, il est donc probable qu'ils effectuent fréquemment de courtes migrations entre les sites actifs et la périphérie. Les femelles passent tout le cycle de vie, après recrutement, dans les agrégations du site actif, mais pourraient effectuer des déplacements occasionnels vers d'autres localisations. Ces déplacements peuvent être associés avec la mue ou les interactions avec les mâles.

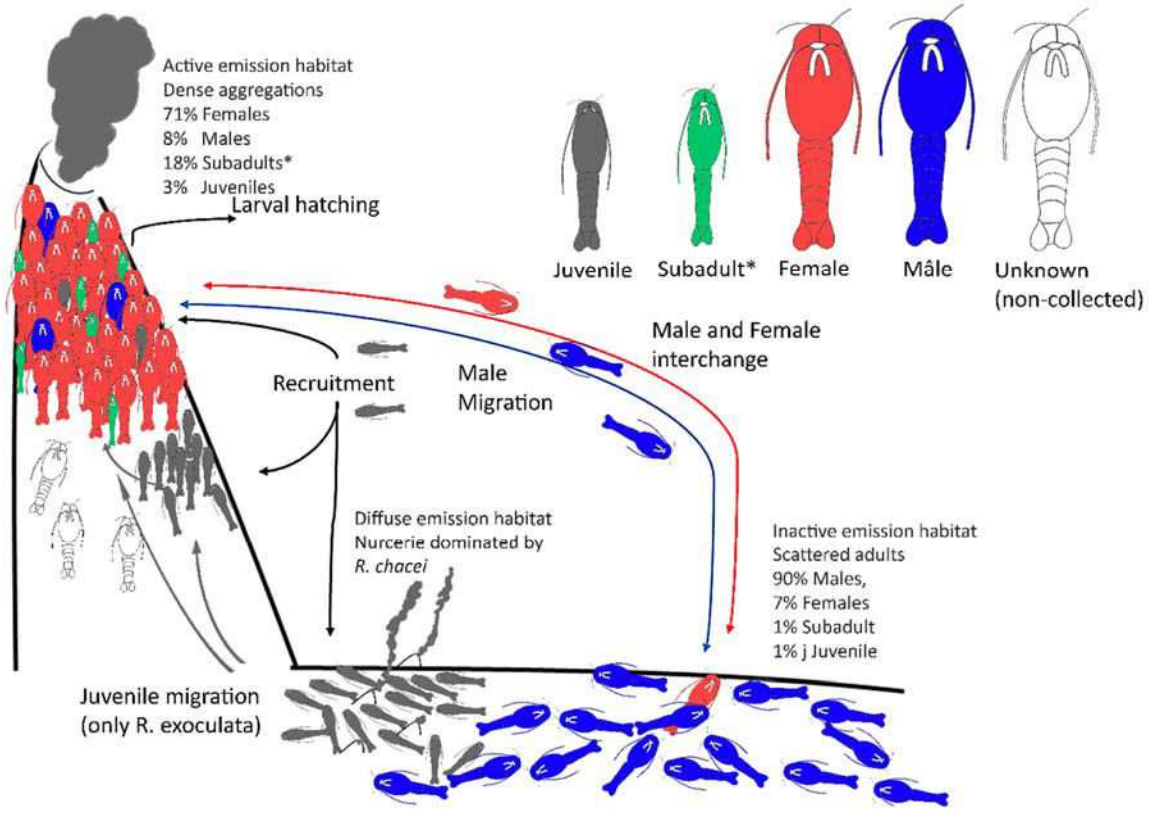

Figure S3. Modèle conceptuel de la distribution de *R. exoculata* sur les différents habitats du champ hydrothermal. Les pourcentages notés sont les estimations générales sur le champ TAG. *subadultes asexués.

Des centaines de crevettes gravides ont été récoltées exclusivement dans la zone active dans les deux champs hydrothermaux, présentant des embryons à différents stades de développement. Ces observations permettent de réfuter l'hypothèse d'une migration des femelles gravides durant le développement embryonnaire. Le contraste entre les femelles gravides trouvées durant Janvier-Mars sur les champs TAG, Snake Pit et Logatchev et leur absence (presque totale) au cours des missions réalisées sur la dorsale médio-Atlantique entre l'été et l'automne favorise l'hypothèse d'une reproduction saisonnière. La proportion de femelles gravides varie beaucoup entre les prélèvements des sites actifs, même au sein d'un même édifice. En général la proportion de femelles gravides est supérieure dans la population du site TAG à celle de Snake Pit (12% vs 4.5% respectivement). La fécondité est positivement corrélée à la taille de la femelle, avec une variation entre 96 et 1879 œufs (Fig S4a). La fécondité relative (œufs par mm de CL de la femelle) de la population sur le site TAG est inférieure à celle de Snake Pit (Fig S4b). De plus, le site TAG présente une proportion importante de pontes avortées (lorsque les femelles perdent les œufs avant l'éclosion). La taille des œufs augmente durant le développement embryonnaire. Des variations ont été détectées aussi dans le volume des œufs entre les champs hydrothermaux, quel que soit le stade de développement. Les œufs de la population du champ TAG sont plus petits que sur le champ Snake Pit (Fig S4c). En général la reproduction dans la population du champ TAG est plus faible qu'à Snake Pit en termes de fécondité, de proportion de pontes avortées, et de taille des œufs.

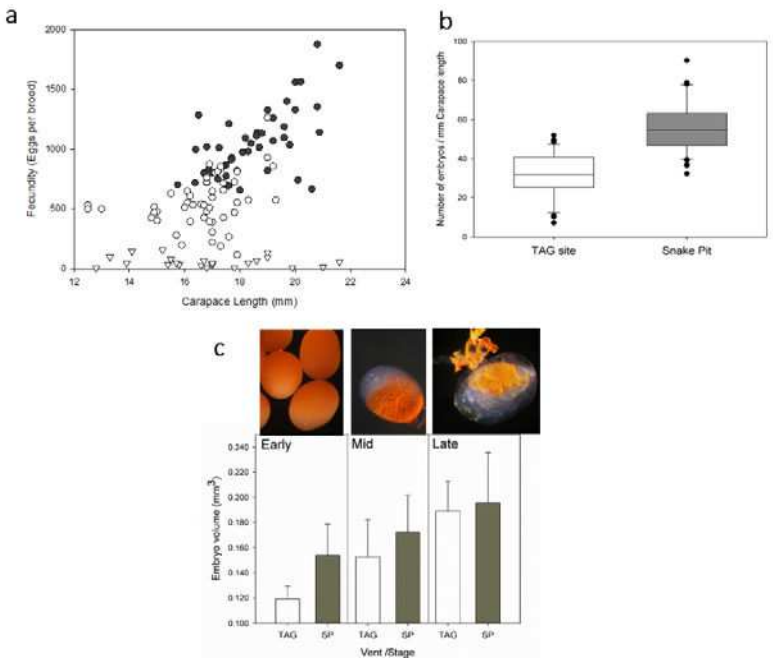

Figure S4. Fécondité et taille d'œufs de *R. exoculata.* a) Relation entre la fécondité (nombre d'œufs) et la taille (longueur de carapace), cercles gris : Snake Pit, cercles vides : TAG,

triangles vides : femelles avortées à TAG; b) Fécondité relative (œufs par mm de la taille de la carapace) ; c) Volume des œufs durant le développement et entre les sites.

Des pontes ont été trouvées infestées par le copépode *Stygiopontius pectinatus* dans les deux populations. Cette espèce a été observée dans la chambre branchiale de *R. exoculata*, chez d'autres invertébrés hydrothermaux et dans le sédiment. La proportion de femelles infestées augmente avec le développement embryonnaire, ce qui est possiblement lié à l'augmentation de la couverture bactérienne sur les œufs (Fig S5). En revanche, l'infestation par le copépode ne semble pas avoir d'effets négatifs sur la reproduction.

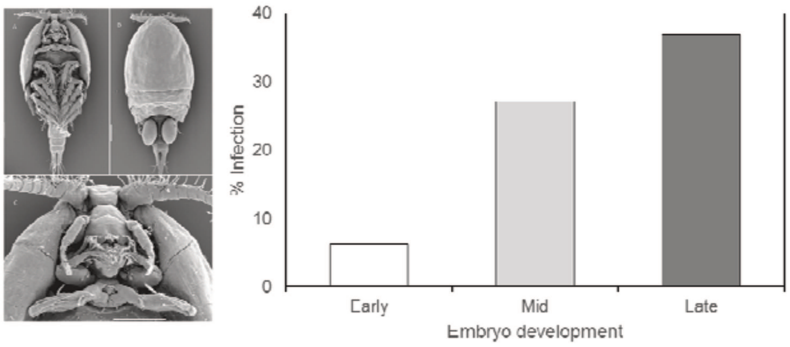

Figure S5. Variation de l'infestation du copépode *S. pectinatus* en relation au stade de développement des œufs. Image : Gollner et al. 2010.

Population structure and reproduction of the alvinocaridid shrimp *Rimicaris exoculata* on the Mid-Atlantic Ridge: variations between habitats and vents fields

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Abstract

Rimicaris exoculata is one of the dominant species in most of the vents on the Mid-Atlantic Ridge (MAR), and lives especially close to vent emissions at depths below 2000 m. Although it is an iconic vent species which play important roles by its abundance, biomass and trophic interactions, many gaps persist in its life cycle and biology. Although many samples had been collected since the discovery of MAR vents, information about sex or stage distribution between the different habitats of the vents is scarce, as well the information about the reproduction. The lack of seasonal reproduction inferred based in gonadal estimation contrast with the perplexing lack of brooding females in 30 years of sampling. This paper describes the population structure and reproduction of this species at two vent fields (Snake Pit and TAG) around 3500 m depth. Samples were collected in January-February 2014, both close to the vent emissions (active emission habitats, AEH) and away from the focused fluid emission (diffuse emission habitat, DEH and Inactive emission habitat, IEH). Sampling design considered the habitat variation (AEH, DEH, IEH), the large scale variation (100s km) between vent fields and the small scale (few m) within the same vent field. Major differences were found in the population structure between the habitats. Females widely dominate the large swarms in the AEH meanwhile DEH are colonized mainly by small juveniles of *R. exoculata* and *R. chacei*, and IEH are inhabited by large males. The population structure of

AEH is similar in both vent fields, but also variations occurs at small-scale. Size structure of the population in the AEH is polymodal at both vent fields, implying a non-continuous recruitment. Males and females exhibit significantly different body size, but non-differences are detected between vent fields for each sex. Similarly male body size did not change between habitats. Hundreds of brooding females were collected from AEH exclusively, bearing embryos at all developmental stages. This contrasts drastically with all previous collections on the MAR, of which a very limited number of brooding females had been reported, and thus may indicate seasonal spawning in this species. Proportion of brooding and hatched females were larger at TAG than in the Snake Pit (12 vs 4.5 % of adult females), however fecundity of was significantly decreased at TAG compared to Snake Pit, with both smaller brood size and smaller egg size. Moreover, more brooding females are found with aborted eggs at TAG. The brood were colonised by the dirivultid copepod *Stygiopontius pectinatus* in both vent fields and the ratio of brood infestation by copepods increase with the embryo stage, probably related with the bacterial colonization in the brood.

Key words: Life cycle, reproduction, *Rimicaris exoculata*, habitat variability

Introduction

The fragmentary and ephemeral nature of the deep-water hydrothermal vent ecosystems is an intriguing scenario for the relicense and connectivity of inhabiting species. Although dominant species are usually endemic to the ecosystem, they tend to show large scale distribution and connectivity between populations (Thaler et al. 2011, Teixeira et al. 2012, Beedessee et al. 2013). Beside of the past and current interest in these systems, the advances in understanding mechanisms and process related with reproduction, dispersion, recruitment and structure of the population are still low and patchy (Hilario et al. 2009). The evidence of complex life history and structure of some hydrothermal vent populations (Marsh et al. 2015) contrast with the gaps in life history of some iconic species.

For instance the alvinocarid shrimp *Rimicaris exoculata* is a dominant species at hydrothermal vents of the Mid-Atlantic Ridge (MAR), especially in vent fields at depths below of 2000 m. This species lives mainly close to the vent emission in dense aggregations of thousands of individuals by square meters (Desbruyères et al. 2000, Copley et al. 2007, Gebruk et al. 2010) and had been studied since the discovery of MAR hydrothermal vents for its particular biology, physiology and symbiotic relationships. However, the informations available about reproduction and population biology is still scattered and

sometimes contradictory. Related with the population structure, major differences in density of shrimp had been found according the vent emission (Copley et al. 1997). Habitats in the base of chimney had been reported as nurseries for juvenile recruitment (Komai and Segonzac 2008). However, it is not clear if the variation in adult shrimp density between habitats is accomplished with a variation in population structure, and the preliminary evidence related of biased sex ratio (Shank et al. 1998) was not tested by additional sampling. For the "nurseries", although there is evidence that juveniles collected in this habitat belong to both *R. exoculata* and *R. chacei* (Komai and Segonzac 2008) proportion between the species or their spatial variation is unknown. In addition juveniles had been reported inhabiting with adult aggregations (Shank 1998, Copley et al 2007) but the stages of these juveniles and their proportion in the populations remain unclear. Other aspects of population structure, as size structure, are based on single sample, pooled samples or preliminary analysis (Gebruk et al. 1997, Vereshchaka 1997).

The reproduction of this species have also many intriguing gaps. Although oocyte size frequency suggest lack of seasonality in the reproduction (Ramirez-Llodra et al. 2000, Copley et al. 2007), very few brooding specimens had been collected since the description of the species (Williams 1986, Ramirez-Llodra, 2000, Gebruk, 2010, Guri et al. 2012). The lack of brooding females also contrast with the large density of the population and the genetic connectivity along vent fields in the MAR (Teixeira et al. 2012), which must be supported by a large larval pool. Egg size had been estimated (Ramirez-Llodra et al. 2000) but the inference on larval feeding based in this estimation was revised recently (Hernandez-Avila et al. 2015). In addition the realized fecundity (number of eggs per brood) had been estimated based on just one specimen (Ramirez-Llodra et al. 2000) due the lack of samples.

Moreover there is very few data related with the variation of *R. exoculata* populations at different spatial scales or their interaction with vent systems. The variation of physical and chemical conditions of vent emission shape the structure of vent communities at various spatial and temporal scales (Sarrazin et al. 2006, Cuvelier et al. 2009). Even vents that belong to the same geological system exhibits variation in community structure between vents (Gebruk et al. 1997, Bachraty et al. 2009), habitats and small scale variation (Van Dover 2002, Cuvelier et al. 2009, Sarrazin et al. 2014), which denote the high level of complexity of these ecosystems. In addition to the variation at community level, dominant megafauna also show variations in the population structure. This variation include differences in habitats for recruitment for motile organisms (Shank et al. 1998, Marsh et al. 2015), changes in population structure between vents (Nye et al. 2013) or habitats (Copley & Young 2006, Marsh et al. 2015) and various scales of temporal variations (Copley et al. 1997, 1999, 2007, Gebruk et al. 2010).

Although less studied, there is recent evidence that support that population of dominant species exihibit spatial and temporal variation at small scale, within a given habitat (at scale of meters and hours respectively) (Copley et al. 1999, Matabos et al. 2015). These variations had been associated with complex life cycles of the vent species, reproductive strategies, tolerance and resource use by different stages and their interaction with environmental variation. This scenario represents a challenge to describe changes in population structure without confounding factors or scales of variation, especially with the limitations of exploring deep-waters.

In this study, we describe the population structure and reproduction of *R. exoculata* on the MAR vent fields of Snake Pit and TAG, their variation between habitats, between vents fields and within vent fields. We intent to untangling different levels of spatial variation in the reproductive parameters and population structure in order review previous hypothesis about the life cycle of this species and bring new clues on the biological response to the physical environment in hydrothermal vents. The current paper is focused on sex ratio, size structure, occurrence of brooding females, fecundity and egg size.

Material and Methods

Sampling

Samples were collected at the hydrothermal vents fields of Snake Pit (23°22.1'N 44°57.1'W, 3470 m depth) and TAG on the MAR (26°08.2'N, 44°49.5'W, 3620 m depth) (Fig. 1a) during the BICOSE cruise held from January 10th to February 11th, 2014. At Snake Pit, six samples were collected in the dense aggregations of shrimps on the walls of vent chimneys close to the fluid emission (therein Active Emission Habitat or AEH). For the TAG field, three samples were collected in the AEH (Fig. 1b). Two samples were collected in dense aggregations of small alvinocaridid juveniles settled in areas with diffuse vent emission, with aggregated shrimp patches around visible shimmering, herein termed "nurseries" (Fig. 1b). Additionally three other samples were collected at the base of the mound, where no active emission emissions were visible (Inactive Emission Habitat or IEH) and characterized by adult shrimps scattered over large areas (Fig. 1c). Distance between samples within the combination of vent field and habitat were from meters to tens of meters.

Shrimps were collected with the suction sampler of the Remotely Operated Vehicle (ROV) Victor 6000. The tip of the suction sampler was pointed as close as possible to the shrimps and maintained

immobile during sampling to avoid disturbance to the population as much as possible. Suction was activated for a few seconds in order to collect individuals from a restricted area. Each sample of the AEH contained between 110-880 specimens. Due to the low density of the shrimps in the DEH of the TAG site, 18 and 38 specimens were collected in two samples. In addition, 37 specimens collected with a shrimp-trap were included in the DEH analyses. Samples collected in nurseries contained between 77-101 juveniles. In total 3 647 specimens were sampled. Additionally, shrimps collected for other purposes during the cruise (samples for experiments in physiology, larval development and dissections) were also examined in order to confirm trends in the analysis.

Identification and measurements

Specimens were identified following Komai & Segonzac (2008) and classified in juveniles (juvenile stages A, B and C according Komai & Segonzac, 2008) and adults. Due to the similarity between juvenile stage A of *R. exoculata* and that of *R. chacei*, morphological identification was confirmed by DNA analysis. Thirty-five juveniles stage A from each "nursery" sample and 10 juveniles stage A from AEH samples were used for molecular identification.

Sex was identified in adults by the occurrence of the appendix masculine on the second pleopod in males, and by the shape of the endopod of the first pleopod (Komai & Segonzac, 2008). Since these sexual characters appear only at the adult stage, sex of juvenile specimens could not be determined. Brooding females are characterized by the presence of embryos held between their pleopods under the abdomen, and by modifications of the pleopods (setae appear that help to maintain the brood). Hatched females (females having just released their larvae, but not molted yet) were identified by their modified pleopods. Young small adults (subadults in Komai Komai & Segonzac, 2008) resemble females (*ie* lack of appendix masculina) and also lack gonadal tissue that could be used for sex determination. In order to estimate the minimal size for confident determination of sex, a subsample of adult shrimps in the small size classes (7 to 15 mm CL) were dissected for verify macroscopic evidence of gonadal tissue. This estimation was consider as the size of onset of sexual differentiation (OSD).

Carapace length (CL) was measured in adults from the anterior margin of the ocular shield to the mid-posterior margin of the carapace with a precision of 0.1 mm (Fig 2a). Morphological changes between juveniles and adults involve rostrum reduction and development of the ocular shield. Therefore, CL measurements have to be adapted for juvenile stages and we measured CL from the anterior tip of the rostrum to the posterior margin of the carapace (Fig 2b). The bias introduced by these

morphological changes in our measurements was however small compared with the total length of the carapace, and had little impact on the reconstruction of the size-classes and mean estimations in this study.

Embryos were removed from brooding females, classified in three developmental stages (early, mid and late stage) and counted. In order to define the embryo classification for this study we consider early embryonic stage from 1-cell embryo to cell proliferation, mid-stage include embryos with separation between the egg envelope and the embryo and early differentiation of larval structures, and late stage include embryo with late development of larval structure and eye spot visible through the egg envelope (Figs 2c-g). For each brood, ten embryos were randomly selected and both their maximum and minimum diameters were measured at a precision of 0.03 mm under a stereomiroscope with a graduated ocular. The volume of embryos was estimated according Oh and Hartnoll (2004), considering a spheroid volume as $v= (4/3) \pi r_1 r_2^2$, where r_1 and r_2 are the half the maximum and minimum axis, respectively. This estimation has a precision of $1.6x10^{-5}$ mm^3.

During examination of the female broods, we found dirivultidid copepods between the embryos or attached at the base of the pleopods. They were identified to the species level by DNA analysis of some specimens.

Genetic identifications

DNA extractions for shrimp juveniles and copepods were performed using the CTAB method (Doyle 1990) on muscle tissue or the complete specimen (copepods). A section of the cytochrome oxidase I gene (COI) was amplified in a 50 µL solution of 1X reaction buffer, 2 mM $MgCl_2$, 0.25 mM dNTP, 1.2 units of Taq polymerase and 0.6 mM of each primer (LCOI1490 and HCOI2198, Folmer, (1994). Amplifications were performed as following: initial denaturation (5 min at 95°C), 40 cycles including denaturation (1 min at 94°C), annealing (1 min at 52 °C) and extension (2 min at 72 °C), followed by a final extension of 7 min at 72°C. All PCR amplifications were conducted on a GeneAmp PCR system 9700 (Applied Biosystems). PCR products were purified and sequenced by Macrogen, Inc. (Netherlands) using the amplification primers. For the phylogenetic reconstructions, the sequences were aligned using MUSCLE (Edgar 2004). For juveniles sequences were aligned with a set of alvinocarid shrimp sequences (descriptions and Genbank codes in Hernandez-Avila et al. 2015). Dirivultidid copepod sequences for comparison were obtained from a subset of sequences obtained by Gollner et al. (2011) (supporting material 1). Neighbor-joining tree trees were constructed using Geneius software. Configurations of the

evolutionary model for each data set were selected according to the best-fit obtained from Model Generator (Keane et al. 2006). The selected model of nucleotide substitution for the COI was HKY for both alvinocaridid shrimps and dirivultidid copepods. Robustness of the inferred trees was evaluated using bootstrap method using 1000 replicates.

Data analysis

OSD was estimated using a similar procedure as that used for determination of the size of sexual maturity in Crustacea (Wenner et al. 1974). Proportion of specimens with gonadal tissue were estimated for the size classes between 7-15 mm CL (larger specimens with clear sex differentiation were not included). The proportion of specimens with gonadal differentiation were plotted against the size class and fitted to the logistic equation:

$$Psd = \frac{1}{1+e^{(a-b*CL)}}$$

Where Psd is the proportion of shrimps with sexual differentiation, CL is the carapace length, and *a* and *b* are constants. The size at which 50 % of the individuals have sexual differentiation was considered as OSD.

For each sample, juvenile ratio, subadult ratio (CL< OSD) and sex ratio (CL > OSD) were estimated. Deviation from a sex ratio of 1:1 was tested with a χ^2 test, using Yates correction in samples with few specimens (n < 30). In order to determine the variations in sex ratio within habitat in each vent, we performed a heterogeneity χ^2 test (Zar 2010). Similarly, size class structures were analyzed for each sample, estimating kurtosis and skewness for size class aggregation and deviation from the mean respectively (Zar 2010). Although histograms were elaborated denoting juveniles, males and females, size structure comparisons were performed including all specimens. Normality test was performed for each sample using the Shapiro-Wilk test (Zar 2010). Unimodal size distribution was tested using the Hartigan dip test (Hartigan & Hartigan 1985) using the package diptest (Maechler 2015) running in R™ (R Core Team 2015). Also the size distribution was analyzed in order to identify discrete size cohorts in the samples using the statistical package mixdist (Du, 2002) running also in R™. The goodness of fit of the identified size cohorts was verified using χ^2 test. The analyses were performed for each sample collected in the AEH and compared for verify the consistency of identified cohorts. Identification of cohorts in other habitats were not performed due sample size (see results).

For the active emission habitat, differences in body size associated with sex and vent fields were tested with multifactorial analysis of variance (ANOVA), after $\log_{10}$ transformation. For this analysis the samples were nested at the factor vent field, representing the variation in body size between samples collected within the same vent field. Similarly, ANOVA test was performed in order to determinate differences in male body size between habitats (AEH vs DEH) at the TAG site, in this case samples were nested at the factor habitat which have a similar effect in controlling the small scale variation. Difference in body size of females between habitats was not tested due the low number of females collected in the DEH. Considerations about assumptions of normality and homoscedasticity in these analyses follows Underwood (1997) and McGuinnes (2002).

In crustaceans, brood size increases as a proportion of female size. In order to compare the reproductive output at the two vent fields, taking into account size differences between brooding females, relative fecundity (number of embryos by mm of CL) were considered. Difference in relative fecundity was tested with a t-test analysis. Variation in embryo size associated with parental female, embryo stage and vent fields was analyzed with a multifactorial ANOVA test. For this analysis the factor parental female is consider as nested to the combination of vent field and embryo stage.

Results

General variation in body size along stages and size of onset of sex differentiation

The smaller specimens found at both vent fields belong to juveniles stage A, with CL range between 4.5 to 8 mm. However the proportion of early juveniles in the AEH was very low, the juveniles found in the AEH but exhibited larger sizes. The size range by juvenile stage was between 5.6 to 11.4 mm CL for stage A, 7.6-11 for stage B and 7.3-11.38 for stage C. Subadults were found only in the active emission habitat, and they exhibit a large range of sizes, from 7.3 to 13.0 mm CL, overlapping with both juveniles and adults. Subadults include both individuals with and without developing gonads. We analyzed the proportion of subadults specimens and adults with differentiated gonads among those with CL between 7.3 and 13 mm, and determined the onset of sexual differentiation at 10.48 mm. This value is consistent with the minimal size measured for adult males (10.4 mm CL), and the low proportion of subadults larger than 11 mm CL. Although the size of some juveniles and subadults may exceed the size for OSD, they can be easily distinguished. OSD was used as minimal size for sex estimation in subadult and adults specimens, in order to avoid bias for wrong sex estimation, without dissecting all

small size females. Subadult and adult females, found mostly in the AEH, showed a range of size between 10.5 to 25.5 mm CL. Similarly, males, collected from both AEH and IEH, exhibited CL between 10.4 to 24.9 mm LC.

Population structure in the AEH from Snake Pit and TAG site

A consistent pattern of population structure was found in the AEH of both vent fields. In all samples, sex ratios were strongly female biased (χ^2, df= 1, p< 0.001 in all cases), females representing between 61 and 97% of sexed specimens in the samples (90% in pooled data) (Table 1). Sex ratios were significantly different between samples at TAG ($\chi^2 het$= 50.61, df= 2, p< 0.001) and at Snake Pit ($\chi^2 het$= 21.01, df= 5, p< 0.001). Despite this variation, females were always dominant in the active emission habitats (Table 2). Proportion of juveniles varied between 0.5 and 5.8 % of the specimens, except for one sample from Snake Pit, where 23.6 % of the specimens were juveniles. Juveniles were mainly in late stages (juveniles stages B and C), while first stage juveniles (stage A) were rare for this habitat. Similarly a large variation in the ratio of subadults below of the OSD was observed, representing between 3.4 to 44.1 % of the samples, especially in the Snake Pit.

Size frequency distributions show variation among samples in both vent fields, both in terms of kurtosis and skewness along samples (Fig. 3). General trend in size frequency of the AEH include bias to the small sizes (skewness 0.54) and slightly leptokurtic distribution (kurtosis 0.41). In some samples the distribution was clearly non-unimodal, in other cases deviation in skewness and kurtosis suggest also mixture of cohorts. This observation were confirmed by the Hartigan dip test, which indicate that the size distribution in the AEH is binomial or polymodal (dip test, D= 0.011, p= 0.008). Base on the analysis of the size cohorts were identified up to 5 different cohorts (Table 2). The size of cohorts were consistent in most of the samples, however some cohorts were lacking in particular samples and variations in the proportion of the cohorts were observed. The cohorts identified were similar between vent fields, corresponding to two cohorts of juveniles and subadults (<12 mm CL) and three cohorts of adult specimens. In both vent fields were observed large proportion of cohorts corresponding to juveniles and subadult stages, however the most early juveniles (5-6 mm CL) cohort was absent.

The analysis of the body size of males and females in the AEH show a significant variation associated to the factor samples (Table 3). Which denote small scale variation in size, within habitat in each vent. Beside this variation, was detected a variation associate with sex. Males were larger

(Mean±Sd 15.76±2.18 mm CL) than females (13.26±2.40 mm CL). No difference was detected between vent fields, denoting that size were similar in the Snake Pit and TAG for males and females respectively.

Differences in sex ratio and size between AEH and IEH at TAG

A major difference in sex ratio was detected between the habitats at the TAG vent field. While females dominated populations in the AEH, the adults specimens collected in the IEH were almost exclusively males (χ^2, df= 1, p< 0.01 in all cases) (Table 1). Small scale variation was not detected for sex ratio in the DEH (χ^2*het*= 0.573, df= 2, p= 0.751), although the samples were collected using two different methods (suction sampler and trap). Shrimps in the IEH also strongly contrasted with those from AEH with a much less dense and more scattered distribution, and the lack of juvenile stages (only one juvenile collected among the three samples). Hartigan test did not reject unimodal distribution for the samples from the IEH (D= 0.028, p=0.9343), however due the high D-value of the statistic and the low number of specimens collected, this results must be consider with precaution. Otherwise the pooled size distribution is non-normal (W-test= 0.965, p= 0.026), potentially composed by at least two cohorts (personal observation). Size frequency distributions of those samples were leptokurtic (kurtosis 0.58 to 3.94) and biased to larger sizes (skewness -1.88 to -0.44). Males did not exhibit significantly different body size between AEH and IEH, although significant differences were detected at small spatial scale (*ie* between samples of a given habitat) (Table 4). The low number of females collected in IEH (7 specimens) did not allow an appropriated comparison of body size of females between habitats. However the females collected in the DEH exhibited a similar range of sizes (10.5-21.50 mm CL) to that of females in AEH (10.5-21 mm CL) at TAG.

Reproduction

Brooding and hatched females were found in almost all samples collected in AEH of both vent fields. However the proportion of these stages among adult females showed large variations (between 0 and 25.6 %). Brooding and hatched females were present in lower proportions at Snake Pit (0 to 12.2 %, pooled proportion 4.49 %) than at TAG (8.4 to 24.7 %, pooled proportion 11.95 %) (t-test= -5.409, p< 0.001, df= 989). Moreover *in situ* observations of shrimps in AEH of both vents suggest aggregative behavior of brooding females which could contribute to the large variation in their proportion at the small scale. Although the proportion of brooding females was relatively low, many non-brooding females exhibited gonads with ripe oocytes, suggesting that the proportion of potential reproductive individuals is higher. Body size of brooding females did not show differences related with vents field

(ANOVA F= 0.919, p= 0.375, df_2= 6). Although a larger size was recorded in Snake Pit (18.33±1.40 mm CL) than TAG site (16.72±1.79 mm CL), this differences are related with the small-scale variation (F= 7.429, p< 0.001, df_2= 122).

Among females, embryos at different stages of development were observed in both vent fields. However, within each female, all eggs were at the same stage of development, except for dead embryos or non-fertilized eggs found in the brood in some cases. Embryos at mid stage of development were more frequent than embryos at early or late stages (Fig. 4A). This probably reflects the fact that our arbitrary classification into early- mid- and late- developmental stages probably encompass a wider developmental range within the mid-stage than in the two others, and should not be interpreted as a sign of synchrony between broods of different females.

Differences in embryo size were detected related with the variation between females, the embryo stage, and the vents (Table 5). Despite the variation between the broods, an increase in the size of the embryo observed during the development at both vent fields. For each developmental stage, embryos at TAG site were smaller than those at Snake Pit (Figure 4B). In early stage (early cleavages from 1 cell-embryo to early cell proliferation) the volume of embryos was 0.154±0.024 mm^3 in Snake Pit, and 0.119±0.024 mm^3 in TAG site. In the mid-stage (cell proliferation and differentiation) the volume of embryos increased to 0.172±0.030 and 0.153±0.030 mm^3 at Snake Pit and TAG respectively (t-test, p< 0.001 in both cases, df= 289 and 122 respectively). In the late stage (when the general larval structures could be identified through the envelope), the volume of the embryos reached 0.196±0.040 mm^3 at Snake Pit and 0.189±0.023 mm^3 at TAG (t-test, p< 0.001 in both cases, df= 196 and 130 respectively). Also a slightly decrease in the minimum diameter of the embryo was observed during the development, from 0.87 of the maximum diameter in early stage to 0.76 in the late stage, which bring a more spheroid shape at end of development. These changes are related with the increase in the polar axis of the embryo, the distribution of the structures inside of the envelope and the water uptake. However, the differences between vent fields are associated with variation in the reproductive effort of the two populations.

Realized fecundity (*ie*, brood size or number of eggs brooded by a female) showed a positive correlation with the female size (Fig. 5A). Reproductive output varied significantly between vent fields. The relative fecundity did not show a variation associated with the stage of the embryos but variations between vent fields was detected. Relative fecundity of females from TAG were lower (31.93±11.68 embryo/mm CL) compared to those of females from Snake Pit (56.20±12.74 embryo/mm CL) (Fig. 5B) (t-

test= 9.556, p< 0.001, df= 91). Moreover, more females with aborted brood were observed at TAG. These females showed few early stage embryos scattered between their pleopods, and were not counted as brooding females in our analyses. The copepods collected from the brooding female were identified as *Stygiopontius pectinatus* according the COI position of the sample in the dirivultidid phylogenetic reconstruction (Gollner et al. 2011). We believe that these copepods are truly associated with broods and not contaminants because they were found mostly deeply inside broods, usually attached to the setae at the basis of the pleopods. No copepods were found on the abdomen of non-brooding females. At Snake-Pit, 33.3 % of the brooding females were infected with copepods, whereas only 18.4 % of the brooding females were infected at TAG. The number of copepods per female brood didn't show a pattern associated with egg stage or vent field, this varied between 1 to 4 individuals. However the proportion of infected females increased with the developmental stage of the embryos, from 6 % in females with early stage embryos, to 38 % in females with late stage embryos.

Nurseries of alvinocaridid shrimps in the diffuse emission habitat

All specimens collected in the nurseries belonged to the juvenile stage A described by Komai and Segonzac (2008) for *R. exoculata* and *R. chacei*. Juveniles of each species were first identified based on their description with dominance in the sample of the *R. exoculata* morphotype. However, DNA analysis showed that the samples were a mixture of juveniles of both species with large dominance of *R. chacei*. Surprisingly, most of specimens identified as *R. exoculata* based on morphology set in the phylogenetic reconstructions with *R. chacei* (69 of the 70 tested specimens collected in the nurseries). In addition, the specimen that set with *R. exoculata* in the COI analysis did not exhibit morphological differences with those that set with *R. chacei*. In contrast, all juveniles collected in the active emission habitat were always identified as *R. exoculata* based on both morphology and COI analysis. Juveniles in nurseries were highly aggregated around visible shimmering on the seafloor. Adults *R. exoculata* from adjacent IEH also passed through the nurseries Size range of juveniles in two samples were 4.50-5.80 mm CL (n= 101) and 5.2-8.1 mm CL (n= 77).

Discussion

Sex ratio and size structure

Our study describes for the first time strongly locally biased sex ratios in *R. exoculata*, where sexes appear to segregate between different microhabitats. Evidence of locally biased sex-ratios have

been previously reported by Shank et al. (1998), which showed that females were dominant in samples collected from active chimney walls at the sites Broken Spur, TAG, Snake Pit and Logatchev. This study reports variations in the sex-ratios observed on the different vents. However, with only one sample per vent site, local variations were not evaluated, and the variations in sex ratios between vents could indeed reflect small-scale local variations, similar to our observations. A similar pattern of female dominated population was also observed in shrimps collected in AEH at Logatchev in 2007 during the Serpentine cruise (IHA, personal observations). No previous records of the population structure of adult specimens in the DEH has been reported, probably because shrimps scattered at the periphery of dense aggregates have been considered as remains of the main population in the AEH, and not individuals occupying preferentially a specific and separated habitat.

Other species of alvinocaridid shrimps show biased sex ratios in relation with the local habitat. For instance, Nye et al. (2013) reported that populations of *Rimicaris hybisae*, which inhabit the same niche as *R. exoculata* on vents of the Mid Caiman trough, have a sex ratio in favor of females in samples collected close to the vent emissions, and that scattered populations are dominated by males at the periphery of the vent, with some degree of local variability. At mussel beds of the Brine Pool site in the gulf of Mexico, the cold seep species, *Alvinocaris stactophila*, has a sex ratio biased to males in the outer section of the mussel bed, whereas the inner part of the mussel bed is dominated by the females (Copley & Young 2006). At hydrothermal vents of the East Scotia Ridge, an opposite pattern is observed for the chirostyloid *Kiwa tyleri.* Areas close to the vent emission are occupied by dense aggregations dominated by males, whereas females are more numerous at the periphery of the vents (Marsh et al. 2015).

