

# Direct evidence for maternal inheritance of bacterial symbionts in small deep-sea clams (Bivalvia: Vesicomidae)

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**Abstract** Bacterial symbiont transmission is a key step in the renewal of the symbiotic interaction at each host generation, and different modes of transmission can be distinguished. Vesicomidae are chemosynthetic bivalves from reducing habitats that rely on symbiosis with sulfur-oxidizing bacteria, in which two studies suggesting vertical transmission of symbionts have been published, both limited by the imaging techniques used. Using fluorescence in situ hybridization and transmission electron microscopy, we demonstrate that bacterial symbionts of *Isorropodon bigoti*, a gonochoristic Vesicomidae from the Guinness cold seep site, occur intracellularly within female gametes at all stages of gametogenesis from germ cells to mature oocytes and in early postlarval stage. Symbionts are completely absent from the male gonad and gametes. This study confirms the transovarial transmission of symbionts in Vesicomidae and extends it to the smaller species for which no data were previously available.

**Keywords** Cold seeps · Gametogenesis · *Isorropodon* · Maternal inheritance · Symbiont transmission · Symbiosis · Vesicomidae

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## Introduction

In animal-bacteria symbioses, symbiont transmission is a critical step in the life cycle of both partners. Three different transmission modes are generally distinguished: vertical-maternal, which is the transfer of symbionts to the host progeny via gametes; horizontal-environmental, which involves reinfection of the new host generation from an environmental stock of free-living forms of the symbiont; and horizontal-lateral in which symbionts are acquired from another host. However, many examples of mixed transmission modes can be found, involving vertical and horizontal transfer, or occasional host switching (reviewed in detail by Bright and Bulgheresi 2010). Among chemosynthetic bivalves from reducing marine habitats, the symbiont transmission modes are diverse and family-specific. Environmental acquisition of gill endosymbionts was demonstrated for members of the family Lucinidae based on molecular techniques and electron microscopy (Gros et al. 1996). Subsequently, a free-living form of the *Codakia orbicularis* endosymbiont was shown to be present in the environment (Gros et al. 2003). Morphological and genetic data suggested horizontal transmission of sulfur-oxidizing symbionts in mussels within genus *Bathymodiolus* (DeChaine et al. 2006; Won et al. 2003). Vertical symbiont transmission has been suggested for Solemyidae (Cary 1994; Krueger et al. 1996) but has been best documented in the family Vesicomidae.

The Vesicomidae comprise at least 100 species occurring worldwide at cold seeps, hydrothermal vents, or associated with organic remains, at depths ranging from 100 to 9,050 m (Bennett et al. 1994; Goffredi et al. 2003; Krylova and Sahling 2006, 2010). These clams host sulfur-oxidizing Gammaproteobacteria within their gill epithelial cells (Distel et al. 1988; Imhoff et al. 2003; Kim et al. 1995; Newton et al. 2007). Organic compounds are produced by symbionts and provided to the host, while the bivalve hosts supply the

symbionts with oxygen and carbon dioxide from the seawater, as well as with reduced sulfur from the sediment (Cavanaugh 1983).

In Vesicomidae, two studies on large species suggest vertical symbiont transmission based on transmission electron microscopy (TEM) (Endow and Ohta 1990) or in situ hybridization (ISH) techniques coupled to 16S ribosomal RNA (rRNA) sequencing (Cary and Giovannoni 1993). Endow and Ohta (1990) compared intracellular prokaryotic cells found in gill bacteriocytes with inclusions found in follicle cells and primary oocytes of *Calyptogena soyoe*. Bacteria in all tissues were similar in terms of general morphology and dimensions, presence of electron-lucent vacuoles, and electron-dense granules in the periplasm. Cary and Giovannoni (1993) provided molecular data from three species, namely *C. magnifica*, *C. phaseoliformis*, and *C. pacifica*. They amplified symbiont-specific 16S rRNA genes from the dissected ovarian tissue, which was made possible by the large size of clams. ISH using a specific 16S rRNA-directed probe coupled with digoxigenin confirmed the presence of symbionts in gonad tissues within what they called follicular cells, but not within oocytes. Further indirect supports for vertical symbiont transmission in Vesicomidae are the congruent host and symbiont molecular phylogenies, which suggest cospeciation and a long-term association between the participants in this symbiosis (Peek et al. 1998). Moreover, genomic data indicate reduced genome size, gene loss, and complementation with host genes (Kuwahara et al. 2007; Newton et al. 2007, 2008), as observed in maternally inherited insect-associated bacteria which are unable to live outside their hosts (i.e. *Buchnera aphidicola*) (Gómez-Valero et al. 2007).

The hypothesis of strict vertical transmission for the Vesicomidae was challenged by studies suggesting a mixed mode of symbiont transmission consisting of predominantly vertical transmission punctuated with instances of lateral symbiont acquisition in *Vesicomya* sp. from the Mid-Atlantic Ridge (Stewart et al. 2008, 2009). Recently, it was suggested that physical proximity may promote such events of acquisition of nonparental symbiont strains in two phylogenetically distant but sympatric vesicomid species: *Christineconcha regab* and *Laubiericoncha chuni* (Decker et al. 2013).

Although the hypothesis that symbiont acquisition occurs mainly via maternal inheritance in Vesicomidae is supported and despite a few recent results indicating occasional lateral acquisition of nonparental symbionts, the exact mechanism remains unclear, and whether smaller species (<1 cm) display similar maternal inheritance as observed in the much larger species is not yet known. Here, we investigate the reproduction features and the details of symbiont transmission in *Isorropodon bigoti*, a vesicomid from cold seeps in the Gulf of Guinea harboring sulfur-oxidizing bacteria (Rodrigues et al. 2012). The occurrence of symbionts is tested in gonads and gametes at all stages of gametogenesis, as well

as in early postlarvae, (i.e., individuals collected after larval settlement and metamorphosis) using fluorescence in situ hybridization (FISH) with group-specific probes, while their intra- or extracellular localization is ascertained using TEM.

## Material and methods

### Sampling, collection, and fixation

*Isorropodon bigoti* (Bivalvia, Vesicomidae) (von Cosel et al. 2001) were collected in February 2011 from a pockmark at the Guinness cold seep site during the WACS cruise aboard RV *Pourquoi Pas?* (ROV *Victor 6000*, dive 433, 1°34.65'S, 8°32.91'E, 582-m depth). The clams (2–6 mm) were collected from the first upper centimeters of the sediment using a blade corer. The Guinness' site (Gulf of Guinea), comprises several pockmarks, most of them inactive. Active seepages are colonized by fauna typical of both bathyal and continental margin zones. Seep specialists include bivalves *Elenaconcha guinness* and *Calyptogena valdiviae* (Vesicomidae), *Acharax* sp. (Solemyidae) and *Thyasira* sp. (Thyasiridae); tubeworms *Lamellibrachia* sp. are also reported (Siboglinidae) (Duperron et al. 2012).

