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ORIGINAL PAPER

Spawning and development in *Osedax* boneworms (Siboglinidae, Annelida)

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Abstract We report observations on spawning and early development in bone-eating worms of the genus *Osedax*. Individual females of *Osedax rubiplumus* were observed at 1820 m depth freely spawning hundreds of oocytes, and females of an undescribed species, *Osedax* "orange collar", were observed spawning in laboratory aquaria. Cytological and molecular analysis of the spawned oocytes of two *Osedax* species revealed no evidence for the bacterial endosymbionts that the female worms require for their nutrition, suggesting that the bacteria must be acquired later from the environment, as they are in other siboglinids. Individual *O.* "orange collar" females released an average of 335 (±130) eggs per day, but the number of oocytes spawned per day varied greatly, suggesting that not all the females spawned daily. Fertilization rates of the spawned oocytes varied from 0 to 100%, though most females showed nearly 100% fertilization rates. Oocytes spawned in the laboratory at 4–6°C were negatively buoyant. If fertilized, these oocytes extruded polar bodies and then after at least four hours cleaved unequally. Subsequent cleavages occurred in a spiral pattern at roughly 2-h intervals, resulting in free-swimming trochophore larvae after 24 h. These lecithotrophic trochophores swam for 9–16 days before settling with several hooked chaetae, similar to those of dwarf *Osedax* males. The larval life span of the *Osedax* species studied in the laboratory appears to be shorter than in closely related Vestimentifera. *Osedax rubiplumus*, on the other hand, has much larger oocytes and so may have greater dispersal potential than these other *Osedax* species. The high fecundity and apparently continuous reproduction of *Osedax* boneworms permits the opportunistic exploitation of sunken vertebrate bones.

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Introduction

Though only recently discovered, bone-eating annelids of the genus *Osedax* (Polychaeta: Siboglindae) are now known to occur worldwide (Rouse et al. 2004; Glover et al. 2005; Fujikura et al. 2006; Braby et al. 2007; Vrijenhoek et al. 2008a). *Osedax* females lack a gut and rely instead on a complex system of 'roots' that house endosymbiotic Oceanospirillales bacteria to extract organic compounds from submerged bones (Goffredi et al. 2005; Goffredi et al. 2007). The females host dwarf males in their gelatinous tubes (Rouse et al. 2004; Rouse et al. 2008; Vrijenhoek et al. 2008b), but little else is known about sex-determination,

reproduction, development and dispersal capabilities of *Osedax*. The whale-fall habitats on which most *Osedax* have been found are dynamic and biologically diverse habitat 'islands' on the sea floor (Smith and Baco 2003). Yet the availability of such habitats may be unpredictable in time and space. Consequently, opportunistic marine invertebrates like *Osedax* are predicted to have a suite of covariable traits that include rapid growth, early maturity, a short adult life, high fecundity, broadcast spawning, no parental care, and long dispersal time, (reviewed by Olive 1985; Ramirez-Llodra 2002; Young 2003). An earlier generation of deterministic life history models (e.g., McArthur and Wilson 1967) have proved to be somewhat simplistic in this regard (Olive 1985; Grant 1990; Stearns 1992). Properly assessing historical constraints on the polarity and covariability of evolutionary changes in a complex suite of life history traits requires a solid phylogenetic framework (Harvey and Pagel 1991; Rouse and Fitzhugh 1994; Hart et al. 1997). Thus, monophyletic groups that show a great range of life history traits are valuable in assessing life history theory (McHugh and Rouse 1998).

Siboglinid annelids—the former phyla Vestimentifera and Pogonophora plus *Osedax*—show a wide range of habitat exploitation, body sizes and reproductive traits (Southward et al. 2005; Rouse et al. 2008) and so provide an ideal group for the study of life history evolution. All siboglinids rely as adults on endosymbiotic bacteria for their nutrition though they live in diverse environments including hydrothermal vents, cold water hydrocarbon seeps, anoxic basins, and large organic food falls. Except for *Osedax*, all other siboglinids appear to have males and females of equivalent size (Southward 1999). Males and females of the giant vestimentiferan *Riftia pachyptila* found at eastern Pacific hydrothermal vents can reach well over one metre in length, and the females freely spawn numerous small (fertilized) oocytes (Hilário et al. 2005). Males and females of the frenulate pogonophores, on the other hand, are small, generally several centimeters long and less that one millimetre in diameter, and the females tend to spawn relatively few large eggs that they brood in their tubes (Southward 1999). *Sclerolinum*—sister group to Vestimentifera (Halanych et al. 2001; Rouse 2001; Rouse et al. 2004)—also is relatively small and thin, but little is known about its reproduction. *Osedax*, are sister group to the *Sclerolinum* plus Vestimentifera clade (refered to as Monilifera in Rouse 2001). Studying the life histories and reproductive traits of siboglinid worms and their associations with diverse microbes and deep-sea habitats is difficult because not one species has been reared through a complete life cycle in the laboratory. Nonetheless, considerable progress has occurred with studies of early development based on spawning wild-caught individuals (Bakke 1976; Marsh et al. 2001; Young et al. 1996).

Here we report the first observations of spawning in *Osedax* (field and laboratory) and describe the zygotes, embryogenesis and larval development for two species from Monterey Bay, California (Braby et al., 2007). To determine if the endosymbiotic Oceanospirillales bacteria might be vertically transmitted from the mother, we examined spawned oocytes histologically and with in situ hybridization techniques. Using *Osedax* maintained in the laboratory, we quantified numbers of oocytes spawned by females, measured fertilization rates, documented developmental rates, and described the development of *Osedax* embryos, larvae, and newly settled larvae. Finally, we showed that the late swimming larval stages and newly settled larvae have morphology similar to that of dwarf males.

