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Published in:
PLoS ONE

DOI:
10.1371/journal.pone.0035975

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Huusgaard, R., Vismann, B., Kühl, M., Macnaughton, M. O., Colmander, V., Rouse, G. W., ... Worsaae, K. (2012). The potent respiratory system of Osedax mucofloris (Siboglinidae, Annelida) - a prerequisite for the origin of bone-eating Osedax? PLoS ONE, 7(4), [e35975]. DOI: 10.1371/journal.pone.0035975
The Potent Respiratory System of Osedax mucofloris (Siboglinidae, Annelida) - A Prerequisite for the Origin of Bone-Eating Osedax?

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Abstract

Members of the conspicuous bone-eating genus, Osedax, are widely distributed on whale falls in the Pacific and Atlantic Oceans. These gutless annelids contain endosymbiotic heterotrophic bacteria in a branching root system embedded in the bones of vertebrates, whereas a trunk and anterior palps extend into the surrounding water. The unique life style within a bone environment is challenged by the high bacterial activity on, and within, the bone matrix possibly causing O2 depletion, and build-up of potentially toxic sulphide. We measured the O2 distribution around embedded Osedax and showed that the bone microenvironment is anoxic. Morphological studies showed that ventilation mechanisms in Osedax are restricted to the anterior palps, which are optimized for high O2 uptake by possessing a large surface area, large surface to volume ratio, and short diffusion distances. The blood vascular system comprises large vessels in the trunk, which facilitate an ample supply of oxygenated blood from the anterior crown to a highly vascularised root structure. Respirometry studies of O. mucofloris showed a high O2 consumption that exceeded the average O2 consumption of a broad line of resting annelids without endosymbionts. We regard this combination of features of the respiratory system of Osedax as an adaptation to their unique nutrition strategy with roots embedded in anoxic bones and elevated O2 demand due to aerobic heterotrophic endosymbionts.

Introduction

The unique bone-eating organism, Osedax (Siboglinidae, Annelida) was first described in 2004 from a whale fall located at 2891 m depth in Monterey Bay, Pacific Ocean [1]. Since its first discovery it has been found on multiple whale falls in the Pacific and Atlantic Oceans, artificially deployed cow bones [2] as well as on other vertebrate bones such as those of teleost [3]. There are five formally described species, with at least a further 12 species known from genetic evidence [4,5]. In addition, convincing fossil traces of Osedax have been found in Oligocene and Pliocene mammal bones [6,7].

Evidence suggests that Osedax females utilize the complex organic compounds of the bone through endosymbiotic aerobic heterotrophic bacteria (Oceanospirillales) located in bacteriocytes in a root system that is embedded in the bone matrix [9,9]. The O2 microenvironment around the embedded root system of Osedax has not been studied, yet this knowledge is crucial for understanding the function of Osedax in its natural habitat. The O2 supply within the bone matrix is presumably strongly diffusion limited, and with whale bone lipid content reaching 45% in Sperm whales (closely related to Minke whales) [10] the bone interior may become O2 depleted due to high heterotrophic microbial activity, including sulphur-reducing bacterial processes that generate sulphide, further reducing O2 below the bone surface. External O2 supply to the roots of Osedax via the bone matrix is thus highly unlikely. Yet, symbiosis with detoxifying sulphide-oxidizing bacteria has so far not been proven, though Osedax has been observed along with sulphophilic species and mats of white Beggiatoa-resembling bacteria (Figure 1) or bones exhibiting ferrous sulphide precipitation [11–14]. The highly folded epidermis of the root structure of Osedax ‘green palp’ has recently been shown to lack a cuticle and possess an extensive microvillous border [15], potentially facilitating the uptake of organic substrates, but also facilitating interaction with sulphide from the root surroundings. In the methane seep-dwelling vestimentiferan Lamellibranca the sediment-embedded posterior body region (also called root) is actually the main source of hydrogen sulphide uptake, and is used for maintaining the chemoautotrophic endosymbionts [16,17]. Respiratory features in other siboglinids involve ciliary or...
muscular ventilation of the chitinous tube and expanded branchial structures with extensive blood vascular systems and large respiratory surface areas [16,18–20]. Whereas the tube of Osedax is gelatinous and only covers the trunk, the vascularized and pinulated palps of Osedax are believed to have a similar function as the anterior branchial plumes of Vestimentiferidae [21]. However, neither these structures nor the blood vascular system have been examined in detail, nor has the O2 consumption been measured previously in any Osedax species.