Ten specimens of *I. bigoti* were fixed on board for fluorescence in situ hybridization (FISH) experiments and histological serial sectioning, using 4 % formaldehyde in twice-filtered seawater (TFSW) (4 °C, 2–4 h), rinsed twice in TFSW and then transferred in a gradient of ethanol (50, 80, and 100 %). Three specimens were fixed in buffered 2.5 % glutaraldehyde (pH 7.4) for 24 h and then rinsed in TFSW for TEM. All samples were stored at 4 °C and dissected in the laboratory under a dissecting microscope.

### Hematoxylin and eosin staining

Fixed *I. bigoti* specimens were rinsed in butan-1-ol (100 %) and in Histo-Clear (EMS, USA) before being embedded in Paraffin Embedding Wax (melting point 52 °C; Peel-A-Way, EMS, USA). The soft tissues (animals without shell) were cut into 8- $\mu$ m-thick sections using a microtome (Jung, Heidelberg, Germany) and deposited on SuperFrost Plus slides (EMS, USA). Standard hematoxylin and eosin staining protocol was used on one in every ten slides and coverslip was mounted with Permount (EMS, USA). Histological and anatomical observations were performed on an Olympus BX61 light microscope (Olympus, Japan). Basophilic cell structures appeared purple and acidophilic appeared pink. Gamete measurements (circular diameter), as well as shell measurements (length and height), were made with ImagePro software. The circular diameter of gametes ( $d$ ) was calculated as follow:  $d=(a \times b)^{0.5}$ , where ( $a$ ) and ( $b$ ) are the lengths of the major and minor elliptical axes, as oocytes may become ellipsoidal during

their growth (Beninger and LePennec 1997; Heyl et al. 2007; Scheltema and Williams 2009; Tyler et al. 2009). In addition, oocyte measurements were restricted to those oocytes in which the nucleus was clearly visible.

#### Fluorescence in situ hybridization (FISH)

FISH and CARD-FISH (CAlysed Reporter Deposition-FISH) were employed to reveal the presence of bacteria in gonads, gametes, and early post-larval stages. The small size of *I. bigoti* allowed internal negative and positive controls (muscle and gill, respectively) in the same histological section on most of the slides. The paraffin wax was removed using Histo-Clear, tissues were rehydrated in decreasing ethanol series (96→70 %) prior to FISH using Cy3- and Cy5-labeled probes, performed as described previously (Duperron et al. 2005) with a hybridization buffer containing 40 % formamide. The probes used in this study were IMedT2-193, targeting the 16S rRNA (small subunit), originally designed for mussel symbionts, used here as mussel symbionts are closely related with vesicomid symbionts (5'-TAGAGG CCTCCTTTA-3'; Duperron et al. 2008; Rodrigues et al. 2012); EUB-338, targeting most Eubacteria (5'-GCTGCCTC CCGTAGGAGT-3'; Amann et al. 1990) and NON-338, as a control for nonspecific binding (5'-ACTCCTACGGGAGG CAGC-3'; Wallner et al. 1993). Additional negative controls were performed using all probe combinations on slides treated with RNase A (2 mg mL<sup>-1</sup>; 15 min).

CARD-FISH experiments using horseradish peroxidase (HRP)-labeled probes (EUB338 and IMedT2-193) were performed as described by Schönhuber et al. (1997) with slight modifications: (1) internal peroxidases were inactivated on slides for 20 min in H<sub>2</sub>O<sub>2</sub> (0.5 % in PBS buffer) after the lysozyme permeabilization step; (2) slides were stabilized in PBS buffer prior to CARD reaction, which was completed according to manufacturer's instructions (TSA Cyanine 5 System, PerkinElmer, USA). After 30 min at 37 °C, the slides were rinsed in the same buffer for 15 min, dipped in H<sub>2</sub>O, and air dried. SlowFade® Gold with DAPI (Molecular Probes) was applied directly on slides to prevent bleaching of fluorescent dyes. Image acquisition was performed using a Leica SP5 confocal microscope (Leica, Germany).

#### Transmission electron microscopy (TEM)

Glutaraldehyde-fixed samples were rinsed in TFSW, dissected (gill, visceral mass, and foot separated) and postfixed in 1 % osmium tetroxide (45 min, RT). After the dehydration step in increasing ethanol series and 1,2-propylene oxide, tissues were embedded in medium Epon resin (Agar 100 Resin, Great Britain). Sections were cut on a Leica Ultracut R ultramicrotome. Semithin sections were stained with toluidine blue, and ultrathin sections (75-nm thick) were contrasted with uranyl

acetate (5 %) and examined under a Zeiss EM912 Omega transmission electron microscope operated at 80 kV.

#### Post-larvae

In parallel, FISH experiments with the same probes were performed on five FISH fixed *Isorropodon* post-larvae (length ~200 µm with shells) collected from colonization devices (CHEMECOLIs) carried on in 2007 for 1 year in Eastern Mediterranean (Gaudron et al. 2010). FISH tissues were embedded in LR White resin. In order to confirm that larvae actually belong to the genus *Isorropodon*, the gene encoding mitochondrial 16S rRNA was sequenced as follows. DNA extraction of ethanol fixed tissue of one post-larva was done using a QIAamp DNA Micro Kit (QIAGEN). Molecular identification was possible by PCR, ~500 bp part of the mitochondrial 16S rRNA gene was amplified using the primer pair 16SarL (5'-CGCCTGTTTATCAAAAACAT-3') and 16SbrH (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi et al. 2002), then gel excised, cloned, and sequenced.

## Results

#### Morphology and anatomy

The shell length of specimen ranged between 2 and 6 mm. The visceral mass was surrounded by gill tissue organized in filaments consisting of two layers of epithelial cells (bacteriocytes) occurring in the lateral zone of gill filaments. The digestive tract was located in the dorsal part of the visceral mass and seemed to form a short, single tube. Gonads were found dorsal to the foot and ventral to the digestive tract and occupied about 25 % of the visceral mass. Both male and female gonads were organized in a similar way and consisted of round-shaped canals (acini). Gametes at different developmental stages could be observed inside acini. All gamete dimensions and main characteristics are summarized in Table 1. Out of thirteen specimens, we identified nine females and two males of similar size range. For two specimens, the sex could not be determined because of lack of gonad tissues on slides. No hermaphrodite was identified.