Materials and methods

Study organisms and collection methods

Field observations and collections were made on *Osedax* species living on whale skeletons deployed at four depths in Monterey Bay, CA, USA: 385, 633, 1,018, and 1,820 m. Exact locations and methods for sinking the carcasses were presented earlier (Braby et al. 2007). Bones from whale-385, whale-633 and whale-1018 (number indicates depth of carcass) were collected with remotely operated vehicle (ROV) *Tiburon* and transported to the surface in closed, insulated containers. Bones from whale-1018 were occupied by an undescribed species of *Osedax* referred to here as *Osedax* "nude palps", similar to the *Osedax* "nude-palp-A" and "nude-palp-B" found by Jones et al. (2008) from other whale carcasses. The *Osedax* studied from whale-385 and whale-633 are other, yet to be described, new species referred to here as *Osedax* "yellow collar" and *Osedax* "orange collar" (see also Braby et al. 2007). Upon recovery at the surface, isolated bones were maintained at atmospheric pressure and supplied with recirculating surface seawater at 4–6°C. Bones with *Osedax* were transported or shipped overnight where they were maintained in chilled flow-through aquaria at Scripps Institution of Oceanography (SIO), or in refrigerated recirculating aquaria at Monterey Bay Aquarium (MBA) and Oregon Institute of Marine Biology (OMIB). Bones from different whale-falls were always maintained in separate aquaria.

Spawning observations

In situ observations and video recordings of spawning *O. rubiplumus* females on whale-1820 were made with a high-definition digital video camera (Panasonic WVE550) aboard the ROV *Tiburon*. Laboratory observations of and digital video recordings (with a

Canon Powershot G9) of streams of zygotes being spawned by females of *Osedax* "yellow collar" were made at SIO.

Fertilization, fecundity estimates and development

Developing *Osedax* larvae were first observed for a small group of worms on a bone held at the Monterey Bay Aquarium and observations on development through the early larval stages were made shortly thereafter at the Oregon Institute of Marine Biology. These early observations were confirmed at SIO followed by detailed fertilization, fecundity estimates and developmental studies using the following methods. Oocytes were gathered with a pipette as they were spawned to give zero points for timing of cleavage stages and fertilization rates. The bottoms of aquaria were siphoned twice daily to obtain further zygotes or early embryos. These were subsequently held in glass dishes with 0.22 μm filtered seawater and maintained in a constant temperature room (4–5°C). Developmental stages were photographed alive with either Nikon Coolpix 4300 or Canon Powershot G9 cameras through a Leica MZ8 stereomicroscope or DMR compound microscope using differential interference contrast (DIC). Cleavage rates were followed for numerous individual embryos observed at hourly or half-hourly intervals with a stereomicroscope kept in the controlled temperature room. Further development was followed by maintaining larvae in glass dishes with daily changes of 0.22 μm filtered seawater maintained at 4–5°C. Scanning electron microscopy was performed on larvae fixed in 1% osmium tetroxide in seawater for 1 h, rinsed, dehydrated in ethanol and critical-point dried and coated with platinum before viewing with a FEI Quanta 600 scanning electron microscope.

To exclude the possibility that laboratory colonies of *Osedax* were contaminated with the larvae of other marine annelids, we conducted molecular identifications to confirm the identity of developmental stages not directly sampled from spawning females. The oocytes, zygotes, embryos and swimming larvae sampled from laboratory aquaria were preserved whole in 95% ethanol for molecular analyses. DNA sequencing of a ~1,250 bp portion of mitochondrial cytochrome-c-oxidase subunit I (mtCOI) was carried out at MBARI following standard protocols for *Osedax* species (Braby et al. 2007; Rouse et al. 2008). The species identities of adult females held in aquaria were verified by sequencing DNA extracted from the tip of one or more palps.

Fertilization status was assessed by gathering streams of oocytes as they were emitted by eight different female *Osedax* "orange collar" on various occasions. Batches of oocytes (at least 20 were gathered) were isolated in petri dishes and those that were uncleaved after 6 h were regarded as unfertilized (after initial longer-term observations). To assess spawning frequency and average daily fecundity, a piece of bone from whale-633 with a known number of female *Osedax* "orange collar" (155 individuals) was isolated in 50 l of water in an aquarium kept at 4°C. After 24 h, the bone with worms was removed and the seawater was filtered through 33 μm mesh. The bone and worms were then placed in fresh seawater in the aquarium for another 24 h and this process was repeated for 7 consecutive days. The residue from each daily sample, containing thousands of oocytes, zygotes and embryos, was resuspended in 200 ml of seawater and six aliquots of 1 ml each were taken and the numbers of zygotes or embryos counted. Total fecundity for the 24 h period was then estimated and divided by the total number of female worms on the bone to give a mean spawning number of oocytes spawned per female per day.

Assessment of vertical bacterial symbiont transmission

Newly spawned oocytes were fixed for light microscopy (LM) at SIO in 3% glutaraldehyde in 0.1 M cacodylate buffer with 0.3 M sucrose (pH 7.8). After several buffer rinses, oocytes were dehydrated in a graded ethanol series, and embedded in Spurr's epoxy resin. Semi-thin sections (1 μm) were cut with a RMC PT-X ultramicrotome, stained with toluidine blue and examined with a Leica DMR compound microscope to determine if bacteria were present. For in situ hybridization to detect the presence of bacterial symbionts, zygotes of *O. rubiplumus* and 3-day old free-swimming larvae of *Osedax* "nude palps" were fixed in 2% paraformaldehyde in PBS with 0.3 M sucrose for 1 h at 4°C, rinsed, and transported to California Institute of Technology. There the larvae were pulled onto a polycarbonate filter and rinsed with 100% ethanol. Hybridization was performed immediately using Cy3-labelled oligonucleotide probes, Gam42a, which encompasses the known Oceanospirillales symbionts of *Osedax*, or sym435 a probe specific to *Osedax* endosymbionts (Goffredi et al. 2007).