Osedax and Vestimentifera are tubicolous species and apart from the ability to retract into their tubes when disturbed, they show very little motile activity. However, Riftia pachyptila has been shown to metabolize much faster than other resting annelids (e.g. double the O2 demand of Arenicola marina Lamarck, 1801) [22]. Moreover, Childress et al. [22] apparently measured the O2 uptake without the presence of sulphide, thereby underestimating the O2 consumption of chemosynthetic endosymbionts. Later, Girguis & Childress [23] showed that O2 uptake was reduced significantly (from 14.35±1.23 µmol O2 g−1 wet wt h−1 to 2.88±0.89 µmol O2 g−1 wet wt h−1) when the activity of endosymbionts was restricted by blocking sulphide supply. This clearly demonstrated that the metabolism of the endosymbionts of R. pachyptila contribute significantly to its O2 consumption. Experiments by Freytag et al. [17] further confirms this as an increase in O2 consumption of ~42% was seen when Lamellibrachia cf. layesi was exposed to hydrogen sulphide at the root area. The increase in O2 consumption is a result of the activation of the metabolism of chemoautotrophic endosymbionts. Since Osedax also carries aerobic endosymbionts and furthermore is hypothesised to be partly exposed to hypoxia or anoxia, knowledge of the O2 uptake for enhancing our understanding of the respiratory systems and physiology of Osedax.

The shallow water species Osedax mucofloris Glover, Kallström, Smith, Dahlgren, 2005, has been found at 30 m and 125 m water depth off the coast of Tjärnö, Sweden [12,13], and at 120 m depth in Bjornafjord, Norway [24]. Currently these are the only records of Osedax from the Atlantic, though other species are found in shallow waters in both the East and West Pacific [4,25]. The 125 m deep locality near Tjärnö is characterized by stable oceanic salinity (34–35%), temperature (5–7°C) and dissolved O2 concentration ranging from 4.6 to 6.3 ml O2 l−1 [13].

In the present study we investigated whether Osedax mucofloris is exposed to an inhospitable hypoxic or anoxic microenvironment by measuring the O2 distribution surrounding the embedded root system. We assessed possible morphological and/or physiological adaptations to the environmental conditions through detailed morphological studies of the respiratory surfaces and the blood vascular system as well as respiratory measurements of O2 consumption. The results are discussed in relation to the unique environment, endosymbionts, and embedded root structure of Osedax as well as compared with studies of related annelids.

Results
Ventilating mechanisms
Ciliary bands and sensory structures (anti acetylated α-tubulin staining and histology). In the description below, we use a dorsal-ventral definition opposite to the one used by Rouse et al. [1,21], based on a new interpretation (Rouse & Worsaae, unpublished).

We found two previously unreported broad longitudinal ciliary bands dorso-laterally on each side of the ovoidic on the anterior part of the trunk (Figure 2A, B, E). The ciliary bundles of the bands consist of multi ciliated cells, appearing pillow-like with numerous, conspicuously short cilia (<10 µm long) projecting outwards from the centre of the elliptical bundles (Figure 2C). The bundles are organized in an anterior-dorsal diagonal pattern within the longitudinal bands (Figure 2B). The ciliary bands narrow toward the posterior part of the trunk and are replaced by single tufts of cilia scattered basally across the trunk surface (Figure 2A). The short cilia appeared immotile, and with several longitudinal nerves running beneath, (Worsaae & Rouse, unpublished) their function may be sensory rather than ventilatory.

Two dense ciliary bands are found on each palp along their epidermal longitudinal lobes on each lateral side, as previously reported [12] (Figures 3A, B, E, 4C, 5A, F, G). The bands consist of multi ciliated cells with 70–90 µm long cilia (Figure 3E). The ciliary bands extend from near the basal part of the palps to the distal tip, beating in metachronal waves.

Two nerves, originating at the anterior part of the brain, innervate each palp. One nerve runs abfrontally, between the two lateral ciliary bands and the other nerve runs laterally underneath one of the ciliary bands (Figure 5A). Further details on the female Osedax nervous system will be described elsewhere (Worsaae & Rouse, unpublished).