#### Oogenesis

The female gonad consisted of a cluster of 15 to 40 acini surrounded by a thin acinal wall. Each acinus is composed of germ cells forming the wall epithelium, oogonia, previtellogenic oocytes initially attached to the inner wall of the acinus, vitellogenic primary oocytes, and mature oocytes which stay in close contact to germline epithelium wall (Fig. 1a). Germ cells were basophilic and constituted a thin epithelium forming the acinus (mean diameter±SD=142.7±45.7 µm; n=31; four

**Table 1** Details on female and male gametes size and main histological features

Sex	Cell type	Gametes size [ $\mu\text{m}$ ]			Main features		
		Mean diameter	Standard deviation	No. of measurements	H&E staining	Shape	Figure
Female	Germ cells	6.4	1.6	23	Basophilic	Cuboid	1a; 1d; 2c
	Oogonia	30.4	8.8	17	Basophilic cytoplasm, acidophilic nucleus	Round	1a; 2e
	Pre-vitellogenic oocytes					Ellipsoidal	1a; 1e
	Vitellogenic oocytes	55.7	8.8	29	Basophilic cytoplasm, acidophilic nucleus	Ellipsoidal	1a; 2g; S1a
	Mature oocytes	97.4	22.6	27	Acidophilic	Round	1a; 1f–g; 2h; S1b–c
Male	Germ cells	3.8	0.4	17	Basophilic	Cuboid	1b; 3a–b
	Spermatogonia	3.9	1.3	44	Basophilic	Round	
	Spermatocytes	1.2	0.2	27	Basophilic	Round	
	Spermatids	1.6	0.5	34	Basophilic	Round	
	Mature spermatozooids	2.3	0.8	69	Acidophilic	Elongated	

specimens analyzed; Table 1). On the transmission electron micrograph (Fig. 2c), germ cells were cuboidal with a prominent nucleus, scant cytoplasm filled with rough endoplasmic reticulum (RER) and few mitochondria. Round oogonia (Ov), ellipsoidal pre-vitellogenic (pVO), and vitellogenic oocytes (VO) (Fig. 1a; Table 1) were anchored to the acinus wall and showed a developed nucleus, as well as a visible nucleolus and scant cytoplasm. Numerous arrays of RER, swollen cisternae in the cytoplasm, electron-dense granules formation, and the nucleus containing scattered clumps of heterochromatin were observed in oogonia (Fig. 2e). In pre-vitellogenic oocytes, numerous mitochondria (Fig. 2g) and visible nucleolus with scattered chromatin within the nucleus were observed. Vitellogenic oocytes (VO) were still in contact with the germline epithelium wall. The VO at the Prophase I of the meiosis appeared pedunculated with developed nuclei forming the germinal vesicle (mean nucleus diameter $\pm$ SD=27.4 $\pm$ 6.7  $\mu\text{m}$ ;  $n=29$ ; Table 1) and filled with an increasing volume of cytoplasm (Fig. 1a). The germinal vesicle and a prominent nucleus occupied most of the cell. The numerous electron-lucent vesicles (vitelline) and some electron-dense lipid and yolk granules could be observed on sections (Fig. S1A). In mature oocytes, the cytoplasm reached the size of the acinus and was still in contact with few germ cells. Mature oocytes were filled with different types of organelles, mainly by lipid droplets (Table 1). Several electron-dense structures could be observed (Fig. S1A–C): germ plasm, which are cloud-like granules (Balbiani bodies) in ooplasm; lipid droplets; Yolk granules; mitochondria in lysis; and other electron-lucent vesicles. The microvilli projections were observed in the outer membrane of the mature oocyte (Fig. S1B). During oogenesis, mitochondria were numerous; in later stages, some of them were in lysis or exocytosis, RER was disappearing (Fig. S1D–F).