Results

Spawning observations

During ROV *Tiburon* expeditions on October 17, 2006, January 2007 and December 20 2007 at 1,820 m depth, we repeatedly observed spawning events involving *O. rubiplumus* females living on the bones of whale-1820, a California grey whale carcass (Fig. 1a; and Supplement Videos 1&2):

Spawning was not synchronous in these dense assemblages of worms, as only occasional individual females

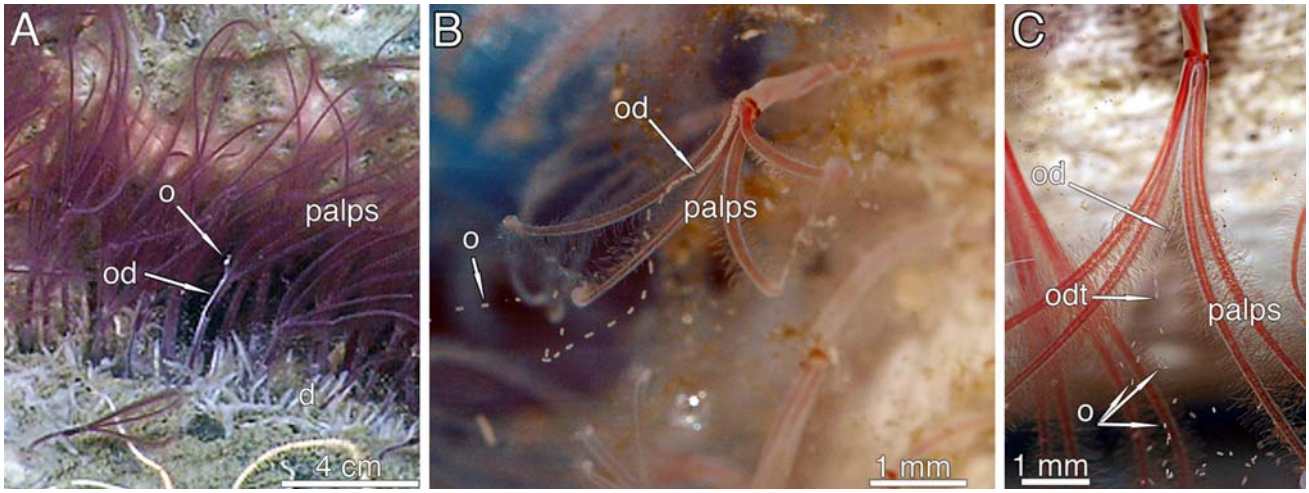

Fig. 1 *Osedax* spawning. **a** *Osedax rubiplumus* females (1,820 m depth). Framegrab from supplemental video S1. The oviduct of one female is filled with oocytes that are emerging from the tip. **b** Female *Osedax* "orange collar" on bone from whale-633, maintained in laboratory aquarium at atmospheric pressure and 4°C, spawning a stream of oocytes. **c** Female *Osedax* "orange collar" on bone from whale-633, in laboratory aquarium at atmospheric pressure with water flow turned off. The stream of oocytes emerging from the oviduct tip is clearly negatively buoyant. Note the ellipsoidal shape of the spawned oocytes. *o* spawned oocytes, *od* oviduct, *odt* oviduct tip

released streams of "eggs" (almost certainly fertilized primary oocytes, see below) from the oviduct extending as part the worms' anterior crown. Each spawning event, which could last several minutes, consisted of a series of emissions (~60 s long) interrupted by pauses of 20–30 s. Prior to each emission, the oviduct visibly filled with a stream of white oocytes (Fig. 1a, S1&2). The oocyte streams appeared as a loosely connected stream following emission, and then rapidly dissipated in the current (S1&2).

Spawning by female *Osedax* "orange collar" was also observed repeatedly in aquaria (Figs. 1b, c; and Supplement Video 3). Females spawned continuous streams of oocytes, with occasional pauses, for periods if up to 4 minutes. The oocytes were emitted at the tip of the oviduct, which, as in most *Osedax* species, extends beyond the trunk as a thin tube that lies among the plume-like palps. As the oocytes were being expelled the female rotated slowly within her tube over a range of about 30°. The oocytes, which had a flattened ellipsoidal shape, separated immediately after being expelled from the tip of the oviduct (Figs. 1b, c, S3).

Females of *Osedax* "yellow collar" from whale-385, *Osedax* "orange collar" (Fig. 1b, c) from whale-633 and *Osedax* "nude palps" from whale-1018 regularly spawned negatively buoyant oocytes. Supplemental video S3 shows oocytes apparently floating upwards from a spawning female of *Osedax* "orange collar", but this is due to the current flow in the tank. Figure 1b and c were taken with the water flow turned off and the oocytes were seen to fall to the bottom. Further evidence to suggest that oocytes, zygotes and early embryos are negatively buoyant (at atmospheric pressure) lies in the fact that large numbers could be obtained by retrieving the water at the bottom of aquaria.

DNA sequencing (mtCOI) was used to confirm (DNA barcode) the identity of the adult female *Osedax* kept in aquaria. The *Osedax* "yellow collar" adult females from whale-385 (Genbank Accession numbers EU267673 and EU267674) and whale-633 (Genbank Accession numbers FJ347628-9, FJ431198-200, FJ431202-4) are the same two undescribed species referred to as *Osedax* "yellow collar" and *Osedax* "orange collar" in Braby et al. (2007). *Osedax* "nude palps" (EU267676 and other as yet unpublished sequences) from Whale-1018 is morphologically very similar to *Osedax* "nude-palp-A" and *Osedax* "nude-palp-B" as mentioned in Jones et al. (2008), but based on pairwise sequence divergences appears to represent another new species (G.W. Rouse et al. in preparation). During early phases of the project, larvae and embryos were being retrieved from the bottom of aquaria and hence we were unsure of their identity. After carefully observing the representative larvae we then obtained mtCOI from them that turned out to be *Osedax* "yellow collar" (EU267672) or *Osedax* "nude palps" (EU267675), respectively. Our subsequent studies were on *Osedax* "orange collar" from whale-633, where oocytes were sampled as they were spawned in aquaria.

Fertilization rate

The rate of fertilization varied among batches of spawned oocytes, though the observations were limited in this study. Of the eight female *Osedax* "orange collar" whose oocyte stream was sampled (with at least 20 oocytes gathered), two females were found to have released unfertilized oocytes. On the other hand, five females were found to have

released fertilized oocytes with 97–100% cleavage within 6 h, while one female released 41% fertilized oocytes.