Pinnales (up to 100 µm wide) project perpendicularly from the frontal palp surface between the lateral ciliary bands, with a density of approximately eight pinnales across the palp per 50 µm palp length (Figures 3A, 5F, G). At the proximal end of the palps, pinnales are less developed than at the distal end, seemingly growing in length (up to 170 µm) synchronously with the growth of the palp (Figure 3A). A sensory cell extends through the centre of the pinnule with a distal perikaryon and a few external, presumably sensory, cilia (Figure 4C, D). Its axon seems to connect with one of the two major longitudinal palp nerves, possibly the...
Figure 2. Confocal laser scanning microscopy (CLSM) of the trunk of *Osedax mucofloris* females. A: Dorso-lateral view of a complete specimen, lateral ciliary bands occupies half the length of the trunk. B: Dorsal view of the anterior part of the trunk, elliptical shaped cilia bundles are directed anteriorly from the lateral part of the trunk. C: Depth coded z-stack, the elliptical shaped cilia bundles constituting the lateral ciliary band are formed by ciliary tufts. D: Close-up of transverse section of a trunk. E: Transverse section of a trunk, note the muscularized dorsal blood vessel. F: Single z-stack image of the trunk musculature, longitudinal muscles beneath circular and diagonal muscles. Abbreviations: ciliary tufts (ct), circular muscles (cm), diagonal muscles (dm), elliptical ciliary bundles (ecb), lateral ciliary band (lc), longitudinal muscles (lm), muscular gap (mg), palp (p), root structure (r), torn ovisac (to), trunk (t), dorsal blood vessel (dbv).

doi:10.1371/journal.pone.0035975.g002
The Respiratory System of *Osedax mucofloris*

**Musculature (F-actin, phalloidin staining).** The longitudinal muscles run along the entire length of the trunk (Figure 2A), originating posteriorly at the trunk basis, and inserting anteriorly at the base of the four palps (Figure 3D). In one individual, it was possible to detect clustering of longitudinal muscles into 14–16 bundles of >20 muscle strands in the posterior part of the trunk, anterior to the root structure (Figure 4A). Along, and around the entire trunk, the muscles are distributed in a dense cylindrical formation with an average of six strands per 50 μm (Figures 2D, E, 3D). The musculature is slightly separated internally to the oviduct (possibly by the nerve cords), as well as randomly along the trunk, creating minor gaps most likely for mucus gland exits or nerves (Figures 2A, 3D). Anteriorly, a gap in the musculature is found ventrally at the position of the brain, as well as at each of the four insertion points of the palps. At each of these four points, the longitudinal musculature divides into two bundles (of each 20–30 strands), encircling the insertion point of the palp (Figure 3D). The longitudinal palp muscles originate at the base and run along the entire length of the palps to their tips, separated by a smaller frontal and a larger abfrontal gap (Figures 3C, D, 5A). No longitudinal musculature was detected in the pinnules.

Around the ovisac and anterior root structure the longitudinal musculature divides, and together with the circular muscles, creates a mesh-like structure (Figure 4A). The musculature extends posteriorly along the roots in a cylindrical formation, supporting the tissue penetrating the bone.

Thin circular muscles are found peripheral to the longitudinal muscles along the entire trunk, with 24 strands per 50 μm (Figure 2D). The circular muscles are most dominant in the posterior part of the trunk and continue into the root structure (Figure 4A). Notably, much thicker diagonal muscle strands were found beneath the circular musculature, but peripheral to the longitudinal muscles (Figure 2F). Attached at the mid-dorsal line, the strands run diagonally around the trunk in an anterior direction and attach at the mid-ventral line. The diagonal muscles are distributed along the entire length of the trunk, lying further...
apart (35–70 μm) in the posterior end than along the rest of the trunk (5–10 μm).

The circular musculature of the palps encircles the longitudinal musculature as a cylinder with 18 muscle strands per 50 μm and is evenly distributed along the entire palp (Figure 3C). Fine circular muscles enclose each vessel of the pinnular loop (Figure 4C, D); they were likewise visible in the semi-thin sections on the outside of the pinnular loop and along the midline of each pinnule (Figure 5F, arrow tips), with a spacing corresponding to those shown with phalloidin staining (Figure 4C, D).

**Blood vascular system**

The trunk of *Osedax mucofloris* encloses two major longitudinal blood vessels (Figures 2E, 6A, B). The dorsal vessel is highly muscularized with circular musculature throughout its length (Figure 2D, E). No lateral connecting vessels between the major longitudinal vessels or epidermal capillaries were found in the trunk. From the trunk, the two blood vessels continue posteriorly into the root structure and increase in diameter (Figure 6A, B). When studied under the light microscope, the muscularized dorsal vessel is visible as a defined tube along the trunk, and continues into the anterior root structure, curling up in the centre anterior to the ovisac. The curling configuration is most likely caused by contraction of the basal part of the trunk. The exact further path of the vessel was difficult to determine. The Confocal Laser Scanning Microscope (CLSM) studies also showed the continuation of the dorsal blood vessel around the ovisac, revealing cylindrical musculature at the ovisac, corresponding in diameter to the musculature of the dorsal blood vessel of the trunk (Figure 4B).