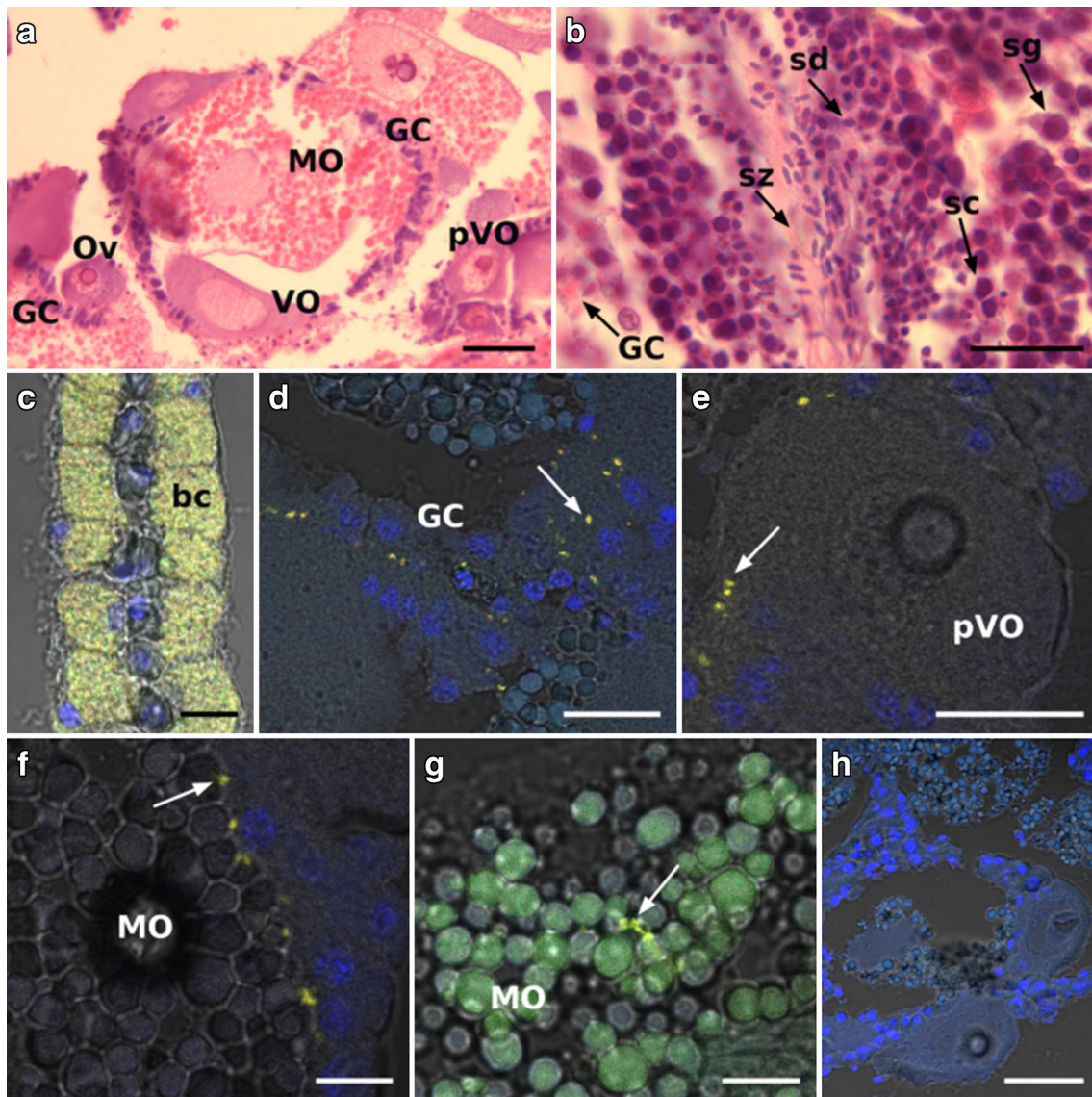
### Spermatogenesis

Testes were also organized in acini in which the germinal epithelium was surrounded by loose connective tissue. Germ cells were located within the inner wall of the round-shaped acinus. The acini were lined with spermatogonia and towards the lumen the following cell types were progressively encountered: spermatocytes, spermatids, and spermatozoa (Fig 1b; Table 1). Because the two males were not fixed in glutaraldehyde, no ultrastructure was available; therefore, the flagellum of the spermatozoa could not be seen.

### FISH

Positive and unambiguous signals were obtained from the gills of all specimens and on each section when using the Eubacteria probe EUB338 and symbiont-specific probe ImedT2-193. CARD-FISH signals were stronger than those in classical FISH (data not shown), but because a single probe could be applied at once, most experiments were done using the basic FISH protocol. Signal patterns were identical for both. Bacteria were abundant in the lateral zone of gill filaments and occupied most of the volume within a single bacteriocyte (Fig. 1c). Groups of bacteria or single bacteria were successfully localized in the female gonad. Bacterial symbionts were present in germ cells (Fig. 1d). Moreover, signals could be detected in the periphery of pre-vitellogenic (Fig. 1e) and mature oocytes (Fig. 1 f–g), most often with uncertain localization within or outside oocytes, although Fig. 1g suggests intracellularly.

Male gonads yielded positive signals using all probes, including NON-338, localized in the lumen of acini, in between the spermatozoa. The male acini on RNase-treated slides still showed the same FISH signal pattern, while there was no signal in bacteriocytes (Fig. 3a–b). Regardless of sex,



**Fig. 1** **a–b:** Hematoxylin and eosin staining of *Isorropodon bigoti* gonad tissues. **a** Cross-section through the female acinus with all stages of gamete differentiation: *GC* germ cells, *Ov* oogonia, *pVO* pre-vitellogenic oocytes, *VO* vitellogenic oocytes, and *MO* mature oocytes. Scale bar=50  $\mu$ m. **b** Cross-section through the male acinus with all stages of gamete differentiation: *GC* germ cells, *sg* spermatogonia, *sc* spermatocytes, *sd* spermatids and *sz* spermatozoa. **c–h:** Fluorescence in situ hybridization on cross-sections of *I. bigoti* tissues. Signals from probes EUB-338 (Cy-3, in green) and IMedT2-193 (Cy-5, in red) fully overlap, yielding a

yellow color. All slides were counterstained with DAPI (host nuclei visible in blue). Scale bars=10  $\mu$ m (**c, f, g**) 20  $\mu$ m (**d, e**) and 50  $\mu$ m (**h**). **c** Gill filament with visible bacteriocytes (*bc*). **d** Germ cells (*GC*) with symbiotic bacteria (white arrow). **e** Pre-vitellogenic oocyte (*pVO*) with symbionts (white arrow). **f** Mature oocyte (*MO*) with symbiotic bacteria (white arrow) close to the cell membrane. **g** Another view of the mature oocyte (*MO*) with symbionts (white arrow) inside the cell between vitellus. **h** Cross-section through the ovary tissue, on slides treated with RNase A, no FISH signal could be detected

no positive signal was observed in muscle tissue using any probe with both FISH and CARD-FISH, and no signal was detected in any tissue when using the NON-338 probe, and on RNase-treated slides using any of the probes (Fig. 1h).

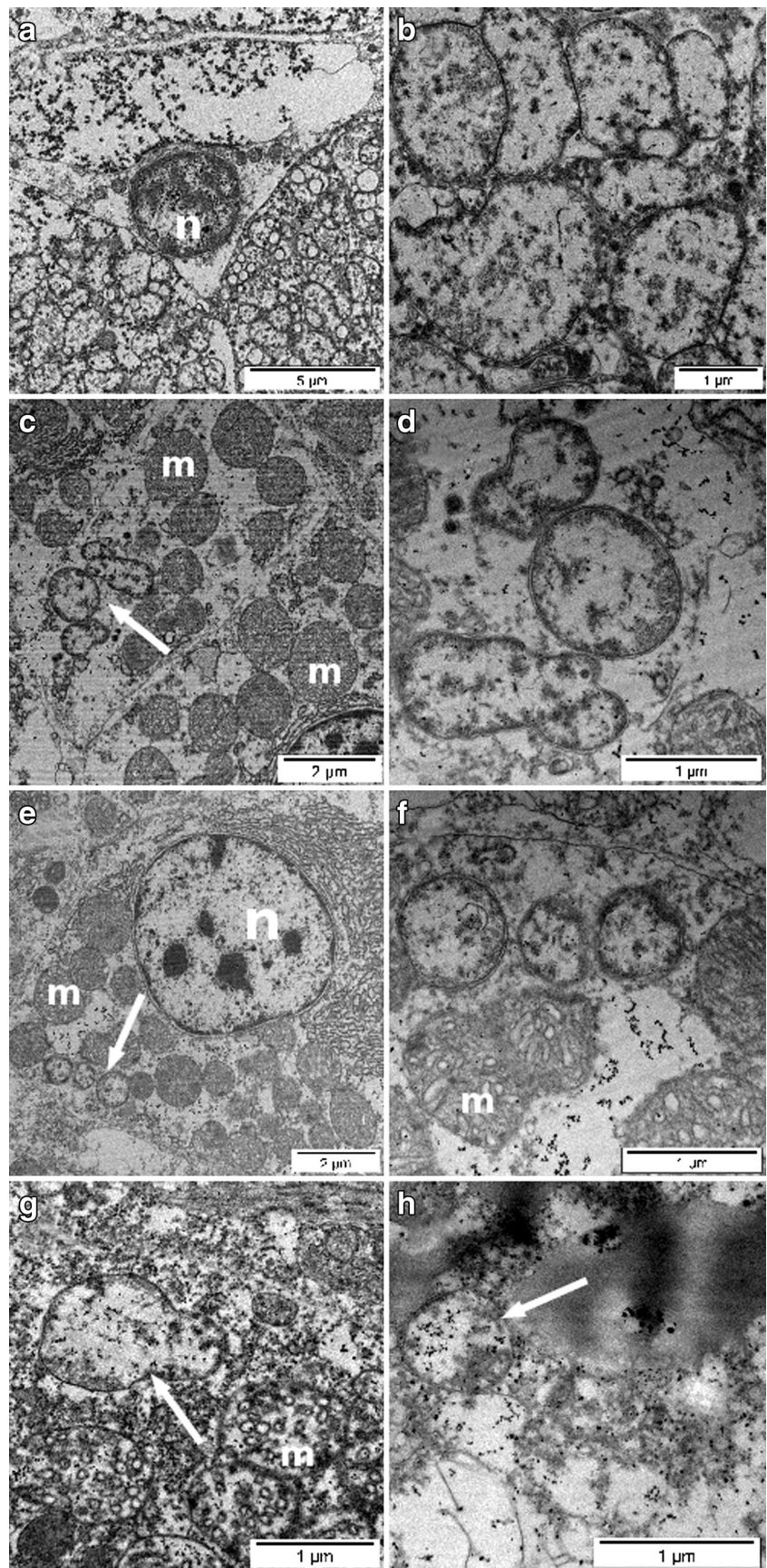
FISH experiments with IMedT2-193, EUB-338 and NON-338 probes showed a typical signal patterns in developing gill epithelium of *Isorropodon* early post-larvae (Fig. 3c). The amplified mitochondrial 16S rRNA gene fragment was 100 % identical to the control