Female fecundity

Our observations of female *Osedax* "orange collar" in situ and in the laboratory (Fig. 1a–c) showed that spawning was not synchronized among females and that, at any one time, only one or a few females were spawning in any cluster of *Osedax*. Our observations on spawning by a group of 155 females (Fig. 2) showed that a large numbers of oocytes were being spawned each day, though we cannot state whether any particular female spawned each day or how much. However, the average spawning rate of oocytes per female per day (over a 7-day period) was 335 (±130). The number of oocytes spawned per day by the females varied significantly (repeated measures ANOVA $P < 0.001$*) over the 7 consecutive day period and ranged from an average of 178 (±11) per female to 579 (±7) per female (Fig. 2).

Larval development

Upon initial release from the female *Osedax* "orange collar" oocytes were markedly elliptical (124 × 67 µm, $n = 8$), though tapered at each end suggesting they had been squeezed in the oviduct. A prominent germinal vesicle (Figs. 1b, c, 3a), was visible in each oocyte, indicating they were primary oocytes (generally fertilized, based on our results above) arrested in meiosis. Over the next two hours they became more spherical (Fig. 3b), but smaller (96 × 63 µm, $n = 6$). Those of *Osedax* "yellow collar" and *O*. "nude palps" were also of a similar size (92 × 72 and 84 × 81 µm, respectively; $n = 5$ for each), though the initial size at spawning was not measured. For each species the oocytes sank to the bottom of the glass culture dishes or aquaria. Fertilized oocytes developed polar bodies (Fig. 3f), and cleaved unequally to a two-cell stage (Fig. 3c, g). First cleavage in *Osedax* "orange collar" occurred in normally developing embryos on average 4.7 (±0.5) hours ($n = 108$) after being emitted from the female oviduct. Subsequent cleavages to four (Fig. 3d, h), eight (Fig. 3e) and 16-cell stages were at 2.1 (±0.1) hourly intervals at 5–5.5°C. Early cleavage was clearly "spiral" (Fig. 3e), with a large "D" quadrant in all three *Osedax* species studied here. Development of *Osedax* "orange collar" larvae raised at 4–5°C in filtered seawater was monitored until settlement. Motile larvae, each with a clearly developing prototroch, appeared after ~24 h (Fig. 3i, j) and were swimming by ~48 h. As in

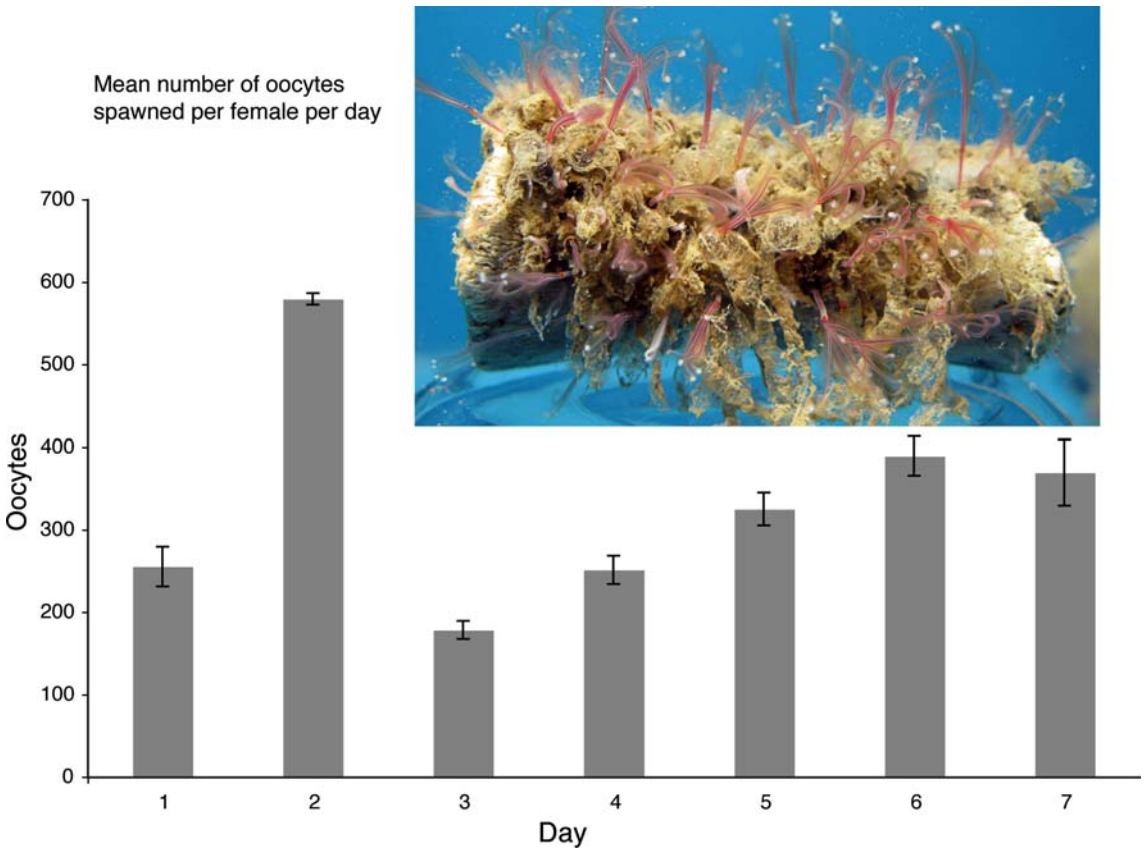

Fig. 2 Histogram of mean number of oocytes spawned per female of *Osedax* "orange collar" over 7 consecutive days of a sample of 155 females. Photograph shows the bone (phalange) from whale-633 with the 155 *Osedax* females