Vent populations show differences in trophic structure according their occurrence in different habitats (Marsh et al. 2015). In *R. exoculata*, juveniles have a different lipid and isotopic signature than adult collected close to the vents, which is considered as an ontogenic switch associated with larval period (Pond et al. 2000). However this difference could be considered also as an effect of the habitat food sources, due other adult alvinodaridids inhabiting the periphery of the vent shown similar isotopic signature than the *R. exoculata* juveniles (Pond et al. 1997). Polymodal size distribution in the AEH suggest that the recruitment of *R. exoculata* is non-continuous. Also similar cohorts were identified in the Snake Pit and TAG sites implying that the pattern of recruitment is similar between vents. Also the lack of early juveniles observed in the nurseries sampled in TAG is consistent with the hypothesis of discrete recruitment. The occurrence of polymodal size distribution had been noted previously (Gebruck

et al, 1997 Copley 1998,) but the estimation of continuous reproduction, based in gonad development (Ramirez-LLodra et al. 2000, Copley et al. 2007), raise questions about the coexistence of both discrete recruitment and continuous reproduction in the population. Also we observed a variation in the occurrence and proportion of the cohorts between samples collected in the same vent field. These results can be explained by the occurrence of microscale spatial population structure that could segregate juveniles and adults within the swarms of the AEH. Also there is evidence of tidal variation in the distribution of the shrimp close the vent emission (Copley et al. 1999) that could affect the identification of small scale variation in the population structure in the AEH. Additional observation could be necessary in order to elucidate additional microscale population structure in the AEH.

The lack of small-size males or juveniles in the IEH, and the skewness to the large size suggest that the recruitment of males at IEH occurs in a late adult stage. Male migration from the AEH could facilitate the recruitment of the IEH, even could be an interchange of males between AEH and IEH that could permit the mating with female in the AEH and the exploitation of different nutrition sources of the AEH and IEH. The large differences in population density and behavior found between habitats could be explained by an interaction between the nutrition source and predation. Shrimps in the AEH harbor symbiotic bacteria in the branchial chamber which depend on the reduced compounds of the vent emission (Zbinden et al. 2008, Petersen et al. 2010, Ponsard et al. 2013). Also the higher temperature close to AEH is likely to increase the metabolism of both bacteria and shrimps, promoting both chemosynthesis and consumption. Thus, living close to the vent emission should enhance the bacterial activity and, in consequence, the shrimp nutrition. This could facilitate the energy needed for demanding tasks as egg production. Moreover, animal communities in the AEH of many MAR vent sites are almost exclusively occupied by *R. exoculata*, which greatly limits the risk of benthic predation (Desbruyères et al. 2000). Although adults in the IEH could also harbor symbiotic bacteria in the modified gill chamber, they probably depend more on organic matter which is more disperse along the periphery of the vents. Moreover, at the periphery of the vent, predation pressure may be higher. Abundant populations of the anemone *Maractis rimicarivora* (Fautin & Barber 1999, Copley et al. 2007), observed at the periphery of the vents could bring a negative control of the population.

Sex differences in body size in *R. exoculata* contrast with other *Rimicaris* species inhabiting AEH, *R. hybisae*, which did not show differences in body size related with sex (Nye et al. 2013). Different combination of body size patterns in crustaceans has been associated with their mating systems (Correa 2003, Baeza & Thiel 2007, Asakura 2009). For free-living shrimps the occurrence of large males is

consistent with mating systems that involve sexual competition for females or precopulatory mate guarding (Bauer 1996, Correa 2003, Asakura 2009). In contrast lack of sexual selection as "pure searching" model or long term mate (monogamy or semimonogamy) are in favor of similar or smaller size in males (Bauer 1996). Moreover mating systems traits in shrimps could change abruptly even within genera (Bauer & Thiel 2011). Although there is no evidence in *R. exoculata* male behavior that suggest direct male competition or mate guarding, an increase in the chance of successful mating by larger size (for instance by more frequent or effective mating performance or by female selection) could be in favor of larger male size in the population (Ward 1983, Shuster 2007, Sato 2012). However behavioral experiments need to be performed for clarify the mating system in *R. exoculata*.

Reproduction

The present study is the first description of the realized fecundity of *R. exoculata*. Despite decades of sampling cruises at hydrothermal vents of the MAR, very few brooding females have been collected (Vereshchaka 1997, Ramirez-Llodra et al. 2000), except by few specimens collected during the SERPENTINE cruise (Gebruk et al. 2010), Guri et al 2012) and the present work. Previous hypothesis to explain the remarkable lack of brooding females proposed the use an additional habitat by brooding females, in order to avoid the exposition of eggs to harmful vent fluids (Ramirez-Llodra et al. 2000). This idea was challenged by observations by Guri et al (2012) of a few brooding females found close to active vent emissions at Logatchev. Our data bring strong support to Guri's observation, confirming that brooding females inhabit the dense aggregations in the AEH, together with juveniles, males and non-brooding females. In addition to the large number of brooding females observed in our samples, all developmental stages were found among brooded embryos from both vents, including hatching Zoea (Hernández-Ávila et al, 2015). This indicates that females remain in the AEH during the entire brooding period and larvae probably also hatch in this habitat. The alternative hypothesis proposed by Ramirez-Llodra et al. (2000) involve a temporal variation in the reproduction of the species, lacked support so far because of the large temporal gaps in sampling periods reported historically. A compilation of *Rimicaris exoculata* samples on the MAR between 1985-2014 shows that large proportion of brooding females are collected between late winter to beginning spring (Table 6). Although many temporal gaps remain and preclude at this stage the determination of a precise reproductive season, the temporal variation hypothesis is supported by data accumulated so far.

Previous histological analyses of the gonads of *R. exoculata* suggested a lack of seasonality in gonadal development in adults (Ramirez-Llodra et al. 2000, Copley et al. 2007), which contrast with the seasonality observed here in realized fecundity. Apparently other mechanism, beside of gonadal development at adult stage, affect the temporal reproduction of this species. However, the data available for both oocyte size frequency and realized fecundity are limited in terms of periods for understand the reproductive rhythms. A hypothesis based on the current data is that asynchronical gonadal development reported previously during summer-autumn is follow by mating and egg brooding during winter. Lack of seasonal reproduction had been suggested for other alvinocaridid species in the in the MAR hydrothermal vents, as *Mirocaris fortunata* and *Rimicaris chacei* (Ramirez-Llodra et al. 2000, Nye et al. 2013). Likewise lack of seasonality was suggested for *Alvinocaris muricola* in the Congo basin (Ramirez-Llodra & Segonzac 2006). However other species shows clear seasonality, as *Alvinocaris stactophila* in the Louisiana Slope cold seep (Copley & Young 2006), or large number of brooding female at specific period of the year as reported for *Rimicaris hybisae* in hydrothermal vents of Cayman Through (Nye et al. 2013). The former two species shows a lack of brooding females in summer samples (July-August for *A. stactophila* and August for *R. hybisae*) contrary to samples collected between late Autumn and early Spring (November-March for *A. stactophila* and January for *R. hybisae*). Which is very similar to the pattern found in *R. exoculata*. As well other vent invertebrates shows seasonality in their reproduction in the MAR, as *Bythograea* crabs (Perovich et al. 2003) with brooding females found in Spring and *Bathymodiolus* mussels (Colaço et al. 2006, Dixon et al. 2006) with spawning reported in January.

Consistent variations in the reproductive output were found between the two vents. At Snake Pit females with aborted brood were not identified, contrasting with 35% of females with aborted brood at TAG. Although brooding females have similar size between vents, the number of embryos per brood in TAG samples and the relative fecundity were also lower at TAG. The size of the embryos was also lower in brooding females from TAG than in brooding females from Snake Pit, especially for early embryos. These findings suggest that the reproductive output at Snake Pit was greater than at TAG, with larger brood size, larger size and better survival of embryos through the development. Since the sampled vent fields are relatively close and also with little difference in depth, we assume that the differences in reproduction between the two populations are related with environmental factors associated with the composition of vent emissions. Shrimp tolerance to metallic elements and dissolved gases in vent fluids depends on detoxification processes through metallothioenins, antioxidants (Gonzalez-Rey et al. 2007) and the symbiotic bacteria of the branchial chamber (Jan et al. 2014). The

increase of metallic elements could redirect metabolic energy to detoxification processes, thus decreasing the potential energy for reproductive function. Moreover embryos could have lower tolerance to toxic compounds than the adults. For instance, TAG fluids show higher iron, cupper and manganese concentration than Snake Pit fluids (Desbruyères et al. 2000, Schmidt et al. 2008) which could be correlated with the lower reproductive output of shrimps from TAG. However both bioenergetics and vent processes are complex and these hypothesis must be explored with physiological experiments and more population data.

The brood of *R. exoculata* represents a new microhabitat for the copepod *S. pectinatus*, previously known to be associated with the branchial chamber of *R. exoculata* or occurring as free living specimens at hydrothermal vents (Gollner et al. 2010, Gollner et al. 2011). As it had been suggested for the branchial chamber of *R. exoculata*, it is possible that this species uses this microhabitat as a refuge and a source of bacteria for feeding (Gollner et al. 2010). We observed an increase of the number of copepods associated with a brood with the development of the eggs, which could be explained by the increase of the bacterial colonization through the brooding period (pers. obs.). Alternatively, this increased colonization rate as embryos develop could simply result from a cumulative probability of encounter with time. In any case the occurrence of copepod did not have a negative effect on the number of eggs or embryo size, indicating that copepods probably do not feed on shrimp eggs.

The contrast between morphological and genetic identifications of juveniles and the dominance of *R. chacei* in the nurseries are striking findings in our study. Komai and Segonzac (2008) had noted the close morphological similarity between juveniles of *R. exoculata* and *R. chacei* at the first stage A. These authors proposed a series of morphological characters for species identification. Specially related with the degree of development of the distolateral tooth in the first segment of the antennular penduncle, the rostrum and the setae in the posterior margin of the telsum. However identifications based on these morphological differences are not consistent with results of the genetic analysis with COI. We assume that the morphological variations proposed by Komai and Segonzac (2008) reflect intraspecific variations present in both species, which were mistakenly interpreted as interspecific variations. The clear separation between *R. exoculata* and *R. chacei* in previous COI phylogenetic reconstructions (Hernández-Ávila et al. 2015, Vereshchaka et al. 2015) makes us confident that genetic identification using COI can be reliably used to distinguish juveniles of both species. Curiously only one *R. exoculata* were genetically identified among the juveniles collected from the nurseries, which appeared to gather mainly *R. chacei* juveniles. This contrasts with the larger juveniles found in AEH, where all genetically

identified individuals belong to *R. exoculata.* It is not possible with our observations to estimate if the lack of *R. exoculata* juveniles in nurseries is related with temporal or spatial variation in species recruitment.

The variation of life-stages, sex-ratios and body sizes along habitats permit a hypothetical reconstruction of the benthic phase of the life cycle of *R. exoculata*. The smaller juvenile specimen in stage "A" (4-5 mm CL) have been found in mixed aggregations beside the vents, sharing the habitat with juveniles of *R. chacei* (Vereshchaka 1996, Shank et al. 1998). These juveniles are in the same range of size as the alvinocaridid post-larvae found in deep-water plankton relatively close to the vents (Herring & Dixon 1998), and could be considered as just recruited to the vents. Larger juveniles stage "A" (6-11 mm CL) and following stages are found mostly in the AEH, which suggest that juveniles make a transition to the AEH in order to continue their life cycle. During this period, juveniles make a series of molts, involving morphological changes leading to the subadult stage (7-13 mm CL): reduction of the frontal eyes and rostrum, development of ocular shield and dorsal structures for light perception (Komai & Segonzac 2008). After acquisition of the subadult stage, sexual differentiation occurs, with the development of gonadal tissue (10.5 mm CL), consistent with the size of the smaller males. A series of transformations then follow until the final adult stage (and sexual maturity), involving the increase of the branchial chamber and change in the relative size of the cephalotorax, consumption of the lipid reserves accumulated during early stages, which occurs at 12 mm CL according Vereshchaka (1997) and is consistent with the minimal size of brooding females (12.5 mm CL). Some males of the population migrate to IEH and settle there as scattered populations adjacent to main vent emission sites. This group is apparently coming from aggregations in AEH because there is not a significant proportion of late juveniles and sub-adults that could suggest that some individuals may continue their life cycle outside of the AEH after juvenile stage "A". It is not clear if the adult males of the DEH migrate to the AEH for nutrition or interactions with other specimens or if their occurrence is related with the interactions with small recruits in the DEH.

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Table 1. Proportion of specimens and sex ratio in different vents and habitats.

Active emission - Aggregate population											
Site	Sample	Dive	%J	%<OSD	% F	%BF	%M	n	M:F	χ^2	p
Snake Pit	S1	Plo1-A3	0.70	5.59	79.72	0.88	13.99	143	0.18	65.94	<0.001
Snake Pit	S2	Plo1-A4	4.10	44.10	49.49	1.04	2.31	390	0.05	167.60	<0.001
Snake Pit	S3	Plo1-A5	4.33	19.06	70.05	5.54	6.56	808	0.09	425.15	<0.001
Snake Pit	S4	Plo5-A1	4.03	39.29	55.16	0.00	1.51	391	0.03	201.64	<0.001
Snake Pit	S5	Plo5-A4	23.64	20.91	37.27	2.56	18.18	110	0.49	7.23	0.007
Snake Pit	S6	Plo5-A6	5.77	39.29	49.73	12.15	5.22	364	0.10	131.22	<0.001
Total			5.20	29.66	59.40	4.49	5.74	2206	0.10	977.77	<0.001
									Heterogeneity	21.01	<0.001
TAG	S7	Plo8-A2	3.17	23.33	69.67	8.58	3.83	600	0.06	353.80	<0.001
TAG	S8	Plo10-A1	0.48	3.38	92.75	13.54	3.38	207	0.04	171.98	<0.001
TAG	S9	Plo10-A2	3.73	14.29	50.31	25.64	31.68	161	0.63	6.82	0.009
Total			2.69	17.56	71.38	11.95	8.37	968	0.12	481.99	<0.001
									Heterogeneity	50.61	<0.001
Inactive emission - scattered population											
TAG	S10	Plo10A5	5.5	0.00	11.11		83.3	18	8.00	9.39	0.002
TAG	S11	Plo10A6	0.00	2.94	8.82		88.24	34	8.50	23.68	<0.001
TAG	S12	Plo12N	0.00	0.00	2.70		97.30	37	36.00	33.11	<0.001
Total			1.20	1.20	7.23		90.36	83	12.50	67.11	<0.001
									Heterogeneity	0.574	0.751
Diffuse emission habitat											
TAG	S13	Plo8A1	100*					77			
TAG	S14	Plo12A2	100*					101			

J, juveniles; <OSD, subadults; f, female; bf, brooding females (related with adult females); m, males.

*Juveniles were dominated by *R. chacei* instead of *R. exoculata*.

Table 2. Identified cohorts for the AEH in the Snake Pit and TAG vent field. Mean and standard deviation is show for each sample, proportion of each cohort are in brackets. χ^2 denote the deviation of the sample from the cohort estimation.

Cohort:	1	2	3	4	5	χ2
Snake Pit						
S1		11.87±1.31(0.52)	14.47±1.59(0.48)			7.3855ns
S2	9.00±0.86(0.50)	11.15±1.06(0.47)		15.93±1.52(0.03)		6.645ns
S3	10.08±0.98(0.32)	12.15±1.17(0.54)		16.59±1.60(0.14)		5.552ns
S4	9.52±0.96(0.50)	11.60±1.17(0.34)	14.40±1.45(0.16)			2.909ns
S5	8.40±0.76(0.37)	11.32±1.03(0.35)	14.36±1.31(0.09)		18.73±1.70(0.19)	6.881ns
S6	9.23±0.72(0.42)	11.21±0.88(0.22)	13.79±1.07(0.15)	16.15±1.26(0.05)	18.64±1.45(0.16)	6.251ns
TAG						
S7	9.84±0.96(0.36)	12.52±1.22(0.42)		15.75±1.54(0.22)		7.738ns
S8	10.36±0.87(0.10)	12.65±1.06(0.56)		15.99±1.34(0.36)		4.688ns
S9	9.29±0.46(0.20)	11.13±0.55(0.11)	13.89±0.69(0.08)		17.62±0.88(0.62)	6.036ns

ns: non-significant.

Table 3. ANOVA of body size of adult stage between vents in the active emission habitats

Source	df	SS	MS	F	p
Vent field	1	0.1695	0.16951	2.856	0.135
Sex	**1**	**0.2989**	**0.29889**	**84.681**	**<0.001**
samples (Vent)	**7**	**0.4154**	**0.0593**	**13.557**	**<0.001**
Vent x Sex	1	4.87E-06	4.87E-06	0.001	0.971
sa(Vent) x Sex	7	2.47E-02	3.53E-03	0.806	0.582
Residual	2003	8.768	4.38E-03		
Total	2020	11.359			

Table 4. ANOVA of male body size between habitats in the TAG site

Source	df	SS	MS	F	p
Habitat	1	0.848	0.848	2.916	0.163
samples (ha)	**4**	**1.163**	**0.291**	**13.984**	**<0.001**
Residual	158	3.284	0.021		
Total	163	5.102			

Table 5. ANOVA of embryo volume associated between vent field, embryo stage and parental female.

Source	df	SS	MS	F	p
Vent	**1**	**0.06043**	**0.06043**	**10.715**	**0.002**
Stage	**2**	**0.24414**	**0.12207**	**21.648**	**<0.001**
V x St	2	0.01511	0.00755	1.340	0.267
Female(V x St)	**94**	**0.52826**	**0.00562**	**15.319**	**<0.001**
Residual	896	0.32869	0.00037		
Total	995	1.18940			

Table 6. Occurrence of brooding females in *R. exoculata* samples in the MAR

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	No date
Rainbow						1998[8]	1997[4]	2005[8]	2005[10]	1998[10]			
						2000[6]	2002[10]						
Broken Spur							1997[4]		1994[10]	1994[3,10]			1994, 1996[3]
							2002[10]		1996[10]				
TAG site	2014*	2014*				2002[10]	1997[4]	1985[1]	1994[3]				
								2005[8]	1994[3,1]				
Snake Pit	2014*	2014*				2002[10]	1997[4]	2003[10]	1994[3]		1995[8]		
							2001[8]				2004[7]		
Logatchev			2007[8,9]				1997[4,5]				1998[10]		
							2001[8]						
Mephisto					2006[8]								

[1]Williams & Rona (1986), [2]Copley (Copley 1998), [3]Vereshchaka (1997), [4]Shank et al. (1998), 5. Gebruk et al (2000), [6]Ramirez-Llodra et al. (2000), [7]Copley et al (2007), [8]Komai & Segonzac (2008), [9]Gebruk et al. (2010), Guri et al. (2012), [10]Lunina & Vereshchaka (2014), *This study. Non-direct estimations and low sampling were omitted. Blue cells, no brooding female reported; green cells, 1-2 specimens reported; pink cell, reported as "many observed"[8] and few specimens collected[8,9]; red cells, hundreds of specimens collected.

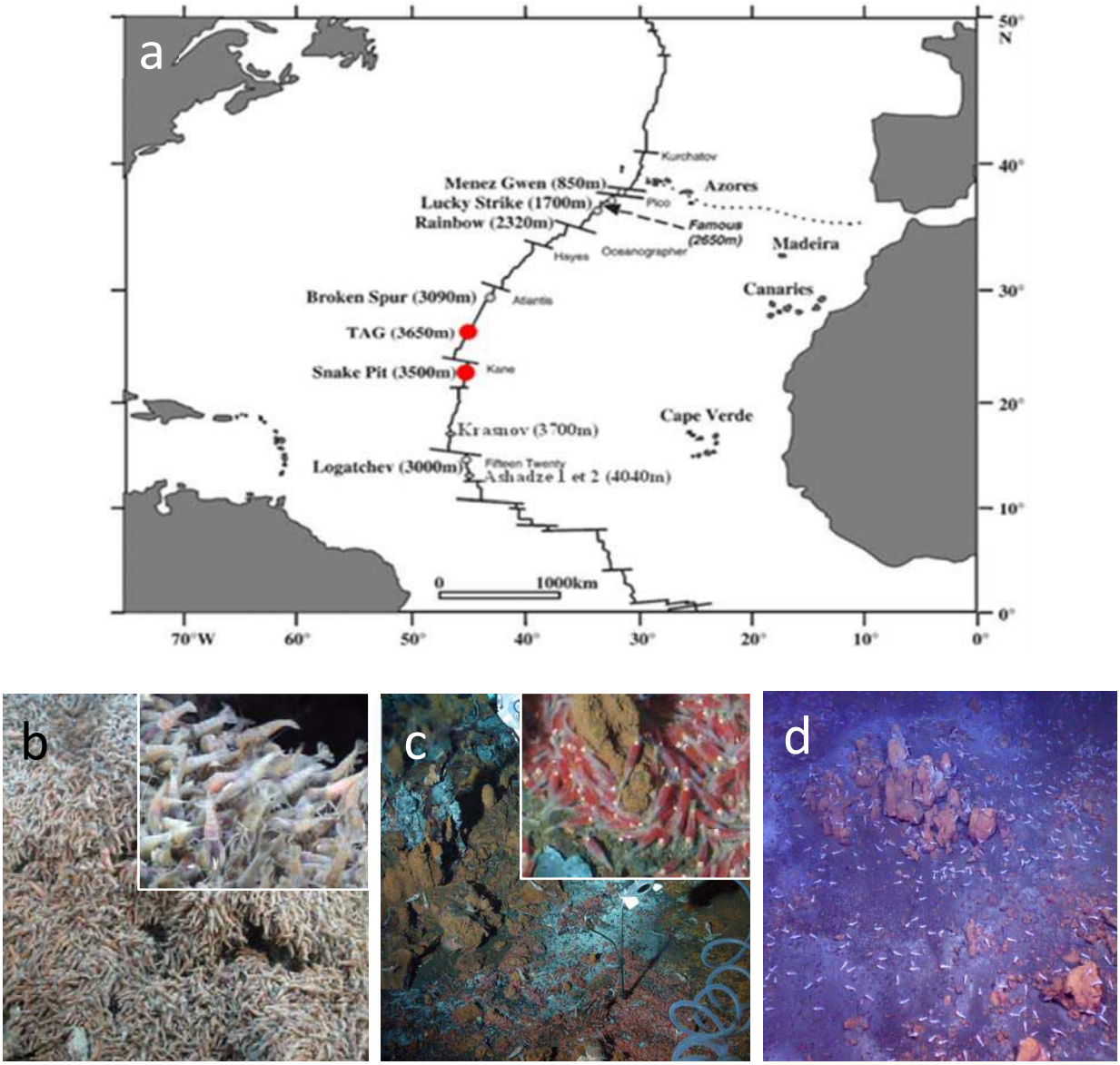


Fig. 1. a. Study area, a. Mid Atlatic Ridge, red dots indicate the vent field sampled in the present study. b. *R. exoculata* swarms in the active emission habitat. c. Difusse emission habitat, showing red aggregation of small juveniles. d. Inactive emission habitat with scattered *R. exoculata* adults (white spots). Pictures b-d are from the TAG vent field.

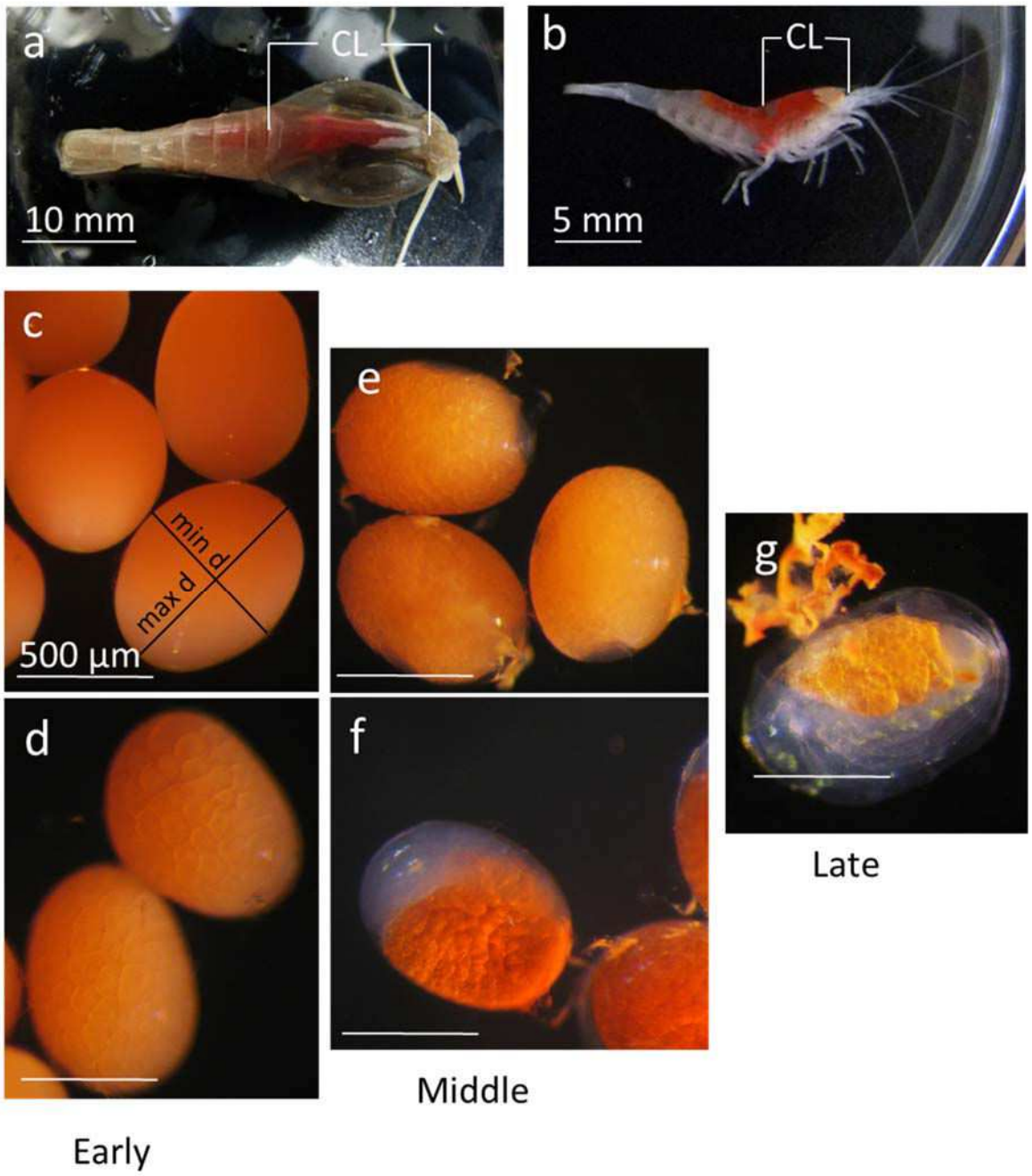


Fig. 2. Measurements and classification included. a,b. *R. exoculata* adult stage and alvicaridid juvenile (either *R. exoculata* or *R. chacei*) respectively, CL indicate carapace length. c-d. Embryo early stage, "max d" and "min d" indicate the maximum and minimum diameter measured. e-f. Embryo mid stage. g. Embryo late stage.

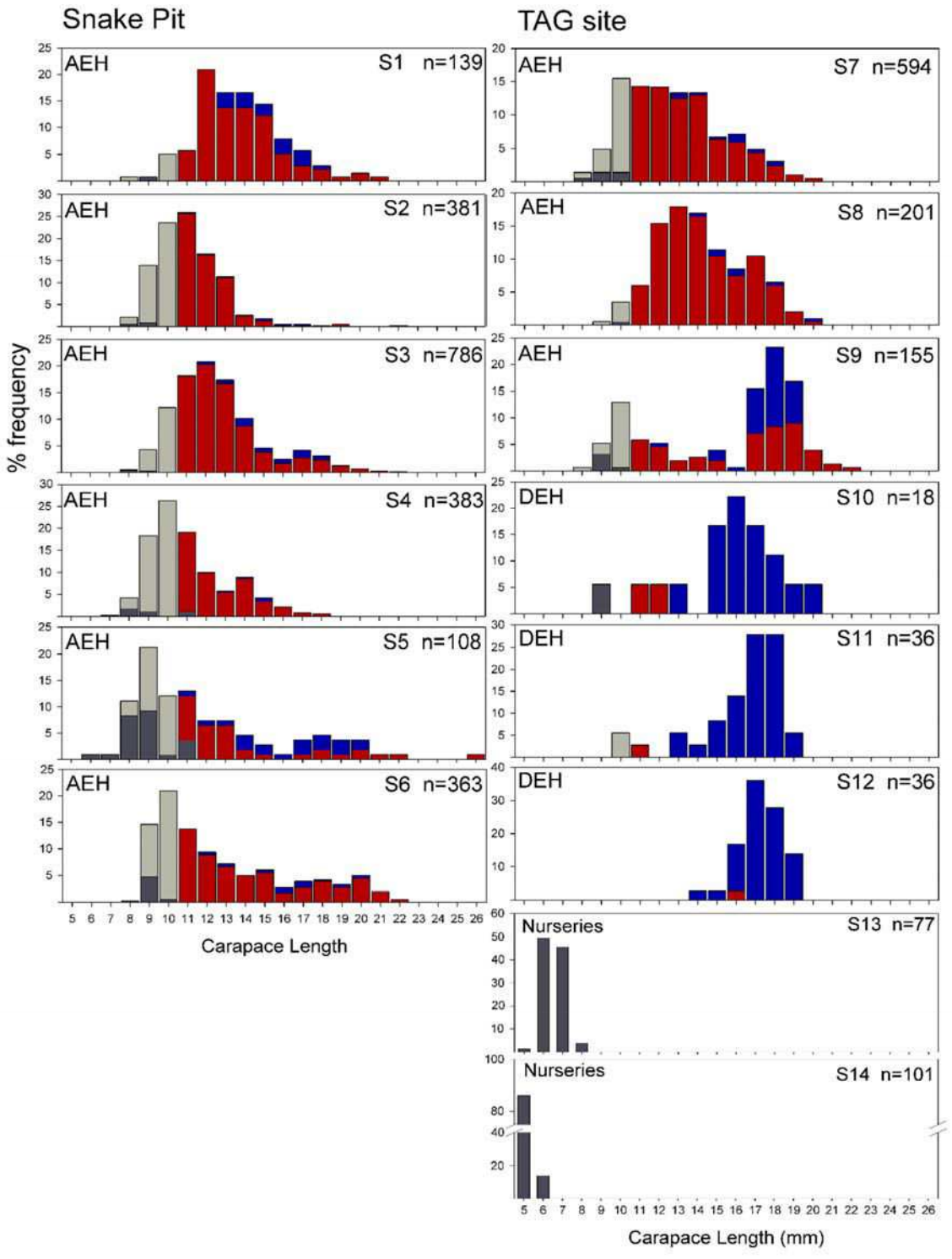
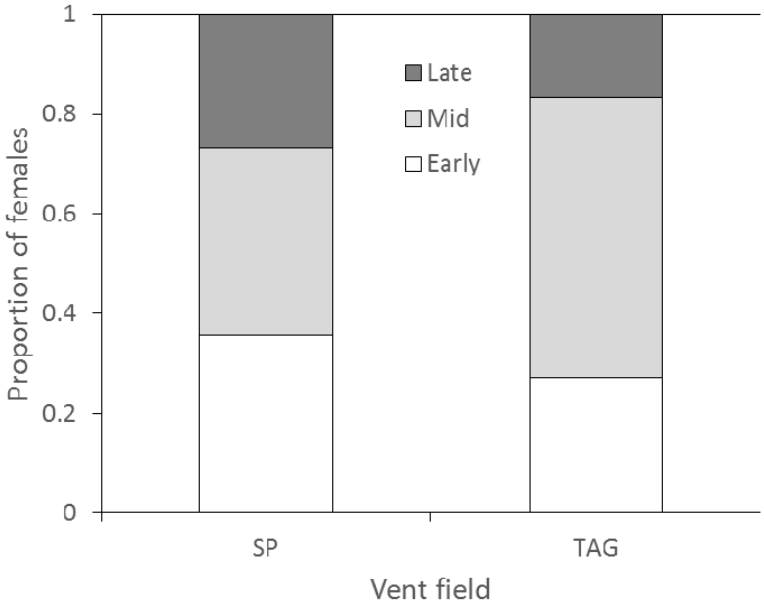


Fig. 3. Size class structure of *R. exoculata* at Snake Pit and TAG site in different habitats. AEH active emission habitat, DEH diffuse emission habitat. Data from nurseries belong to a mixed population of *R. exoculata* and *R. chacei*. Red: females; Blue: males; light grey: subadults below OSD; dark grey: juveniles.

A



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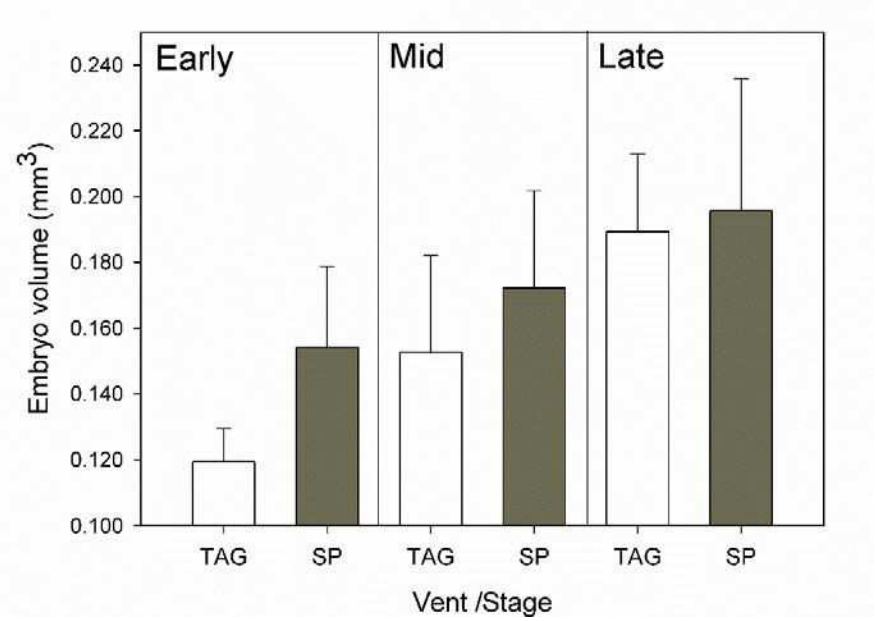
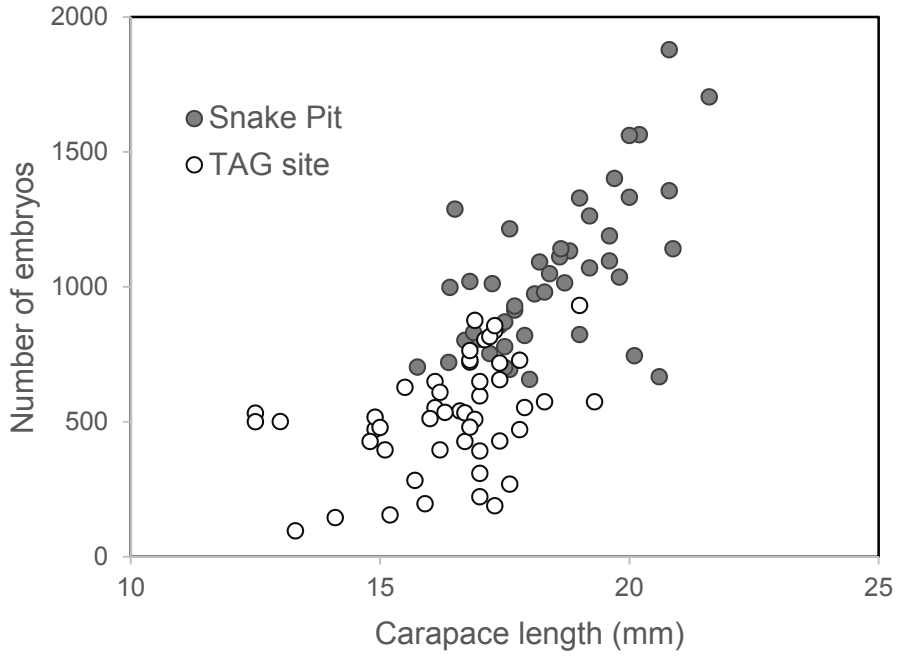


Fig. 4. Developmental stages of eggs in broods of *R. exoculata*. A, proportion of brooding females at each developmental stage. B, Size of the embryos at different developmental stages in the two vent field.