sequence from adult *Isorropodon* specimen (accession numbers: KJ499840-1).

#### TEM on bacterial symbionts

All three specimens fixed for TEM were females. Thiotrophic symbionts observed in gill epithelium served as a reference for comparison with candidate bacteria found in gonads. Bacteriocytes in the gill tissue were filled with vacuoles

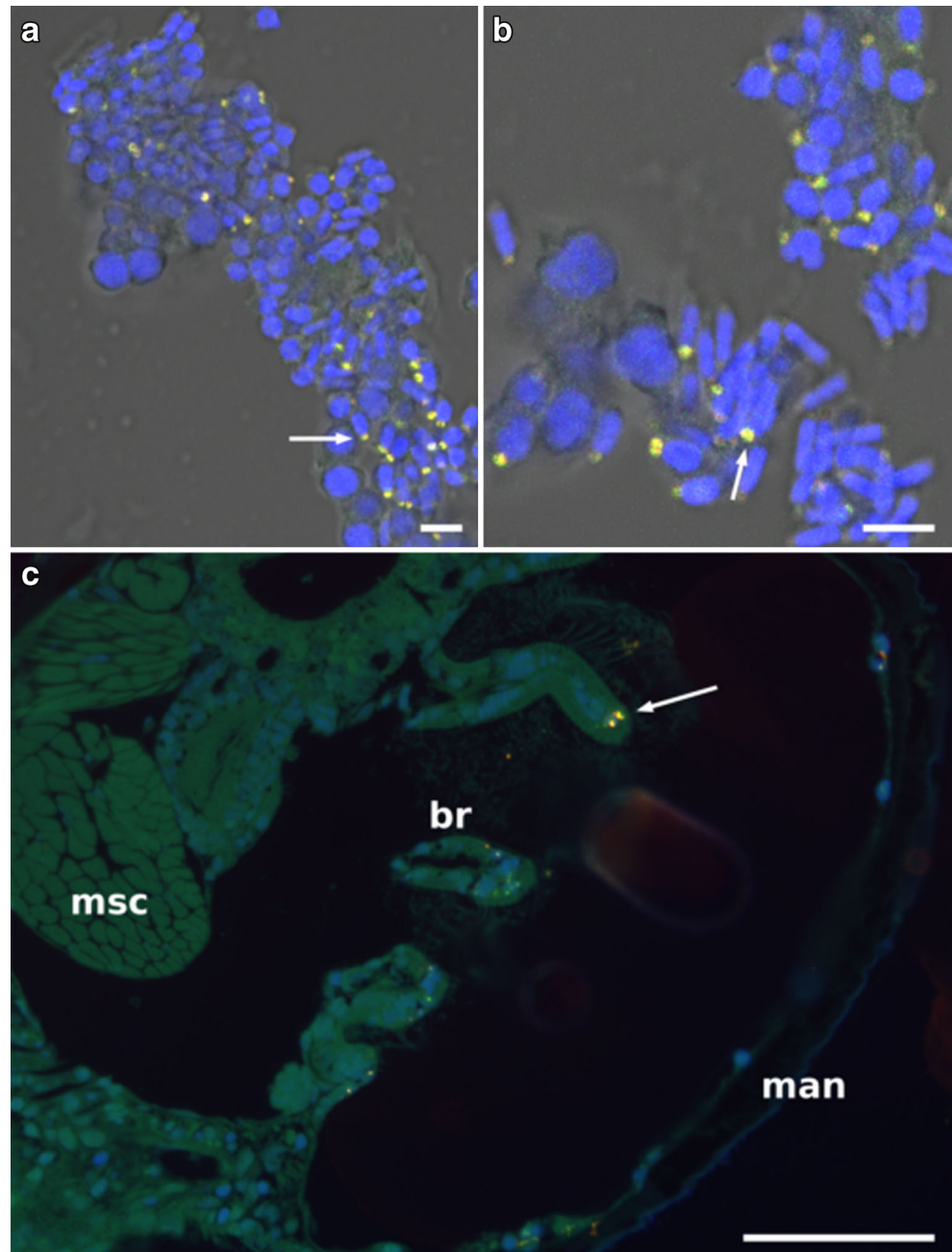
**Fig. 2** Transmission electron micrographs of *Isorropodon bigoti* tissues with endosymbiotic bacteria. **a** Cross-section through the basal part of gill bacteriocyte showing a general organization of the cell (*n* nucleus) and distribution of endosymbionts. **b** Ultrastructure of symbiotic bacteria inside the gill bacteriocyte. **c** A general view of germ cell with endosymbionts (*white arrow*). **d** Three bacterial cells inside one germ cell. **e** A general view of oogonium with endosymbionts (*white arrow*). **f** Three symbiotic bacteria close to the cell membrane of oogonium. **g** Symbiotic bacterium (*white arrow*) among mitochondria (*m*) inside the vitellogenic oocyte. **h** Endosymbiotic bacterium (*white arrow*) inside the mature oocyte



enclosing between 4 and 8 oval or coccoid electron-lucent bacteria with a typical Gram-negative double membrane (Fig. 2a–b). Some bacteria displayed another electron-lucent vesicle in their periplasm (Fig. 2b). Sizes ranged from 1 to 2  $\mu\text{m}$  in diameter and from 2 to 3  $\mu\text{m}$  in length (mean diameter  $\pm$ SD =  $1.43 \pm 0.3 \mu\text{m}$ ;  $n=40$ ). The shape was often irregular. Putative prokaryotic cells displaying morphological characteristics similar to those observed in gills were observed in the cytoplasm of female gametes of different stages in all three clams. Single bacterial cells

were enclosed in a vacuole. Groups up to four intracellular bacteria were observed on a single section in germ cells (Fig. 2c–d), oogonia (Fig. 2e–f), pre-vitellogenic oocytes, vitellogenic (Fig. 2g), and mature oocytes (Fig. 2h). Inside oocytes, most of the bacteria were seen close to the periphery as seen on FISH images, yet intracellular. Bacteria found in female gonads were smaller than those in gill bacteriocytes (mean diameter  $\pm$ SD =  $1.05 \pm 0.29 \mu\text{m}$ ;  $n=20$ ) and had more regular round shapes.