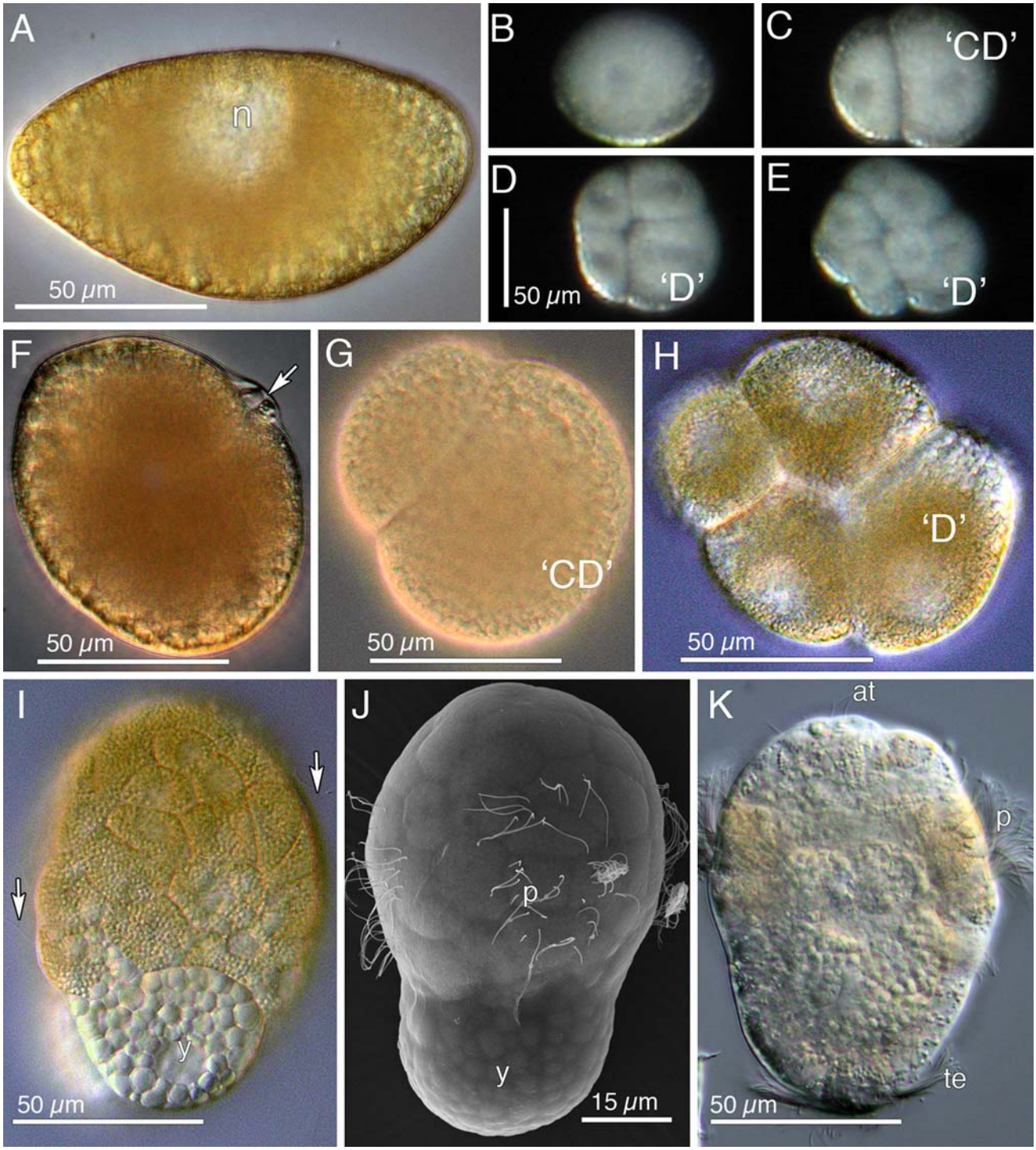

Fig. 3 Early development of *Osedax* spp. **a** Differential interference contrast (*DIC*) micrograph of *O.* "orange collar" primary oocyte 10 min after spawning. At this time the oocyte has a distinct elliptical shape. **b** After one hour the oocyte becomes spherical. **c** Dark field micrograph of a 2-cell embryo ~7 h after spawning. **d** Dark field micrograph of a 4-cell embryo ~7 h after spawning. A large 'D' blastomere is visible. **e** Dark field micrograph of an 8-cell embryo ~9 h after spawning. Cleavage is clearly spiral. **f** DIC micrograph of *O.* "orange collar" zygote two hours after spawning with polar lobe (*arrow*). **g** DIC micrograph of a 2-cell *O.* "orange collar" embryo, ~5 h post-spawning. **h** DIC micrograph of slightly compressed four-cell *O.* "nude palps" embryo, ~7 h post-spawning. **i** DIC micrograph of 2-day *O.* "nude palps" early trochophore. Trochoblasts that give rise to the prototroch are visible anteriorly, as are some cilia of the prototroch (arrows). A large yolk-filled cell (*y*) is present posteriorly. **j** Scanning electron micrograph (SEM) of 2-day *O.* "nude palps" trochophore at the same stage as Fig. 1d. The first cilia of the prototroch are emerging from trochoblasts. k. DIC micrograph of a 4-day *O.* "yellow collar" trochophore with an apical tuft, well-developed prototroch and telotroch. *at* Apical tuft, *'D'* D blastomere, *n* nucleus, *p* prototroch, *te* telotroch, *y* yolk-filled cell

other trochophores, cilia initially appeared in four patches of trochoblast cells (Fig. 3j). Early trochophores had a distinct region of yolk granules in a single large cell at the posterior end (Fig. 3i). After 4 days the larvae had more cilia in the prototroch (Fig. 3k) and a posterior ciliary tuft, equivalent in position to a telotroch but not forming a complete ring. Trochophores such as the nine-day *Osedax* "orange collar" larva in Fig. 4a were active swimmers with a well-developed prototroch of compound cilia and a prominent telotroch. The larvae were lecithotrophic and some trochophores ceased swimming and settled, with a reduced prototroch, as early as 10 days (Fig. 4b), though others settled later as in the 12-day larva seen in Fig. 4c, or even as many as 16 days after spawning. Settled larvae had shorter prototrochal cilia, two segments and paired bundles of long-handled hooked chaetae, as seen in *Osedax* males (Fig. 4d). Some larvae developed hooked chaetae while still planktonic (not shown). The settled larvae of *Osedax* "orange collar" (Fig. 4b, c) showed marked similarity to the *Osedax* dwarf males that were removed from female tubes