A



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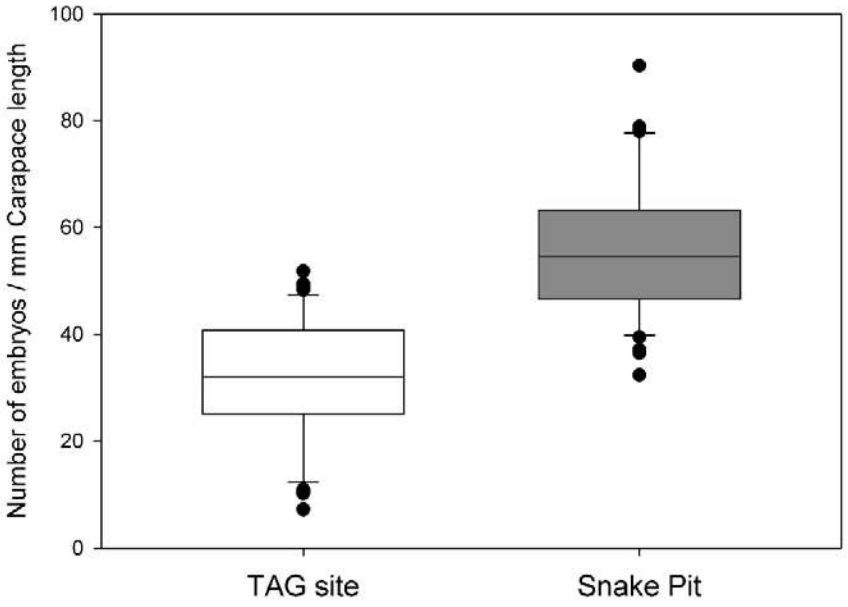

Fig 5. Fecundity in *R. exoculata* from Tag and Snake Pit. A, Number of embryos related with the female size (carapace length). B, Relative fecundity, number of embryo per unit of size (mm of carapace length).

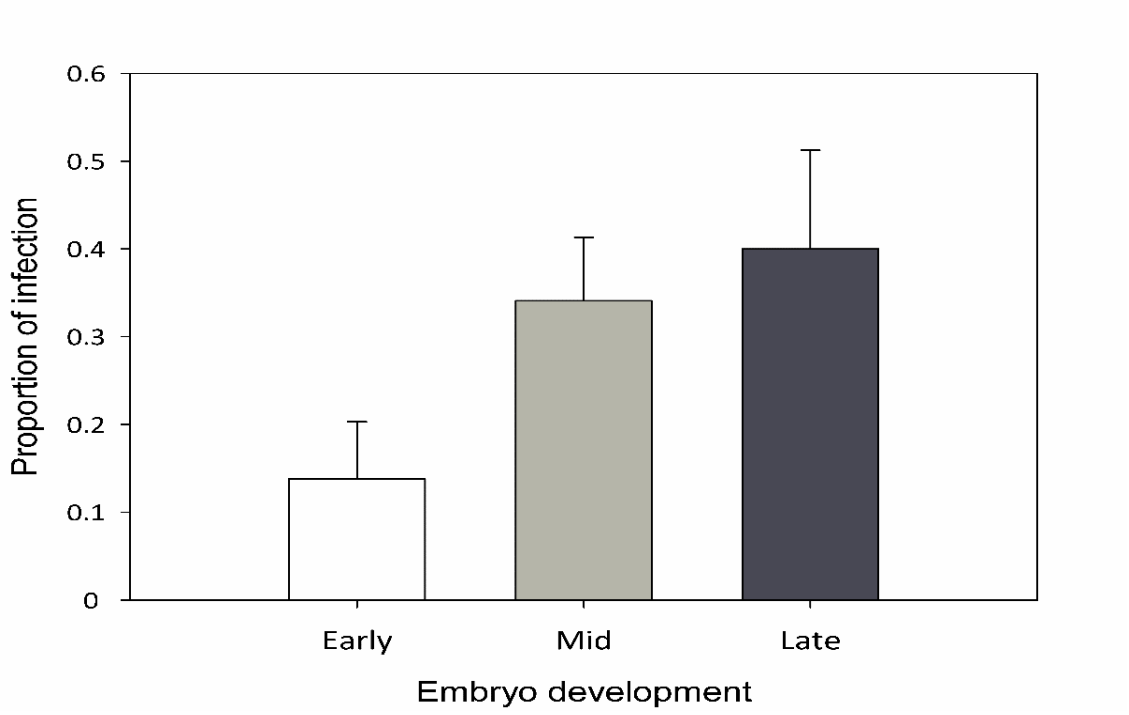

Fig. 4. Proportion of brooding females with broods infected with *S. pectinatus* according to embryos developmental stage (both field pooled).

Chapter 3

Bacterial communities associated with eggs of *Rimicaris exoculata* in deep-water hydrothermal vents: A specific host-symbiont relationship that changes according embryo development and vents

Les assemblages bactériens associés aux œufs de Rimicaris exoculata dans sources hydrothermales profondes : Une relation hôte-symbionte spécifique qui change durant le développement embryonnaire et entre les sources hydrothermales

Synthèse

Les écosystèmes des sources hydrothermales profondes sont généralement dominés par une faune qui abrite des bactéries symbiotiques chimiotrophes. Les relations entre les hôtes et les bactéries symbiotiques ont un profond effet sur la colonisation, la biomasse, la distribution des populations des sources hydrothermales et dans les transferts d'énergie dans l'écosystème. La relation symbiotique des espèces hydrothermales peut démarrer au début du cycle de vie (dans les œufs avant la fécondation, ou aux stades embryonnaires ou larvaires) ou après le recrutement. Le processus d'acquisition des bactéries symbiotiques peut être facilité par le transfert des symbiontes des parents à la descendance durant la reproduction (transfert vertical) ou par la colonisation sélective des assemblages microbiens environnants (transfert horizontal).

La crevette *Rimicaris exoculata* est une espèce dominante des sources hydrothermales de la dorsale médio-Atlantique, en particulier dans les champs hydrothermaux situées à plus de 2000m de profondeur. Les stades adultes de cette espèce abritent des bactéries chimiotrophes dans leur chambre branchiale et leur tube digestif. Les assemblages bactériens chez la crevette sont diverses, mais généralement dominés par des *Epsilonproteobacteria* et *Gammaprotebacteria* dans la chambre branchiale et par des Deferribacteres, Mollicutes et *Epsilonproteobacteria* dans le tube digestif. Des expérimentations récentes montrent que les assemblages bactériens collaborent à l'alimentation de la crevette par transfert direct de carbone organique généré par chimiosynthèse. De plus, il a été proposé que l'activité métabolique des symbiontes puisse favoriser les processus de détoxification qui permettent à la crevette d'habiter l'environnement toxique autour des sources hydrothermales.

Les assemblages bactériens dans les deux compartiments (la chambre branchiale et le tube digestif) varient entre les sources hydrothermales et le stade de vie de la crevette. La composition des éléments libérés par l'émission hydrothermale a été proposée comme la cause principale de la variation de bactéries symbiotiques entre les sources hydrothermales. De plus, les bactéries qui habitent la chambre branchiale sont renouvelées après chaque mue de l'exosquelette de la crevette. Le cycle de mue

de la crevette entraîne un renouvellement constant (et apparement rapide) de la cuticule, avec la production d'une surface nouvelle de l'hôte, suivie de recolonisation et du développement des assemblages bactériens.

Récemment, la colonisation de bactéries épisymbiotiques a été décrite sur les œufs de *R. exoculata,* montrant que la relation hôte-symbionte chez la crevette peut commencer au stade embryonnaire. Le processus de reproduction et d'incubation des œufs permet tester si l'occurrence de bactéries sur les œufs représente une association spécifique, d'analyser son évolution durant le développement embryonnaire et sa variation entre les différentes sources hydrothermales. Chez les crevettes Caridés, les femelles effectuent une mue avant l'accouplement au cours de laquelle s'opère une modification de la morphologie de leurs appendices abdominaux (pléopodes) pour favoriser le maintien des œufs. Après l'accouplement, les œufs sortent des pores génitaux vers l'abdomen et sont mélangés avec les spermatophores. Les œufs fécondés sont couverts d'une couche de mucus qui les maintient en une masse et permet leur adhésion aux pléopodes. Les pléopodes fournissent ainsi un support mécanique qui maintient les œufs attachés sous l'abdomen de la femelle durant tout le développement embryonnaire. La colonisation bactérienne est observée sur la surface des œufs, les pléopodes et sur de petites sections de l'abdomen. Les surfaces de colonisation bactérienne ne changent pas durant le développement embryonnaire car la femelle ne mue pas avant l'éclosion des œufs. Par conséquent les œufs et pléopodes sont exposés aux mêmes conditions de colonisation durant le développement embryonnaire.

Dans ce travail, nous analysons les assemblages bactériens qui colonisent la surface des œufs et des pléopodes, et les comparons entre des crevettes dont les œufs sont à des stades de développement embryonnaire différents et provenant de deux sources hydrothermales. Les objectifs de cette étude sont i) de comparer les assemblages bactériens des œufs avec les assemblages qui colonisent une structure exposée aux mêmes conditions de colonisation, mais non-associé aux embryons (les pléopodes), ii) d'estimer les changements des assemblages bactériens durant le développement embryonnaire, iii) de comparer la composition des bactéries épisymbiotiques des œufs entre différentes sources hydrothermales. Les prélèvements ont été effectués durant la mission BICOSE (Janvier-Février 2014) sur les champs hydrothermaux de Snake Pit (3460 m de profondeur) et TAG (3630 m de profondeur). Les femelles gravides ont été capturées dans les agrégations denses de crevettes, proches de la source hydrothermale, avec l'utilisation d'un aspirateur manipulé par un robot sous-marin télécommandé (ROV Victor 6000). Les crevettes ont été ramenées à bord dans la chambre de compensation isobare PERISCOP,

dans des conditions de température et de pression similaires à celle du milieu profond. Après une décompression contrôlée, les femelles gravides dont les œufs présentaient différents stades de développement embryonnaire ont été sélectionnées. Les spécimens ont été manipulés pour séparer les œufs et les pléopodes de chaque femelle. Douze prélèvements ont été sélectionnés selon différentes combinaisons de traitement en prenant en compte la structure (œufs et pléopodes), le stade de développement (début, milieu et avancé) et la source hydrothermales (champs Snake Pit et TAG). D'autres prélèvements ont été sélectionnés pour des analyses complémentaires (q-PCR, FISH et MEB).

Les assemblages bactériens ont été étudiés par extraction d'ADN et amplification du gène 16S ribosomal (16S). La séparation des séquences d'ADN amplifiées a été effectuée par clonage, qui permet l'insertion de plasmides avec des séquences individuelles du 16S dans des cultures d'*Escherichia coli* et le séquençage du segment de vecteur contenant le gène. Les séquences obtenues (n= 1050) ont été traitées et triées en unités taxonomiques opérationnelles (OTU, > 97% similarité). L'assignation des OTUs a été réalisée avec les bases de données de référence. De plus, les reconstructions phylogénétiques ont été effectuées avec les OTUs et les séquences associées, qui incluent les séquences déjà récupérées chez *R. exoculata*. Des courbes de raréfaction ont été obtenues pour estimer l'efficacité de l'échantillonnage et comparer la diversité des OTUs entre les échantillons. La similarité entre les assemblages a été estimée par l'indice de Sorensen, avec la présence-absence des OTUs. L'analyse d'ordination des prélèvements a été effectuée par l'analyse de 'Cluster' supportée par le test de similarité de profil (SIMPROF). L'analyse de similarité (ANOSIM) a été utilisée pour confirmer les différences des assemblages bactériens entre œufs et pléopodes et les différences entre les stades de développement embryonnaire des œufs.

De plus, les surfaces des œufs et pléopodes ont été observées par microscopie électronique à balayage pour déterminer la configuration de la colonisation bactérienne sur ces surfaces. Des dépôts minéraux trouvés sur la surface des œufs ont été analysés par spectroscopie de rayons X. L'hybridation fluorescente *in situ* (FISH) a été effectuée sur les membranes séparées des œufs au stade avancé, pour confirmer la présence des OTUs les plus fréquemment identifiées dans les approches par clonage. Concernant les analyses de FISH, des sondes moléculaires spécifiques ont été conçues pour détecter spécifiquement les OTUs fréquentes. Les images de FISH ont été obtenues avec un microscope Zeiss Imager.Z2, équipé avec un module ApoTome® et la technologie d'illumination Colibri, et la capture des images a été effectuée avec la camera AxioCam MRm.

Divers assemblages bactériens ont été trouvés sur les œufs et les pléopodes. En général la composition bactérienne est dominée par des *Epsilon*- et *Gammaproteobacteria*, suivi par des

Alphaproteobacteria, le groupe CBF, le groupe RE1, les *Zeta*- et *Deltaproteobacteria*, et le *Deinococcus-Thermus*. L'analyse cluster et le test SIMPROF montrent un premier groupement associé à la structure, ce qui supporte l'hypothèse d'un assemblage bactérien spécifiquement associé aux œufs. Les assemblages bactériens sont plus similaires entre œufs de différentes crevettes (provenant de la même source ou de différentes sources, séparées par 300 km environ) qu'entre les œufs et pléopodes de la même crevette (Fig. S1). Un autre groupe significatif est associé aux stades embryonnaires. La composition bactérienne des œufs au stade avancé est différente de celle des stades initiaux et intermédiaires. Les groupements d'échantillons liés à la structure et au stade embryonnaire (dans les œufs) ont été confirmés par les analyses ANOSIM. Certaines OTUs associées à la variation entre les assemblages sont les *Alphaproteobacteria* et le groupe RE1. De plus, les changements de composition observés sont associés à la variation de la diversité et la structure, avec plus de diversité et d'égalité dans les assemblages des œufs que sur les pléopodes, et encore plus pour les œufs de stade avancés.

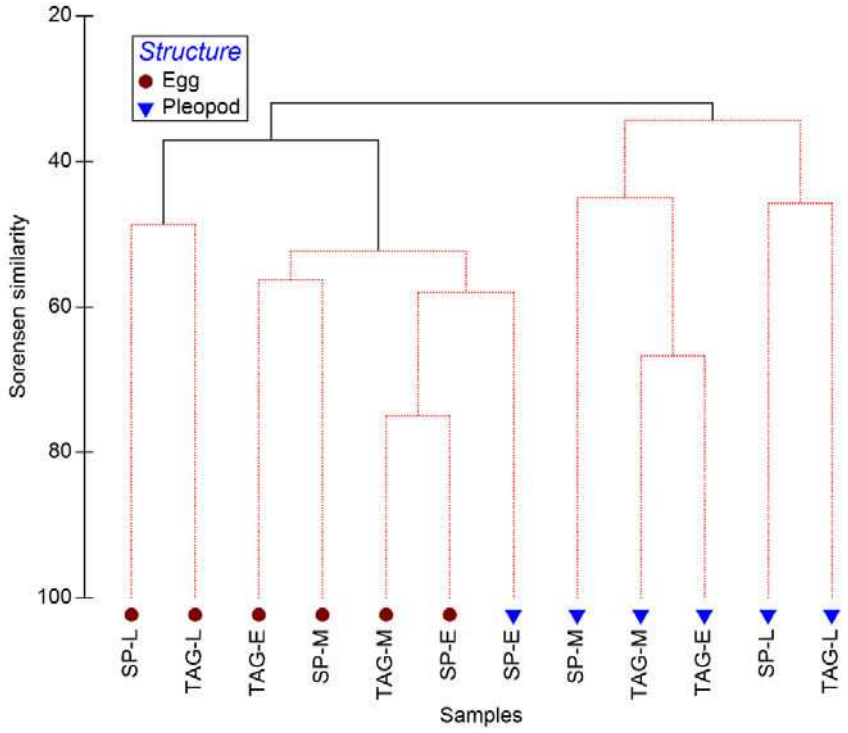

Figure S1. Analyse Cluster des combinaisons de structure, de stade de développement et de site avec le test SIMPROF. Les clusters en ligne continue sont statistiquement supportés (α <0.95), SP : site Snake Pit, TAG : site TAG, E : début de développement, M : milieu, L : avancé ; les œufs sont représentés par les cercles rouges et les pléopodes par les triangles bleus.

La composition des bactéries épisymbiotiques des œufs des champs de Snake Pit et TAG montrent des différences avec les assemblages trouvés sur les œufs du site Logatchev (précédemment rapporté). La plus grande différence réside en la présence de *Gammaproteobacteria* méthanotrophes parmi les assemblages des œufs de Logatchev, absentes à Snake Pit et à TAG. L'hypothèse d'une réponse des assemblages bactériens à la variation de la proportion de sulfure d'hydrogène : méthane dans l'émission hydrothermale peut expliquer le patron observé. D'après les activités métaboliques pour des OTUs similaires rapportées dans la littérature, les processus métaboliques qui pourraient se produire à la surface des œufs comprennent la réduction des sulfures et du soufre, ainsi que de l'hydrogène et du fer. L'activité métabolique proposée est cohérente avec les conditions environnementales des sources hydrothermales de Snake Pit et TAG et similaire à l'activité métabolique estimée pour la chambre branchiale des crevettes adultes.

Les analyses en microscopie électronique montrent que les œufs ont une couverture bactérienne faible au début du développement embryonnaire, avec des bacilles dispersés et quelques filaments. Au milieu du développement, la densité de bactéries augmente de façon irrégulière et avec un début de dépôt minéral. Enfin, au stade avancé, la couverture est plus uniforme sur les œufs et sur les filaments connectifs, et les dépôts minéraux s'épaississent (Fig. S2). Les morphotypes bactériens trouvés incluent des bacilles (avec attache latérale et apicale), des filaments minces (largeur 1 µm environ) et des gros filaments (largeur > 1 µm). Les dépôts minéraux sont composés d'oxydes de fer, qui recouvrent les bactéries dans certains endroits. Le contraste entre la faible couverture au début du développement et la diversité des OTUs suggèrent que la plupart des épisymbiontes effectuent leur colonisation juste après l'extrusion des œufs, possiblement par transfert horizontal ou par transfert durant l'accouplement.

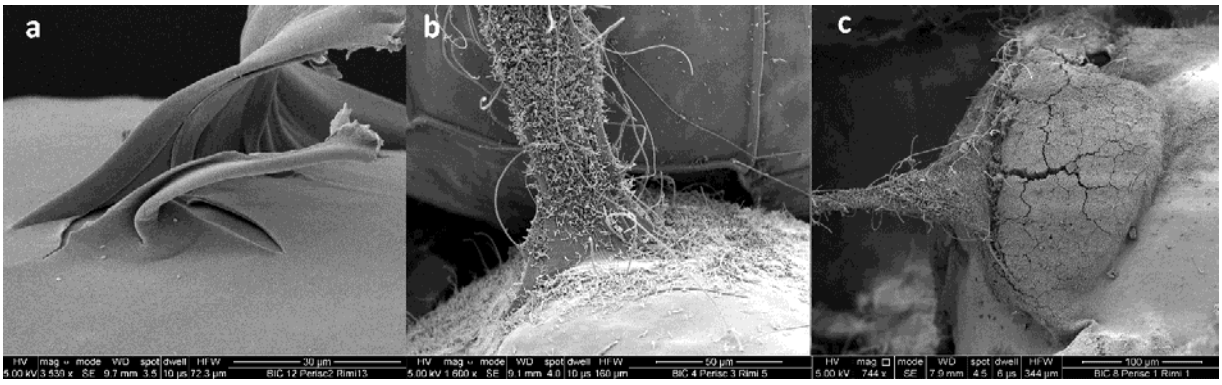

Fig. S2. Couverture bactérienne sur la surface des œufs de *R. exoculata*. a) début de développement, b) milieu de développement, c) développement avancé.

Les bactéries épisymbiontes peuvent faciliter la détoxification et l'inhibition des pathogènes chez les œufs. Cependant, le transfert d'éléments organiques des épibiontes à l'hôte pourrait se produire chez les œufs. La perméabilité sélective de la membrane des œufs et de la couche de mucus pourrait faciliter l'échange des éléments entre l'embryon et les bactéries. La larve des Alvinocarididés passe son premier stade avec un régime alimentaire lécitotrophe et une durée prolongée, et dépend des réserves lipidiques pendant une partie importante de sa dispersion. La production d'éléments organiques par les bactéries durant le développement embryonnaire pourrait permettre la réduction de la consommation des réserves lipidiques de l'embryon et maintenir les ressources énergétiques pour le stade larvaire.

Preliminary manuscript under elaboration and pending of complementary experiments

Bacterial assemblages associated with *Rimicaris exoculata* eggs in deep-water hydrothermal vents: A specific host-symbiont relationship that changes according embryonic stage and vents.

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Abstract

The episymbiotic bacterial community on embryos of the deep-water hydrothermal shrimp *R. exoculata* was analyzed in order to determinate its diversity, its variation along embryonic development and the difference between vent sites. The bacterial assemblages retrieved from eggs at different developmental stages and vent sites were compared with non-symbiotic colonization of a shrimp surface (pleopods) under the same colonization conditions. Samples were collected at Snake Pit (3460 m depth) and the TAG site (3630 m depth) vent fields from the Mid-Atlantic Ridge using a remote operated vehicle (ROV), and pressurized chambers. Bacterial assemblages were characterized by cloning, and the occurrence of bacteria and mineral deposits on the egg surface was described using scanning electron

microscopy. Diverse bacterial assemblages were found on both eggs and pleopods. In general the diversity was dominated by *Epsilon*- and *Gammaproteobacteria*, followed by *Alphaproteobacteria*, CBF group, RE1 group, *Zeta*- and *Deltaproteobacteria*, and *Deinococcus*-*Thermus*. SIMPROF test show a primary clustering of samples associated with the structure, suggesting the occurrence of a specific bacterial assemblage on eggs. Despite the limitations of the cloning approach to reflect the bacterial diversity, bacterial assemblages retrieved in our samples seems to be more similar between eggs from different females, including those living in separated sites (about 300 km apart), than between eggs and pleopods of the same parental female. Also, bacterial assemblages on eggs change according the embryo stage. The bacterial diversity from eggs at Snake Pit and TAG site was different to the one retrieved on eggs of *R. exoculata* reported from another vent (Logatchev site, 3037 m depth). On TAG and Snake Pit, the methanotrophic *Gammaproteobacteria* were not retrieved. The hypothesis of variation in the ratio of sulfide:methane at the vent emission as a control of symbiotic bacterial assemblages is proposed to explain the variation in egg epibionts between vents. The epibiotic bacteria on eggs could contribute to detoxification and pathogen inhibition. However, we discuss other potential roles of the episymbionts for the eggs development.

Keywords: hydrothermal vents, symbiosis, bacteria, embryo, Mid-Atlantic Ridge.

Introduction

The deep-water hydrothermal vent ecosystems are dominated by fauna living with symbiotic chemoautotrophic bacteria. Vestimentiferan worms, *Bathymodiolus* mussels, alvinellid polychaetes, calyptogenid clams, galatheids, chirostyloid and alvinocarid shrimps are some of the iconic vent taxa that host symbiotic bacteria (Grassle 1987, Childress and Fisher 1992, Van Dover 2000, Nakagawa and Takai 2008). These hosts obtain organic compounds generated by bacterial chemoautotrophy, reducing the dependence of other sources of nutrition (Duperron et al. 2005, Dubilier et al. 2008, Childress and Girguis 2011, Hügler and Sievert 2011). The relationship between the vent species with symbiotic

bacteria have a profound impact on colonization processes, biomass, distribution of populations around the vents and finally in the energy transfer of the global deep sea ecosystem (Govenar 2012).

Symbiotic bacteria can be located in specialized cells of the gills (endosymbiosis) (Windoffer and Giere 1997, Duperron et al. 2005) or in trophosome, internal organ that could represent a large portion of the host (Dubilier et al. 2008). Symbiosis over the host tissue (episymbiosis) can include farming and harvesting of bacteria (Thurber et al. 2011, Zwirglmaier et al. 2015) or transcuticular transfer of organic compounds to the host (Ponsard et al. 2013). In both cases, the symbiosis could also involve detoxification processes, allowing the increase of the host tolerance to the vent fluids (Powell and Somero 1986, Campbell et al. 2003, Campbell et al. 2006). The symbiotic relationship usually start early in the life cycle, even for egg at pre-cleavage stage, at embryonic stage (Cary and Giovannoni 1993, Goffredi et al. 2014) or immediately after recruitment (Nussbaumer et al. 2006). The process of early acquisition of symbiotic bacteria could be promoted by transfer from the parents to the next generation during reproduction (vertical transfer) or by selective colonization from the surrounding microbial communities including from conspecific host (horizontal transfer) (Cary and Giovannoni 1993, Won et al. 2003, Nussbaumer et al. 2006). Despite the environmental and host related factors, the symbiotic relationships could be described as a dual, host-specific and compartment-specific relationship. The distribution of symbiotic bacteria are restricted to a particular host and localized in specific structures, usually forming distinctive assemblages (Dubilier et al. 2008).

The alvinocaridid shrimp *Rimicaris exoculata* is a dominant taxa of hydrothermal vents at the Mid-Atlantic Ridge (MAR), below 2000 m depth (Desbruyères et al. 2000). This species forms large aggregations of thousands of shrimps per m^2 close to vent emission, and also occupy other habitats around the vent field (Copley et al. 1997, 2007, Hernandez-Avila et al. in prep). Adult stages host episymbiotic bacteria on its branchial chamber and on its mouthparts, which are modified and adapted to the colonization and growth of the symbiotic bacteria (Segonzac et al. 1993, Zbinden et al. 2004, Corbari et al. 2008b). Moreover, the shrimp hosts symbiotic bacteria in its gut, showing a distinct epibiotic microbial communities (Zbinden and Cambon-Bonavita 2003, Durand et al. 2010, Durand et al. 2015). The symbiotic bacterial assemblages are diverse in both compartments, however is usually dominated by *Epsilon-* and *Gammaproteobacteria* in the branchial chamber and by Deferribacteres, Mollicutes and *Epsilonproteobacteria* in the gut (Zbinden et al. 2008, Durand et al. 2010, Petersen et al. 2010, Jan et al. 2014). There are evidences that bacterial assemblages, especially from the branchial chamber, bring nutrition to the shrimp by direct transfer of organic carbon generated by

chemosynthesis (Ponsard et al. 2013). In addition the metabolic activity of the bacteria could also facilitate the detoxification process of the shrimp from the toxic emission of the vent (Zbinden et al. 2008, Guri et al. 2012, Ponsard et al. 2013, Jan et al. 2014).

The symbiotic bacterial assemblages from both compartments (branchial chamber and gut), show different levels of variations. Geographical variation of the bacterial composition have been detected in both the epibionts of the branchial chamber and the ones of the gut (Petersen et al. 2010, Guri et al. 2012, Durand et al. 2015). The microbial communities hosted in the branchial chamber are particularly dynamic due to the short molting cycle of the shrimp. As the bacteria colonize the cuticule in the branchial chamber and mouthparts, the episymbionts are eliminated with each molt (Corbari et al. 2008b). The molting cycle of the shrimp generates a constant cycle of production of new host surface implying symbionts recolonization, development and generation of mineral deposits at the surface of the branchial chamber (Corbari et al. 2008a, Corbari et al. 2008b).

Recently, colonization of episymbiotic bacteria was described on eggs of *Rimicaris exoculata*, denoting that the bacteria-host relationship in this species could start at embryonic stage (Guri et al. 2012). The non-pathogenic colonization of bacteria on eggs have been found in other vent and non-vent decapods (Fisher 1983a, Harper and Talbot 1984, Goffredi et al. 2014). However, it is not clear if this process is produced by a specific interaction between eggs and bacteria, or by non-symbiotic colonization of the egg surface. The molting cycle and reproductive process of caridean shrimps bring an interesting scenario to test the specific association between bacteria and embryos, their evolution during the embryonic development and the variation in different vents.

In caridean shrimps, females perform a molt before matting, during which a change of abdominal appendices configuration occurs (pleopods) (Bauer 1976, Correa 2003, Bauer 2013). After matting, the eggs are extruded to the abdomen, mixed with the sperm and covered with a mucus layer that keep the eggs attached together and to the pleopods (Fisher and Clark 1983). During the brooding, the pleopods bring mainly a structural support (Bauer 2013). Bacterial colonization in/on the brood of *R. exoculata* occurs on the eggs surface, pleopods and on small sections of the abdominal surface (IHA, pers. obs.). Since the eggs are attached to the pleopods during the brooding period, they are both exposed to the similar conditions.

We then analyzed the bacterial assemblages that colonize the eggs surface and the pleopods, at different stages of the embryonic development and collected from females inhabiting two different vent

fields, Snake Pit (3460 m depth) and TAG (3630 m depth). Although the colonization of eggs is suspected to be a specific symbiotic relationship, the bacterial assemblages retrieved on the surface of pleopods is assumed to be generated by an opportunistic process of colonization. The goal of this study was i) compare the bacterial assemblages found on the egg surface with those retrieved on the pleopods of the parental female; ii) determinate the variation of bacterial assemblages along the embryonic development, iii) compare the colonization of bacterial communities on eggs from two different vents fields.

Materials and Methods

Collection and treatment

Brooding females of *R. exoculata* were collected at two vent fields of the MAR, the Snake Pit site (23°22'N; 44°57'W, 3460 m depth) and the TAG site (26°08'N; 44°49.5'W, 3630 m depth) during the BICOSE cruise (January-February 2014). Specimens were collected in large swarms inhabiting active emission habitats, using a suction sampler manipulated by the remote operated vehicle (ROV) Victor 6000. In order to avoid sampling effect related with uncontrolled decompression, pressurized recoveries of the specimens were performed using the PERISCOP devise. The specimens were collected with the slurp gun of the ROV and transferred to an isobaric chamber PERISCOP before start the ascent to the surface (Shillito et al. 2008). On board of the ship (R/V *Pourquoi Pas?*) samples were processed for dissection and fixation.

Brooding females were selected from each site according the developmental stage of the embryos in the brood. The eggs were classified in three developmental stages: Early, from cleavage to first cell divisions; Mid, early development of embryonic structures; Late, full development of the embryo, larval structures visible through the egg envelope, close to hatching. Eggs were removed from the brood and some of the pleopods were dissected. Twelve samples at different combinations were selected according structure (eggs and pleopods), developmental stage (early, mid and late), and vent (Snake Pit and TAG site) in order to describe the composition of bacterial assemblages. Other samples were also collected for complementary experiments of scanning electron microscopy (SEM) and fluorescent hybridization *in situ* (FISH). Samples for DNA analyses were frozen to -80°C, to FISH analyses

samples were fixed 3% formol samples were stored in PBS/Alcohol at -20°C, and for SEM analyses the samples were fixed in 2.5% glutaldehyde in seawater and kept at 4°C.

DNA extraction and PCR amplification

DNA from eggs and pleopods were extracted using the Fast DNA Pro Soil-Direct Kit (Qbiogen, Santa Ana, CA, USA) following manufacturer's instructions. Bacterial 16S rRNA gene fragments were PCR-amplified using the following thermocycler set: 1 initial denaturalization at 94°C during 15 min, 30 cycles of 1 min at 94°C, 1:30 at 48°C, 2 min at 72°C, and final extension at 72°C during 6 min. PCR solution for the amplification (50 µl vol) were prepared using 1X GoTAG buffer, 2mM MgCl, 0.2 µM dNTP, 1.25 units of GoTag (Promega), 20 pmol of each forward and reverse primer (8F and 1492R (Lane 1991)) and 1 µl DNA solution. For each sample, PCR amplifications were performed 3-4 times in order to obtain enough samples. Replicates from the same sample were pooled and purified using NucleoSpin® Gel and PCR clean up (Macherey-Nagel, Germany) following manufacturer's instructions.

Cloning and sequencing

The purified PCR products were cloned using the TOPO XL Cloning kit (Invitrogen, Carlsbad, CA, USA) following the instructions of the manufacturer. The plasmid insertion was controlled by amplification using M13F and M13R primers in 16 random samples. For each sample, clones were randomly selected and each stabbed into a well of a 96-well plate containing LB-agar with ampicillin (100 µg ml^{-1}). Plates were sent to Macrogen Inc. (Netherlands) for sequencing.

Sequence processing, OTU discrimination and taxonomic affiliation

Sequences were cleaned manually and aligned using Geneious (Kearse et al. 2012). Low quality and short sequences were discarded. Alignments were analyzed using Mothur (Schloss et al. 2009) in order to identify OTUs (sequences with < 3% of distance) and to obtain an initial taxonomic affiliation, based on both Wang and Kmer methods (see Mothur manual). Sequences of close related OTUs were compared by alignment in order to verify their discrimination. One representative sequence of each OTU was compared with sequence databases using BLAST (Altschul et al. 1990) to identify related

sequences and identify putative taxonomic affiliations. For each bacterial division, phylogenetic reconstruction were performed including the OTUs and the related sequences (according BLAST search). Taxonomic affiliations were estimated according to the consistency of the different approaches (Mothur affiliation, BLAST search and phylogenetic reconstruction).

Data analysis

The data analyses were performed considering the limitations of the cloning protocols, in terms of lack of precise quantitative estimations of sequences and bias potentially introduced by rare OTUs (Qiu et al. 2001, Bent and Forney 2008). In order to bring an appropriate comparison of the samples, we performed analysis based on presence – absence of the OTUs, and using statistical approaches that could help to mitigate the limitations of this molecular approach.

Rarefaction curves were elaborated for each sample in order to estimate the coverage of sample library and to compare the diversity between samples, using EstimateS ver 9.0 (Colwell et al. 2004, Colwell 2013) including 1000 replications. Based on presence-absence of the OTUs along the samples, a similarity matrix was calculated using the Sorensen Index:

$$S_{ab} = \frac{2\,C}{A+B}$$

When C is the number of shared species (OTUs) between samples “a” and “b”, A and B are the number of OTUs in the sample “a” and “b”, respectively. OTUs represented by a single sequence were discarded of the similarity estimations for avoid bias introduced by extreme rare OTUs. Information about the number of sequences associated to the OTU was not included because the differential amplification of some sequences could introduce bias in the OTU frequencies, as well the cloning process itself (Qiu et al. 2001, Bent and Forney 2008).

A similarity profile (Simprof) test (Clarke et al. 2008) was performed in order to determinate if the patterns of bacterial lineages (OTUs) observed on the samples represent non-random assemblages associated with the sampled structure (egg and pleopods), the stage of development (early, mid and late) or the vent field (Snake Pit and TAG sites). For this test, a hierarchical cluster of the samples is estimated based on the similarity matrix calculated using the Sorensen index. Posteriorly, simulated clusters were estimated with random placement of the retrieved OTUs along samples, including frequent and rare OTUs. This process was repeated 10 000 times to generate a large sample of

simulated clusters generated by random distribution of the retrieved OTUs, including putative biased rare OTUs. Each node of the cluster (and the sample discrimination related), was statistically compared with the simulated set of permutated clusters. This analysis permit discriminate the putative introduction of random bias by the occurrence of rare (or even frequent) OTUs in our samples. Also, this method permit to test *a priori,* the occurrence of samples groups according to their similarity instead of testing a factor fixed by the experimental design. Cluster segregation of samples was also tested with a Anosim test, both analyses were performed using Primer/Permanova 6.0 (Clarke and Gorley 2006). In addition, for referential purpose the ecological indices of evenness and Simpson diversity were estimated using the proportion of sequences by OTU as a proxy of abundance.