The ventral blood vessel also continues into the root structure, but as a narrower and less defined vessel, the path of which was even more difficult to determine, than that of the dorsal vessel. Blood vessels of different sizes, as well as multiple obvious capillaries within the root tissue were observed (Figure 6B–E). Larger vessels extend out from the area of the ovisac and divide into thinner vessels (diameter: 7–30 μm). Capillaries were detected in the periphery of the root tissue (Figure 6E), with distances between the detected capillaries ranging from 65–220 μm.

Anteriorly, each palp encloses a pair of blood vessels created by invaginations of the inner lamina of the basement membrane of the epidermis (Figures 5F, G, 7C). Imaging of live specimens...
confirmed the presence of two blood vessels, running along the entire length of the palps.

The pinnules are largely filled by a blood cavity lined with a membrane, which fuses in between the two blood cavities along

most of the pinnule length, thereby creating the pinnular loop (Figures 5B-G, 7C). The palps of the histological sections were ~300 μm in diameter at the base and the palp epidermis was ~50–75 μm thick. The palp diameter and the thickness of the

Figure 5. Diagram of a transverse section of the basal part of palps (A), DIC light micrographs of benzidine stained palps (B–D) and transverse 1.2 μm sections of O. mucofloris palps stained with toluidine blue (E–G). A: Diagram of a transverse section at the basal part of the palp region. Circular musculature (continued green lines) encircles the longitudinal musculature (green broken lines) in a cylinder formation. Two gaps separate the longitudinal muscle bands. Black lines illustrate the motile lateral ciliary bands and two main palp nerves run along each palp as shown (blue dots). B: Pinnular loop filled with blood. C: Pinnular loops, broken lines and arrows indicate the assumed direction of blood flow. D: Midsection of palp, longitudinal blood vessels and pinnular loops visible. E: Transverse section of a pinnule, the pinnular loop enclosed by a membrane fusing in the centre. F: Transverse section of the distal part of a palp, arrow tips shows circular musculature. G: transverse section of the distal part of a palp, the two longitudinal blood vessels obvious. Abbreviations: circular muscles (cm), epidermis (ep), lateral ciliary band (lcb), left dorsal palp nerve (ldpn), left ventral palp nerve (lvpn), membrane fusion (mf), musculature (m), palp blood vessel (pbv), pinnule (pin), pinnular loop (pl), right dorsal palp nerve (rdpn), right ventral palp nerve (rvpn).

doi:10.1371/journal.pone.0035975.g005
epidermis were both found to decrease towards the distal end of the palp.

Pinnules vary in diameter along their length and along the palp, with a median diameter of \(40 \pm 10\) mm for the pinnules and \(20-40\) mm for each blood vessel. The diffusion distance across the pinnule epithelium was measured on the semi-thin sections to be 1–2 mm, and for the epidermis of the distal part of the palps, the diffusion distance was measured to be \(30\) mm.

**Oxygen consumption**

When corrected for background respiration, the measured O\(_2\) consumption (MO\(_2\)) of *O. mucofloris* ranged almost across a factor of ten from \(220 \pm 93\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\) to \(2053 \pm 1950\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\) (Table 1), depending on the approach used for measurement. MO\(_2\) measured on *O. mucofloris* inhabiting sectioned bone pieces (B1–B3) vs. MO\(_2\) measured *O. mucofloris* inhabiting bones in cuvettes (C1, C2), resulted in two distinctly different ranges of MO\(_2\). The MO\(_2\) of C1 and C2 was \(220 \pm 93\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\) and \(238 \pm 29\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\), respectively, while the MO\(_2\) of B1–B3 range from \(976 \pm 201\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\) to \(2053 \pm 1950\) \(\mu\)g O\(_2\) g\(^{-1}\) h\(^{-1}\). Methods and ranges are commented further in the discussion.