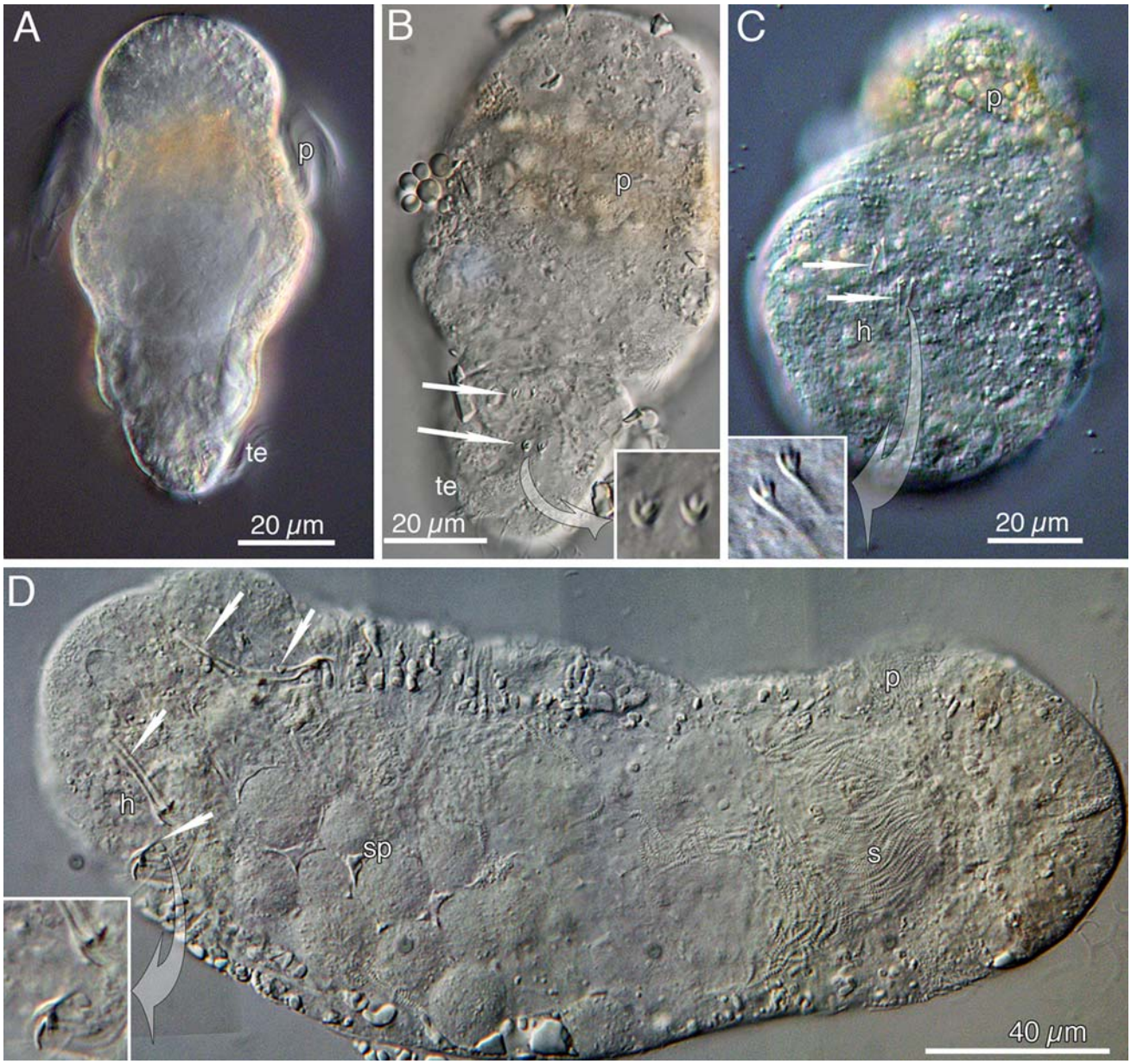

Fig. 4 *Osedax* larvae and dwarf male. **a** DIC micrograph of a 9-day *O.* "orange collar" trochophore with well-developed prototroch. Telotroch 'patch' visible posteriorly. **b** Recently settled 10-day *O.* "orange collar" larva with chaetae (one pair enlarged in *inset*). **c** Recently settled 12-day *O.* "orange collar" larva with chaetae (one pair enlarged in *inset*). Two segments appear to have developed. **d** DIC micrograph of *O.* "orange collar" dwarf male (squeezed). Mature sperm are visible in the head region (*inset*) and posterior hooks similar to those seen in settled larvae. *h* Hooked chaetae, *p* prototroch, *sp* spermatids, *s* sperm, *te* telotroch, *y* yolk-filled cell

(Fig. 4d). Both had similar long-handled hooks with a few recurved teeth, a ciliary band (prototroch), and an anterior patch of gold pigment in the region of the prototroch. Dwarf males of *Osedax* are characterized by the presence of sperm and spermatids in their bodies as seen in a male of *Osedax* "orange collar" (Fig. 4d), but these were absent in the settled larvae.

Symbiont transmission

Microscopic evidence revealed that the *Osedax* oocytes and larvae lack symbionts. Images of these negative results are not shown here. Whole spawned oocytes and larvae, as well as sections of both retrained and spawned oocytes revealed no evidence for bacteria upon application of fluorescent in situ hybridization (FISH) with probes directed against the symbiont 16S (SSU) rRNA, a method that works well with symbiont-bearing tissues of adult *Osedax* (Goffredi et al. 2005, 2007). Light microscopy confirmed the absence of bacteria-like cells in stained sections through spawned oocytes ($n = 5$) of *O. rubiplumus* or *Osedax* "yellow collar" as well as two-day old larvae ($n = 5$) of *Osedax* "nude palps".

Discussion

Spawning females of *O. rubiplumus* and *Osedax* "orange collar" released streams of oocytes in episodic bursts that lasted for several minutes. Females were not synchronized in their release and only one, or sometimes two females, were observed in an observed area to spawn at any one time. This spawning process by *Osedax* females shares some similarities with vestimentiferan tubeworms. Van Dover (1994) noted that spawning is not synchronous in dense colonies of *R. pachyptila* at 2500 m depth. The "eggs"—which were subsequently found to generally be fertilized oocytes (Hilário et al. 2005)—are initially released in "clouds", which rapidly separated, similar to what we observed in *O. rubiplumus* and *O.* "orange collar" (see Supplemental Videos). Unlike *Osedax* females, which host dwarf males in their tubes, *R. pachyptila* males and females are equal in size, but females store sperm in spermathecae and, like *Osedax*, release oocytes from their oviducts (Hilário et al. 2005).