**Fig. 3** a–b Fluorescence in situ hybridization on cross-sections of *I. bigoti* male acini on RNase A treated slides. False positive signals from probes EUB-338 (Cy-3, in green) and IMedT2-193 (Cy-5, in red) fully overlap, yielding a yellow color (white arrows). Slides were counterstained with DAPI (DNA visible in blue). Scale bars = 5  $\mu\text{m}$ . c The general larvae organization on fluorescence in situ hybridization cross-sections: muscle tissue (*msc*), mantle (*man*), and gill tissue (*br*). FISH experiments with IMedT2-193, EUB-338 and NON-338 probes showed a typical signal pattern in gill epithelium (white arrow) of *Isorropodon* larvae. Scale bar = 50  $\mu\text{m}$



## Discussion

To date, information available supporting vertical transmission of symbionts in deep-sea clams was based on transmission electron microscopy (Endow and Ohta 1990) or in situ hybridization (ISH) techniques coupled to 16S rRNA sequencing (Cary and Giovannoni 1993). FISH and CARD-FISH techniques (Amann et al. 1991; Schönhuber et al. 1997) are more sensitive than ISH, yielding bright and localized fluorescent signals. PCR-based detection of symbionts in gonad tissue (Cary and Giovannoni 1993; Krueger et al. 1996) is also a sensitive technique, yet restricted to relatively large animals, where the entire gonad can be isolated without contamination by gill bacteria. Here, we investigated symbiont transmission in a small clam in which the physical separation of gonad tissue was not possible. Yet, save for its small size, the general anatomy of *I. bigoti* was similar to that of larger Vesicomidae (von Cosel et al. 2001).

## Reproduction

*I. bigoti* is a gonochoric species displaying male and female acini in separate specimens. No evidence for hermaphroditism was found, although simultaneous or successive hermaphrodites were reported in *Calyptogena phaseoliformis* and *C. magnifica* (Le Pennec and Beninger 2000). For 2 out of 13 specimens analyzed, the sex was not possible to determine. Presence of gametes at different stages suggests a continuous spawning ability. In *C. kilmeri*, reproduction was suggested to be seasonal, while in *C. pacifica*, oocytes were ready for spawning all over the year (Lisin et al. 1997). Specimens used in our study were collected during a single cruise; therefore, seasonality could not be explored more in details. The sex ratio was skewed towards females. Mature oocytes displayed a size similar to that of larger vesicomid clams (Table 1).

Testes from two male *I. bigoti* showed a typical morphology of bivalve gonad (Beninger and Le Pennec 1997) (Fig. 1b). Again, the cooccurrence of all stages with mature spermatozoa suggests a continuous rather than seasonal reproduction.

## Symbionts in adult host tissues

The general gill organization and bacterial FISH signals were similar to those of other vesicomid clams (Rodrigues et al. 2012; Duperron et al. 2012). Individual bacteria, positive with the symbiont probe, were also localized in the female gonad. By using FISH, we confirmed the actual occurrence of symbionts in gonad tissue in Vesicomidae (Cary and Giovannoni 1993). We observed the intracellular occurrence of these bacteria within oocytes at different stages, but the more advanced the developmental stage of a gamete, the fewer bacteria could be observed within a single gamete. Our results support that

bacterial symbionts are present at all steps of female gametogenesis from germ cells to mature oocytes, providing a way for vertical transfer through female gametes (transovarial inheritance). Oocytes derive initially from germ cells, within which bacteria are present, though smaller and less abundant than in gill bacteriocytes. Bacteria seem to remain intracellular during all gametogenesis. This suggests a strictly intracellular route of inheritance from mother to offspring, rather than contamination of developing gametes by environmental bacteria which would have entered the acinus. Bacteria were detected in about 1 out of 10 mature oocytes. On a given FISH slide, only one or a few bacteria were seen, and TEM confirmed the occurrence of single bacterial cells within vacuoles in both germ cells and oocytes, with very few dividing stages. FISH signals in mature oocytes were scarce because of low total number of bacteria dispersed in a more and more voluminous three-dimensional structure. The volume of oocytes is indeed much higher than that of germ cells, and symbionts do not seem to divide actively within developing gametes; the chance of seeing a FISH signal in oocytes is thus lower, as their diameter is at least 10 times the thickness of slides. The TEM observation of single bacterial cells within vacuoles in both germ cells and oocytes further supports this hypothesis, and very few dividing stages were seen. This lack of dividing stages may suggest the temporary inhibition of bacterial cell divisions during oogenesis.

In male acini, all IMedT2 signals cohybridized with those of NON probe, even on RNase-treated slides. This indicates nonspecific binding of fluorescent dyes or probes and that the signal is an artifact. No observed signal could be considered as a putative symbiont (Fig. 3a–b). The symbiotic bacteria were absent in all other organs and tissues of *I. bigoti* regardless of sex.

## Symbiont translocation and possible consequences on symbiont transmission

Contrary to insect bacteriomes, specialized organs housing endosymbiotic bacteria which are close to gonads, the symbiont-harboring organs in Vesicomidae are far from each other, and we do not know how bacteria pass from one to the other.

One could hypothesize that symbionts pass from gill to gonads through other tissues during early development. In *Riftia pachyptila* (Annelida, Siboglinidae), bacterial symbionts indeed colonize the developing tube of the settled post-larvae and enter the host through the skin, a process that continues through the early juvenile stages during which the trophosome is established from mesodermal tissue (Nussbaumer et al. 2006). There, bacteria penetrate and cross several tissues in which they will not be retained. Alternatively, because both gonad and gill are epithelial tissues, a broad contamination of epithelia might occur at an



early stage, and the bacteria are retained only in the gill and gonad. In this case, bacteria never occur in any other internal tissue. Wide infection of epithelium is for example reported in juvenile *Bathymodiolus puteoserpentis* and *B. azoricus* mussels (Wentrup et al. 2013).