**Oxygen levels around Osedax**

Micro sensor measurements of O\(_2\) concentrations in proximity to the bone interface were conducted, through agar-filled holes, on *Osedax*-colonized bone fragments in cuvettes. The obtained profiles of O\(_2\) concentration in the aerated seawater, and inwards showed steep O\(_2\) gradients towards both the bone and tissue surface (Figure 8A, B). Anoxic conditions or very low O\(_2\) levels were found at the bone surface, within the bone, and in proximity to the embedded root tissue of *O. mucofloris* (Table 2). The average
Figure 7. Schematic illustration of O₂ distribution in the internal and external environments of Osedax mucofloris. A: Osedax mucofloris extends its palps and pinnules, with large respiratory surfaces, into the overlying O₂-rich water in order to uptake O₂. Osedax mucofloris extend its palps and pinnules, with large respiratory tubes showed a 50% decrease in the O₂ concentration in the bone matrix as well as in the bone surface. Hydrogen sulphide is produced by anoxic bacterial processes within the bone matrix during decomposition of organic content using sulphate. B: Schematic illustration of assumed blood flow in palp and pinnules, longitudinal section. Blue vessels carrying venous blood through afferent vessels, red vessels carrying arterial blood through efferent vessels. C: Schematic illustration of assumed blood flow in palp and pinnules, transverse section. Likewise blue vessels carry venous blood through afferent vessels, red vessels carry arterial blood through efferent vessels. Note that the palp blood vessels are created by an invagination of the basement membrane. Green indicates musculature.

Discussion

Ventilation and branchial structures

The presence of highly vasculated palps and pinnules, the former densely ciliated showed that the anterior crown is the main site for O₂ uptake (Figure 7A–C). Uptake of O₂ over the trunk surface is possible, but a thicker epidermis, short and seemingly immotile ciliary bands and no obvious respiratory structures suggest that the trunk is a minor site of O₂ uptake. Oxygen does, to some extent, diffuse from the surrounding water into the mucus tube, thereby supplying the dwarf males.

The well-developed longitudinal musculature of the trunk serves to retract the trunk and palps into the tube and bone, presumably as protection from predators. As no regular retraction patterns of trunk musculature were detected, retraction into the tube is not considered a significant mode of ventilation for required O₂. Furthermore, the tube only surrounds the trunk, tightly fitting to the base of the trunk and the surface of the bone, preventing any water exchange to the ovisac and roots from the bone surface. The longitudinal musculature is moreover able to stretch the extensive branchial structures (palps) into the aerated water in order to increase the O₂ uptake.

The circular trunk musculature in Osedax mucofloris is weakly developed, as found in several other annelids [26,27]. The diagonal musculature revealed in the present study, may compensate for the weak circular musculature by having a similar supportive function. Diagonal musculature has most likely been mistaken for circular musculature in several annelids [26,27], including previous studies of Osedax [15,21].

Our study demonstrates the respiratory challenges faced by Osedax, as no oxygen was measured in the bone matrix as well as in the immediate vicinity of root structures and ovisac. Furthermore, we found no ventilating structures such as ciliary bands on the root epidermis, nor dense capillaries beneath the epidermis. Therefore, the epidermis of the embedded root structure is unlikely to facilitate ventilation or local O₂ supply to the Osedax root system. The O₂ necessary to maintain the metabolism and heterotrophic endosymbionts of the roots may instead primarily be transported via the blood vascular system from uptake at the aerated palps (Figure 7A). However, the microvillar root epidermis in Osedax does constitute a large surface area to volume ratio, which besides uptake of organic compounds may also facilitate uptake of e.g., hydrogen sulphide in the bone matrix. This is the case for the ‘root’ surface of the vestimentiferan Lamellibrachia, where hydrogen sulphide is absorbed and then transported via the circulatory system to the chemosynthetic endosymbionts in the trophosome [16,17,28].

Previous studies of other close relatives of Osedax such as Vestimentifera and Sabellidae have shown anterior branchial structures to be of vital importance when tube-bound tissue is not
Likewise, the anterior branchial structures of *O. mucofloris* seem morphologically adapted to facilitate efficient $O_2$ uptake form the surrounding water. As with the branchial plume of Vestimentifera [31,32], the anterior palps and pinnules of *O. mucofloris* have a large surface area to volume ratio and short diffusion distances. Furthermore, $O_2$ uptake is optimized by the two ventilating ciliary bands on each palp, a feature also seen in other Siboglinidae and in Sabellida in general [20,31,33].

A rough estimate was made of the surface area of the anterior crown, calculated from palp length given by Glover et al. [12], and the dimensions of palps and pinnules of *O. mucofloris* found in the present study. This results in a weight specific branchial surface area (SBSA) of $\sim 22 \text{ cm}^2 \text{ g}^{-1}$ fixed mass, which is similar to the SBSA of *Riftia pachyptila* and higher than the SBSA of fish, crabs and other annelids [31]. This is especially obvious when compared to the SBSA of e.g., *Arenicola marina* (4.00 cm$^2$ g$^{-1}$) [34], although this species can also take up $O_2$ across its general epidermis.

The estimated diffusion distance in *O. mucofloris* (pinnule epidermis $1-2 \mu m$, palp epidermis $\sim 30 \mu m$) is furthermore comparable to what has been found for *R. pachyptila* (pinnules $\sim 2.00 \mu m$; branchial filaments $\sim 25 \mu m$) and *Ridgeia piscesae* (pinnules $\sim 1.00 \mu m$; branchial filaments $\sim 17 \mu m$) [31,32].