Results and Discussion

SEM observations

The colonization of bacteria on the eggs along the embryonic development show similar patterns in both vent fields. The surface of the eggs at the early stage was colonized by few and scattered bacteria (Fig. 1). The morphotypes identified at this stage were small isolated rod-shaped bacteria, mostly attached laterally, and 1 µm thick filaments with a proximal attachment to the eggs and projected over 10s to 100s µm. These clearly remind the ones observed on the gill chamber after molting (Zbinden et al. 2004, Corbari et al. 2008b). The bacteria were retrieved on the mucus coat embedding the eggs and also on the projections of mucus that maintain the eggs attached to the female body. In the small sections were this mucus layer is broken (allowing the observation of the egg envelope itself) no bacteria was observed directly attached to the egg envelope (Supplementary material 2). In addition, the eggs were almost clear of mineral deposits, except by thin and scattered ferric-like deposits found on some eggs. At mid stage of embryonic development, the eggs show different degrees of bacterial colonization, usually with patches of bacteria over the eggs and the connective mucus. In general, the bacterial coverage were denser with more bacterial filaments, including thin (< 1 µm) and thick (> 1 µm) filaments and rod-shape erected bacteria as found in the branchial chamber of the adults (Zbinden et al. 2004). Many of the erected rod-shape bacteria were observed with a basal constriction, with a pedal-like structure of attachment. Other erected bacteria show few divisions. Thick bacterial filaments were sometimes observed with rod-shape bacteria

attached (both laterally attached and erected). In addition, in some spots, they are crusts of mineral deposits of different thickness (up to 5 µm) with the bacteria. The spectrometric analysis of the crust shows that the deposits are mainly composed of ferric oxides.

On the eggs at late stage of embryonic development, bacteria cover almost entirely the eggs, especially on the eggs found in the external part of the brood. The morphotypes identified are the same than those found at the mid-stage eggs, however the distribution is more uniform along the egg and the connective mucus. Similarly, there are more mineral deposits which tend to form thick crusts (5-7 µm thick) and covering even the connective mucus. In some spots of mineral deposits, it seems that the crust covers a part of the bacterial biofilm, but still there are bacterial cells visible over the crust. All these observations remind the bacterial colonization observed in the branchial chamber of adults specimens at late molt stages (Corbari et al. 2008b).

Bacterial diversity on eggs and pleopods

The libraries included a total of 1050 sequences of the 16SrRNA gene, separated in 57 OTUs. For each library we observed a high ratio of recovered sequences (mean 91±6 %) and high phylotype coverage (94%). The rarefaction curves by sample show that the libraries give a comparable representation of the bacterial diversity, they tend to stabilize with the accumulation of sequences. In general, the retrieved sequences were dominated by *Gamma* and *Epsilonproteobacteria* in all bacterial assemblage libraries, followed by *Alphaproteobacteria*, CFB group, RE1 group, *Zeta* and *Deltaproteobacteria* and *Deinococcus-Thermus* group (Table 2). Most of the OTUs were affiliated to epibiotic bacteria OTUs previously identified on *R. exoculata,* including those identified on egg samples obtained from the Logatchev site (Guri et al. 2012). Other sequences were affiliated to epibiotic bacteria found on Alvinocarididae and other hydrothermal vent fauna. The few OTUs without putative taxonomic affiliation were related to bacteria previously reported as epibiont of the hydrothermal vent galatheid *Shinkaira crosneri*.

The *Epsilonproteobacteria* group was composed by three major clusters (Fig. 2a). The most frequent was a large cluster described as “hydrothermal invertebrates epibionts group” (Petersen et al. 2010, Guri et al. 2012), affiliated to *Helicobacteraceae* and related to cultured species of *Sulfurovum,* including species from hydrothermal vents (Inagaki et al. 2004, Giovannelli et al. 2016). This cluster is associated with an autotroph metabolism that include sulfur oxidation and nitrate reduction (Campbell

et al. 2006). For instance the hydrothermal epibiont *S. riftiae* share close similarity with some OTUs identified (OTU11: 96.7%, OTU19: 94.4%, OTU02:92.9%), and show a metabolism based on nitrate-reducing, sulfur and thiosulfate oxidizing (Giovannelli et al. 2016). As well *S. lithotrophicum* is a sulfur-oxidizing bacteria (Inagaki et al. 2004). This lineage is the main one also retrieved on the gill chamber of adults of *R. exoculata* (Petersen et al. 2010). Other *Epsilonproteobacteria* sequences were affiliated to Campylobacteraceae, in particular two clusters affiliated to *Campilobacter* and *Arcobacter*, respectively. The two former clusters include also sequences retrieved from epibiotic bacteria of hydrothermal vent species (including adults of *R. exoculata*) and in sediments of hydrothermal vent and other reducing environments, and show metabolic diversity that include sulfur oxidization and nitrate reduction (Wirsen et al. 2002, Guri et al. 2012, Vetriani et al. 2014). In addition, were collected some OTUs affiliated to the "Reduced Environment 1 (RE1) group" proposed by Perner et al. (2007), composed in part by clones previously misplaced within *Epsilonproteobacteria* (Campbell et al. 2006, Perner et al. 2007). In our samples, the former group was collected more on eggs than pleopods, especially the most frequent RE1 OTU (OTU07) was retrieved almost exclusively (28 of 29 sequences) in late-stage eggs from both sites. But this could be due to the low number of clones treated here.

Gammaproteobacteria were also widely distributed in all samples, in particular a cluster affiliated to Thiotricaceae that included three OTUs with 30% of total libraries. This family is frequently found as ectosymbiont of hydrothermal vent invertebrates and includes sequences affiliated to *Leucothrix* (Goffredi 2010) and *Cocleimonas* (Goffredi et al. 2014). On eggs and pleopods, the retrieved sequences affiliated to Thiotricaceae are separated in two groups: a first group affiliated to sequences retrieved from epibiotic bacteria in *Shinkaia crosnieri* epibionts and a dominant cluster affiliated to sequences from the branchial chamber and eggs of *R. exoculata* (Fig. 2a). Both genera *Leucothrix* and *Cocleimonas* include sulphur-oxidizing bacteria capable of depositing sulfur intracellularly (Grabovich et al. 1999, Tanaka et al. 2011). In addition, one OTU affiliated to Alcanivoracaceae was identified, based on sequences collected only in late-stage eggs. Related sequences of the former OTU were found on hydrothermal vent substrates of the Eastern Pacific Rise and from bacterial mats in iron rich sediments of a submarine volcano (Sudek et al. 2009, Sylvan et al. 2012).

Alphaproteobacteria were quite diverse (9 OTUs) but represented only 5% of the libraries. However most of sequences of this group (92%) were retrieved from eggs samples (in both vent fields) instead of pleopods. The majority of OTUs were affiliated with Rhodobacteraceae, except by one OTU affiliated to Hyphomicrobiaceae (Fig. 3b). In addition, the *Zetaproteobacteria*, a division recently

included as part of the *R. exoculata* ectosymbionts on specimens from the Rainbow site (Jan et al. 2014), were also more frequent on eggs than pleopods. The single cultivated species from this division so far, *Mariprofundus ferrooxydans*, is a neutrofilic iron oxidizer bacteria associated with microbial mats in hydrothermal vents (Emerson et al. 2007, Singer et al. 2011). In adult specimens of *R. exoculata,* the *Zetaproteobacteria* were observed in close association with the iron hydroxide layer that accumulates between the shrimp's molts (Jan et al. 2014). Also *Zetaproteobacteria* were retrieved in a gut sample of *R. exoculata* (Durand et al. 2015). These findings sustain that *Zetaproteobacteria* is a regular members of the *R. exoculata* epibiont assemblages. Also supporting the hypothesis of iron-oxidizing processes in the epibiont assemblages (Zbinden et al. 2004, Corbari et al. 2008a, Corbari et al. 2008b, Zbinden et al. 2008, Jan et al. 2014).

Deinococcus-Thermus bacteria are reported for first time associated with *R. exoculata*. The OTU retrieved is related to the genera *Truepera* (according to SILVA classification), which have a single species so far (*T. radioviotrix*, a radiation-resistant chemoautotroph from the Azores hot spring (Albuquerque et al. 2005)). However, this OTU form a cluster with sequences collected from deep-water reducing environments, such as sediments collected in *Ridgeia piscesae* habitats at the Juan de Fuca Ridge (Forget and Juniper 2013) and epibiontic assemblages collected in tubes of *Lamellibrachia* sp. from eastern Mediterranean cold-seeps (Duperron et al. 2009)(Fig. 2b).

For the CFB group, most of the OTUs (5 of the 6 OTUs identified) were exclusively found either on eggs or on pleopods, but not on both. A cluster related to *Tenacibaculum,* which contain most of retrieved sequences (27 of 39 sequences in 2 OTUs, fig. 3b), is also affiliated to sequences identified in the branchial chamber of adult shrimps (Petersen et al. 2010). The less represented division was however an interesting *Deltaproteobacteria* (1 OTU, 2 sequences) closely associated with *Desulfocapsa sulfoxigens* (97.5% similarity). This is a marine chemoautotroph that use elemental sulfur as only energy source in presence of ferrihydrite (Finster et al. 1998). This OTU were found in Snake Pit and TAG site (egg and pleopod respectively), but also is consistent (99.6% identical) with the OTU obtained from the branchial chamber of *R. exoculata* adults collected at the Snake Pit site by Hügler et al. (2011)(Fig. 3a).

Variation in bacterial assemblages between eggs and pleopods

Because all samples have been treated using the same protocol, some general inferences can be estimated, even if cloning approach can lead to molecular bias and limitations. Although the proportion

of the bacterial groups is similar along the samples, the structure of the bacterial assemblage diversity estimated by OTU exhibit some differences. The cluster analyses of the bacterial assemblages show the occurrence of differences between eggs and pleopods. The bacterial assemblages retrieved from the eggs samples grouped in a single cluster, separated from a group formed by most of the pleopod samples (Fig. 4, 5). Only one pleopod sample was grouped in the same cluster as the eggs. Despite the limitations on the lack of a quantitative estimation of the bacteria and the relatively low number of OTUs, the SIMPROF test show that the clustering of the samples according to the structure is statistically supported. The probability of generating similar cluster of samples by random occurrence of the OTU along the samples is lower than 1% (under 10 000 permutations). The ANOSIM test also detect a difference in the structure of the bacterial assemblages between eggs and pleopods (Rho= 472, p= 0.009). These differences are remarkable considering the design of the experiment, bacterial assemblages are more similar between eggs from different females, including those living at different vents sites (about 300 kms apart), than between eggs and pleopods of the same parental female (separated in a scale lower than few mm). Despite the proximity occurring between egg and pleopods during the brooding period, the exposition to the same colonization conditions and potential bacterial transfer between the structures, differences have been noticed. These results suggest the occurrence of a specific assemblages of bacteria colonizing the eggs surface, as occurring in the branchial chamber and digestive system of adult shrimps (Corbari et al. 2008b, Jan et al. 2014, Durand et al. 2015). This level of specificity to the host compartment is suspected to be driven by bacterial-host molecular interactions that allow the selection of bacteria on the host surface (Distel et al. 1988, Boutet et al. 2011). Future quantitative analysis, as quantitative PCR, and analyses of Fluorescent *in situ* Hybridization could contribute to corroborate these trends.

In addition, a variation in the bacterial assemblages that colonize the egg was detected between the stages of embryo development. Two groups were detected. The first group was composed of the bacterial assemblages of eggs at early- and mid-stage and one pleopod (from the parental female of the early-stage egg, the less colonized one), and the second group comprise the bacterial assemblages from egg at late stage from both vent sites. The differences in the composition of the bacterial assemblage on eggs are also consistent with variation of the evenness and Simpson diversity. The late-stage development eggs host a bacterial assemblage more diverse in term of OTU richness and morphologies, but also with evenness and higher score of the Simpson diversity. For the cluster of samples composed by pleopods, non-significant differences of the assemblages were detected within this cluster (Simprof; $p > 0.05$). This variation is associated to the evolution of the bacterial assemblages during the

developmental period. Although a precise estimation of the brooding period of *R. exoculata* is not available, it is globally estimated to occur in a range of 2-3 month as other alvinocaridid shrimps (Copley and Young 2006). The SEM analysis of the egg surface also show a pattern of colonization (and mineral deposition) analogous to the one reported for the branchial chamber of adult shrimps along their molt cycle (Corbari et al. 2008b). However, despite the very low coverage of bacteria observed at early stage, the OTUs diversity is relatively high compared to the late stage one. For instance, at TAG, there is no variation of the OTU diversity according to the stage of development while at Snake Pit, an increase of OTU number was observed at the late stage (lower than 30%, supporting material 1). These results suggest that the colonization could start rapidly, with a large proportion of the bacterial assemblage colonizing from the beginning of the egg development. The assemblages in other developmental stages would then be generated by the growth of the early established colonies and the recruitment-extinction process occurring in few species. This remind the observations of the branchial chamber colonization (Corbari et al 2008).

Dissection of the male and female reproductive systems did not show evidence of any bacterial assemblages that could be associated to a vertical transmission of symbionts (Hernandez-Avila et al. *per. obs*.). However, it is possible that a bacterial transfer during matting process of the shrimp occurs. Since the mating occur soon after of the female molt, it is expected that colonization of bacteria is low at this stage (Corbari et al. 2008b). The male pleopods (the first and second pair used during the spermatophore transfer) are exposed to bacterial colonization, as the females pleopods observed in this study. The manipulation of spermatophores during mating could bring some of the bacteria that colonize the eggs, in a kind of hybrid horizontal-vertical transmission model.

Although the epibiotic bacterial assemblage did not shown clear variation between the vent fields of TAG and Snake Pit, these assemblages are different from those collected on eggs of *R. exoculata* from Logatchev site, described by Guri et al. (2012). At Logatchev vent field, the epibiotic assemblages on eggs were widely dominated by *Gammaproteobacteria* including a cluster affiliated to methanotrophic symbionts. Other bacteria divisions (*Alpha-*, *Delta-* and *Epsilonprotobacteria*, and Bacteroidetes group) were poorly represented. Although it must be confirmed by a quantitative approach, the *Epsilonproteobacteria* and *Gammaproteobacteria* were both the most frequent groups in the assemblages on eggs from Snake Pit and TAG site (although with some variation between samples) and methanotroph *Gammaproteobacteria* were here not retrieved. In addition, more diversity of both *Alphaproteobacteria* and CFB groups was observed and the *Zetaproteobacteria*, *Deinococcus-Thermus*

and RE1 groups, not identified at Logatchev. The occurrence of geographical variation in the composition of the assemblages reminds the symbiotic assemblages in adult stages of *R. exoculata*. Although the bacterial assemblages are clearly distinct for each compartments of the shrimp (branchial chamber and gut), there are geographical variations in the bacterial composition (Petersen et al. 2010, Guri et al. 2012, Jan et al. 2014, Durand et al. 2015).

The variation on the egg bacterial assemblages from different vents could be correlated to changes in the end-member emissions chemical composition. Salerno et al. (2005) proposed, based on the comparison of endosymbionts in *Bathymodiolus* spp., that the ratio CH_4:H_2S could drive the dominance of sulfoxidizer symbionts (in environments with CH_4:H_2S< 1) or methanotrophs symbionts (CH_4:H_2S> 2). In the case of *R. exoculata* eggs*,* the methanotroph related *Gammaproteobacteria* were retrieved only from Logatchev site, which show a CH_4:H_2S end-member ratio > 2, instead of TAG and Snake Pit sites that show a CH_4:H_2S ratio <<1 (Desbruyères et al. 2000). Similarly, the methanotroph related *Gammaproteobacteria* detected on the eggs of the yeti crab *Kiwa puravida* (Goffredi et al. 2014) is in agreement with a high methane emission in a hybrid "hydrothermal seep" ecosystem (Levin et al. 2012). Although the methanotrophs like bacteria on eggs did not dominated the epibiotic assemblages in any case, as occurring in the model proposed by Salerno et al. (2005) for *Bathymodiolus* mussels, their occurrence could also be driven by a methane threshold. In the branchial chamber of *R. exoculata*, the methanotroph bacteria were detected in sites with high methane emission (Zbinden et al. 2008), although with variations (Guri et al. 2012, Jan et al. 2014).

These epibiotic bacteria could also contribute to detoxification and pathogen inhibition on eggs (Guri et al. 2012, Goffredi et al. 2014). Early papers show that egg epibiotic bacteria could control the growth of pathogenic fungi in decapod crustaceans (Fisher 1983b). However, the occurrence of a chemoautotrophic assemblage, similar to the one retrieved in the branchial chamber suggest that the functional interaction host-bacteria could be similar to this compartment. The transfer of organic compounds generated by chemosynthesis, as the one demonstrated in the branchial chamber (Ponsard et al. 2013), is a process to be explored in this embryo-bacteria interaction in next future. The selective permeability of the egg membrane and mucus coat (Fisher and Clark 1983) could facilitate compound transfers between the episymbiotic bacteria and embryo. The alvinocaridid larvae spent the early stage as lecitotrophic during long term, depending on lipid reserves during extended period (Hernández-Ávila et al. 2015). The putative transfer of organic compounds from bacteria to embryos during brooding

could enhance the larval survivorship by reduction in the consumption of lipid reserves during embryonic development and then favor long dispersal.

A series of additional experiments will be implemented in order to corroborate the current results and bring more detailed and robust information about the bacterial assemblages. These experiments include a precise estimation of the sequence abundance in the bacterial assemblage using real-time polymerase chain reaction (qPCR). Also experiments of *in situ* Fluorescence Hybridization (FISH) are schedule in order to analyze the occurrence of the difference bacterial groups on the egg surface. Other future experiments, as analysis *in vivo* of the consumption and localization of isotope-labelled inorganic carbon, could explore the hypothesis of carbon transfer from the episymbiotic bacteria to the embryo.

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Table 1. Samples of eggs and pleopod used for DNA extraction and scanning electron microscopy

Vent Field	Sample label	Sample	Egg stage	DNA extraction	SEM analysis
Snake Pit	Female « 1 »	Eggs	Early	●	●
Snake Pit	Female « 2 »	Eggs	Mid	●	●
Snake Pit	Female « 3 »	Eggs	Late	●	●
Snake Pit	Female « 1 »	Pleopod	Early	●	
Snake Pit	Female « 2 »	Pleopod	Mid	●	
Snake Pit	Female « 3 »	Pleopod	Late	●	
TAG	Female « 4 »	Eggs	Early	●	●
TAG	Female « 5 »	Eggs	Mid	●	●
TAG	Female « 6 »	Eggs	Late	●	●
TAG	Female « 3 »	Pleopod	Early	●	
TAG	Female « 4 »	Pleopod	Mid	●	
TAG	Female « 5 »	Pleopod	Late	●	

Labels correspond to the parental female for eggs and pleopods.

Table. 2. Distribution of sequences by bacterial groups along the samples

	Snake Pit						TAG site						
	Pleopods			Eggs			Pleopods			Eggs			
Division \ Stage	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late	Total
Epsilonproteobacteria	44	58	40	37	62	20	94	59	15	35	21	27	512
Gammaproteobacteria	21	22	25	39	14	40	1	27	45	37	54	20	345
Alphaproteobacteria	2			8	5	6			2	10	12	12	57
CFB*	2	5	9		2	5			13	13		3	52
RE1 group	3	1		2	1	12			7		1	16	43
Zetaproteobacteria	4			3							6	2	15
Deinococcus-Thermus			2			4		1	2	1			10
Deltaproteobacteria					1			1					2
Others	5		2		1	2			3			1	14

*Cytophaga-Flavobacteria-Bacteroidetes

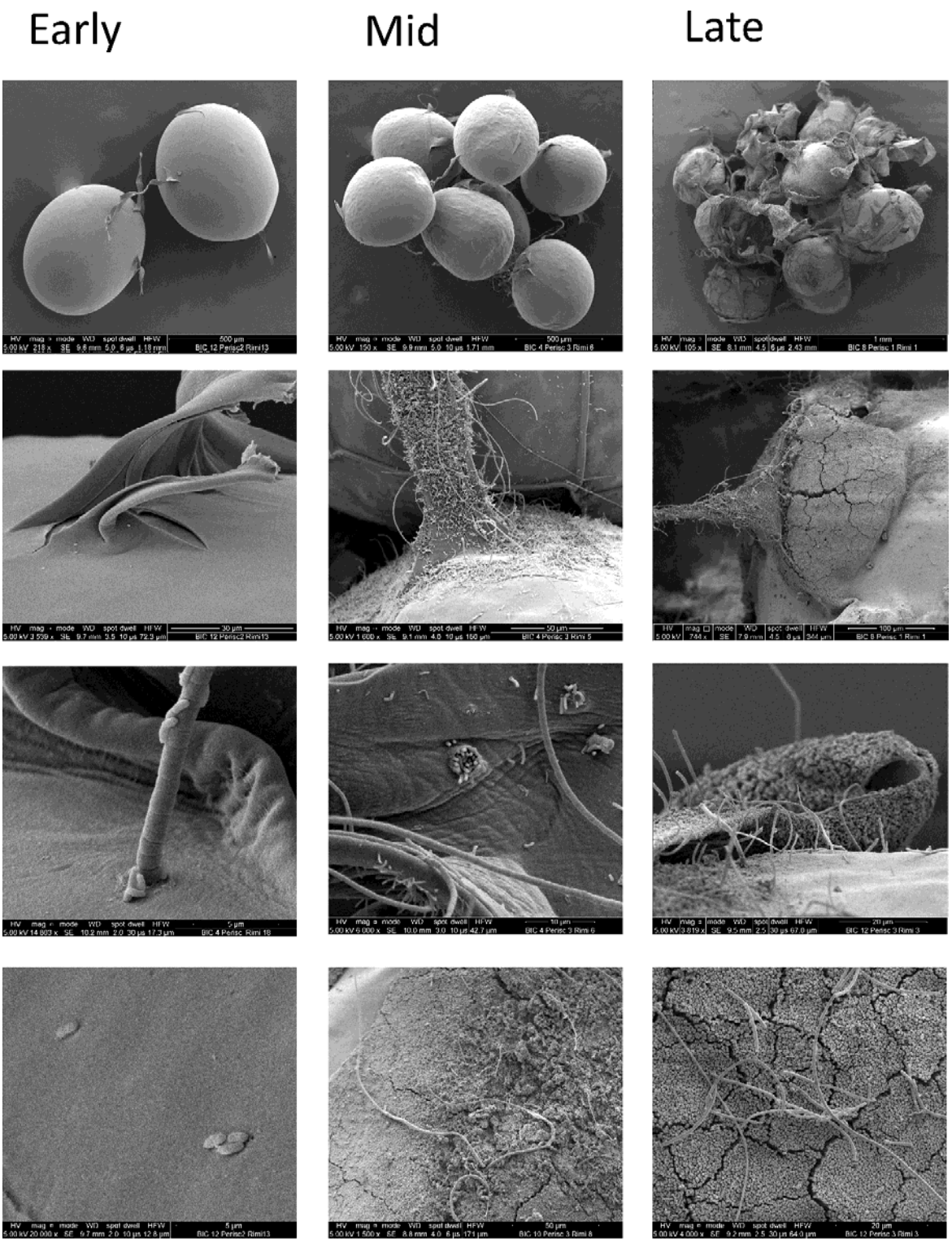


Fig. 1. SEM pictures of epibiotic bacteria in the egg surface of *R. exoculata* at Snake Pit and TAG site. Images are sorted in columns (early, mid and late stages) and row (Snake Pit and TAG). The crust observed in close view are deposits of ferric oxides.

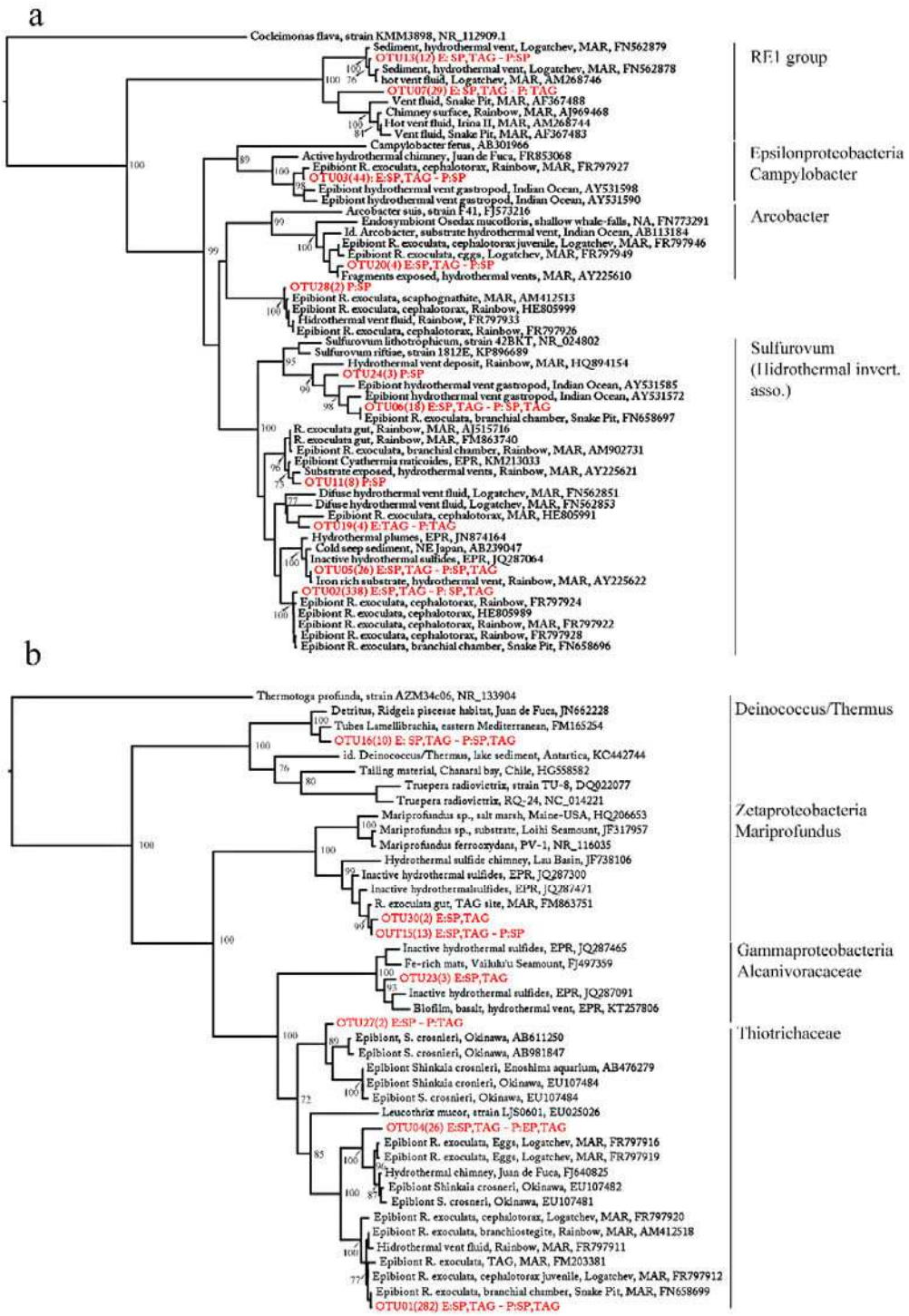


Fig. 2. Phylogenetic reconstructions of the 16S bacterial genes for the bacterial groups. a. *Epsilonproteobacteria* and RE1 group (519 bp). b. *Gammaproteobacteria*, *Zetaproteobacteria* and *Deinococcus-Thermus* (596 bp). Reconstructions based on neighbor-joint trees using HKY model, including bootstrap (10 000 permutations). Bootstrap support <70 omitted. OTUs from the present study in red. E, eggs; P, pleopods; SP, Snake Pit; TAG, TAG site

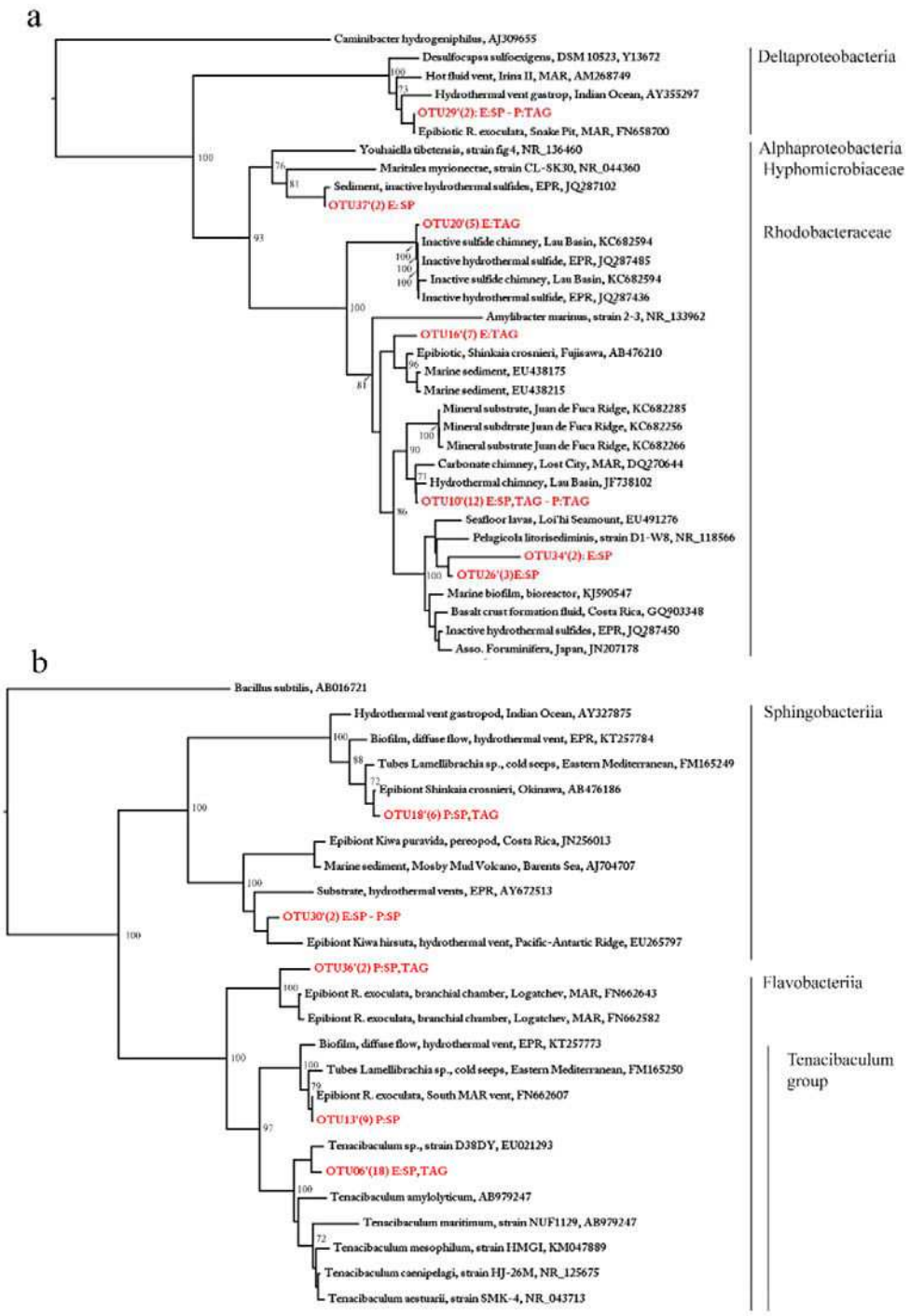


Fig. 3. Phylogenetic reconstructions of the 16S bacterial genes for the bacterial groups. a. *Alpha-* and *Deltaproteobacteria* (659 bp). b. CFB group (543 bp). Reconstructions based on neighbor-joint trees using HKY model, including bootstrap (10 000 permutations). Bootstrap support <70 omitted. Codes as Fig. 2.

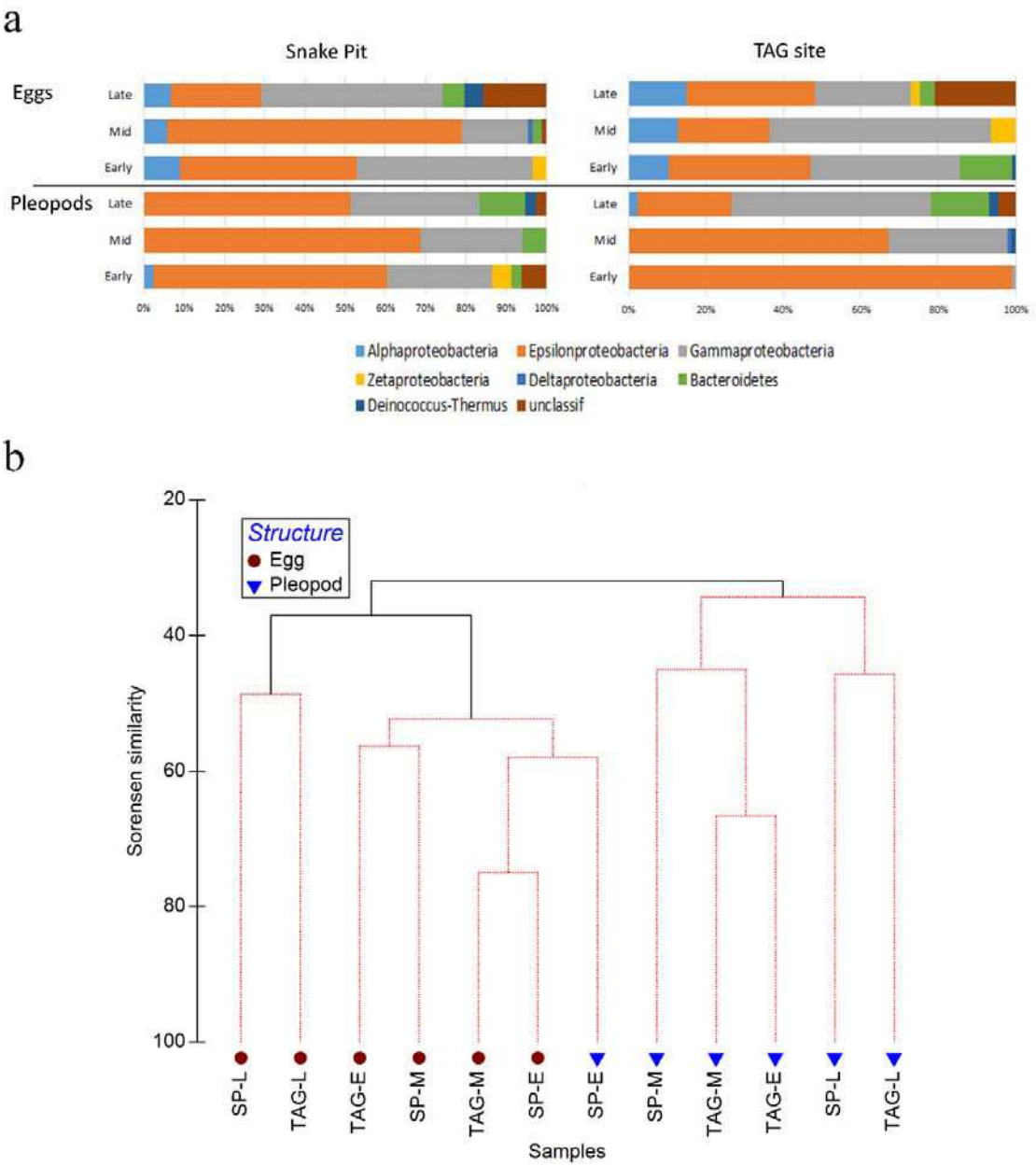


Fig. 4. a. Proportion of sequences by bacterial groups along the samples. b. Cluster analysis supported by the SIMPROF test solid lines denote groups with significant differences ($p < 0.05$), dotted red lines are not supported. Red dots, eggs; blue triangles, pleopods; SP, Snake Pit; TAG, TAG site; E, early stage; M, mid stage; L, late stage.