However, our results on *Isorropodon* larvae suggest that bacteria are restricted to the gill epithelium as early as the post-larval stage (~200  $\mu\text{m}$  shell length, see Fig. 3c). Symbiont translocation may have already occurred at the veliger stages during dispersion before the settlement of the post-larvae. This evidence of transmission seems to be very difficult to document in larvae of deep-sea bivalves. Due to their small size, we could identify neither gonad tissue nor the sex of animal.

A third possibility for symbiont translocation would be that bacteria are released from the gill epithelium into the pallial cavity and then enter the female gonad through the genital duct. In this case, the mechanism could be similar to what is reported between the squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri* (reference). In this association, the specialized light organs called crypts are colonized by only few bacteria which are recognized by the epithelium and allowed to pass through the pores and inoculate crypt spaces (McFall-Ngai 2000). In the case of *I. bigoti*, the parental gill symbiont would be always transmitted maternally to the progeny if we consider the first hypothesis. In the second situation, acquisition of nonparental bacteria would be possible if free-living forms occur in the environment and are able to establish in the epithelia. The third situation is more complex. If bacteria pass through the pallial cavity, then the gill symbiont would be the most likely transmitted because it is a dominant bacterium in the pallial cavity, but it is not impossible that another vesicomid symbiont present could enter the mantle cavity and colonize gonads instead of the maternal one. This could occur in particular when several Vesicomidae species cooccur in a single aggregate (Decker et al. 2013). In all cases, the possibility of additional secondary, postfertilization, acquisition of symbionts must be further investigated.

#### Evolutionary consequences of symbionts in female gonads

Environmentally acquired symbionts are assumed to derive from a genetically diverse free-living population. In this case, selection can freely act against deleterious alleles that have arisen by chance in the symbiont genome, and the risk of their accumulation is lower (Funk et al. 2001; Moran 1996; Ohta 1973). In transovarial inheritance, only few symbionts seem to be transmitted within a given oocyte, which produces a bottleneck. Limited populations and transmission bottlenecks lead to accelerated evolution and to symbiont genome reduction (McCutcheon and Moran 2012; Peek et al. 1998; Kuwahara et al. 2007; Newton et al. 2007). Recent genome sequencing of two vesicomid symbionts suggests that the

symbiont genome (~1.1 Mb) has experienced some level of size reduction (Kuwahara et al. 2007; Newton et al. 2007).

We herein present strong evidence for maternal transmission of symbionts in *I. bigoti*, although the fate of bacteria from fertilization to larval settlement remains to be documented. This transmission mode does not exclude additional acquisition of laterally transmitted bacteria. Indeed, recent work has emphasized the existence of occasional lateral transmission in some Vesicomidae. The phylogenetic trees of symbionts that are exclusively vertically transmitted are congruent with those of their hosts over long evolutionary time (Peek et al. 1998). However, a comparative analysis of three *Vesicomya* sp. genes from hydrothermal vents at the Juan de Fuca Ridge, with four symbiont loci revealed unexpected symbiont phylotypes occurring at low frequencies and highly divergent from previously described symbionts of the same host lineage (Stewart et al. 2008). Recently, it was shown that when two phylogenetically distant vesicomid species, *Christineconcha regab* and *Laubiericoncha chuni*, cooccur at a given aggregate in the Gulf of Guinea, each species harbored its symbiont plus a few bacteria corresponding to the symbiont of the other species in their gills (Decker et al. 2013). These studies do not confirm whether the unexpected, nonparental, symbionts can actually be transmitted to the progeny. For this, the presence of these lineages should be investigated in gonad tissue.

#### Conclusions

Bacterial symbionts of *I. bigoti*, a gonochoristic bivalve from the Guinness cold seep site, occur within female gonad cells at all stages of gamete differentiation from germ cells to mature oocytes. They are completely absent from the male gonad and gametes. Post-larvae (200- $\mu\text{m}$  shell length) display low amounts of these bacteria in their gills. This study strongly suggests the transovarial inheritance of symbionts within family Vesicomidae. Future studies should attempt in vitro fertilization experiments and investigate larval development to test the possibility of additional postfertilization symbiont acquisition.