Interestingly, the present study showed that each pinnule is equipped with a distal sensory cell and external sensory cilia. These structures may sense disturbance in the water to avoid predators, as *Osedax* need not sense food items or reproductive indicators in the water [1,8,35]. Additionally, sensing of currents may also be beneficial in order to orientate the palps e.g., for optimal uptake of dissolved $O_2$. *Riftia pachyptila* does not have sensory structures on the pinnules, but does have long, separate sensory filaments that lack ciliary bands and pinnules. These structures, with unknown function, are placed between pinnulated filaments [33].
Blood vascular system

The quantity and complexity of capillaries in the root structure of *O. mucofloris* reflects the high O2 demand of *Osedax*, presumably for the metabolism of its heterotrophic endosymbionts and production and development of eggs. Similar capillaries are visible in the exposed osic of *O. franksi* [figure 2F in [1]] and *O. roseus* (figure 4E in [21]), while the present study shows the presence of capillaries supplying the more distally placed root tissue and bacteriocytes. However, the extent of capillaries does not match the extensive capillary network of the trophosome in other Siboglinidae [20,33]. This is in accordance with the original description [1] mentioning the lack of a discrete trophosome, the *Osedax* trophosome instead being diffuse and beneath the epidermis of the embedded tissue.

The present study supports the basic histological findings with regard to the circulatory system of the palps in *O. roseus* [21]. The increased level of detail in the present study suggests similarities with some previous siboglinid studies [31,32,36]. The pinnule loop is most likely lined by an extension of the outer lamella of the basement membrane, which is in accordance with the findings of Norrevang [36]. No indication of a capillary plexus between the two elements of the loop was observed, as otherwise seen in *Riftia pachyptila* and *Ridgea piscesae* [31–33]. In accordance with Norrevang’s [36] observations of blood flow in *Siboglaum*, the blood of *Osedax* flows through an afferent vessel into the palp, through the pinnule loops and back through an efferent palp vessel (Figures 7B–C). This circulatory system carries oxygenated blood to the trunk and root system. In the present study of *O. mucofloris* and previous studies of *Osedax* [1,21] the main trunk blood vessel has been shown to be muscularized, and is now regarded as dorsal (Rouse & Worsaae, unpublished). This is in accordance with other annelids [37] and siboglinids [20,33], where the muscularized dorsal vessel, possibly assisted by a heart, create blood flow in an anterior direction in the dorsal trunk vessel and posterior in the ventral trunk vessel. Rouse et al. [1] reported an anterior lying dorsal heart in the original description of the genus, not noted in any descriptions since and would now appear to be an error (Rouse, pers. obs.).

The main blood flow within the palp vessels may be generated by the circular body wall musculature of the palp. However, the thin circular musculature of the pinnules surrounding each branch of the looped blood vessel (Figure 4C, D) most likely assists local blood flow, as suggested for the tentacular vessels of *Riftia pachyptila* [22]. Musculature in anterior appendages has been found in several Vestimentifera, in the form of splanchnic muscles located in the branchial lamella and filament vessels [33].

As with the trunk of *O. mucofloris*, the anterior vestimentum of Vestimentifera lacks branching vessels between the ventral and the muscularized dorsal vessel [33]. The resemblance between the blood vascular systems adds new evidence to the hypothesis that the trunk of *Osedax* and the vestimentum of Vestimentifera are homologous regions [21].

Oxygen consumption

The present study shows that *Osedax mucofloris* has a higher weight specific O2 consumption (MO2) than other resting annelids (Figure 9) [18]. This may reflect an elevated demand of the embedded tissue due to presence of heterotrophic aerobic endosymbionts, which have a higher metabolism than regular tissue. The measured MO2 actually corresponds to that found for *Riftia pachyptila* [22,23] (Figure 9), possessing a vast amount of chemoautotrophic bacteria in their trophosome. Furthermore, a high MO2 may also reflect oxidative sulphide detoxification. The high MO2 corresponds well with the large SBSA and elaborate branchial structures, which are both usually associated with animals exhibiting a high O2 demand. This correlation is also found in *Artemia marina* with smaller SBSA and lower MO2 (red square, Figure 9).