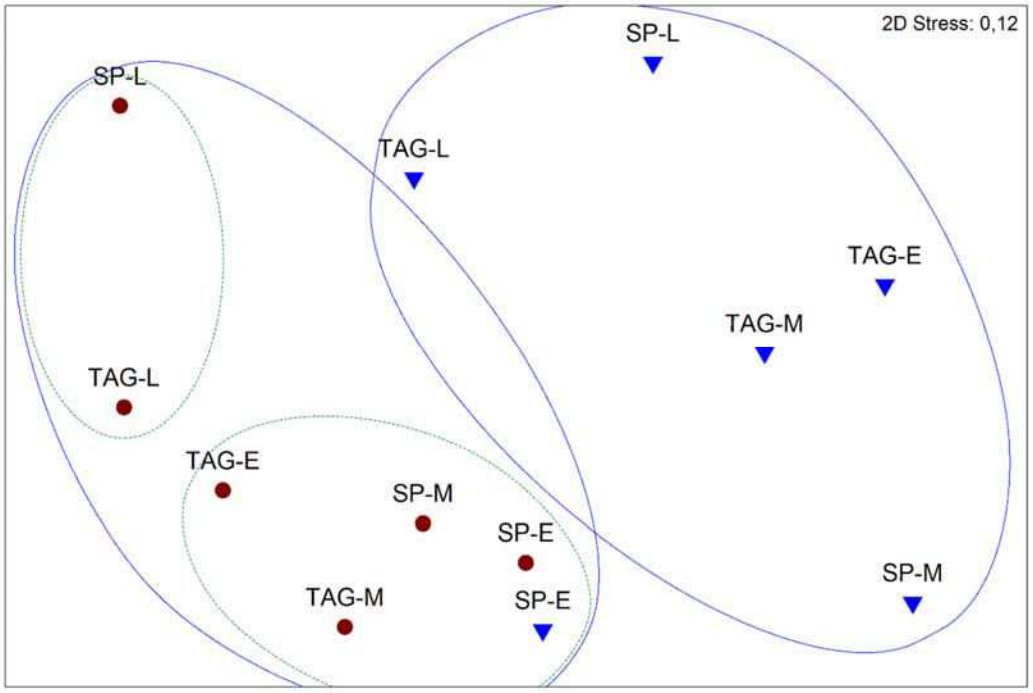
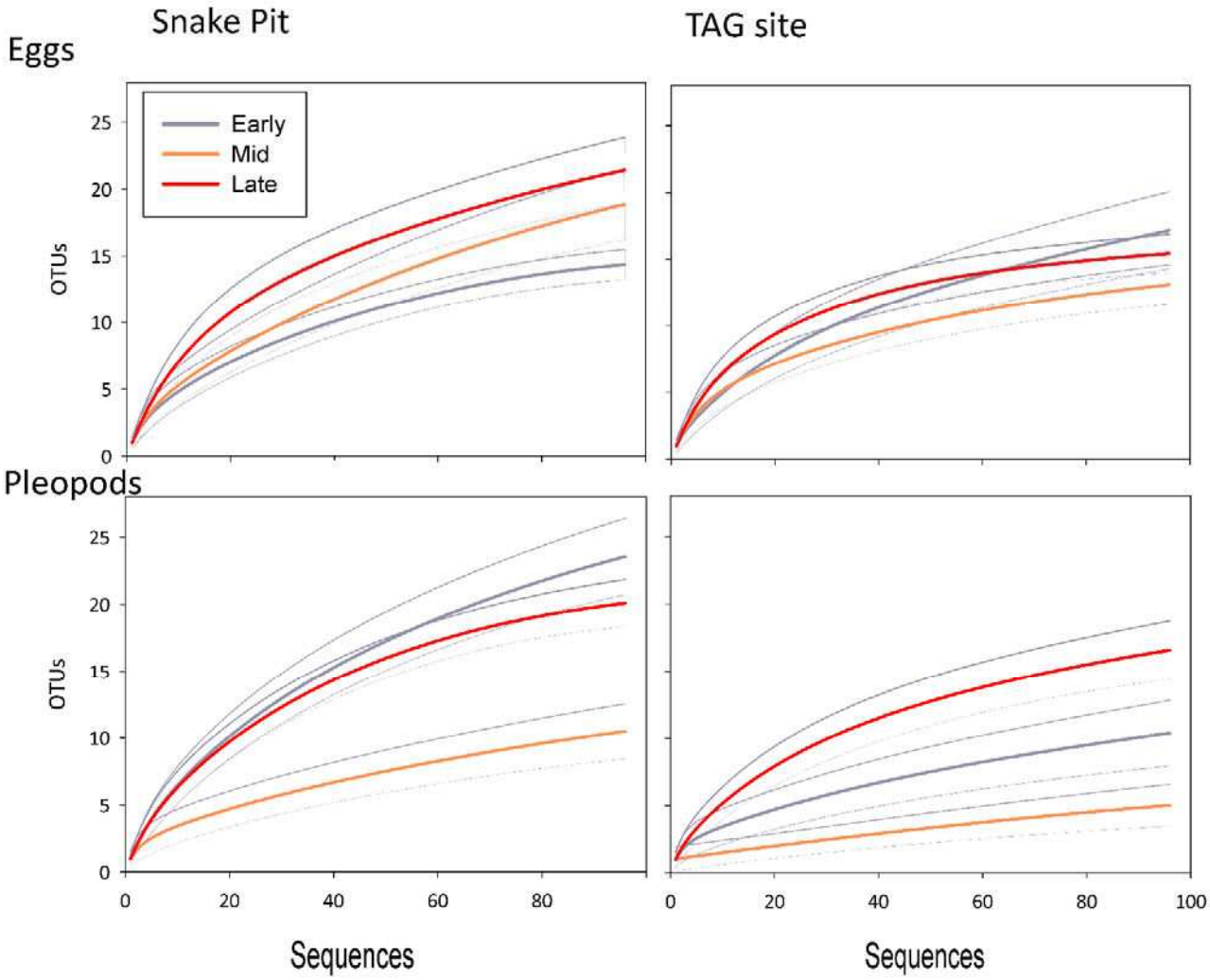
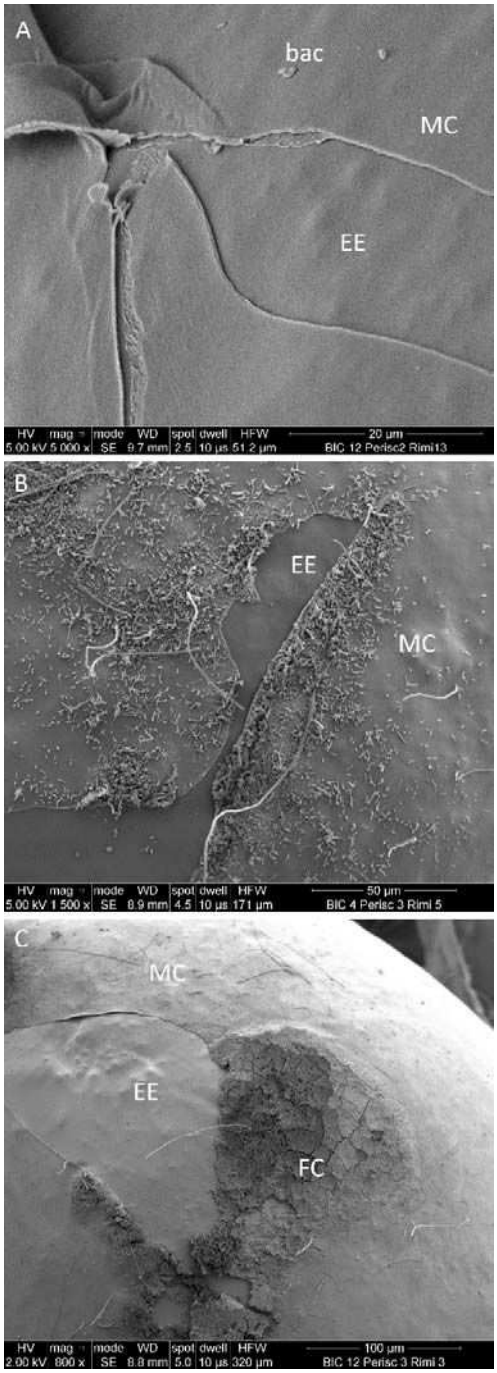


Fig. 5. Non-metric multidimentional scaling of sampling according similarity (Sorensen). Contours refer to the significant groups detected with the SIMPROF test. Codes as Fig. 4.



Supplementary material 1. Rarefaction curves of OTUs collected by sample. Dotted gray lines are confidence intervals of each estimation.



Supplementary material 2. SEM images of *Rimicaris exoculata* eggs, the pictures in areas with the mucus coat (MC) lacerated, showing the egg envelope (EE) deprived of bacteria. Eggs at early (a), mid (b) and late stage (c) stage from Snake Pit (b) and TAG (a,c). bac, bacteria; FC, ferric crust.

Chapter 4

Rimicaris hybisae/*R. chacei* species complex: complex life cycle or evidence of early speciation in hydrothermal vents?

Complexe d'espèces Rimicaris hybisae/R. chacei : cycle de vie complexe ou spéciation récente sur les sources hydrothermales

Synthèse

La dorsale médio-Atlantique et la fosse des Caïmans présentent des écosystèmes de sources hydrothermales profondes très intéressants avec certaines similarités dans la structure de leurs communautés faunistiques. Dans les deux régions, les crevettes *Rimicaris* dominent les habitats proches des émissions hydrothermales, *R. exoculata* sur la dorsale médio-Atlantique (dans les sources situées à plus de 2000 m de fond) et *R. hybisae* dans la fosse des Caïmans. Ces crevettes possèdent des bactéries symbiotiques et forment des agrégations de milliers d'individus par m^2 à proximité de la source hydrothermale. Des individus sont également trouvés dans différents habitats à la périphérie de la cheminée hydrothermale. Bien que les sites de la dorsale médio-Atlantique et de la fosse des Caïmans soient colonisés par des espèces proches (dans leur phylogénie), aucune espèce hydrothermale dominante n'est commune aux deux régions. Au sein de chaque région, le flux génétique entre les sources hydrothermales est important pour plusieurs espèces, dont *Rimicaris* spp. Pour ces espèces, il est supposé que la dispersion larvaire est importante entre les sources de la même région, bien que la colonisation entre les différentes régions ne soit pas détectée.

Cependant, les précédentes analyses phylogénétiques montrent que *R. hybisae* est très proche de *R. chacei*, une espèce qui habite la dorsale médio-Atlantique. Certains auteurs proposent que les deux espèces représentent une seule unité évolutive. La crevette *R. chacei* habite la périphérie des sources hydrothermales de la dorsale médio-Atlantique, et vit en populations dispersées (10 individus par m^2) (Fig. S1). La morphologie des deux espèces est très similaire, mais elles conservent des différences importantes dans le développement de la chambre branchiale et les structures de vision dorsales. Un résultat encore plus intrigant est l'observation d'un recrutement massif de petits juvéniles de *R. chacei* sur le site TAG (dorsale médio-Atlantique) durant la mission BICOSE (Janvier-Février 2014, Fig. S1b). Ce recrutement semble incompatible avec la faible densité des adultes de *R. chacei* et la très faible occurrence de femelles gravides observées depuis la description de l'espèce. Un recrutement massif peut être plus compatible dans le cas d'une espèce qui forme de grandes agrégations, avec une reproduction importante, comme c'est le cas pour *R. hybisae*. Déterminer si le complexe *R. chacei*/*R. hybisae* est une seule espèce ou deux

espèces séparées est nécessaire pour émettre des hypothèses sur les causes du recrutement massif observé sur le site TAG.

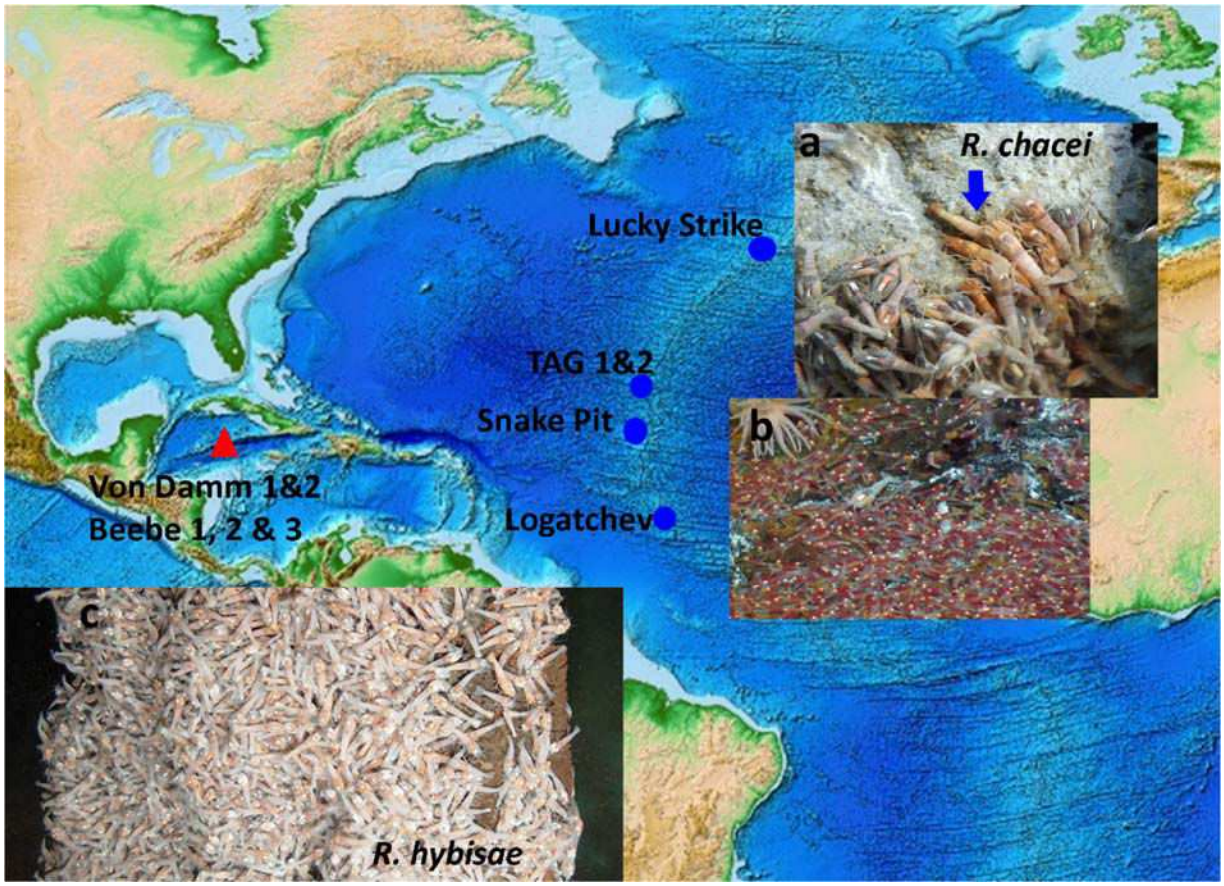


Figure S1. Distribution des prélévements de *R. chacei* sur la dorsale médio-Atlantique (bleu) et *R. chacei* sur la fosse des Caïmans (rouge). a) Adultes de *R. chacei* proches de l'agrégation de *R. exoculata* (site TAG), b) recrutement massif de juvéniles de *R. chacei* sur le site TAG, c) Agrégations de *R. hybisae* sur les édifices de la fosse des Caïmans.

L'hypothèse qui propose que *R. hybisae* et *R. chacei* font partie de la même espèce, ci-après appelée 'hypothèse de l'espèce unique', a plusieurs implications dans l'interprétation de la connectivité régionale des sources hydrothermales, le cycle de vie et la variabilité morphologique. Cette hypothèse implique que la dorsale medio-Atlantique et la fosse des Caïmans sont des écosystèmes hydrothermaux connectés par l'échange de cette mégafaune endémique et dominante. Cette 'espèce unique' domine la biomasse, formant de denses agrégations proches des sources hydrothermales de la fosse des Caïmans, mais vit en densité faible sur la dorsale médio-Atlantique. Selon cette même hypothèse, les différences

morphologiques de la chambre branchiale et de la structure dorsale de vision doivent être interprétées comme une expression phénotypique différentielle entre les deux régions. En revanche, si *R. hybisae* et *R. chacei* sont des espèces séparées (hypothèse « deux espèces »), cela implique qu'il n'existe pas de connectivité entre les différentes régions (la dorsale médio-Atlantique et la fosse des Caïmans). L'interprétation du cycle de vie et de la morphologie peut être considérée par deux modèles d'espèces séparés. Le niveau important de similarité génétique entre les espèces (dans l'hypothèse des deux espèces) peut s'expliquer par une spéciation récente, un taux de mutation faible ou un échange génétique durant le début de la spéciation.

Dans ce travail, nous explorons les données publiées (séquences COI), auxquelles nous ajoutons nos propres séquences, pour estimer laquelle de ces deux hypothèses, 'espèce unique' ou 'deux espèces', est la plus probable. Dans ce but, nous employons deux approches différentes : i) une analyse génétique populationnelle, en comparant la diversité génétique du gène cytochrome oxydase I (COI) entre les différentes populations de la fosse des Caïmans et de la dorsale medio-Atlantique ; ii) la reconstruction phylogénétique en utilisant deux gènes mitochondriaux : COI et 16S ribosomal (16S), et trois gènes nucléaires : 18S ribosomal (18S), 28S ribosomal (28S) et histone 3 (H3).

Pour l'analyse de génétique populationnelle, des séquences du COI ont été obtenues de spécimens de *R. chacei* récoltés sur les champs Snake Pit et TAG (dorsale medio-Atlantique, n= 79) durant la mission BICOSE. Nous comparons ces séquences à des séquences déjà obtenues par différentes études sur des spécimens de *R. chacei* provenant des sites Lucky Strike et Logatchev (dorsale médio-Atlantique, n= 175), et sur des spécimens de *R. hybisae* récoltés sur les sites Beebe et Von Damm (fosse des Caïmans, n= 193). Ce jeu de données a été analysé, et comparé avec les patrons de flux génétiques attendus sous les hypothèses d' « espèce unique » ou de « deux espèces ». Pour cela, nous avons effectué l'analyse du réseau d'haplotypes COI, l'estimation de la distance génétique entre populations (index de fixation, ϕst), l'analyse de la variation de l'index de fixation en fonction de la distance et les analyses de la variance moléculaire (AMOVA). De plus, une reconstruction phylogénétique a été effectuée avec cinq spécimens de *R. chacei* (4 adultes et 1 juvénile, des sites TAG et Snake Pit), deux spécimens de *R. hybisae* (adultes, sites Beebe et Von Damm), d'autres Alvinocarididés : *R. exoculata, Mirocaris fortunata, Alvinocaris chelis* et *Nautilocaris saintlaurentae*, et d'autres familles de crevettes (Caridés et Péneidés). La reconstruction phylogénétique a été réalisée avec les séquences concaténées des gènes COI, 16S, 18S, 28S et H3. Enfin, deux dates de calibration temporelle ont été inclues pour estimer la date de séparation hypothétique entre *R. chacei* et *R. hybisae*.

Bien que *R. chacei* et *R. hybisae* partagent certains haplotypes, le réseau d'haplotypes bien structuré (Fig. S2a) et l'indice de fixation entre les populations ne soutiennent pas l'hypothèse de « l'espèce unique », en particulier en comparant avec le profil typique de structure populationnelle des Alvinocarididés. De plus, sous l'hypothèse de « l'espèce unique », la variation de l'index de fixation en fonction de la distance n'est pas expliquée par les modèles qui prédisent une association par la distance (modèles « Stepping-stone » ou « Isolation-by-Distance ») ou les modèles qui prédisent l'absence d'association par la distance (modèle « Island » et connexes) (Fig. S2b). En revanche, la variation de l'index de fixation est cohérent avec deux modèles « Island », c'est à dire deux groupes de populations séparés par espèce, sans variation au sein de chaque groupe, ce qui favorise l'hypothèse des « deux espèces » (Fig S2c). Enfin, le test AMOVA confirme aussi une variation significative entre les espèces et non une variation entre populations au sein de chaque espèce.

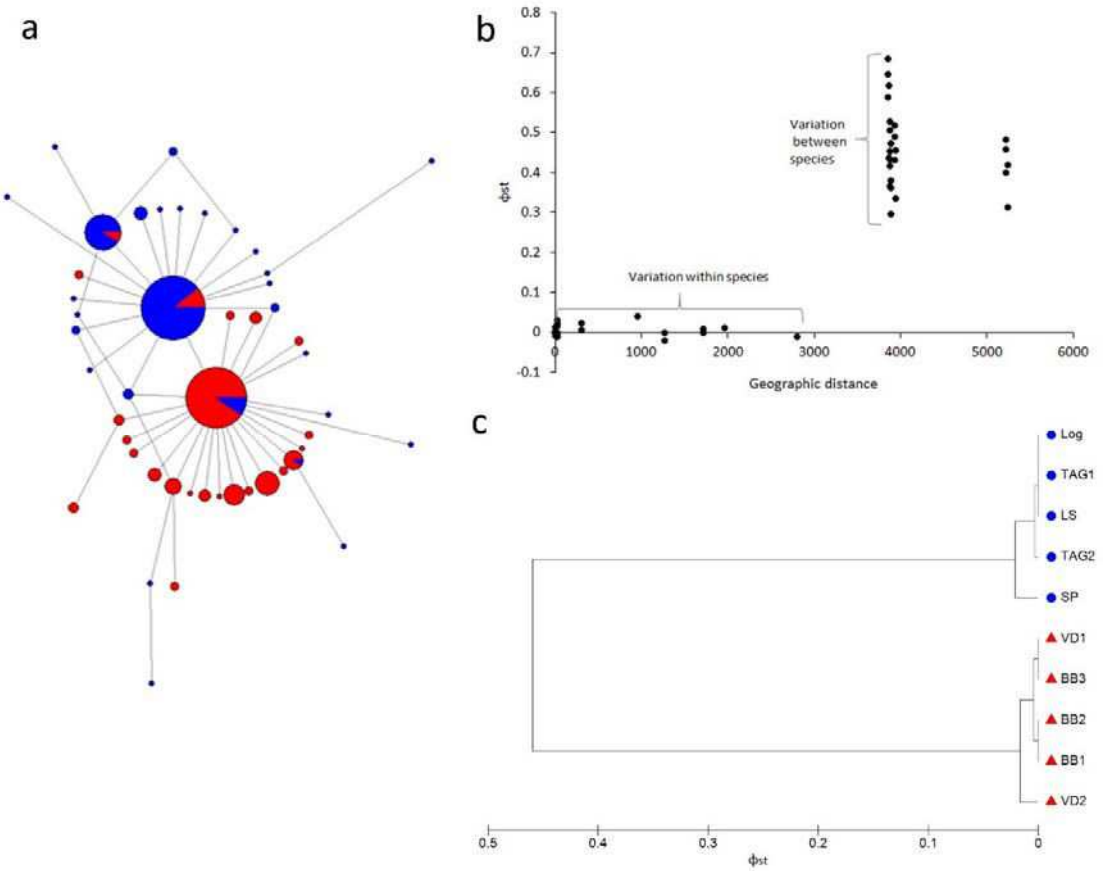


Figure S2. Génétique populationnelle de *R. chacei* et *R. hybidae* estimée avec le gène COI. a) Réseau d'haplotypes (bleu, *R. chacei ;* rouge, *R. hybisae*), b) variations de l'indice de fixation concernant la distance des populations, inclus la variation entre les espèces et au sein de la même espèce, c) Analyse de cluster entre les populations en relation avec l'indice de fixation (bleu, *R. chacei* ; rouge, *R. hybisae*)

La reconstruction phylogénétique avec les cinq gènes (mitochondriaux et nucléaires) et la calibration de la date permet la discrimination de *R. chacei* et *R. hybisae* (Fig. S3). La date du plus récent ancêtre commun entre les deux espèces a été estimée à 2.8±1.2 Ma, ce qui est récent en comparaison de la date de début de la radiation des Alvinocarididés (98±15.6 Ma). De plus, la distance proche entre *R. chacei* et *R. hybisae* détectée dans la reconstruction phylogénétique non-calibrée et la date estimée de l'ancêtre commun suggèrent un taux de mutation faible par rapport aux autres crustacés.

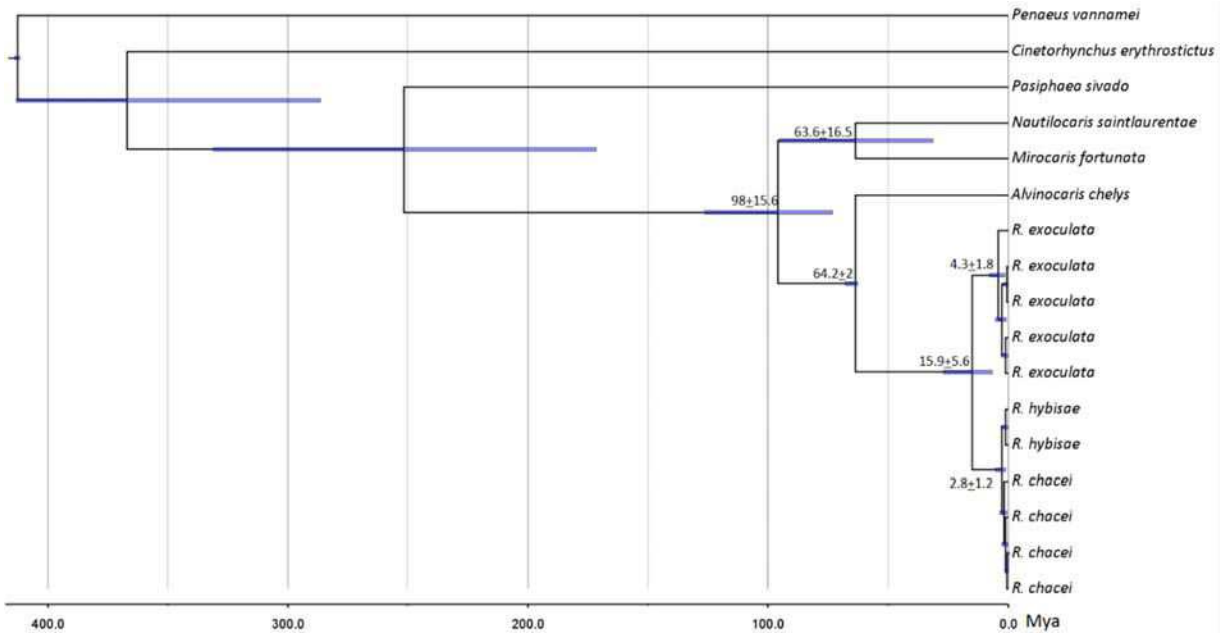


Figure S3. Reconstruction phylogénétique des Alvinocarididés, focalisée sur le clade *R. chacei*/*R. hybisae*, basé sur les gènes COI, 16S, 18S, 28S et H3. L'échelle temporelle s'exprime en millions d'années.

Les résultats favorisent l'hypothèse des « deux espèces », *R. chacei* qui habite en populations dispersées sur la dorsale médio-Atlantique et *R. hybisae* qui forme agrégations denses sur la fosse des Caïmans. Cependant, la distance proche entre les espèces et les haplotypes partagés entre les deux limitent sa discrimination en reconstructions phylogénétiques avec un seul gène, comme COI, 18S et 16S. Ces deux espèces semblent constituer de bons modèles pour l'analyse des processus évolutifs associés à la diversification dans les sources hydrothermales. Ce cas souligne l'importance de l'utilisation de différentes approches pour la délimitation d'espèces, surtout dans les complexes d'espèces.

L'observation de recrutement massif de *R. chacei* sur la dorsale medio-Atlantique, en dépit de la faible densité des adultes, peut s'expliquer par la structure génétique de ses populations. L'absence de structure génétique entres les populations de *R. chacei* est cohérente avec le modèle 'Island', qui assume que les migrants (dans ce cas, les juvéniles) viennent de populations différentes, en dépit de la distance. Comme les sources hydrothermales sont soumises à des événements d'extinction locale et de (re)colonisation (suite à la diminution de l'activité hydrothermale), il est approprié de considérer les modèles de métapopulation associés avec le modèle « Island ». Dans ce contexte, le patron génétique entre les populations de *R. chacei* ressemble au modèle « Migrant-pool » de Stalking. Ce modèle propose que le stade post-recrutement provienne d'un échantillonnage aléatoire de la métapopulation totale (le migrant-pool). Le 'migrant-pool' peut être représenté littéralement par un « larval-pool » généré par la contribution de chaque population. La durée larvaire prolongée des Alvinocarididés peut faciliter les processus d'accumulation des larves des différentes populations dans plancton. Enfin, l'agrégation de larves de différentes populations peut générer un recrutement massif comme observé sur le site TAG.

Un recrutement massif de juvéniles de *R. chacei* doit être compensé par une mortalité importante pendant la transition juvénile-adultes, pour aboutir à la densité populationnelle faible observée chez les adultes. Les vidéos enregistrées des agrégations de juvéniles durant la mission BICOSE montrent que les juvéniles sont affectés par la prédation des anémones hydrothermales (cf. *Maractis rimicarnivora*) et par le cannibalisme des adultes de *R. chacei*. Cependant, peu d'informations sont disponibles sur la biologie de *R. chacei*, et les hypothèses proposées doivent être considérées avec précaution en l'absence de preuves complémentaires. Plus de recherches sont nécessaires pour générer un modèle plus précis du cycle de vie et la dynamique populationnelle de cette espèce.

Rimicaris hybisae/*R. chacei* species complex: single species with complex life cycle or evidence of recent speciation in hydrothermal vents?

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Abstract

Phylogenetic analyses show that the vent shrimp *Rimicaris hybisae* from the Mid-Cayman Spreading Center (MCSC) is closely related to *R. chacei* on the Mid-Atlantic Ridge (MAR), suggesting that they could represent a single evolutionary unit. This hypothesis may deeply affect our interpretations of the connectivity between MAR and MCSC, as well as our understanding of the life cycle and phenotypic variations of these shrimps. It was also of particular relevance in a recent observation of massive *R. chacei* recruitment at the TAG vent field on the MAR, where local populations documented so far appear too scarce to supply sufficient numbers of recruits. We explored the genetic variation of *R. chacei* and *R. hybisae* in order to estimate if they belong to one or two separated lineages. For this purpose we compared the genetic diversity of the cytochrome oxidase I gene (COI) among different populations of the MCSC and MAR vents. In addition we performed time-calibrated phylogenetic reconstructions including two mitochondrial genes: COI, 16S ribosomal (16S), and three nuclear genes: 18S ribosomal (18S), 28S ribosomal (28S) and Histone 3 (H3). Although *R. chacei* and *R. hybisae* shared some haplotypes of the COI gene, the haplotype network and differentiation indexes ϕst between populations were not consistent with the single lineage hypothesis. In addition, the variation of ϕst with increasing distances between populations did not fit expectations of a single lineage under any of the standard models of populations (Island or Isolation by distance). Instead, the variation of ϕst was more consistent

with patterns expected from two island models in two separated lineages, which was also supported by AMOVA tests. Our phylogenetic reconstruction using multiple mitochondrial and nuclear genes and age calibration further indicate that *R. chacei* and *R. hybisae* belong to different lineages, which would have diverged from a common ancestor about 2.8±1.2 Ma ago. This very recent speciation event possibly combined to low mutation rates would explain the lack of genetic difference between *R. chacei* and *R. hybisae*. In addition, our result would support a migrant-pool metapopulation model, where the planktonic larval pool incorporates propagules from different source population, which would agree with our observation of massive local recruitment in *R. chacei*. Considering the contrasting ecological niche of *R. chacei* and *R. hybisae*, with the later species undergoing metamorphosis and transition to a symbiosis-based life style, and the co-ocurrence of *R. chacei* with *R. exoculata*, the ecological counterpart of *R. hybisae* on the MAR, the low genetic differentiation of R. *hybisae*/*R. chacei* raises a number of questions regarding the mechanisms involved in the acquisition of the symbiotic way of life.

Introduction

Since the discovery of the exuberant faunal assemblages developing at hydrothermal vents and cold seep ecosystems, species identification has been challenged by various phenomena, including bizarre morphologies species, cryptic species, aberrant life cycles, strange behavior or a combination of the above. In many cases, morphological delimitations of species has been hiding cryptic diversity, as in vesicomid clams [1], some polychaete worms [2], limpets [3,4] and gastropods [5]. On the other hand, some species show morphological plasticity, and the different morphotypes have been misinterpreted as different species. Eventually, however, these morphotypes have been recognized as taxonomic synonyms belonging to the same species [6,7]. In addition, some species have complex life cycles that involve juvenile stages morphologically different from their adult counterparts, and that inhabit different microhabitats [8,9], further increasing the problem of species identification in these ecosystems. The introduction of genetic approaches has improved considerably the species delimitation problem above [10]. Still, coupling genetic approaches with standard morphological taxonomy into an 'integrative taxonomy' approach to define species is not always a straightforward path [11]. Particularly, cases involving species complexes must be addressed carefully because species delimitation could have a pivotal effect in our perception of the deep-sea biogeography, connectivity, population genetic

structure, community structure, colonization and overall ecological and evolutionary dynamics of these systems [12-15].

A taxon in which species delimitation is indeed a complex problem is the family Alvinocarididae within the species rich and ecological dispar infraorder Caridea [16]. Alvinocaridid shrimps inhabit deep-water hydrothermal vents and cold seeps around the world, and some of these species can dominate the biomass of these ecosystems, particularly at hydrothermal vents [17]. Since the description of the first species in 1982 [18], there has been a long debate about the evolution, phylogenetic relationships, and the concept of the species in these shrimps. Although the monophyly of the family Alvinocarididae is well supported, its phylogenetic placement within the infraorder is still not fully resolved [19,20]. Early hypothesis placed the origin of alvinocaridid shrimps during the Miocene (6.2-9.9 Ma) [21], but current estimations on the age of this clade based on molecular characters trace their origin back to the period Late Mesozoic-Early Cenozoic (51.5-69.7 Ma) [22]. Phylogenetic reconstructions support the division of this family in three clades, recognized as subfamilies, Mirocaridinae, Alvinocaridinae and Rimicaridinae [7]. However, the position of some species in these clades and the recognition of several species as 'true' species is a point of debate [7,12]. The identification of species based only on morphological traits has been problematic in this group. In some cases, morphological variations between adult and juvenile stages have been confounded with species level variation [6,9], or with phenotypic variation in adults stage [21]. In other cases, nearly cryptic phenotypes with allopatric distributions are recognized as different species [7,12]. Although most of these cases have been resolved with the implementation of phylogenetic reconstructions based on genetic sequences, some complicated clades remain with species complexes [7,12].

Additional problems of species identification are concerning early stages of alvinocaridid species. One remarkable case is the identification of juveniles of *Rimicaris* shrimps inhabiting the Mid-Atlantic Ridge (MAR), in particular, the distinction between the most common species, *R. exoculata* and *R. chacei*. Early papers had described juvenile shrimps as different species (*Iorania concordia* and *R. aurantiaca*) [23,24]. Posterior phylogenetic reconstructions of cytochrome oxidase I (COI) sequences recognized these juveniles as *R. exoculata* [25]. More recently, Komai and Segonzac [8] described four juvenile stages (A, B, C, D or subadults) in *Rimicaris*, and proposed morphological differences between juveniles of *R. exoculata* and *R. chacei*. However, we recently compared these morphological characteristics to molecular COI data in stage A juveniles and found that they were indeed not reliable to identify species in these young stages [Hernandez-Avila *et al*. in prep]. This lack of reliable

morphological characteristics in early planktonic [26,27] or recruited [25] stages complicates our understanding of alvinocaridid larval life and recruitment processes because insights gained from field sampling, such as larval nutrition based on lipid profiles [28], or larval orientation based on eye morphology [29], cannot be attributed to a species. Molecular approaches thus cannot be circumvented to distinguish species in early life-stages [30], and accurately decipher the life cycle of vent species, especially when close relatives with contrasting ecology coexist.

In January 2014, we observed discrete but dense (100s individuals.dm^{-2}, figure 1) aggregations of small bright red juvenile alvinocaridid shrimps around low temperature diffusion at the periphery of the main vent emission of the TAG vent field, which we named "nurseries". They were not close to dense adult populations, but few scattered adults of both *R. exoculata* and *R. chacei* were crawling among these juveniles. Initially, because their high densities reflected the dense *R. exoculata* populations observed at TAG, as well as the high recruitment levels expected from the high connectivity found in this species [12], we expected that juveniles in these nurseries were *R. exoculata*. However, after closer examination of their morphology (based on characteristics reported in Komai and Segonzac [8]), the juveniles were identified as stage A belonging to both *R. exoculata* and *R. chacei*. Unexpectedly, COI sequencing finally revealed that the nurseries harbor almost exclusively *R. chacei*. These nurseries were different from the juvenile aggregates observed close to dense adult population on vent chimney walls, such as those reported by Shank et al. [9] and also observed during our cruise in 2014, which harbor stage B *R. exoculata* juveniles [Hernandez et al, in prep]. These dense aggregations of *R. chacei* recruits were puzzling considering the low densities of adults of this species (<10s ind.m^{-2}) and the few brooding females observed [31]. This contrast in abundance of juvenile stages compared to their adult counterpart might be explained either by a different, yet unidentified location of the main part of the adult population, or by high post-recruitment mortality in juveniles which would result in a drastic reduction of the local adult population.