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## References

- Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microb* 56:1919–1925
- Amann R, Springer N, Ludwig W, Gortz H, Schleifer K (1991) Identification in situ and phylogeny of uncultured bacterial endosymbionts. *Nature* 351:161–164
- Beninger PG, LePennec M (1997) Reproductive characteristics of a primitive bivalve from a deep-sea reducing environment: giant gametes and their significance in *Acharax alinae* (cryptodonta: Solemyidae). *Mar Ecol-Prog Ser* 157:195–206
- Bennett B, Smith C, Glaser B, Maybaum H (1994) Faunal community structure of a chemoautotrophic assemblage on whale bones in the deep northeast pacific-ocean. *Mar Ecol-Prog Ser* 108:205–223
- Bright M, Bulgheresi S (2010) A complex journey: transmission of microbial symbionts. *Nat Rev Microbiol* 8:218–230
- Cary SC (1994) Vertical transmission of a chemoautotrophic symbiont in the protobranch bivalve, *Solemya reidi*. *Mol Mar Biol Biotech* 3:121–130
- Cary S, Giovannoni S (1993) Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *Proc Natl Acad Sci U S A* 90:5695–5699
- Cavanaugh CM (1983) Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. *Nature* 302:58–61
- DeChaine EG, Bates AE, Shank TM, Cavanaugh CM (2006) Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. *Environ Microbiol* 8:1902–1912
- Decker C, Olu K, Arnaud-Haond S, Duperron S (2013) Physical proximity May promote lateral acquisition of bacterial symbionts in vesicomid clams. *PLoS ONE* 8:e64830. doi:10.1371/journal.pone.0064830
- Distel DL, Lane DJ, Olsen GJ, Giovannoni SJ, Pace B, Pace NR, Stahl DA, Felbeck H (1988) Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences. *J Bacteriol* 170:2506–2510
- Duperron S, Nadalig T, Caprais JC, Sibuet M, Fiala-Medioni A, Amann R, Dubilier N (2005) Dual symbiosis in a *Bathymodiolus* sp. Mussel from a methane seep on the Gabon continental margin (southeast Atlantic): 16S rRNA phylogeny and distribution of the symbionts in gills. *Appl Environ Microbiol* 71:1694–1700
- Duperron S, Halary S, Lorion J, Sibuet M, Gaill F (2008) Unexpected co-occurrence of six bacterial symbionts in the gills of the cold seep mussel *Idas* sp. (Bivalvia: Mytilidae). *Environ Microbiol* 10:433–445
- Duperron S, Rodrigues CF, Léger N, Szafranski K, Decker C, Olu K, Gaudron SM (2012) Diversity of symbioses between chemosynthetic bacteria and metazoans at the Guinness cold seep site (Gulf of Guinea, West Africa). *MicrobiologyOpen* 1:467–480
- Endow K, Ohta S (1990) Occurrence of bacteria in the primary oocytes of vesicomid clam *Calyptogena soyoae*. *Mar Ecol-Prog Ser* 64:309–311
- Funk DJ, Wernegreen JJ, Moran NA (2001) Intraspecific variation in symbiont genomes: bottlenecks and the aphid-*Buchnera* association. *Genetics* 157:477–489
- Gaudron SM, Pradillon F, Pailleret M, Duperron S, Le Bris N, Gaill F (2010) Colonization of organic substrates deployed in deep-sea reducing habitats by symbiotic species and associated fauna. *Mar Environ Res* 70:1–12
- Goffredi S, Hurtado L, Hallam S, Vrijenhoek R (2003) Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomidae) of the “*pacifica/lepta*” species complex. *Mar Biol* 142:311–320
- Gómez-Valero L, Silva FJ, Simon JC, Latorre A (2007) Genome reduction of the aphid endosymbiont *Buchnera aphidicola* in a recent evolutionary time scale. *Gene* 389:87–95
- Gros O, Darrasse A, Durand P, Frenkiel L, Moueza M (1996) Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. *Appl Environ Microbiol* 62:2324–2330
- Gros O, Liberge M, Heddi A, Khatchadourian C, Felbeck H (2003) Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* environment). *Appl Environ Microbiol* 69:6264–6267
- Heyl TP, Gilhooly WP, Chambers RM, Gilchrist GW, Macko SA, Ruppel CD, Van Dover CL (2007) Characteristics of vesicomid clams and their environment at the Blake Ridge cold seep, South Carolina, USA. *Mar Ecol-Prog Ser* 339:169–184
- Imhoff JF, Sahling H, Sling J, Kath T (2003) 16S rDNA-based phylogeny of sulphur-oxidising bacterial endosymbionts in marine bivalves from cold-seep habitats. *Mar Ecol-Prog Ser* 249:39–51
- Kim YW, Yasuda M, Yamagishi A, Oshima T, Ohta S (1995) Characterization of the endosymbiont of a deep-sea bivalve, *Calyptogena soyoae*. *Appl Environ Microbiol* 61:823–827
- Krueger DM, Gustafson RG, Cavanaugh CM (1996) Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). *Biol Bull* 190:195–202
- Krylova EM, Sahling H (2006) Recent bivalve molluscs of the genus *Calyptogena* (Vesicomidae). *J Mollus Stud* 72:359–395
- Krylova EM, Sahling H (2010) Vesicomidae (Bivalvia): current taxonomy and distribution. *PLoS ONE* 5:e9957. doi:10.1371/journal.pone.0009957
- Kuwahara H, Yoshida T, Takaki Y et al (2007) Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutani*. *Curr Biol* 17:881–886
- Le Pennec M, Beninger PG (2000) Reproductive characteristics and strategies of reducing-system bivalves. *Comp Biochem Phys A* 126:1–16
- Lisin SE, Hannan EE, Kochevar RE, Harrold C, Barry JP (1997) Temporal variation in gametogenic cycles of vesicomid clams. *Invertebr Reprod Dev* 31:307–318
- McCutcheon JP, Moran NA (2012) Extreme genome reduction in symbiotic bacteria. *Nat Rev Microbiol* 10:13–26
- McFall-Ngai MJ (2000) Negotiations between animals and bacteria: the “diplomacy” of the squid-vibrio symbiosis. *Comp Biochem Phys A* 126:471–480
- Moran NA (1996) Accelerated evolution and Muller’s ratchet in endosymbiotic bacteria. *Proc Natl Acad Sci U S A* 93:2873–2878
- Newton ILG, Woyke T, Auchtung TA et al (2007) The *Calyptogena magnifica* chemoautotrophic symbiont genome. *Science* 315:998–1000
- Newton ILG, Girguis PR, Cavanaugh CM (2008) Comparative genomics of vesicomid clam (Bivalvia: Mollusca) chemosynthetic symbionts. *BMC Genomics* 9:585
- Nussbaumer AD, Fisher CR, Bright M (2006) Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* 441:345–348
- Ohta T (1973) Slightly deleterious mutant substitutions in evolution. *Nature* 246:96–98
- Palumbi S, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (2002) The simple Fool’s Guide to PCR, version 2.0. Department of Zoology, Kewalo Marine Laboratory, University of Hawaii, Honolulu
- Peek AS, Feldman RA, Lutz RA, Vrijenhoek RC (1998) Cospeciation of chemoautotrophic bacteria and deep sea clams. *Proc Natl Acad Sci U S A* 95:9962–9966
- Rodrigues C, Cunha M, Olu K, Duperron S (2012) The smaller vesicomid bivalves in the genus *Isorropodon* (Bivalvia, Vesicomidae, Pliocardiinae) also harbour chemoautotrophic symbionts. *Symbiosis* 56:129–137
- Scheltema RS, Williams IP (2009) Reproduction among protobranch bivalves of the family Nuculidae from sublittoral, bathyal, and

- abyssal depths off the New England coast of North America. *Deep Sea Res II* 56:1835–1846
- Schönhuber W, Fuchs B, Juretschko S, Amann R (1997) Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Appl Environ Microbiol* 63:3268–3273
- Stewart FJ, Young CR, Cavanaugh CM (2008) Lateral symbiont acquisition in a maternally transmitted chemosynthetic clam endosymbiosis. *Mol Biol Evol* 25:673–687
- Stewart FJ, Young CR, Cavanaugh CM (2009) Evidence for homologous recombination in intracellular chemosynthetic clam symbionts. *Mol Biol Evol* 26:1391–1404
- Tyler PA, Marsh L, Baco-Taylor A, Smith CR (2009) Protandric hermaphroditism in the whale-fall bivalve mollusc *Idas washingtonia*. *Deep Sea Res II* 56:1689–1699
- Von Cosel R, Salas C, Høisæter T (2001) Vesicomylidae (Mollusca: Bivalvia) of the genera *Vesicomya*, *Waisiuconcha*, *Isorropodon* and *Callogonia* in the eastern Atlantic and the Mediterranean. *Sarsia* 86:333–366
- Wallner G, Amann R, Beisker W (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* 14:136–143
- Wentrup C, Wendeberg A, Huang JY, Borowski C, Dubilier N (2013) Shift from widespread symbiont infection of host tissues to specific colonization of gills in juvenile deep-sea mussels. *ISME J* 7:1244–1247
- Won YJ, Hallam SJ, O'Mullan GD, Pan IL, Buck KR, Vrijenhoek RC (2003) Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiulus*. *Appl Environ Microbiol* 69:6785–6792