Our data show that *O. mucofloris* inhabiting bone in cuvettes (purple and yellow dots, Figure 9) have a lower MO2 than *O. mucofloris* inhabiting sectioned cow bone (green, blue, red dots, Figure 9). Along with high standard deviations, this highlights the difficulties of measuring O2 consumption on the embedded worms. The variations are most likely caused by the difference in blind respiration measurements and the large biological activity present on decaying bone, which was difficult to quantify. Future studies should thus be carried out to more precisely determine the

Table 2. O2 concentration measured by micro sensors at the bone or tissue surface of *Osedax mucofloris* inhabiting whale bone in two cuvettes, WB1 and WB2.

<table>
<thead>
<tr>
<th>Bone surface</th>
<th>O2 concentration (µmol l⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Site 1</td>
<td>0.00</td>
</tr>
<tr>
<td>Site 2</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 1 Site 4</td>
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<td>0.00</td>
</tr>
<tr>
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<td>0.00</td>
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<tr>
<td>WB 2 Site 5</td>
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<tr>
<td>Tissue surface</td>
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<td>WB 1 Site 3</td>
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<tr>
<td>WB 2 Site 3</td>
<td>0.00</td>
</tr>
<tr>
<td>WB 2 Site 4</td>
<td>0.67</td>
</tr>
</tbody>
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doi:10.1371/journal.pone.0035975.t002

Table 3. O2 concentration measured by micro sensors in a mucus tube wall and at the epidermis of palps of *Osedax mucofloris*.

<table>
<thead>
<tr>
<th>Mucus tube wall</th>
<th>O2 concentration (µmol l⁻¹)</th>
<th>Palp</th>
<th>O2 concentration (µmol l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td></td>
<td>Site</td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td>Free water 297.86</td>
<td>Base I</td>
<td>0.00</td>
</tr>
<tr>
<td>Site 2</td>
<td>Surface 221.94</td>
<td>Base II</td>
<td>0.57</td>
</tr>
<tr>
<td>Site 3</td>
<td>Breached tube 122.65</td>
<td>Middle</td>
<td>5.73</td>
</tr>
<tr>
<td>Site 4</td>
<td>Centre 170.54</td>
<td>Tip</td>
<td>148.99</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0035975.t003
from Cammen [18], the regression line (log R = 1.682 - 0.850 * log W) calculated from measurements of resting nonventilating annelids only. Dots: B1 (red), B2 (blue), B3 (green), C1 (purple), C2 (yellow). Triangles: Previous measured O2 consumption of R. pachyptila. No sulphide present in water when measuring: Red, blue [22] and green [23]; sulphide present in water during measurement: Purple [23]. Red Square: O2 consumption of resting Arenicola marina [52].

doi:10.1371/journal.pone.0035975.g009

MO2 of O. mucofloris and the contribution of other O2-consuming surfaces.

Osedax adaptations to the bone environment

The bone matrix exhibited strong O2 depletion with anoxic or low O2 levels at its surface and with areal O2 consumption rates comparable to in situ diffusive O2 uptakes measured in sediments proximate to whale falls [38]. The respiratory and circulatory system of Osedax is well suited to these environmental challenges. The elaborate branchial structures of the palp and pinnules of O. mucofloris facilitate a high O2 uptake from the surrounding seawater that, in combination with the blood vascular system, apparently enables sufficient oxygenation of the embedded tissue and endosymbionts. A similar, well-developed respiratory system is also found in other members of Siboglinidae (for review see [39]), also exhibiting high surface area to volume ratio in the branchial structures in particular. Based on these morphological and functional similarities among several siboglinids it seems possible that a well-developed anterior branchial structure may be a plesiomorphic condition among siboglinids and possibly a prerequisite for housing aerobic symbionts while dwelling in anoxic habitats. This respiratory system may therefore very well be a preadaptation present in the common ancestors of Osedax and other siboglinids, responsible for the successful colonization and evolution of Osedax in the unique bone environment.

An extended root surface (and high area to volume ratio) is likewise found in both Lamellibrachia (e.g., [16]) and Osedax (yet branched). However, the main function has so far been interpreted as very different. Whereas the Osedax root surface is suggested to mainly facilitate uptake of organic compounds (with less focus on the possible congruent uptake of sulphide) [9], the root of Lamellibrachia is found to be the main respiratory surface of hydrogen sulphide necessary for the chemosynthesis of the symbionts [16,17,28].

The measured O2 depleted bone environment as well as ferrous sulphide precipitations (Figure 8E) supports the suggested exposure of Osedax to sulphide. Furthermore the large root surface in the related Lamellibrachia is highly efficient in hydrogen sulphide uptake [17]. Since the main trophic source of Osedax compared to other siboglinids (including Lamellibrachia) seems to be heterotrophic bacteria rather than sulphide-detoxifying chemoautotrophic bacteria [16], Osedax may instead possess physiological adaptations to detoxify sulphide.