Recent molecular studies suggested that *R. hybisae*, a species forming high density populations at the Mid-Cayman Spreading Center (MCSC) might be genetically identical to *R. chacei* [7,12,30]. If these two shrimps are indeed one species, then the dense populations at the MCSC might be the source of the abundant recruits we observed on the MAR. *R. hybisae* is one of the dominant taxa of the vents at the MCSC, between 2300 m and 4960 m depth, where neither *R. exoculata* nor *R. chacei* occur [32,33] (Fig. 1). This species lives both in dense aggregations (1000s ind.m^{-2}) close to the vent emissions or scattered at the periphery of the vent chimney [33] thus occupies a similar ecological niche to *R.*

exoculata on the MAR. In addition, as *R. exoculata*, *R. hybisae* have an enlarged gill chamber that hosts epibiotic bacteria that could provide their host with organic carbon from chemosynthesis [34,35], have a reduced stomach, and shown episymbiotic bacteria in its gut [36](Durand per. com.). Although *R. hybisae* is phylogeneticaly more closely related to *R. chacei*, it appears to be much more similar to *R. exoculata* in terms of biology and ecology. *R. hybisae* is also quite similar to *R. chacei* morphologically, despite conspicuous external differences in the size of the branchial chamber, occurrence of pores in the carapace and the shape and position of adult light-perception structures [32]. These morphological differences might well be phenotypic plasticity, as well reported phenomenon in deep-sea marine invertebrates [10]. Given the contrasting ecological characteristics and distribution patterns in Atlantic *Rimicaris* shrimps, there is a need to further examine their genetic identity and assess the status of the complex *R. hybisae*/*R. chacei* as one or two distinct species, since this would have different implications regarding their dispersal potential, connectivity, life-cycle and evolution.

Considering *R. chacei* and *R. hybisae* as the same species assumes that populations in the MCSC and MAR vent systems are exchanging migrants and maintain genetic homogeneity. This 'single species hypothesis' for the complex *R. chacei*/*R. hybisae* implies contrasting ecological profiles at the two geographic locations, with shrimps at the MCSC forming dense aggregates (1000s ind.m^{-2}) on chimney walls close to fluid emissions, whereas at MAR vent fields they would be restricted to low density patches (10s ind.m^{-2}) located more at the periphery of actively venting areas. The 'single species hypothesis' would also implies that morphological differences between shrimps at the MCSC and on the MAR indeed reflect phenotypic variability perhaps driven by the use of a different ecological niche. The shrimps would then have a quite complex life cycle, with a strong dispersal potential bridging two locations 4000 km apart, and exhibit alternative ecological niches that could be due to competition with *R. exoculata* on the MAR.

If *R. chacei* and *R. hybisae* are two distinct species, their separated distribution range and their differences in ecology and morphology would be the result of recent evolutionary processes such as allopatric speciation. Although recent genetic evidences advocate against the 'two species' hypothesis [7,12,30], they were not totally conclusive. The lack of genetic discrimination in COI gene could reflect sister species that arose from a recent speciation event and did not accumulate discriminative mutations in particular genes. Differences in gene sequences between two species usually arise from the interaction between the time of speciation event and the mutation rate of the gene. If the mutation rate of the gene is low or if there is a recent speciation event in the clade, the result of the phylogenetic

reconstruction may not separate closely-related species, particularly those generated recently [37]. Also genetic exchange early or even late in the speciation process could prevent the generation of distinct species lineages in phylogenetic reconstructions in more complicated models, as admixture or restricted genetic flow between related species [38,39].

In order to elucidate the massive recruitment of *R. chacei* observed at TAG, we investigated the hypothesis that *R. hybisae* and *R. chacei* are the same species, by further exploring genetic separation between shrimps from the MCSC and MAR. For this purpose we implemented two different approaches: 1) we compared the genetic diversity of the COI gene among different population of the MCSC and MAR vents, including newly recruited juveniles from TAG; 2) we performed phylogenetic reconstructions using five different genes, two mitochondrial (COI, 16S) and three nuclear genes (18S, 28S and Histone 3).

Materials and Methods

Data sets and collection of samples

Collections of adults and juveniles of *R. chacei* were carried out during the BICOSE cruise (Jan-Feb 2014) using a suction sampler attached to the ROV Victor 6000 in different locations of the Snake Pit (23.4°N 45°W) and TAG vent fields (26.1°N 44.8°W) at depths between 3468-3637 m (Table 1). Samples of *R. chacei* were collected beside of the vent emission in mussel beds and in nurseries of small juveniles. In addition five specimens of *R. exoculata* were selected from collections taken in the dense aggregations close to the vent emissions in both vent fields. On board of the *R/V Porquoi pas?* the samples were sorted and fixed in 96% ethanol or frozen at -80 °C until laboratory analysis.

In order to compare the genetic variation between *R. chacei* and *R. hybisae* we analyzed two different data sets. The first data-set includes a compilation of sequences of the cytochrome oxidase subunit 1 mitochondrial gene (COI) of *R. chacei* and *R. hybisae* from three sources: 1) sequences of *R. chacei* (n= 96) from the Logatchev (14.7°N 45°W) and Lucky Strike (37.3°N 32.3°W) vent fields at the MAR, obtained from Teixeira et al. [12]; 2) 193 sequences of *R. hybisae* from the Von Damm Spire (18.38°N 81.8°W) and Beebe vent field (18.55°N 81.72°W) at the MCSC, from Plouviez et al. [40]; 3) sequences of *R. chacei* (n= 84) obtained from the shrimps collected during the BICOSE cruise on the MAR at two locations in the TAG vent field vent field and scattered specimens collected at the Snake Pit site.

The second data set consists in five specimens of *R. chacei* selected from the BICOSE cruise collection at the TAG site vent field and Snake Pit site, and two specimens of *R. hybisae* collected at the Von Damm and Beebe sites in the MCSC in 2010 by the R/V *Atlantis* (gently provided by C. Van Dover). In this set of samples, genomic extraction and gene amplifications were performed in order to obtain partial sequences of two mitochondrial genes (COI and 16S ribosomal) and three nuclear genes: two ribosomal (18S and 28S genes) and one coding gene for histones (H3). In order to reveal the placement of the two studied species within the genus and family to which they belong, we selected other alvinocaridid species. *R. exoculata* (5 specimens) was included in the analysis because there is evidence that this species has a relatively close phylogenetic position to *R. chacei*/*R. hybisae* [7,21,32]. *Alvinocaris chelys* was included as a member of the subfamily Alvinocaridinae, and both *Mirocaris fortunata* and *Nautilocaris saintlaurentae* were included to represent the subfamily Mirocaridinae. Three other species from different shrimp families were included as outgroups in the phylogenetic reconstructions (Table 1). Also sequences for the genes COI, 16S and 18S were obtained from the description of *R. hybisae* [32] to compare with the corresponding genes in our second data set.

DNA extraction and sequencing

DNA was extracted from abdominal tissue, mouthparts or pleopods using the CTAB method (cetyl trimethyl ammonium bromide [41]). Gene amplifications were obtained through polymerase chain reactions (PCR) conducted using a GeneAmp PCR system 9700 (Applied Biosystems), with specific sets of primers, solutions and thermal cycles for each gene fragment detailed in Table 2. PCR products were purified and Sanger sequencing were produced by Macrogen, Inc. (Netherlands). Sequencing was performed using the same primers as those used for PCR amplification for each gene, except for the 18S gene which was sequenced with the following primers: 18S-3F, 18S-bi, 18S-1F, 18S-5r, 18S-1373F, 18S-505r [42-44].

Alignment and Data Analysis

Variation of the COI gene fragment in populations of *R. chacei* and *R. hybisae*

Sequences of COI of *R. chacei* and *R. hybisae* were aligned using MUSCLE [45] and verified manually in order to correct minor inconsistencies. COI sequences from published studies varied in

length or location along the gene. We therefore focused our analysis on the portion of the gene that was common for all studies and sequences were trimmed to a 450 base pairs section of the COI gene for the alignment. As expected, this procedure eliminates information compared with studies that use longer COI sequences [40], and homogenizes some of the haplotypes identified in previous analyses. Indeed, this approach did reduce to a third haplotype diversity previously identified with the same data set for MCSC [40]. Therefore we verified that this trimming procedure did not have a significant effect on our estimation of the genetic structure of the populations in this case (see results).

The haplotypes were identified and sorted using DnaSP [46]. To compare genetic diversity between populations and species we estimated the number of haplotypes (nh), the haplotype (h) and nucleotide (π_2) diversities [47], and the mean number of pairwise differences between populations [48]. Mutation-drift equilibrium was tested using the Tajima *D index* [48] and the Fu *Fs-Test* [49] for each population, in order to detect the occurrence of selective or demographic processes and their variation between populations or species. Although population genetics of both species were already described [12,40], we repeated these analyses in order to verify the consistency of our estimations, the effect of reduction of sequence length for *R. hybisae,* as well as and to include new information about the population of *R. chacei* sampled at TAG and Snake Pit.

Since *R. chacei* and *R. hybisae* share some COI haplotypes, it is important to estimate the proportion of shared haplotypes and the relevance of those haplotypes (in terms of haplotype frequency). In order to visualize the most parsimonious branch connections between the haplotypes, median-joining network was created using Network 5 [50]. In order to represent the distribution of haplotypes among the populations, the proportion of specimens of each population were represented as pie chart in each haplotype node.

To address the species and population differentiation we estimated the fixation index (ϕ_{ST}), following Hudson *et al*. [51], between species/populations pairwise combinations. In addition, exact tests of differentiation proposed by Raymond & Rousset [52] were conducted between species and populations. Samples of the populations under study were collected at different spatial scales between 0.01s to 1000s km, from 0.01 to 10s km for *R. hybisae* and from 0.01 to 1000s km for *R. chacei*. Assuming the single species hypothesis, the variation of the ϕ_{ST} between populations would reflect some of the different models of population gene flow identified in vent fauna [53]. These models describe two main patterns of genetic variation, showing correlation with geographic distance between populations (Stepping-Stone or Isolation-By-Distance) or not (Island models).

Stepping-Stone and Isolation-by-Distance models predict an increase of the genetic variation between populations separated by increasing distances. In these models, levels of the genetic exchange increase in neighboring populations compared to more distant populations which accumulate more genetic variation. These models are usually associated with species exhibiting limited dispersal in natural populations. Examples of Stepping-Stone Process or Isolation-By-Distance in hydrothermal vent species are found in the vestimentiferan tubeworm *Riftia pachyptila* [54], the vetigastropod *Lepetodrillus elevatus* [4] and vent amphipods [53]. On the other hand, the Island Model predicts no association between the genetic distance and the geographic distance between populations. Under this model, a wide dispersal of migrants allows high level of connectivity between neighboring and distant populations. In addition, the Island Model has derivate models according to the occurrence of extinction and foundation in the populations (ie Migration-pool, Propagule-pool or Metapopulation coalescence). Cases of genetic structures consistent with an Island Model in hydrothermal vents are found in alvinocaridid shrimps, *Bathymodiolus* mussels and *Calyptogena* clams [53,55].

Under the 'two species' hypothesis, significant genetic distance is expected between populations of different species, might their populations lie in close or distant geographic range. In addition, gene flow restriction between species would be reflected by a gap between the ϕst index estimated between populations of the same species and ϕst index estimated between populations of different species, which would not be explained by the distance between populations. In this case, each species can show additional variation in the genetic distance associated with their corresponding models of genetic flow.

In order to test if the genetic distance between populations was associated with the geographic distance, we performed a Mantel permutation test using IBD software [56], contrasting the matrix of genetic distance between populations (expressed as ϕst) and the matrix of geographic distance between populations. In addition, an indicator matrix associated with the species was included. The geographic distance was estimated using Geographic Distance Matrix Generator [57] using vent field coordinates. In order to estimate the significance of the Mantel test, 10 000 permutations were performed. The same analysis was performed including only *R. chacei* in order to compare the results of the Mantel test obtained using a single species with those estimated with all populations, at the same spatial scales (0.01 to 1000 kms).

We are aware that the isolation by distance analysis considers only the minimum straight distance between the populations, and ignores the occurrence of topographic barriers, depth variations,

unidentified vent fields, vent rising plumes and the channeling effect of the ridge axial valley between the vents, oceanographic currents and dispersal mechanisms of the species. However, alvinocaridid shrimps populations are able to maintain high genetic flow over 1000s km [12,55] and colonize cold seeps and hydrothermal vents from the eastern Atlantic, MAR and Gulf of Mexico [12]. Moreover, they exhibit similar biological traits having a lecithotrophic larvae with extended development [30], suggesting similar potential for wide dispersal, and ability to colonize hydrothermal vents in a wide range of depths (see introduction).

A cluster analysis based on the matrix of genetic differentiation (ϕst) was performed using Primer-Permanova v6 [58] to visualize the assemblage of populations, based on their fixation index and their association with the species. In addition, to test if the variation in ϕst is associated to the occurrence of two species, we perform an analysis of molecular variance (AMOVA, [59]). The former analysis determines the genetic variation associated between populations of the same species, the variation between species and the residual variation (within populations). Although the groups of populations between species are the same than the groups by vent systems (MCSC vs MAR) and the systems are widely separated, exclusion of geographical factors is supported by the isolation-by-distance analysis. The analysis AMOVA was computed using Arlequin ver 3.5.2 [60] using 10 000 permutations to determinate the significance of the statistic.

Species discrimination using multiple genes

To perform phylogenetic reconstructions, alignments of each gene (COI, 16S, 18S, 28S, H3) were conducted using MUSCLE and verified manually. Consistency in the phylogenetic reconstruction of each gene was verified using neighbor-joining tree implemented in Geneious [61], supported by 10 000 bootstraps. Then, sequences were analyzed in a single phylogenetic reconstruction [62] based on the following aligments: COI (561 bp), 16S (496 bp), 18S (1869 bp), 28S (755 bp) and H3 (302 bp). The evolutionary models for the phylogenetic reconstruction and their parameters were estimated using Model Generator [63] according to the Bayesian criteria for each gene. The models selected for each gene were: the generalized time reversible model (GTR) for COI, the Hasegawa, Kishino and Yano model (HKY) for the 16S gene, and the Tamura-Nei model (TrN) for the 18S, 28S and H3 genes. In all cases, we applied a gamma distribution of substitution rates. Phylogenetic trees were estimated using Bayesian Inference (BI) in BEAST version 1.8 [64], and $4x10^8$ generations of trees were calculated with sampling

each 5000 generations. The first 20% of the trees obtained from the analysis were discarded, the rest of the trees were summarized using Treeannotator [64] to estimate the posterior probabilities. In addition, a Maximum-Likehood (ML) tree was constructed, considering the GTR+G model, using PHYML [65]. The opology of the ML tree was obtained by 1000 bootstraps. Robustness of the nodes for the inferred trees was evaluated using posterior probabilities for the BI and bootstraps proportions for the ML tree.

Time calibration was included in the previous phylogenetic reconstruction in order to estimate the date of the most recent common ancestor for the *Rimicaris* species studied here. Two calibrations were used according to the estimations of Yang et al. [22], which are based on multiple fossil records. The first calibration was included in the root, considering the date of separation between penaeids and carideans shrimps 411.4 Ma ago (also consistent with Ma et al. [66]). The second calibration was included in the node *Alvinocaris*+*Rimicaris* (62.5 Ma). Although there is concern about the phylogenetic position of some *Alvinocaris* species, there is a consensus that Alvinocaridinae and Rimicaridinae are reciprocally monophyletic taxa [7,21,30]. These calibrations permit an estimation of the date of origin of Alvinocarididae (including the clade Mirocaridinae), and of the divergence times between the three species of *Rimicaris* included in the present analysis. To estimate the effect of the calibration of the node *Alvinocaris*+*Rimicaris* (for instance, time constrains in the date estimation), a phylogenetic reconstruction was performed using only root calibration. Dating estimations were performed using uncorrelated log-normal clock model [67].

Results

COI diversity and differentiation in the *R. chacei/hybisae* complex

A total of 48 haplotypes were identified from the 368 specimens of both species. Most of the haplotypes were singletons (64.6 %) or doubletons segregated in a single species (10.4 %). However, two haplotypes were common in all populations of both species and were also the most abundant haplotypes, representing 65.7 % of the individuals. In addition, two other haplotypes were present in populations of both species representing 11.8 % of the individuals. Haplotype diversity was high in most populations, however both the pairwise differences (π_1) and the nucleotide differences (π_2) were low. The former indicates that the populations show a large number of haplotypes but they are defined by few mutations (Table 3). As expected from the high number of haplotypes and the low level of pairwise differences, both Tajima (D) and Fu (Fs) tests had significantly negative scores, suggesting a scenario of

expansion of the population after a bottleneck event. However in two MAR populations (Logatchev and Snake Pit) the neutral model was not rejected, probably due to the low sampling effort and the low number of haplotypes observed in these populations.

Despite the occurrence of shared haplotypes, the network plot shows a segregation of the haplotypes according to species and vent system (Fig. 2). The general structure is represented by three main haplotypes present in almost all populations of both species. Most of the additional haplotypes are separated from the two main haplotypes by few mutations (mostly one or two), forming a double star-like configuration. The two main haplotypes are separated by one mutation. One section of the network is dominated by the haplotypes from populations of *R. hybisae* at the MCSC whereas haplotypes from the populations of *R. chacei* from the MAR prevail in the other section of the network. Fixation indexes (ϕst) confirms the segregation of haplotypes by species observed in the haplotype network configuration. Populations within each species did not show any evidence of genetic differentiation (ϕst test, $p>0.05$ in all cases). However, there is a genetic differentiation between populations from different species (ϕst between 0.297 and 0.685, $p< 0.001$ in all cases).

The Mantel test between the fixation index matrix and the geographic distance matrix using indicator species matrix shows a significant correlation between the fixation index and the species indicator, controlled by the geographic distance (Mantel test, $r= 0.994$, $p= 0.006$). This suggests that the differences between populations are rather associated with their assignation to a different species than to the geographic distance between populations. The pattern of variation of ϕst according to the geographic distance was not progressive. Instead, a steep increase was associated with the change of species (Fig 3a). The scores of the fixation indexes between populations of *R. hybisae* were similar to those between the populations of *R. chacei*, despite the fact that the distances between the populations at the MCSC is at scale from 0.01 to 10s km and the distances between populations at the MAR vent systems is at the scale of 0.01 to 1000s km. The Mantel test calculated using only the populations of *R. chacei* did not show a correlation between ϕst and the geographic distance. Both analyses suggest that the differences between populations of *R. hybisae* and *R. chacei* are not associated to an isolation by distance process assuming a single species model, and support the hypothesis of both taxa being separate evolutionary units.

Average clustering of the populations based on their genetic differentiation shows two consistent groups separating the two species (Fig 3b). This supports the previous pattern observed with the isolation by distance approach. This finding also supports the absence of genetic structure among

populations of each species, with a panmixic distribution of the haplotypes within each species. Although the clustering approach does not involve a phylogenetic reconstruction, it clearly shows the distinction between *R. chacei* and *R. hybisae* based on genetic differentiation. The analysis of the molecular variance of the COI gene also confirms that genetic variation patterns are associated with the species (Table 4). Other important source of genetic variation was found within populations, whereas the variation between populations was not significant. Although the species share major haplotypes and the non-shared haplotypes are very similar, there is a significant genetic differentiation between the species, which is detected by the genetic structure of the populations. However, this high similarity in the COI gene precludes its use for species identification purpose in *Rimicaris* shrimps.

Species discrimination using multiple genes and divergence time estimation

Our phylogenetic tree based on 5 genes (Fig. 4) is consistent with previous results and supports the monophyly of the Alvinocaridid family, the branching topology within Alvinocarididae, including the separation of the clade Alvinocaridinae/Rimicaridinae from Mirocaridinae and the basal position of the later taxa [7,21,30]. The phylogenetic reconstruction shows a clear separation between *R. exoculata* and the other *Rimicaris* species herein studied, but very short distance was observed between *R. chacei* and *R. hybisae* (Fig 4). However, the node that separes the specimens of *R. chacei* and R. *hybisae* is well supported, and the nodes within these clades had low support (BI posterior probability ≤ 0.65, ML boostraps proportion < 60). Although the specimens of *R. hybisae* and *R. chacei* are separated in different clades and the basal node is well suported, the distance between the clades is very short.

A better resolution in the distal secction of the phylogenetic reconstruction is obtained with the inclusion of time calibration in the evolutionary model (Fig. 6), with a better distinction between the clades of *R. chacei* and *R. hybisae*. Dating estimation places the origin of Alvinocarididae in the cretaceous (98±15.6 Ma) and the origin of both clades Mirocaridinae and Alvinocaridinae at early cenozoic (around 64 Ma). *Rimicaris* species herein studied shared the last common ancestor during the miocene (15.9±5.6 Ma). For the clade *R. chacei*/*R. hybisae*, we estimate a date for the last common ancestor at 2.8±1.2 Ma. Phylogenetic reconstruction using time calibration only in the root of the three bring similar estimation dates. However, higher standard deviations and confidence intervals were observed (supplementary material). These results permit to assume that the calibration in the clade

Alvinocaris/*Rimicaris* based on previous studies did not constrain the date estimation but contribute to the estimation of more precise dates.

Discussion

R. hybisae and *R. chacei* belong to distinct evolutionary units

The descriptive statistics of population genetics of the Alvinocaridid shrimp species studied here were in agreement with previous analyses conducted on similar databases [12,40]. Our results confirm that the reduction of the alignment length from the sequences of *R. hybisae* obtained by Plouviez et al. [40], or the inclusion of additional populations to the data set of *R. chacei* analyzed in Teixeira et al. [12] did not have an effect on the estimation of connectivity and population structure of both species in their respective vent systems. However, the reduction of the aligment length by a third when triming the sequences from Plouviez et al. [40] resulted in the merging of a third of the haplotypes intially reported, thus affecting the resolution of genetic diversity. The lack of divergence between the two taxa in phylogenetic reconstructions using COI gene was also supported by previous analyses, although the same fragment of the COI gene considered in the presented study permit the discrimination of most other species of the family Alvinocarididae [7,12,30]. Moreover, shared haplotypes between *R. chacei* and *R. hybisae* were also observed with longer COI fragments, as well as in partial sequences of 16S and 28S genes ([32], and present study).

The populations of both *R. chacei* and *R. hybisae* show similar characteristics: high haplotype diversity, low nucleotide diversity and negative scores in mutation-drift equilibrium. In addition, for each species the distribution of the haplotypes is panmixic, without any genetic structure along their respective distribution ranges. Alvinocaridid shrimps usually show high genetic flow between populations. A lack of population structure has been reported in *Alvinocaris* [68], *Shinkaicaris* [68] and *Rimicaris* [55,69-71] based on COI gene and/or microsatellite approaches, across different ecosystems (hydrothermal vents or cold seeps), habitats, or different spatial scales in species distribution. The only known case of significant variation of the fixation index associated to geographic locations in alvinocaridid shrimps [12], using COI and microsatellites, indeed appears to reflect differences in local ecosystems (hydrothermal vents vs cold seeps) rather than geographic distance between populations.

Our analysis of the genetic structure between populations of *R. chacei*/*R. hybisae*, revealed variations that are more consistent with the pattern expected with two separated lineages. The

populations did not show a gradual increase in the genetic differentiation associated with the geographical distance. Instead, we observed a gap between the low values observed among populations within each taxa and much higher values of fixation index between populations of the two species, and not reflecting the geographical distribution of the populations. Similarly, the fixation index between populations of different species is not affected by the geographic distance, suggesting that even potential genetic flow between the two species is not enhanced between populations in closer geographic distance. The lower values of the fixation index between population of different species were observed between one populations of *R. hybisae* in Von Damn Vent (VD2) with populations of *R. chacei* and are associated with the lower frequency of the *R. hybisae* dominant haplotype in VD2 (Supporting material 1).

R. hybisae and _R. chacei_ diverged very recently

Our phylogenetic reconstruction using five genes with date calibration brought a well-supported tree with a separation between *R. chacei* and *R. hybisae*. These species form the most distal clade of the three, denoting the recent evolutionary history in this clade (compared to other members of the tree). Our date estimation proposes that *R. exoculata*, *R. chacei* and *R. hybisae* shared a most recent common ancestor 16.6±6.1 Ma ago, long before the date previously suggested by Shank et al. [21] for the separation of *R. exoculata*/*R.chacei* (0.42-0.5 Ma). Considering the relatively low level of sequence divergence observed between species (mean 0.41 % for COI gene), this result is also consistent with the hypothesis of low mutation rates in Alvinocarididae [21,69]. In addition, we estimated the date of the radiation of Alvinocarididae at 98±15.6 Ma, during the middle Cretaceous. Although Yang et al. [22] gave a more recent estimation, between late Cretaceous and early Tertiary, which is well supported by multiple fossil calibration, it concerns the origin of the clade *Alvinocaris*+*Rimicaris*+*Opaepele*, excluding the more basal clade *Mirocaris*+*Nautilocaris* ([7,21], present study). The origin of Alvicarididae is consistent with the hypothesis of a recent evolution (≤ 100 Ma) of the extant deep-water hydrothermal vent fauna and previous estimations in other hydrothermal vent taxa [15,72,73]. Moreover, we estimated a recent common ancestor between *R. chacei* and *R. hybisae* that lived 2.8±1.2 Ma ago. According to this estimated date and the differences in the COI sequences between the two species, the mutation rate for the COI gene must be at least 10 times lower than the rate estimated for shallow water shrimps [74] or land crabs [75]. Considering the matrix COI distance between the specimens of *R. chacei*-*R. hybisae* analyzed in the population data set (0.41±0.20%), and the estimated divergence date

(2.8 Ma), we could expect a divergence rate around 0.20 % Ma^{-1}. However, a tree-based estimation of the substitution rate is not possible in this case due to the shared COI haplotypes between the species and the lack of species discrimination using only COI gene. The analysis of other potential sister species in the family (for instance the *R. exoculata*/*R. kairei* group and other potential sister taxa) should permit a better estimation of the molecular clock in Alvinocarididae.

Phylogenetic reconstructions using single sequences of both mitochondrial and nuclear genes did not permit a clear distinction between *R. chacei* and *R. hybisae* [7,12,30]. Differences previously reported between sequences of both species seem to be due to misidentification of the specimens from which the sequences available in the Genbank database were obtained. For Instance Nye et al. [32] report a 7.39 % divergence between sequences of the COI gene of *R. hybisae* and both *R. chacei* and *R. exoculata*. However, some sequences obtained from specimen identified as *R. chacei* cluster within the clade of *R. exoculata* instead of the clade of *R. chacei/hybisae* [7], which questions initial species assignation. In the present study we found a divergence between 7.13 to 7.49 % between *R. exoculata* and *R. hybisae* and much lower divergence between *R. hybisae* and *R. chacei* (0.41 %). Similarly, the position of *R. chacei* (previously classified as *Chorocaris chacei*) in the phylogenetic reconstruction of Aznar-Cormano et al. [19] sets within the clade *Mirocaris*/*Nautilocaris* instead of *Rimicaris*, which contradicts other reconstructions [7,21,30,32]. A potential source of inconsistencies in the sequences of *R. chacei* is the misidentification or mislabeling of specimens in collections of MAR samples due to the morphological similarity between *R. chacei* and juveniles of *R. exoculata* [8] or even (superficially) with adults of *Mirocaris fortunata*, which also inhabit in the MAR vent fields. Genetic introgression had also been suggested to explain the position of some of the genbank sequences of *R. chacei* within the *R. exoculata* clade [7], but the population data obtained by Teixeira et al. for both *R. exoculata* and *R. chacei* [12,69] did not support the occurrence of interbreeding between these two species.

The lack of genetic discrimination in some alvinocaridid species underlines the need for more integrative approaches in species delimitation of the family. The recent descriptions of alvinocaridid species usually include the analysis of genetic sequences of some specimens (mostly COI and 18S) to support the morphological delimitation [76-78]. Although in previous cases the occurrence of both morphological and genetic discrimination support the erection of a new species, short genetic distance between two “species” in phylogenetic reconstructions using a single gene does not mean that they must be treated as a single evolutionary unit. Special attention must be paid to species complex because the estimation of connectivity of deep-water ecosystems can be affected by species misidentification

[12,79]. Each potential case must be revised with larger samples and/or genetic approach with better resolution, also integrating the information about morphology and ecology in order to access the species delimitation. Untangling species complex could permit identify interesting processes in these ecosystems as crypticism, morphological plasticity, or early speciation as we propose here for *R. chacei/R. hybisae*.

Two species with low genetic differentiation, yet strong ecological differences

Evidences supporting a separation of *R. chacei* and *R. hybisae* as different species are still low considering the definition use in phylogenetics to conclude the separation of two lineages [80], and the close distance between the two species observed in our analyses. A more integrative approach, including morphological, biological and ecological characteristics, could give support to our hypothesis of species delimitation [81,82]. The two species exhibit morphological differences in the size of the branchial chamber, the position and shape of dorsal photoreceptors and the presence of pores in the carapace (see [8,32]). Moreover, they occupy different habitats in different vent systems. *R. chacei* occurs only in habitats with diffuse vent emission in the MAR field vents whereas *R. hybisae* shows dense aggregations close to the vent emission at the MCSC, although scattered shrimps are also found beside the vent source [33]. The difference in the ecological niche between *R. hybisae* and *R. chacei* is all the more relevant as *R. hybisae* seems to have a similar niche to that occupied by *R. exoculata* on the MAR which is related with their trophic behavior. Indeed, like *R. hybisae* at MCSC vent sites, *R. exoculata* forms dense aggregations with a complex population structure [Hernandez-Avila et al. in prep] and represents a dominant species in terms of abundance and biomass. *R. chacei* does not colonize the top of the vent emission, even in shallower vent fields such as Lucky Strike where *R. exoculata* is absent [83]. So far, *R. chacei* has only been found in scattered populations occupying habitats with mild environmental conditions along the hydrothermal fluids mixing gradient. The evidence obtained from the variations of fixation indexes between and within species, and along the distribution range of each species supports the morphological and ecological evidences that *R. chacei* and *R. hybisae* are separated evolutionary units. The close similarities in the phylogenetic reconstructions suggest that *R. chacei* and *R. hybisae* are sister species, sharing a recent common ancestor not shared by other species [38]. These species could represent a good model for the study of recent speciation processes in hydrothermal vent species and their relationship with the distribution along vent systems and habitats.

The recent divergence between *R. hybisae* and *R. chacei,* posterior to the separation from a common ancestor with *R. exoculata*, raises a number of questions regarding the colonization history of these *Rimicaris* species in the Atlantic Ocean, as well as the mechanisms leading to the symbiotic adaptations observed in *R. hybisae* and *R. exoculata* (ie in the gill chamber, stomach, gut). Our results suggest that exchanges between populations of the ancestor of *R. hybisae* and *R. chacei* were maintained well after the formation of the Cayman Trough (50Ma, Eocene [84]), and the closure of the Panama Ithmus deep-water section (9-15 Ma) [85-87]. In this context of evolutionary divergence with geographic overlap [88,89], two alternative evolutionary scenarios may have led to the emergence of contrasting ecological characteristics in *R. chacei* and *R. hybisae*. In the first scenario, the ancestor of *R. hybisae*/*R. chacei* would have been similar to *R. chacei*, living in moderate vent conditions, and relying on a mixed nutrition involving both symbiotic bacterial chemoautotrophy and a scavenger/predator behavior. At MCSC, this ancestor would have specialized on an exclusively symbiotic chemoautotrophic nutrition favored by morphological adaptations (modifications in size and shape of scaphognatite and exopodite, gill chamber enlargement, stomach reduction) and colonization of microhabitats close to the vent emission. This more efficient nutrition would have led to high biomass populations on the MCSC, while specialization on symbiotic food supply did not occur at MAR vent sites, perhaps because *R. exoculata* already occupied this ecological niche. Populations of the *R. chacei/R.hybisae* ancestor at the MCSC and MAR subsequently diverged by allopatry, by the niche partitioning or both. In the second scenario, the ancestor of *R. hybisae*/*R. chacei* would have already been specialized on symbiotic food supply. A reversal towards the mixed trophic regime would have occurred in the *R. chacei* lineage, and again the two current species would have emerged through allopatric speciation and/or niche partitioning. The co-occurrence of another species specialized on symbiotic chemoautotrophic nutrition on MAR vent sites, *R. exoculata*, which separated from the R. chacei/R. hybisae lineage well before the two later diverged, questions the ancient colonization history of shrimps on the MAR, as well as the mechanisms involved in symbiotic nutrition specialization. Different scenarios involving species competition for resources or differential extinctions could explain contemporary colonization patterns. For instance a symbiotic ancestor of *R. chacei*/*R. hybisae* would question its co-ocurence on the MAR with an ancestor of *R. exoculata*: were both lineages already specialized on symbiotic nutrition? were they already coexisting at MAR vent sites ? If they did, how could they partition resources? In fact, a scenario involving a *R. hybisae*/*R. chacei* ancestor with mixed trophic regime would appear more likely, and it would suggest that specialization on symbiotic nutrition evolved at least twice in Rimicaridinae. However, a more complete phylogenetic analysis among species of the Rimicaridinae clade, including

both species specialized on symbiotic nutrition and with mixed trophic regimes, as well as species inhabiting different ridge systems would be necessary to give support to hypotheses about the colonization history of MAR vent sites. This is beyond the scope of this study.

Colonization processes in *R. chacei*

Our observation of massive recruitment of *R. chacei* at the TAG vent field during the winter 2014, together with data on the population genetics of *R. chacei* are consistent with the Migrant-pool model [53,90]. In this case the genetic structure of one population is the result of random sampling within the entire metapopulation, with a mixed larval pool that is generated by the contribution of each population. The extended larval development of alvinocaridid shrimps [30] could facilitate a putative process of accumulation in the larval phase. The accumulation of a large larval pool could then result in a massive recruitment as we observed at the TAG site for *R. chacei*. Post-recruitment life apparently results in a drastic reduction in the number of individuals leading to scattered adult populations. This reduction may involve high post-recruitment mortality. Such hypothesis of high post-recruitment mortality is supported by video observation we made while sampling during the BICOSE cruise. Early juveniles were affected by predation from vent anemones and by cannibalism from *R. chacei* adults (supporting material). However, too many gaps remain in the biology and life-cycle of *R. chacei*, and the hypothesis proposed must be considered with caution due to the lack of additional evidence. Moreover, explorations have to be leaded as it may not be excluded that the main *R. chacei* ecosystems have not yet been identified.

Not only *R. chacei* adults are rare compared to stage A juveniles, but we also completely lacked late juvenile stage in this species. Conversely, stage B and C juveniles of *R. exoculata* were sampled among dense adult populations found on chimney walls, whereas stage A of this species appeared to be extremely rare. Post-recruitment processes may thus involve early segregation of the two species, either due to the use of different microhabitats, or due to different recruitment timing. Before recruitment, “chorocaris-type” planktonic post-larval stages [28], which are morphologically identical to *Rimicaris* stage A juveniles, and would thus represent the settlement stage, appear to be a mixed pool of both species. Although rare stage A *R. exoculata* were identified at the nurseries [Hernandez et al, in prep], most juveniles of this species were found in patches nested among adults on chimney walls, and were at stage B. No stage A juveniles nor *R. chacei* juveniles were found among dense adult *R. exoculata* on

chimney walls. Although significant recruitment of *R. exoculata* stage A juvenile remains unknown, it appears that their recruitment in adult populations at stage B also coincides with the first morphological modification (reduction of grooming apparatus in *R. exoculata* but not in *R. chacei* [8]) related with the adaptation to a symbiotic life. Perhaps recruitment segregation and exposure of juveniles to specific microhabitats (and microbial community) is the trigger to the development of the symbiotic way of life in *R. exoculata*, which is not present in *R. chacei*.

More systematic sampling of the different juvenile stages, along with the characterization of their respective microhabitats, and careful examination of the development of the symbiosis in post-recruitment stages of both species are required to gain a better picture of their life cycle and population dynamics, and to improve our understanding of the mechanisms involved in the development of their symbiotic way of life.