Van Dover (1994) observed that the spawned oocytes of *R. pachyptila* are negatively buoyant at 2,500 m depth. Experimental observation on *Riftia* oocytes found them to be neutrally buoyant at 250 atmospheres and positively buoyant at lower pressures (Marsh et al. 2001). Oocytes of the vestimentiferans *Seepiophilia jonesi* and *Lamellibrachia luymesi* are buoyant at 1–100 atmospheres (Young et al. 1996), whereas *L. satsuma* oocytes are neutrally buoyant at atmospheric pressure (Miyake et al. 2006). Unfortunately, the currents in Monterey Bay at 1,820 m depth on October 17, 2006 were too fast to observe whether *O. rubiplumus* oocytes would sink or float at ambient pressures. Oocytes produced by *O.* "yellow collar", *O.* "orange collar" and *O.* "nude palps" in the laboratory sank at atmospheric pressure. Comparative evidence from vestimentiferans suggests that lower pressures should result in increased buoyancy (Marsh et al. 2001). Though further observations are needed to document whether *Osedax* oocytes are positively or negatively buoyant at native pressures, the present observations suggest that early developmental stages of *Osedax* may stay relatively close to the bottom.

Vestimentiferans may use egg buoyancy to compensate for a much longer period of embryogenesis (8 days in *L. luymesi*, 21 days in *R. pachyptila*) during which they have no locomotory ability. The very rapid development of cilia in *Osedax* larvae (cilia appear after 24 h) is remarkable for this family, especially considering that *Osedax* larvae were reared at a lower temperature than *L. luymesi* (Young et al. 1996). Staver and Strathmann (2002) have suggested that time to first swimming is a factor influenced by evolution in which some species devote more effort to cell multiplication and others to cell differentiation early in development. It is interesting that *Osedax* develop ciliation earlier than some other polychaete embryos reared at more than twice the temperature (Staver and Strathmann 2002).

As with the other Siboglinidae studied to date, there is no evidence for vertical transmission of symbiotic bacteria in *Osedax* species. Free-living sulfur-oxidizing bacteria infect vestimentiferan larvae transdermally in an event that coincides with development of the trophosome, a specialized organ that houses the symbionts (Nussbaumer et al. 2006). Though the homology of *Osedax* "roots" with the vestimentiferan trophosome has yet to be established, metamorphosis may be similarly coincident with, or induced by, infection with a suitable strain of Oceanospirillales bacteria (Goffredi et al. 2005, 2007). We hypothesize that further development and metamorphosis of those *Osedax* larvae destined to become females requires both infection by bacteria and bone suitable for settlement.

There is circumstantial evidence now available (Rouse et al. 2008; Vrijenhoek et al. 2008b) that male *Osedax* are morphologically similar to late-stage larvae, but make sperm instead of continuing with somatic development. This would appear to be induced by the female *Osedax* in some way, possibly analogous to that seen in bonelliid echiurans (Balzer 1935; Jaccarini et al. 1983). This is further supported here with the rearing of *Osedax* larvae that develop hooked chaetae as seen in *Osedax* males and bearing a striking resemblance to them without having developing sperm. With the feasibility of rearing *Osedax* in culture

now demonstrated, it may now be possible to develop experiments to explore the sex determination mechanisms in *Osedax*.

There are currently few data available on spawning or fertilization rates for any siboglinids. A laboratory maintained colony of *O. mucofloris*, spawned at a rate of 5 "eggs" per female per hour according to unpublished data (Wiklund et al., cited in Dahlgren et al. 2006). For a sample of 155 *Osedax* "orange collar" females we found that the average spawning rate per day per female was 335 oocytes, a rate of around 14 per hour, or nearly triple that reported for *O. mucofloris*. *Osedax* "orange collar" females are somewhat larger (see Fig. 1b, c) than those of *O. mucofloris* (see Glover et al. 2005: Fig. 1), perhaps explaining the greater fecundity in this species. *O. rubiplumus* is by far the largest *Osedax* species found to date (Rouse et al. 2004, 2008), so its fecundity may be considerably greater. However, it should be noted that the size of spawned oocytes of *O. rubiplumus* (151 µm × 121 µm; Rouse et al. 2004) are much larger than those of *Osedax* species studied to date, (this study; Fujikura et al. 2006; Glover et al. 2005; Rouse et al. 2008), so overall fecundity may not be dramatically higher, even though reproductive investment may be larger.

We found that five of eight female spawning samples had fertilization rates near 100%, while one had 41% and two released unfertilized oocytes. These results suggest fertilization is internal in *Osedax*, and the usual proximity of the dwarf males to the trunk oviduct (Rouse et al. 2004, 2008; Vrijenhoek et al. 2008b) supports this hypothesis. How sperm are transferred to the female and where fertilization occurs in *Osedax* spp. is not yet known, nor is it known whether sperm are stored by females. It has been shown that that not all spawning *Osedax* females have harems of dwarf males (Rouse et al. 2004, 2008; Vrijenhoek et al. 2008b), and this may have been the case for the two females found here to be spawning unfertilized oocytes. Variability in fertilization rates is known for internally fertilizing Vestimentifera (Hilário et al. 2005) and Frenulata (Bakke 1976) and sperm availability is presumably a limiting factor in fertilization success. The close proximity of *Osedax* dwarf males (when a "harem" is of adequate size) to female *Osedax* may ensure a greater fertilization success rate than seen in other siboglinids, which must obtain spermatozeugmata or spermatophores spawned by males freely into the water (Bakke 1976; Hilário et al. 2005). It should be noted that some vestimentiferans, such as *Ridgeia piscesae*, also have efficient sperm transfer from males to females via direct contact between the sexes (Southward and Coates 1989; MacDonald et al. 2002) when aggregations of individuals are dense.