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Table 1. Species, locations and sequences used in phylogenetic analysis

Species	Vent System	Vent Field	Depth (m)	n[1]	Reference
Diversity of the COI gene between *R. chacei* and *R. hybisae*					
R. chacei	Mid-Atlantic Ridge (MAR)	Snake Pit	3466	9	This study[2]
		TAG1	3637	35	This study[2]
		TAG2	3637	35	This study[2]
		Logatchev	3028	16	[12]
		Lucky Strike	1700	80	[12]
R. hybisae	Mid-Cayman Spreading Center (MCSC)	Von Damm 1	2290	26	[40]
		Von Damm 2	2293	55	[40]
		Beebe 1	4966	40	[40]
		Beebe 2	4942	39	[40]
		Beebe 3	4945	33	[40]
Phylogenetic reconstruction based on concatenated sequences of COI, 16S, 18S, 28S and H3 genes					
R. chacei	MAR	TAG	3637	1	This study
	MAR	Snake Pit	3468	4	This study
R. hybisae	MCSC	Beebe	4960	1	This study
	MCSC	Von Damm	2290	1	This study
R. exoculata	MAR	TAG	3624	4	This study
	MAR	Snake Pit	3468	1	This study
Alvinocaris chelys	Taiwan	Gueishandao	276-300*	1	[19]
Mirocaris fortunata	MAR	Lucky Strike	3450	1	This study[3]
Nautilocaris saintlaurentae	Wallis and Futuna	Fatu Kapa	1554	1	This study[3]
Outgroups (Family)					
Pasiphaea sivado (Pasiphaeidae)				1	[19]
Cinetorhynchus erythrostictus (Rhynchocinetidae)				1	[19]
Penaeus vannamei (Penaeidae)				1	[19]

[1]number of sequences or concatenated sequences (accession numbers in supporting material). [2]Part of sequences analyzed also in Hernandez-Avila et al (in prep) for barcoding purpose. [3]COI and 18S included also in [30].*Based on the species description.

Table 2. Solutions and thermal set for PCR amplifications of the different genes

Solution[1]	COI	16S	18S	28S	Histone 3
MgCl2 (mM)	2	3.4	2	3.4	3.4
dNTP (mM)	0.25	0.26	0.4	0.26	0.26
Primer forward (mM)	0.6 LCO[91]	0.3 CariF[19]	0.5 18S1[42]	0.3 C1[92]	0.3 F1[93]
Primer reverse (mM)	0.6 HCO[91]	0.3 CariR[19]	0.5 1498r[42]	0.3 D2[92]	0.3 R1[93]
Taq Pol (U)	1.25	1.25	1.25	1.25	1.25
Thermal set					
Time[2] (min:seg)	1/1/2	0 :35/0 :35/0 :50	1/1/2	0 :35/0 :35/0 :50	0 :35/0 :35/0 :50
Temp. Annealing (°C)	52	55	51	55	58°C
Cycles	35	35	30	40	40

[1] Prepared in 1X reaction buffer, final volume 50 µl. [2]time set for denaturalization/annealing/extension at 94°C/(see table)/72 °C, in addition a previous step of 5 min of denaturalization at 94°C and a posterior step of 7 min of elongation at 72°C.

Table 3. Genetic diversity of *R. chacei* and *R. hybisae* populations among vent fields estimated from the COI sequences. N, sample size; nh, number of haplotypes; k, number of polymorphic sites; h and h sd, haplotype diversity and its standard deviation; π_1, mean pairwise differences; π_2, nucleotide diversity. Significant Tajima and Fu tests are in bold.

Species	*R. chacei*					*R. hybisae*				
Vent field	Lucky Strike	Snake Pit	TAG1	TAG2	Logat-chev	Beebe1	Beebe2	Beebe3	Von Damm1	Von Damm2
n	80	9	35	35	16	40	39	33	26	55
nh	15	2	14	11	4	8	13	8	8	23
k	13	1	12	10	4	7	13	7	7	23
h	0.58	0.22	0.80	0.71	0.68	0.50	0.59	0.66	0.57	0.94
h sd	0.06	0.17	0.06	0.07	0.09	0.09	0.09	0.09	0.11	0.02
π	0.93	0.22	1.40	1.04	0.82	0.62	0.76	0.86	0.75	1.97
π^2	0.0021	0.0005	0.0032	0.0024	0.0018	0.0014	0.0017	0.0019	0.0017	0.0044
D test	**-1.98**	-1.08	**-1.64**	**-1.75**	-0.28	**-1.78**	**-2.36**	**-1.45**	**-1.89**	**-1.94**
Fs test	**-8.99**	-0.263	**-10.56**	**-7.74**	-0.707	**-5.62**	**-13.64**	**-4.44**	**-5.70**	**-19.94**

Table 4. Analysis of molecular variance between populations grouped in species.

Source of variation	df	Sum of Scares	Variance	% Variation	ϕindices		p
Among Species	1	66.918	0.36134	40.7	ϕct	0.4070	**0.018**
Among Populations	8	4.588	0.00141	0.16	ϕsc	0.0026	0.1793
Within populations	358	187.982	0.5209	59.14	ϕst	0.4086	**<0.0001**
Total	367	259.489	0.88784				

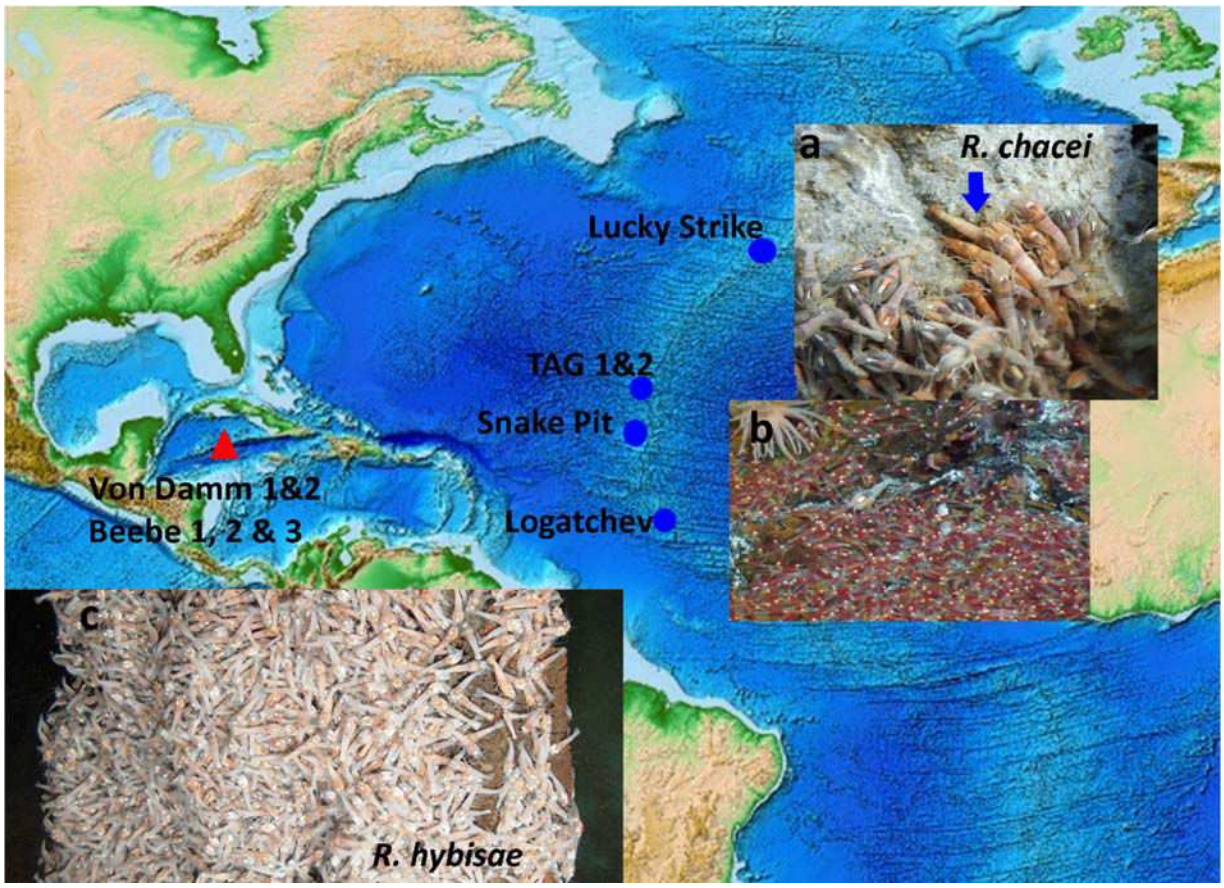

Figure 1. Regional distribution of populations of *Rimicaris hybisae* and *R. chacei* considered in the present study. Details of vent field locations in the MCSC in [40]. Inserted photos illustrate assemblage characteristics and microhabitats for each species, and different life stages: adults of *R. chacei* (a) occur in small scattered patches at the periphery of dense *R. exoculata* populations, in areas of mild vent influence, whereas juveniles (b) formed dense patches around low temperature diffusions at the periphery of the main active structure; adults of *R. hybisae* (c) form dense aggregates close to the vent emission.

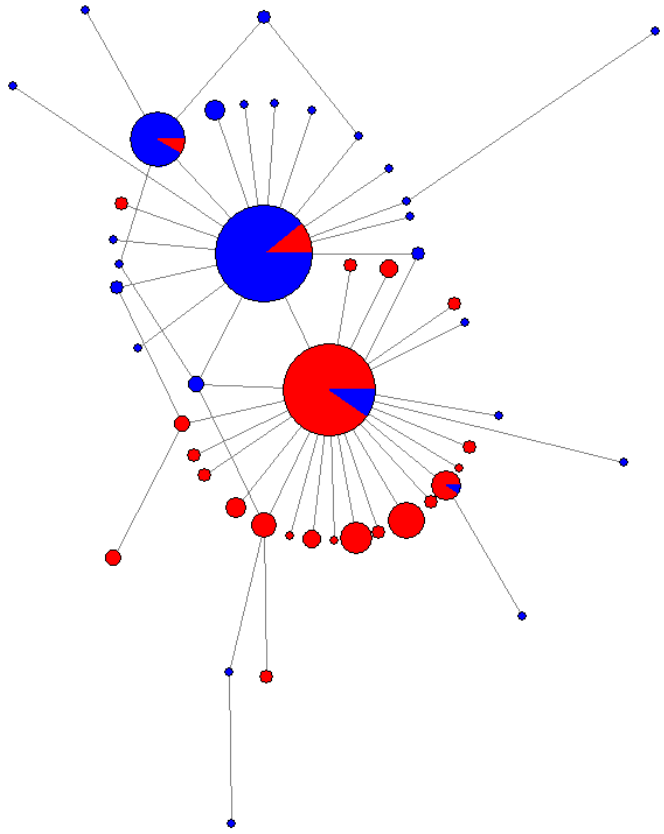

Figure 2. Haplotype network of a 450 bp portion of the COI gene of *R. chacei* and *R. hybisae*. Circles represent the haplotypes and their size is according to the haplotype frequency (the two major haplotypes gather 110 and 102 specimens, top and bottom respectively). Species/vent system: *R. hybisae*/MCSC, red; *R. chacei*/MAR, blue.

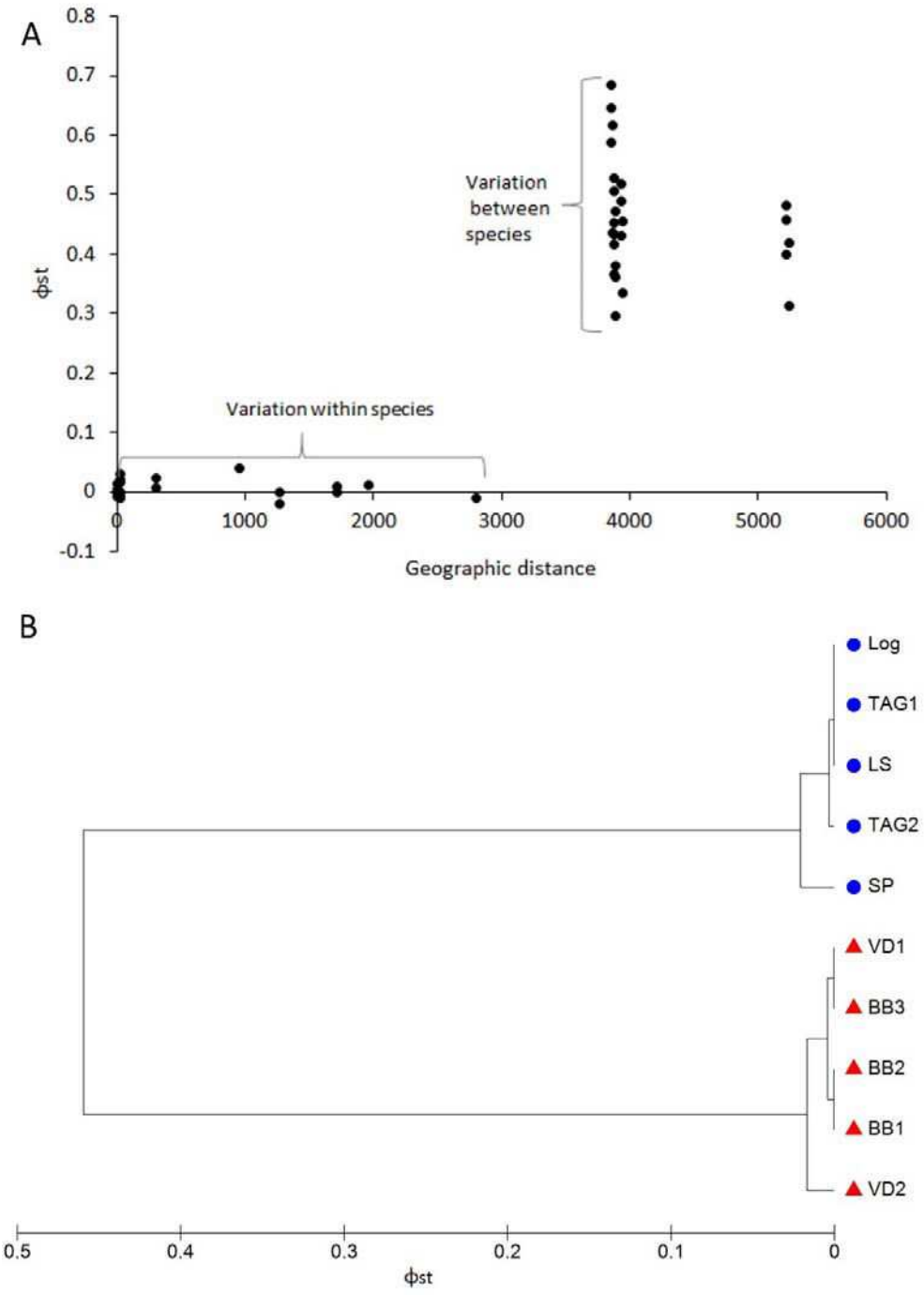

Figure 3. Genetic differenciation between species hypotheses and vent systems. A, Relationship between the fixation index (Fst) and the geographical distance between populations of *R. chacei* (at MAR) and *R. hybisae* (at Cayman through). Comparisons within species and between species are indicated. B, Group average cluster of the populations based on their genetic differentiation. Symbols indicate the species: blue dots*, R. chacei*; red triangles, *R. hybisae*; LOG, Logatchev; LS, Lucky Strike; VD, Von Damm; BB, Beebe.

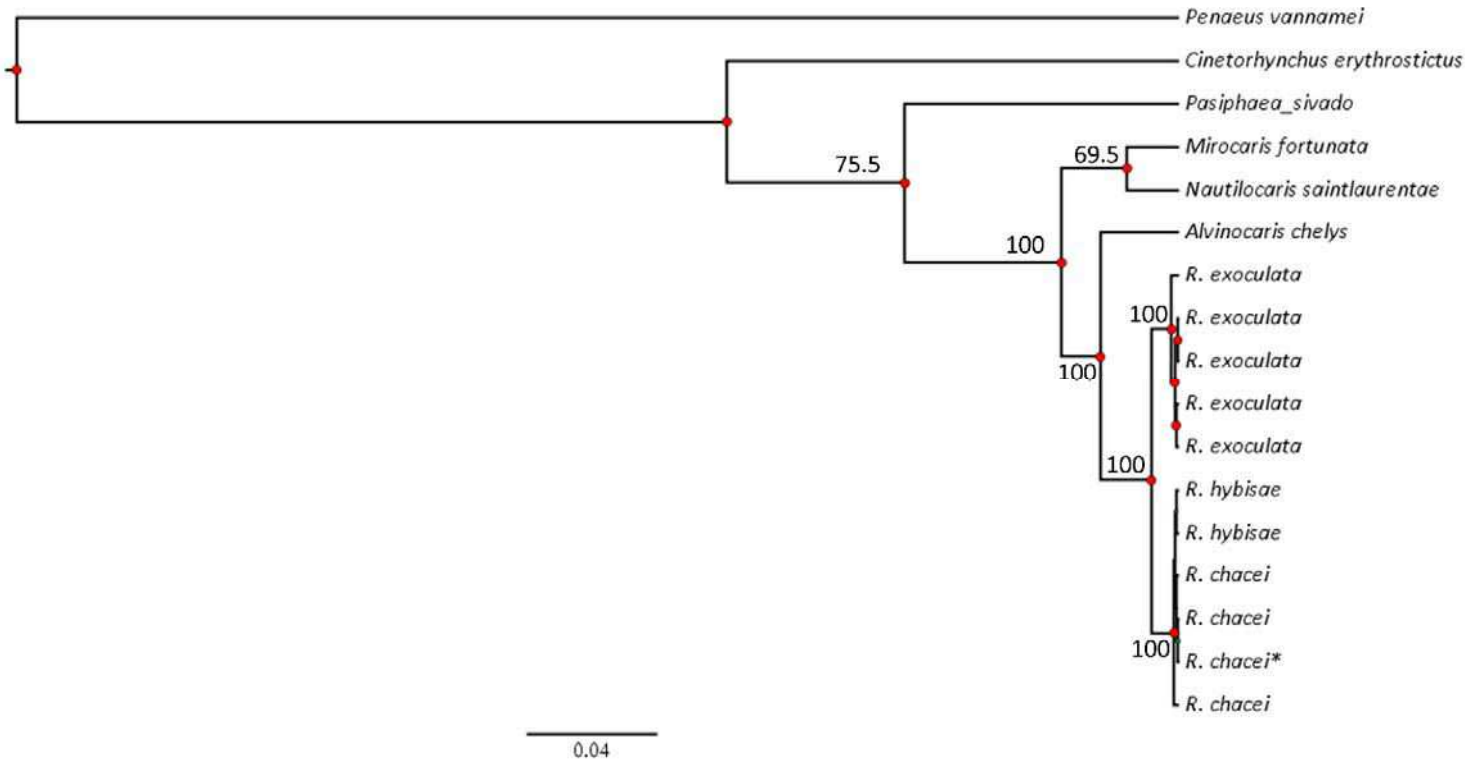

Figure 4. Phylogenetic reconstruction of *R. hybisae*/*R. chacei* and other alvinocaridid species based on the Bayesian Inference of concatenated sequence (genes: COI-16S-18S-28S-H3), without time calibration. Red nodes indicate Bayesian posterior probability= 1, annotations indicate bootstrap proportion based on Maximum Likehood, distal nodes omitted. *Two identical haplotypes for all genes. . *P. vannamei, C. erythrostictus* and *P. sivado* set as outgroups.

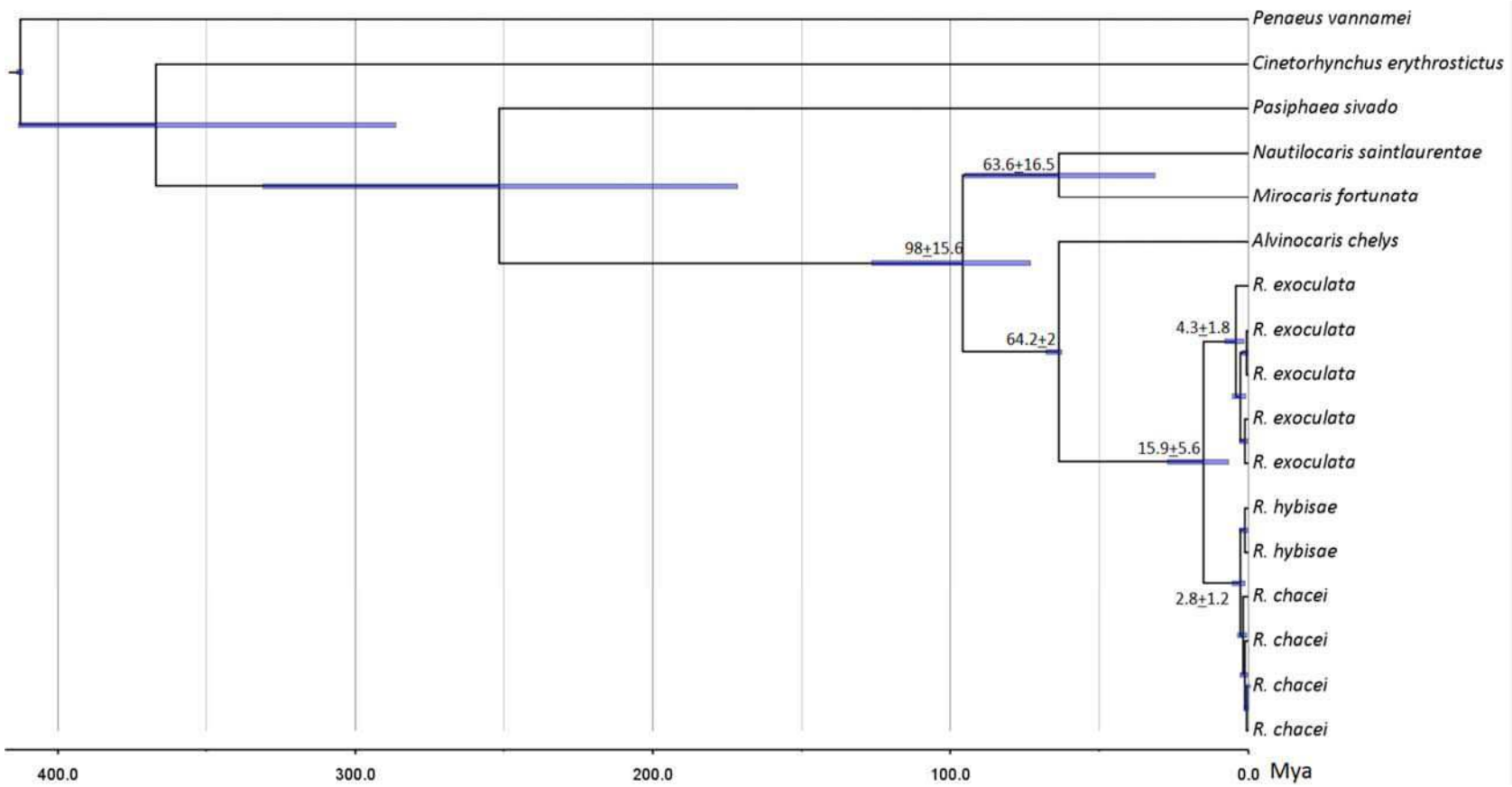

Figure 5 Time-calibrated phylogenetic reconstruction of *R. hybisae*/*R. chacei* and other alvinocaridid species based on the Bayesian Inference of concatenated sequence (genes: COI-16S-18S-28S-H3). Date calibration was included in the root and in the clade *Alvinocaris+Rimicaris* according Yang et al. [22]. Scale in Ma; node bars represent 95% confidence interval of the date estimated.

Supporting material 1. Table 1. Distribution of COI haplotypes along the populations of *R. hybisae* and *R. chacei* for the present study

Vent System	Mid Cayman Spreading Center					Mid Atlatic Ridge						Genbank references		
Population	Von Damm1	Von Damm2	Beebe1	Beeb2	Beebe3	Logatchev	Lucky Strike	Snake Pit	TAG1	TAG2	Total	MCSC[1]	Log & LS[2]	Snake Pit and TAG[3]
H1	17	3	28	25	19	2	6		2		102	KJ566968	KC840930	KU948498
H2	1		5	1	1						8	KJ566969		
H3	3	3	1	2	3	8	50	8	15	17	110	KJ566971	KC840928	KU948491
H4	1	9	2	2	2						16	KJ566973		
H5		6	1		3				1		11	KJ566975		
H6	1	7		1	3						12	KJ566980		
H7		3			1						4	KJ566986		
H8		2			1	5	13	1	5	9	36	KJ566987	KC840929	KU948492
H9	1	1									2	KJ566989		
H10	1	1									2	KJ566992		
H11	1	1									2	KJ566993		
H12									1		1			KU948493
H13									1		1			KU948494
H14										1	1			KU948495
H15										1	1			KU948496
H16									1		1			KU948497
H17									1		1			
H18						1	1		2	1	5		KC840931	KU948499
H19							1		2		3		KC840934	KU948500
H20		1	1								2	KJ566974		
H21		3	1	1							5	KJ566976		
H22		2	1								3	KJ566977		
H23		1		1							2	KJ566978		
H24		1		1							2	KJ566979		

H25		3		1							4	KJ566981		
H26		2		1							3	KJ566982		
H27		1		1							2	KJ566983		
H28		1		1							2	KJ566984		
H29		1		1							2	KJ566985		
H30		1									1	KJ566986		
H31		1									1	KJ566994		
H32		1									1	KJ566995		
H33								1			1		KC840932	
H34								1			1		KC840933	
H35								2			2		KC840935	
H36								1			1		KC840936	
H37								1			1		KC840937	
H38								1			1		KC840938	
H39								1			1		KC840939	
H40								1			1		KC840940	
H41										1	1			KU948501
H42										1	1			KU948502
H43									1		1			KU948503
H44										1	1			KU948504
H45										1	1			KU948505
H46									1		1			KU948506
H47									1	1	2			KU948507
H48									1	1	2			KU948508
Total	26	55	40	39	33	16	80	9	35	35	368			

*Haplotypes obtained from [1]Plouviez et al. 2015, [2]Teixeira et al. 2013, [3]Present study.

Table 2. Genbank references for the sequences included in the phylogenetic reconstructions.

Family	Species	Vent Field	Genes					Source
			COI	16S	18S	28S	H3	
Alvinocarididae	*R. chacei*	TAG						This study
		Snake Pit						This study
		Snake Pit						This study
		Snake Pit						This study
		Snake Pit						This study
	R. hybisae	Von Damm						This study
		Beebe						This study
	Rimicaris exoculata	TAG						This study
		TAG						This study
		TAG						This study
		TAG						This study
		Snake Pit						This study
	Alvinocaris chelys	Gueishandao	KP215334	KP215292	KP215307	KP215322	KP215349	[19]
	Mirocaris fortunata	TAG	KT210460		KT210458			[30], This study
	Nautilocaris saintlaurentae	Fatu Kapa	KT223501	KP725559	KP725762	KP725938	KP726116	[19]and [30]
Pasiphaeidae	*Pasiphaea sivado*		KP759487	KP725631	KP725826	KP726010	KP726190	[19]
Rynchocinetidae	*Cinetorhynchus erythrostictus*		KP759393	KP725512	KP725712	KP725890	KP726076	[19]
Pennaeidae	*Pennaeus vannamei*		AY781297	AF192089	EU920969	EU921006	EU921075	[19]

Summary of Main Findings, Conclusions and Perspectives

Summary of Main Findings, Conclusions and Perspectives

Advances regarding the study of alvinocaridids first larval stage morphology and previous experiments performed on this larval stage allow us to propose a model of larval dispersal capabilities of these shrimps. The larval life of alvinocaridids starts with a lecitotrophic larva which has an extended development. The duration of the first stage is unknown, however preliminary experiments (Koyama et al. 2005, Watanabe et al. In press) suggest that it could be in a range of 1-3 months. Experimental approaches where *Mirocaris fortunata* Zoea I were submitted to different temperature and pressure combinations show that the larva is able to tolerate a wide range of pressure conditions, but do not survive in warm temperature (°20 C) (Tyler and Dixon 2000), limiting its occurrence to deeper waters. As early larvae do not depend on external food of source (ie phytoplankton) and do not tolerate surface water temperature, the assumption that the larvae inhabit the photic layer is not supported anymore. In addition, the few collections of alvinocaridid early life stages are limited to deep-waters, near hydrothermal vent and cold seeps. The potential habitat for the early dispersal of alvinocaridid could be the bathypelagic environments, along the ridge valleys.

As other caridean shrimps with extended development, alvinocaridid could have six or more larval stages (between zoeal and decapodids stages)(Anger 2001, Guerao and Cuesta 2014). The occurrence of intraspecific variations in the number of stages would be possible, which could facilitate the recruitment to the corresponding hydrothermal vent or cold seep habitat. Other decapod crustaceans show a large number of larval stages and intraspecific variations (eg. between 11-17 in the caridean *Macrobrachium rosenbergii*)(Anger 2001), and variations in order to change the pelagic larval period (Diaz and Bevilacqua 1987).

During the larval development, probably at the second zoeal stage, a shift is expected from lecitotrophic to planktotrophic nutrition. Although the eggs accumulate a large amount of lipid reserves (Pond et al. 1997c), these reserves could be depleted after an extended lecitotrophic period of 2-3 months of larval dispersal. In addition, successive larval stages require an energetic budget for larval growth and development (Anger 2001). Late stage larvae and early juveniles of alvinocaridids typically show a lipid composition that includes “bathypelagic wax esters” (with high proportion of fatty alcohols 16:0 and 18:1) (Pond et al. 1997a, Pond et al. 1997c, Pond et al. 2000a). The occurrence of polyunsaturated fatty acids and isotopic signatures in juveniles also suggested a link with the photic zone (Pond et al. 1997c, Stevens

et al. 2008). However, these lipid signatures are compatible also with bathypelagic shrimps (Pond et al. 2000b). Up to date, all collections of alvinocaridid larvae and post larvae have been retrieved in the bathypelagic environment and near bottom deep-waters (Gebruk et al. 1997, Pond et al. 1997a, Herring and Dixon 1998). The current evidence suggests that the putative planktotrophic stage of alvinocaridid shrimps could also have a bathypelagic dispersal.

A very large potential for larval dispersal is expected in alvinocaridid shrimps, both in terms of the duration of the pelagic larval period and the achieved distance. A conservative estimation of a development including 6 larval stages, with similar scale of duration than the first stage (1-3 months), encompasses a putative larval duration of one year or more. In terms of distance, a model of shallow-water dispersal proposed by McVeigh (2016) for *Alvinoncaris muricola* in the Gulf of Mexico and the Western Atlantic, shows distances travelled between 612-858 km for three months of the larval period. Although, considering that a bathypelagic dispersal could reduce later estimations, the inclusion of longer periods of dispersal could compensate the lower current speeds that usually occur in deep-water. Specific distances could depend on the species distribution, the timing of larval release and the physical dynamic of each system. However, it seems that the potential distance traveled during the larval period could be at the scale of 100s km (Fig. 10). The large potential of dispersal in alvinocaridids is consistent with the high gene flow between populations (Teixeira et al. 2012, Beedessee et al. 2013, Thaler et al. 2014, Plouviez et al. 2015, Yahagi et al. 2015).

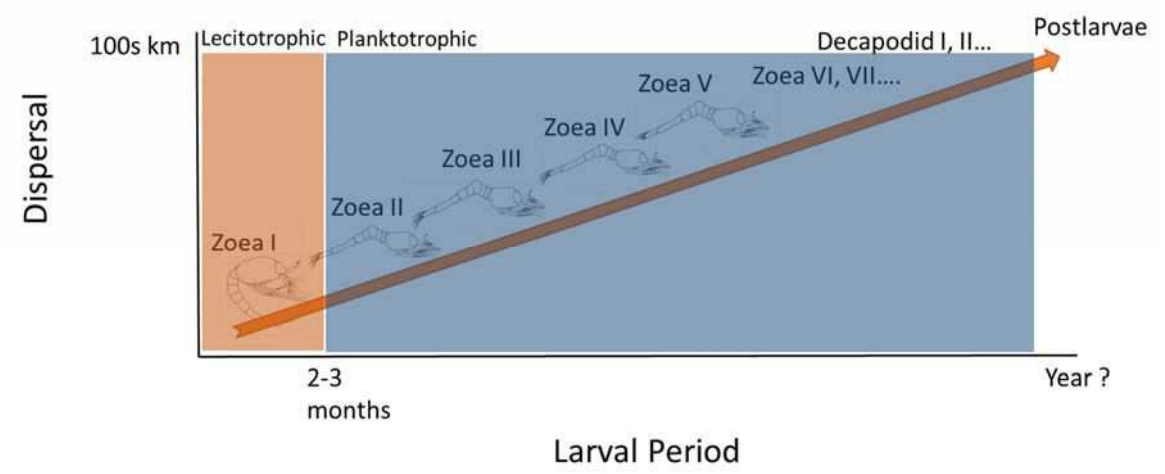

Figure 10. Model of the larval period and dispersal for alvinocarid shrimps through a putative lecitotrophic-planctotrophic development. Stages after Zoea I are unknown.

The population genetic analyses of *R. chacei, R. hybisae* and others alvinocaridids reveal a pattern of genetic flow between populations that is consistent with a migration-pool model (Slatkin 1977, Vrijenhoek 1997). Under this model, it is proposed that the accumulation and mixing of larvae in the bathypelagic environment could represent a genetic pool (larval-pool) generated by the contribution of the benthic populations. The larval-pool could contribute to benthic populations by the recruitment of post-larval stages and by the mix of migrants form different origins, promoting high genetic flow. In this case, the larval stages would not only represent developmental and dispersal phase of the life cycle, but they would also represent the genetic pool of the populations (Fig. 11). The occurrence of massive juvenile recruitments for species with low adult population density, as was observed for *R. chacei,* supports the occurrence of large larval pool for this species. It is presumed that a larval accumulation is necessary in order to generate the large number of recruits. Also, predictions about the number of hydrothermal vent sites along the Mid-Atlantic Ridge (Beaulieu et al. 2015), that still have to be identified, could increase the number of habitats for colonization and more putative populations that could contribute to the larval pool. A larval pool could also explain the resilience of species with very large populations such as *R. exoculata*. Although the model proposed could show variations according to the recruitment pattern and the life cycle of each species, the common larval traits observed in alvinocaridids and their similar pattern of population genetics suggest that alvinocaridids could share a general mode of larval dispersal.

Although this model is supported by the available results, it is necessary to corroborate some assumptions and elaborate experiments that could bring more precise information about the larval dispersal. For instance, it is important to perform *in vivo* experiments of larval maintenance in order to have more accurate estimations of the duration of larval periods, especially at temperature and pressure conditions of the bathypelagic habitat. Also, obtaining other larval stages by *in situ* sampling could bring information about the larval traits and would allow us to test the occurrence of a planktotrophic larval period after the first lecithotrophic stage. Information about the larval traits and duration must be incorporated in the elaboration of models of larval dispersal and ecosystem connectivity (McGillicuddy Jr et al. 2010, Young et al. 2012, McVeigh 2016). Recent works on larval connectivity are focused on estimating separate dispersals from single locations (Young et al. 2012, McVeigh 2016). Modeling approaches could be of interest to test our model of a unique larval pool by analyzing the dispersal and mixing processes of a larval pool generated by larval releases from multiple origins, using simulation of

trajectories based on ocean currents. More exploration and *in situ* sampling is also required to discover new sites, new habitats and complete our models.

Regarding the post recruitment stage, it was demonstrated that *R. exoculata* perform a partition of its population in different habitats according to the sex and the maturity stage. Part of this segregation is related to juvenile aggregations that were reported previously in hydrothermal vents of the Mid-Altlantic Ridge (Vinogradrov and Vereshchaka 1995, Shank et al. 1998). In the present work, we described for first time a strong segregation of adult shrimps: the large swarms of shrimps close to vent emissions are largely dominated by adult females and young specimens (juveniles and subadults), whereas the habitat at the vent periphery is occupied almost entirely by adult males. The variations in maturity stage, sex and size along the habitat allow us to draw a hypothetical model of habitat use during the shrimp life cycle. Early juvenile recruits (5-6 mm CL) are not abundant at the periphery of the vent, but they could occur next to the adult aggregations living close of the vent emission, or even inside of the aggregations. After recruitment, juveniles grow and molt in two successive stages with overlapping sizes (7.3-11.38 mm CL, for both stages) during which many morphological changes occur in relation with the symbiotic life style of the future adults. Then they molt to the subadult stage. Subadult size is also overlapping with juveniles stage (7.3-13 mm CL), with an onset of sexual differentiation at 10.5 mm CL for both sexes, during the progressive transition to the adult stage. Although males and females have a similar range of size (males 10.4-24.9 mm CL, females 10.5-25.5 mm CL), males are slightly larger (15.76±2.18 mm CL) than females (13.26±2.4 mm CL). Males would initially recruit as juvenile stage into the massive aggregations close to the vent emission. After both symbiotic and sexual differentiation, most of the adult males would migrate at the periphery of the vents and occupy this habitat in a scattered pattern. Regarding the shrimp biology and their high nutritional dependence on bacterial symbionts, it is possible that the males perform short migrations between the periphery and the vent emission in order to supply their symbionts with reduced compounds necessary for chemosynthesis, or they could sustain on diffusing fluid emission. Also, preliminary observations suggested that females would migrate at the vent periphery in order to perform matting (Tyler and Young 1999). However, except for an eventual location in other habitats, our results indicate that females seems to spend most of the post-recruitment period aggregated close to vent emission, including the brooding period (Fig. 11, 12).