We were not able to assess the spawning output and timing between spawning events of individual females of the *Osedax* species studies here. Monitoring isolated individual females will be required to resolve this. The large variation in average number of spawned oocytes for the sample of 155 female *Osedax* "orange collar" (Fig. 3) suggests that a given female may not spawn every day. At present we do not have estimates of longevity for any *Osedax*, so we cannot estimate lifetime fecundity of females, but it is likely to be high. The proportion of the female body that is dedicated to ovary is large in *Osedax* (Rouse et al. 2004, 2008; Glover et al. 2005; Fujikura et al. 2006) correlating with high reproductive output. It is also known that bones can be colonized very quickly after being exposed (Rouse et al. 2008), so large numbers of larvae appear to be present waiting for recruitment opportunities, in Monterey Bay at least. The likely high fecundity and continuous reproductive mode of *Osedax* permits the exploitation of widely scattered and ephemeral vertebrate bones (Jones et al. 2008; Vrijenhoek et al. 2008a).

The three undescribed *Osedax* species maintained in the laboratory for this study spawned oocytes that are comparable in size to those of *O. mucofloris* (Glover et al. 2005) and slightly smaller than those of *O. japonicus* (100 µm diameter, Fujikura et al. 2006). The oocytes of all these relatively shallow-water species are notably smaller that those of the deep-water species, *O. rubiplumus* and *O. frankpressi*, which produce ellipsoidal zygotes measuring 121 × 151 and 117 × 146 µm, respectively (Rouse et al. 2004). Assuming an ellipsoid shape (prolate spheroid), the oocytes spawned by *O. rubiplumus* have nearly five times the volume of the oocytes spawned in the species examined in the laboratory. If *Osedax* larval lifespan correlates with egg sizes (because they depend on yolk reserves), *Osedax* species with large eggs should have greater dispersal potentials. Also, the deeper-dwelling *Osedax* species live in colder water (3–4°C) than the shallower species (5–7°C) and this may also be a factor in larval lifespan. *Osedax roseus* was described from whale-1018, the same whalefall that hosts *O.* "nude palps", and produces relatively large ellipsoidal oocytes that measure 89 × 132 µm (Rouse et al. 2008), so spawned oocyte size is not simply related to depth. A detailed phylogenetic analysis of *Osedax* is needed to provide a framework for understanding the evolution of oocyte size in the group.

The cleavage rates for embryos documented here for *Osedax* were similar to some previously known siboglinids. Timing from spawning to first cleavage in *L. satsuma* was found to be 4 h followed by cleavage at 2-hourly intervals thereafter (Miyake et al. 2006). In other seep-dwelling vestimentiferans this takes up to 14 h with subsequent divisions at 4-hourly intervals (Young et al. 1996). In contrast the hydrothermal vent vestimentiferan *R. pachyptila* has an average of 1.8 days per cleavage (Marsh et al. 2001). Little has been published on other siboglinids, though Bakke (1976) reports slow cleavage rates in the frenulate

Siboglinum fiordicum, similar to those for *R. pachyptila*, and much faster rates in *Sclerolinum brattstromi* (every 7 h).

Photographic evidence presented by Fujikura et al. (2006) suggests that *O. japonicus* females retain embryos and may be brooders. Unlike the extensible oviducts of other known *Osedax* species, the oviduct of *O. japonicus* does not extend beyond the base of the palps, so embryo retention might occur in this species, but their maternal origins have yet to be investigated. The embryos were determined not to be dwarf males (Fujikura et al. 2006). We occasionally found embryos attached to the enlarged globular tubes of *O.* "orange collar", *O.* "yellow collar" and *O.* "nude palps" females (G.W. Rouse, personal observation), but we suspect that this finding may be an artifact of laboratory conditions, as tubes in aquaria accumulate mucous with time, whereas freshly-collected females possess thinner tubes that are less likely to 'trap' spawned oocytes. The vast majority of oocytes that we observed were spawned freely, both in situ and in aquaria (Fig. 1a–c and supplemental videos).

The maximum lifespan of *Osedax* "orange collar" larvae (up to 16 days to settlement) is shorter than the documented lifespans of vestimentiferan larvae (21–45 days, Young et al. 1996; Marsh et al. 2001; Miyake et al. 2006). *R. pachyptila* spawns zygotes that are 105 µm in diameter (Carey et al. 1989) and, assuming similar energy content per unit volume, has about three times the energy content of *O.* "orange collar" larvae. Other vestimentiferans spawn similar-sized zygotes. Marsh et al. (2001) raised *R. pachyptila* larvae at 2°C for up to 34 days, and so the longer larval life span (given the larger egg volume and lower temperature) seems congruent with our results. Miyake et al. (2006) raised their *L. satsuma* larvae (egg diameter 100 µm) at 16°C and while some settled after 12 days, others swam for 45 days. Development in the other major siboglinid clade, Frenulata, has only been studied in species that brood larvae. The time taken to develop to a ciliated trochophore stage is not known, but embryos take up to a month at 6–8°C to become segmented larvae that may swim briefly before settling and burrowing into sediment (Bakke 1976). Faster development of the *Osedax* species reported here may also be influenced by available oxygen, as we could not control for this with our experimental set-up, which used surface seawater. The levels of oxygen likely to be encountered by *Osedax* larvae at 300–1,000 m are 5–10% of this value (Braby et al. 2007) and may result in a slower developmental rate at depth.

Deep ocean currents are estimated to disperse relatively long-lived vestimentiferan larvae hundreds to thousands of kilometres between disjunct hydrothermal vents or cold seeps (Marsh et al. 2001; Miyake et al. 2006). However, comparatively reduced dispersal times and distances for *Osedax* may be efficient if suitable bones occur at closer intervals. The California margin is a migration corridor for a variety of large cetaceans and mean nearest-neighbor distances among Grey whale-falls may be as low as 5 km (Smith and Baco 2003). Furthermore, some *Osedax* species are not whale-fall specialists (Jones et al. 2008), so non-migratory small cetaceans and pinnipeds may also provide bones that can sustain these opportunistic worms. However, the *Osedax* species with larger egg sizes, such as *O. rubiplumus* and *O. frankpressi*, may have greater dispersal potential than the relatively shallow-water dwelling species studied here.

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