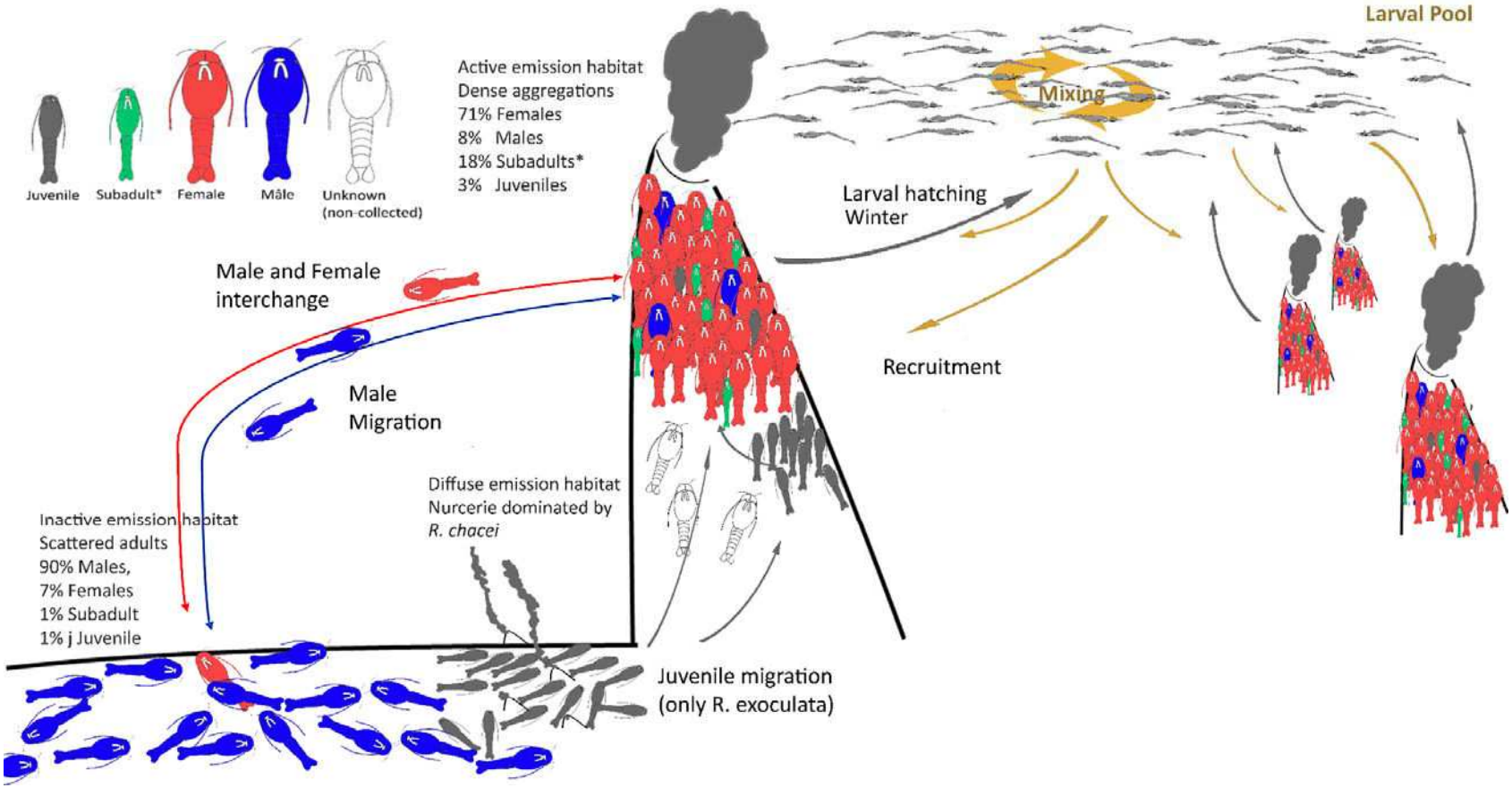

Figure 11. A model of post-recruitment spatial distribution of *R. exoculata* and metapopulation larval pool model of connectivity, proposed for alvinocaridids in hydrothermal vents. Proportion of sex and stade are based on pooled estiation of specimens collected in the TAG vent field. Note that the connectivity between the systems is established indirectly, via the larval pool.

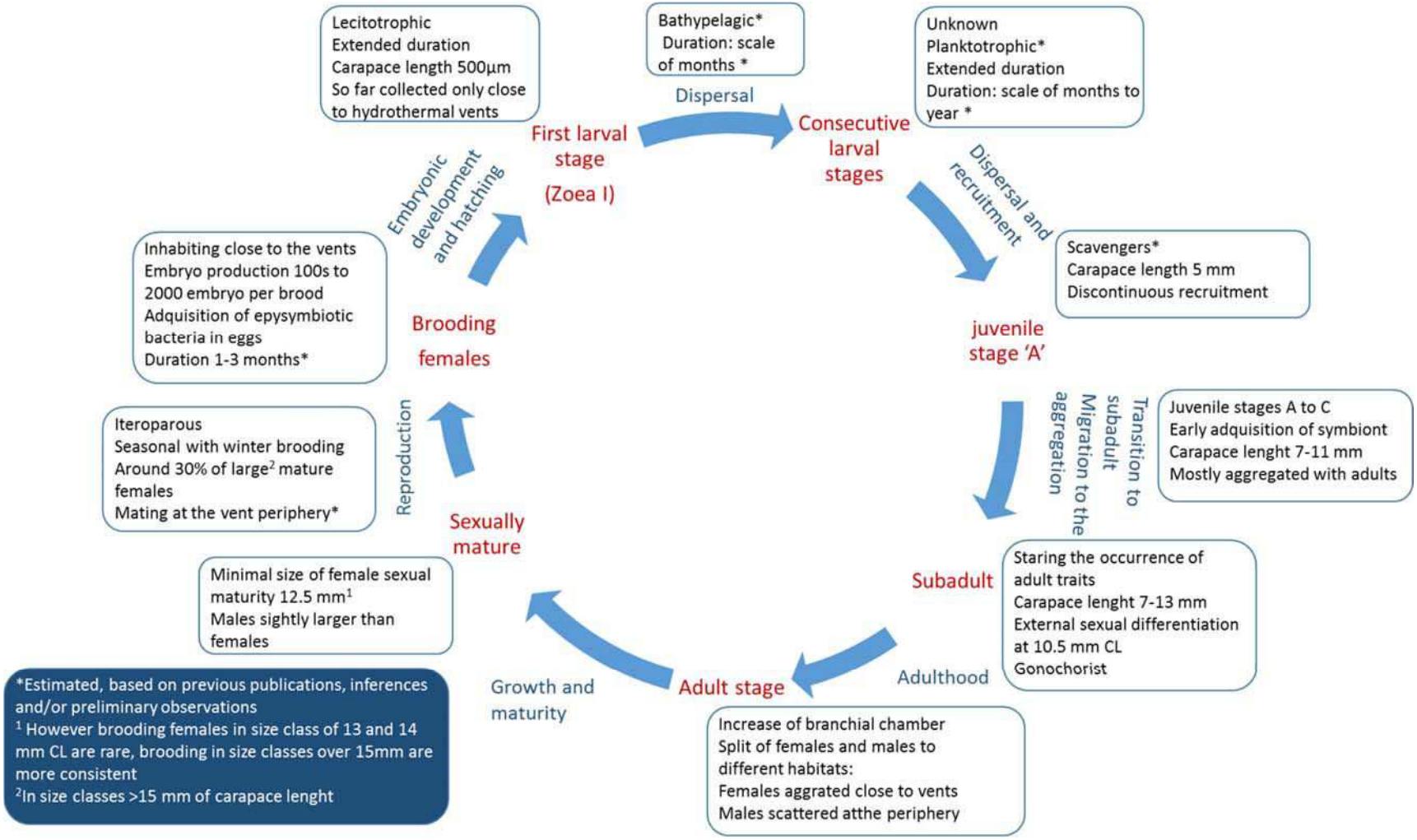

Figure 12. Schematic diagram of the life cycle of *R. exoculata* with a compilation of previous (sustained) and current observations. Stages are presented in red and life-cycle processes in blue. Blocks show main features of each stage or process.

This partition of males and females among different habitats in the vent field could have an effect on the symbiotic bacterial activity and so the nutrition of the shrimp, especially males that seem to spend at least some time out of the vent fluid-seawater mixing gradient. Habitat partition in vent species have been associated with changes in the trophic regime inferred from isotopic signatures (Marsh et al. 2015). The variation in the composition and concentration of reduced compounds in fluids along the vent field affect the symbiotic bacterial assemblages diversity and activity (Salerno et al. 2005). To test this hypothesis, current analyses are still in progress using specimens of *R. exoculata* collected from different habitats (close to the vent emission and at the vent periphery) during the BICOSE cruise. The samples were processed in order to analyze the bacterial assemblage diversity in different compartments (branchial chamber, stomach, hepatopancreas and hindgut). Preliminary cloning results show significant variation in the composition of the bacterial assemblage according to habitats for both the branchial chamber and the hindgut (Fig. 13). Males at the vent periphery show lower OTU diversity in their branchial chamber than specimens living in the vent aggregations. In addition, the OTU diversity in the hindgut is higher in specimens at the vent periphery. Both compartments show a shift in the bacterial assemblages between the habitats. These changes denote an effect of the habitat partition of the population in the assemblage of symbiotic bacteria. Additional analyses will have to be performed in order to determine precise distribution of the bacteria on histological samples (via FISH), and the relation between changes of epibiotic assemblages with metabolic pathways of the bacteria using functional genes analyses. *In vivo* experimentations will have to be conducted during next cruises to study the efficiency of the trophic symbioses in males.

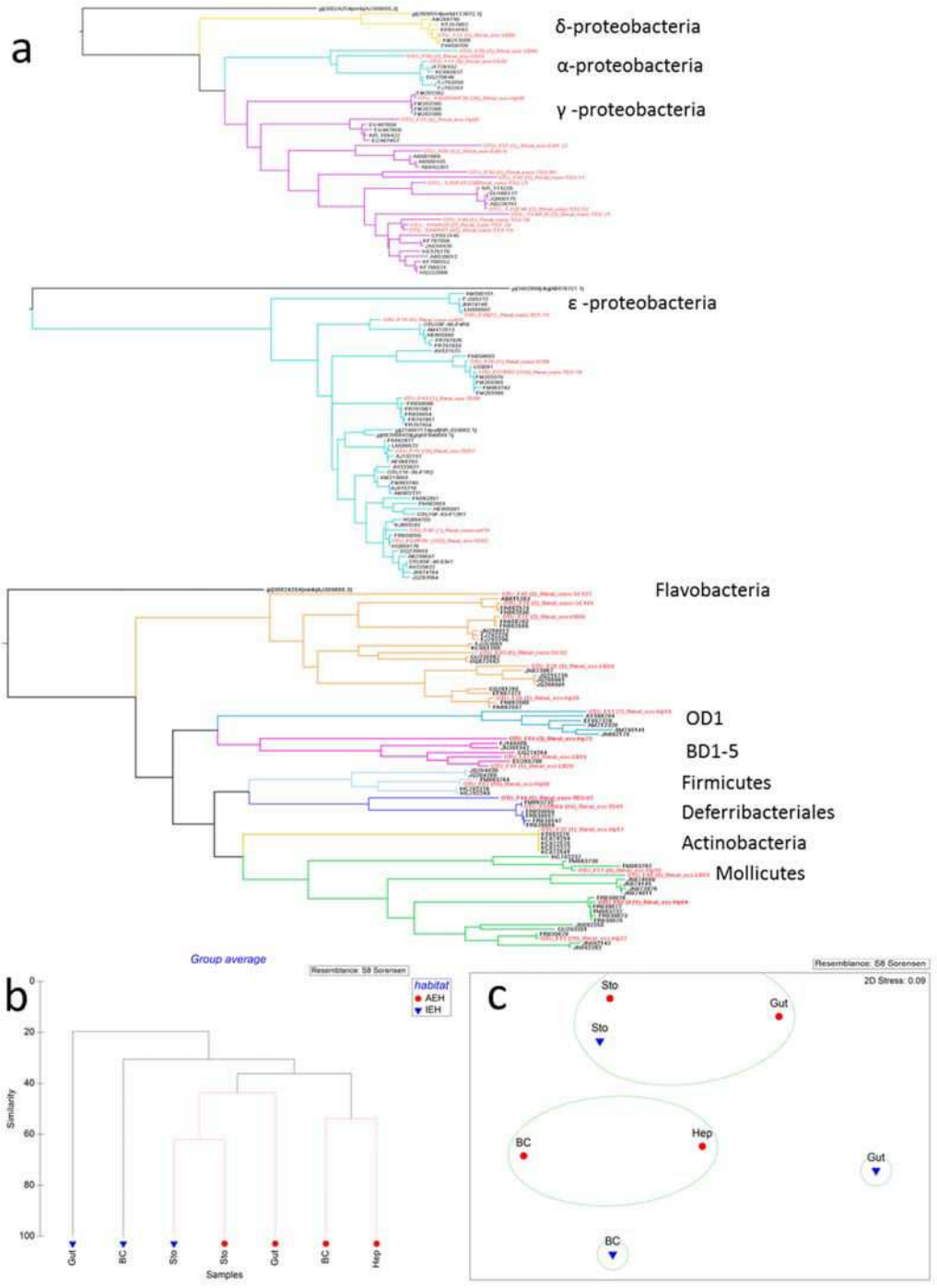

Figure 13. Bacterial assemblages of *R. exoculata* at the TAG site with differences between compartments and habitats. a, Phylogenetic reconstructions (16S gene) of the OTUs identified (red labels); b, Cluster with SIMPROF test of Sorensen similarity (solid lines supported by $\alpha < 0.1$); c, MDS of samples according assemblage similarity, contours refers to significant groups of the SIMPROF test. Abbreviations in b and c, AEH (red circle): Active emission habitat (close to the vent), IEH (blue triangle): Inactive emission habitat (periphery), BC: branchial chamber, Hep: hepatopancreas, Sto: stomach, Gut: Hindgut. (Preliminary results).

Regarding the reproduction of *Rimicaris exoculata*, the previous hypothesis of brooding females migration out the active vent area (Tyler and Young 1999, Ramirez-Llodra et al. 2000) is rejected by the results obtained in the present study. Brooding females were found in the shrimp aggregation close to the vent emission in both vent fields of TAG and Snake Pit. This confirmed previous reports of Gebruk et al. (2010) and Guri et al. (2012) at the Logatchev vent field. The occurrence of a seasonal reproduction in *R. exoculata,* with a brooding period during winter, is also proposed in the present study. Other alvinocaridid shrimps show both seasonal (Copley and Young 2006) and continuous reproduction (Ramirez-Llodra et al. 2000). However, the underlying ecological link for each strategy is not clear. Although a coupling with the seasonal surface productivity for species having seasonal reproduction has been suggested (Copley and Young 2006), the occurrence of a lecitotrophic and extended larvae do not support this hypothesis. The analysis of large scale transport processes in relation with seasonality of the reproduction could bring a better idea about possible link between reproductive strategies and oceanographic processes. In addition, differences in the reproductive output are observed between the vent field in terms of egg production by female, egg size and number of aborted females. It is expected that these differences could be due to physicochemical conditions of the vent fields, but other vent need to be explored to determinate a clearer pattern. The size population structure shows multiple cohorts at both TAG and Snake Pit vent, suggesting a discontinuous recruitment, consistent with a previous study (Copley 1998) and with the hypothesis of seasonal reproduction of the species. Since the number, size and proportion of cohorts were similar between vents, the population dynamics is presumed to be mostly similar at larger scale.

The occurrence of brooding females close to the vent emission also shows that embryonic stages are able to tolerate high concentration of reduced compounds and other toxic elements released by the vent emissions. In addition, the eggs harbor diverse episymbiotic bacterial assemblages, which in a first approach, seemed to be different from the bacterial assemblages retrieved on a non-symbiotic structure exposed to the same conditions. Bacterial assemblages found at the surface of the eggs evolve during the embryonic development which reminds the epibiotic development in the adult gill chamber along the molt cycle. Also, a comparison of the episymbiotic bacteria detected on eggs from Snake Pit and TAG site with those retrieved from eggs from the Logatchev site (Guri et al. 2012) show differences, including the occurrence of methatrophic bacteria at Logatchev. The symbiotic bacteria on eggs could be driven by a threshold in the ratio of sulfide:methane of the vent emission, as have been proposed for endosymbionts of *Bathymodilus* mussels (Salerno et al. 2005) and episymbionts of the branchial chamber of *R. exoculata*

(Guri 2011). Currently, additional experiments are in progress, in order to corroborate the patterns observed, determinate the distribution of bacterial groups on egg surfaces and confirm the occurrence of the metabolic pathways associated with the bacterial assemblages.

The epibiotic bacteria could contribute to detoxification and pathogen inhibition on eggs. However, the hypothesis of the potential transfer of organic compounds between epibiotic bacteria and embryo is also proposed Fig. 14). The selective permeability of the egg membranes and mucus coat (Fisher and Clark 1983) could facilitate the transfer of organic carbon between embryo and the episymbiotic bacteria, as it occurs in the branchial chamber of adult shrimps (Ponsard et al. 2013). The putative exchange of organic compounds from bacteria to embryos during brooding could enhance the larval survivorship, as they would compensate lipid reserves consumption during embryonic development.

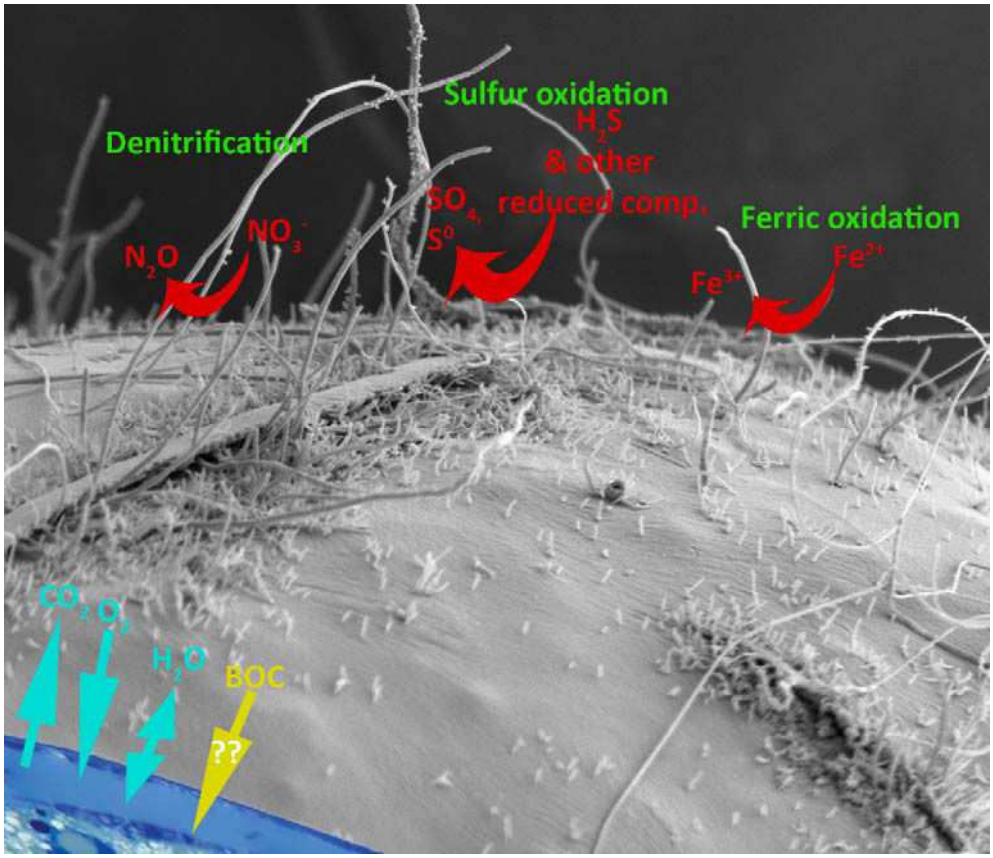

Figure 14. Hypotheses on metabolic pathways of the episymbiotic bacterial assemblages occurring on eggs, and putative relations with embryos. On top are represented the three general pathways of bacteria (to be confirmed). In the corner are embryonic exchanges with the surrounding environment including respiration and water intake (blue) and the proposed incorporation of organic carbon produced by bacteria (BOC). SEM image: egg at mid-stage of development from the Snake Pit site. Left corner: transversal section of a *R. exoculata* egg,

modified from Guri et al. (2012). Metabolic pathways proposed are based on results obtained for the branchial chamber (Hügler et al. 2011, Ponsard et al. 2013, Jan et al. 2014).

In terms of evolution and speciation, sister species of *Rimicaris* can occupy very distinct ecological niches in different hydrothermal vent systems, as observed for *R. chacei* and *R. hybisae*. The low genetic distance observed between these two taxa is explained by a recent event of speciation and low mutation rate. Other potential groups of sister species, as *R. exoculata* and *R. kairei* (Hernández-Ávila et al. 2015, Vereshchaka et al. 2015), show similar ecological niche but allopatric distribution. This genus has a very interesting evolutionary history, showing important variation in morphology (at least gill chamber and digestive tract), distribution, ecological niche and symbiotic relationships. The evolutionary processes related to these adaptations are presumed to have occurred recently, compared to the early radiation date estimated for the whole family. This genus seems to represent a good model to study recent or even current evolution, including undergoing speciation processes of hydrothermal vent species, including the acquisition and specialization toward symbiotic association with bacteria that enable them to dominate the biomass of some hydrothermal vent systems. A strong recommendation is then to take into account not only a multi genetic approach but also detailed analyses of morphology, behavior, reproductive patterns and ecology.

Finally, this work brings a contribution to the understanding of *R. exoculata* life cycle and related species, regarding larval life, population biology, connectivity and symbiotic relationship with epibiotic bacteria. The results obtained here show additional levels of complexity of the biology of these species that need to be explored in future works. Despite advances in the study of deep-water hydrothermal vent ecosystem, the life cycle of vent species still has to be explored, in particular for species that dominate the biomass of these systems. Understanding the life cycle of vent species is essential to understand the ecosystem dynamics and elucidate the mechanisms associated to the resilience, succession and functioning of benthic communities.

Different hypothesis have emerged about the larval dispersal, population biology, reproduction, symbiosis and evolution, revealing the high degree of life cycle complexity in alvinocaridid shrimp in general, and *R. exoculata* in particular. This work aimed to bring some advances in these topics. However, it is necessary to perform additional experiments in order to test the hypotheses proposed and better understand the life cycle and distribution of these species. Future approaches proposed include deep sea currents studies to better understand dispersal events; performing additional cruises in order to obtain

in situ samples of each larval stages; hatching larvae maintenance under *in vivo* experimental controlled conditions using pressurized chambers, to better understand their capabilities of adaptation and so migration. The conceptual model of larval dispersal therein proposed could then be included in simulation models, specially using multiple larval origins, to link the larval biology with the physical mechanisms that allow the connectivity between vent systems. Future collections of *R. exoculata* populations from other vents and seasons could bring additional information about the population dynamics and their relation with symbiosis. In addition, the symbiotic relationships at embryonic stage could be explored trough the identification of the metabolic pathways, by the characterization of functional genes, and by *in vivo* experiments using isotope-labeled inorganic carbon. Finally, at evolutionary scales, the phylogenetic relationships of alvinocarid shrimps, especially in the genera *Rimicaris*, must be revisited with an integrative approach including multiple genes, morphological and ecological traits, in the context of the speciation processes and their relationship with the acquisition of a symbiotic relationship and their ecology.

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Resumen

Las emisiones hidrotermales profundas albergan comunidades de elevada biomasa basadas en quimioautotrofía, soportadas por la actividad metabólica de bacterias de vida libre y bacterias simbiontes asociadas a invertebrados marinos, especialmente megafauna. El conocimiento de los mecanismos de dispersión y el ciclo de vida de las especies de ambientes hidrotermales es escencial para comprender los procesos ecológicos de ambientes hidrotermales asociados a la distribución, la estructura comunitaria y la variación temporal. En este estudio, presento algunos avances relacionados a la dispersión y el ciclo de vida de una especie dominante de los sistemas de emision hidrotermal de la dorsal medioatlántica. La aproximaciones metodológicas aplicadas en este estudio incluyen el estudio de la morfología larvaria, el análisis de la biologia poblacional y de la reproducciôn, así como genética molecular con fines de identificación, reconstructión filogenética, genética de poblaciones y análisis de diversidad de bacterias. La mayoría de las observaciones y análisis presentados en el presente estudio fueron realizados con muestras colectadas en enero y febrero de 2014 durante el crucero oceanográfico BICOSE en la dorsal medioatlántica (campos TAG y Snake Pit). El análisis morfológico de la larva Zoea I de cuatro especies (*R. exoculata, Mirocaris fortunata, Nautilocaris saintlaurentae* y *Alvinocaris muricola*) permite concluir que el primer estadio larvario de la familia Alvinocarididae es lecitotrôfico con una duración del desarrollo extendida, permitiendo la dispersión a grandes distancias sin requerimiento de una fuente externa de nutrición. Se propone para estas especies una dispersión batipelágica y un cambio a un estadio planctotrófico durante el periodo larvario. En relación a la biología poblacional, fue observada una variación en la estructura poblacional entre hábitats en relación al sexo y el estado de desarrollo. Las agregaciones densas de camarones encontradas cerca de las emisiones hidrotermales están compuestas principales de hembras y juveniles, mientras la mayoría de adultos dispersos encontrados en la periferia de las chimeneas fueron machos. Varias cohortes de tallas fueron identificadas en ambas poblaciones, lo cual denota un reclutamiento discontinuo. Una gran cantidad de hembras ovígeras fueron observadas cerca de la emisión hidrotermal, lo cual contrasta con la casi completa ausencia de hembras ovígeras en muestreos previos y sugiere una reproducción estacional con incubación y desove durante el invierno. La superficie de los huevos de *R. exoculata* está colonizada por bacterias episimbiontes. Los análisis de clonación muestran que los ensambles bacterianos parecen ser específicos, lo cual sugiere una relación simbiótica. Además estos ensambles cambian en relación al desarrollo embrionario. Los ensambles de bacterias observados en los huevos son similares a las comunidades episimbiontes encontradas en la cámara branquial de los adultos, sugiriendo la ocurrencia de procesos de detoxificación o nutrición similares. En otras especies del género *Rimicaris*, interrogantes en relación al ciclo de vida, la conectividad entre sistemas hidrotermales y la especiación han surgido recientemente. Estudios genéticos sugieren que dos especies alopátricas y con diferencias en morfología y ecología, *R. hybisae* y *R. chacei* representan una especie única. Esta hipótesis se encuentra relacionada además con el origen de un reclutamiento masivo de *R. chacei* encontrado en el campo TAG, a pesar de la baja densidad de adultos. Análisis de genética poblacional y reconstrucciones filogenéticas utilizando varios genes muestran que *R. chacei* y *R. hybisae* son linajes separados producto de una especiación reciente o en proceso. Estas especies, al igual que *R. exoculata* y otros alvinocarídidos, muestran patrones de conectividad asociados al modelo de migración colectiva (migration pool). Implicaciones de estos hallazgos y perspectivas de futuras investigaciones son discutidas en relación a experimentos adicionales y muestreos de campo necesarios para una caracterización completa del periodo larvarios de los alvinocarídidos, la variación en las relaciones simbióticas con bacterias entre sexos y estados de maduración que ocupan hábitats diferentes, así como la caracterización cuantitativa y funcional de la episimbiosis en huevos y los procesos evolutivos relacionados con la especiación en el género *Rimicaris*.

Palabras claves: Emisiones hidrotermales profundas, ciclo de vida; estructura poblacional, reproducción, dispersión, simbiosis, especiación, Alvinocarididae.

Larval dispersal and life cycle in deep-water hydrothermal vents: The case of *Rimicaris exoculata* and related species

Abstract

Deep-water hydrothermal vent host high-biomass communities based on chemoautotrophy supported by the metabolic activity of free-living and symbiotic bacteria associated to invertebrates, especially megafauna. Knowledge on the mechanisms of dispersal and the life cycle of vent species is essential to our understanding of the vent communities in terms of distribution, structure and temporal variation. In this study, I present some advances regarding the dispersal and life cycle of a dominant species of the Mid-Atlantic Ridge (MAR) vent ecosystems, the alvinocaridid shrimp *Rimicaris exoculata,* and other related species. The methodological approaches applied include morphological descriptions of larvae, analysis of population biology and reproduction, and molecular genetics for species identification, phylogenetic reconstructions, population genetics and bacterial diversity. Most observations and studies presented here were conducted on samples collected in January-February 2014, during the BICOSE cruise on the MAR. Based on the analysis of Zoea I larvae of four species (*R. exoculata, Mirocaris fortunata, Nautilocaris saintlaurentae* and *Alvinocaris muricola*), we conclude that the alvinocaridid first larval stage is lecitotrophic with extended development, allowing large dispersal without external food requirement. A bathypelagical larval dispersal and a shift to a planktotrophic stage during the larval period is proposed. In terms of population biology, collections performed at the TAG and Snake Pit vent fields show variations in the population structure among habitats, according to sex and life stage. Large aggregations of shrimps found close of the vent emission comprise mostly females and young individuals, whereas scattered adults found at the vent periphery were mostly males. Multiple cohorts were found in both vents fields, denoting a discontinuous recruitment. Brooding females were observed in significant numbers close to the vent emission, which contrasts with their constant lack in previous field studies and suggests a seasonal reproduction with a brooding period the winter season. In addition, differences in the reproductive effort were detected between vent fields, including egg number, egg size and proportion of aborted females. The egg surface of *R. exoculata* is colonized by episymbiotic bacteria. Cloning approaches show that the bacterial assemblages on eggs seem to be specific, suggesting their symbiotic role, and evolve according to the egg development. The bacterial assemblages on eggs and their variation during the embryonic development remind the episymbiotic communities found in the branchial chamber of adults, suggesting similar detoxification or nutrition role. In other *Rimicaris* species, questions about life cycle, vent connectivity and speciation have been raised recently. Genetic studies suggest that two species with contrasting distribution, morphology and ecology, *R. hybisae* and *R. chacei,* are the same species. This question is related also with the source of a massive recruitment of *R. chacei* found at TAG vent field, despite the low density of adults. Analysis of population genetics and phylogenetic reconstructions with multiple genes show that *R. chacei* and *R. hybisae* are separate lineages with recent or undergoing speciation. These species, as *R. exoculata* and other alvinocaridids, show a genetic population model associated with a migration pool. An extended larval period could contribute to the wide dispersal and high genetic flow between populations. Implications of these findings and perspectives of future research are discussed in terms of additional experiments and field sampling required to characterize the larval period of alvinocaridids, the variations of symbiosis of the different life stages and sexes inhabiting different habitats, the quantitative and functional characterization of the episymbiosis on eggs, and the evolutionary processes associated with the speciation in *Rimicaris*.

Keywords : Deep-water hydrothermal vents, life cycle, population structure, reproduction, dispersal, symbiosis, speciation, Alvinocarididae.

Dispersion larvaire et cycle de vie dans les environnements hydrothermaux profonds: le cas de la crevette *Rimicaris exoculata* et d'espèces proches

Résumé

Les écosystèmes hydrothermaux profonds hébergent des communautés présentant de fortes biomasses, issues de l'activité chimiotrophique des microorganismes, avec de nombreux exemples d'associations symbiotiques entre ces derniers et les organismes de la mégafaune dominante. La connaissance du cycle de vie de ces espèces, y compris de leurs symbiontes, et de la façon dont elles sont capables de disperser et de coloniser de nouveaux sites est incontournable pour la compréhension du fonctionnement des communautés hydrothermales.

Dans cette étude, sont présentées de nombreuses avancées portant sur la distribution, la reproduction, la dispersion et le cycle de vie d'une espèce dominante des écosystèmes hydrothermaux de la dorsale Médio-Atlantique, la crevette alvinocarididdé *Rimicaris exoculata*, et des espèces proches. Les outils méthodologiques utilisés incluent la description morphologique de larves, l'étude de la structure de populations et de leur état de reproduction, des approches moléculaires appliquées à l'identification des espèces via la reconstruction phylogénétique, la génétique populationnelle et l'étude de la diversité bactérienne. La plupart des observations et analyses ont été réalisées grâce aux prélèvements de la mission BICOSE qui s'est déroulée de Janvier à Février 2014 sur la dorsale Médio-Atlantique.

L'analyse morphologique détaillée des premiers stades larvaires (zoé I) de quatre espèces d'Alvinocarididae (*R. exoculata, Mirocaris fortunata, Nautilocaris saintlaurentae* et *Alvinocaris muricola*), indique une combinaison de traits caractéristiques de cette famille et unique parmi les crevettes Caridés. Le premier stade larvaire lécithotrophe présente vraisemblablement une durée de développement prolongée, avec une transition vers la planctotrophie au cours des stades ultérieurs. La capture de ces larves près du fond suggère par ailleurs une dispersion bathypélagique.

L'étude réalisée sur les populations de *R. exoculata* des champs hydrothermaux de TAG et Snake Pit met en évidence une ségrégation spatiale des sexes et des stades de vie. Les femelles, les sub-adultes et les juvéniles occupent la paroi des fumeurs actifs, tandis que les mâles se retrouvent majoritairement dispersés à la périphérie inactive des sites. L'identification de plusieurs cohortes d'individus, retrouvées au niveau des habitats des deux champs hydrothermaux indique par ailleurs un recrutement discontinu. Enfin, l'observation, pour la première fois, d'un grand nombre de femelles gravides sur les deux champs hydrothermaux, suggère une reproduction saisonnière, avec quelques différences mineures en terme de fécondité entre les populations des deux champs.

Les embryons portés par les femelles jusqu'à l'éclosion des larves sont exposés aux fluides hydrothermaux. Nos résultats, encore partiels, d'analyses par clonage d'assemblages bactériens se développant sur les œufs au cours de cette phase d'incubation indiquent une spécificité qui pourrait être le reflet d'une fonction symbiotique s'établissant à un stade précoce du cycle de vie de la crevette. La similarité de ces assemblages bactériens avec ceux colonisant le céphalothorax des crevettes adultes, suggère un possible rôle de détoxification et/ou de nutrition.

Enfin la découverte, sur TAG, d'importantes « nurseries » de post-larves appartenant à *R. chacei*, espèce cohabitant avec *R. exoculata* mais relativement peu abondante, pose la question de l'origine de ce recrutement. Cette question s'inscrit également dans le débat taxonomique récurrent des délimitations d'espèces chez les Alvinocarididdés. Ainsi, de récents travaux de génétique suggèrent que *R. chacei* pourrait être identique à *R. hybisae*, une espèce des sites hydrothermaux de la Ride des Caïmans, qui paradoxalement, présente une écologie et un développement symbiotique beaucoup plus similaire à celui de *R. exoculata* que de *R. chacei*. Nos analyses de génétique populationnelle et une reconstruction phylogénétique réalisée avec plusieurs gènes suggèrent que *R. chacei* et *R. hybisae* représentent bien deux lignées distinctes, issues d'un événement de spéciation récent. Nous suggérons également que le recrutement massif de post-larves de *R. chacei* pourrait refléter un modèle de dispersion larvaire « en pool », où la durée de vie larvaire prolongée permettrait la formation d'un pool de larves planctoniques, lieu de brassage génétique entre les différentes populations adultes benthiques.

La portée de ces résultats et les perspectives de recherche sont discutés concernant en particulier la dispersion et la phase larvaire des alvinocarididdés, le développment de la symbiose aux différents stades de vie, la caractérisation des différents habitats occupés par ces stades de vie, et les processus évolutifs associés à la spéciation chez *Rimicaris.*

Mots clés : Sources hydrothermales profondes, cycle de vie, structure de population, reproduction, dispersion, symbiose, spéciation, crevettes Alvinocarididae.