Though beyond the scope of the present paper, microanalytical approaches such as microsensors [40,41] and functional imaging techniques [42] could yield a more complete mapping of the chemical microenvironment of Osedax, including the exact levels of sulphide exposure and spatio-temporal dynamics of O2 in the root system. It would also be interesting to know whether Osedax have sulphide-binding properties of their haemoglobin as found in Vestimentifera [43,44], as they might use them to transport toxic sulphides from the area surrounding the root structure to e.g., the palps, somehow releasing the toxic compounds to oxygenated seawater or detoxifying them.

Materials and Methods

Sampling and aquarium setup

In January 2009, 3 replicate experimental sampling devices, using cow and whale bone, for recruitment of female O. mucofloris (Figure 10) were placed at 125 m depth off the coast of Tjärnö, Sweden (58°52.976N; 11°05.715E) in close vicinity to a minke whale carcass sunk in October 2003 [13]. One device for morphological and reproductive studies was successfully retrieved in May 2009. A second device was retrieved in November 2009 for in vivo studies (respirometry and microsensor analysis) and additional morphological studies. Additionally, a piece of cetacean

Figure 9. O2 consumption of O. mucofloris females set in relation to O2 consumption of resting annelids as well as recent data of O2 consumption of R. pachyptila. Graph modified from Cammen [18], the regression line (log R = 1.682 - 0.850 * log W) calculated from measurements of resting nonventilating annelids only. Dots: B1 (red), B2 (blue), B3 (green), C1 (purple), C2 (yellow). Triangles: Previous measured O2 consumption of R. pachyptila. No sulphide present in water when measuring: Red, blue [22] and green [23]; sulphide present in water during measurement: Purple [23]. Red Square: O2 consumption of resting Arenicola marina [52].

doi:10.1371/journal.pone.0035975.g009

Figure 10. Overview of bone types in experimental sampling device for recruitment of Osedax. The experimental sampling devices were placed at 125 m depth off the coast of Tjärnö, Sweden (58°52.976N; 11°05.715E) in close vicinity to a minke whale carcass sunk in October 2003 [13].

doi:10.1371/journal.pone.0035975.g010
bone deposited at 123 m in the same area in May 2008 was retrieved in January 2009 and used for preliminary investigations and for designing experimental setups. In January 2009, the bottom water had a salinity of 34.6%, a temperature of 8.4 °C, and an O2 content of 78.7% atmospheric saturation.

Placement and retrieval were carried out with a remotely operated vehicle (ROV, Sperre Subhunting, Norway) operated from the R/V Lofthela (Sven Lovén Centre for Marine Science, University of Gothenburg, Sweden). Upon retrieval, the sampling device was placed in a cooling box with ambient aerated water and transported to the Marine Biological Section, University of Copenhagen within 8 hours. In the laboratory bones were kept in aerated seawater under in situ conditions, in aquaria (Vol 60 litre) with aeration, protein skimmers and a recirculating pumping system. The Osedax "yellow-collar" shown as comparison in Figure 4 was sampled in November 2009 in Monterey Bay, California at 305 m from a Grey whale skeleton and stained in accordance to the protocol described below. All necessary permits were obtained for the described whale fall experiment; received from Karin Pettersson at the County Administrative Board Vastra Gotalands Lan, Oct. 2003. The field studies did not involve endangered or protected species.

Fixation

Specimens were carefully dissected from the bone and fixed for immunohistochemistry, benzidine staining and histology. Prior to dissection, Osedax muciferi were anesthetized for 3–10 minutes in a 1:1 solution of seawater and MgCl2 (isotonic to seawater). Anaesthetized animals were gently dissected with scalpels and fixed at 4 °C over night in 4% paraformaldehyde in 0.13 M phosphate-buffered saline (PBS) with 5% sucrose, pH 7.4. Subsequently, the animals were rinsed 4–6 times for 30 min in PBS with 5% sucrose and were then stored at 4 °C in PBS with 0.05% sodium azide (Na3N4).

Immunohistochemistry

Four specimens were stained following the protocol of Worsaae & Rouse [45]. First staining included the primary antibodies monoclonal mouse anti-acetylated τ-tubulin (Sigma T6793, 1:200) & polyclonal rabbit anti-serotonin (Sigma: S5545; 1:100/1:400) or monoclonal mouse anti-acetylated τ-tubulin & Anti-FMRFamide (ImmuNoStar: 20091, 1:100). This was complemented by secondary antibodies; anti-mouse CY5 [Jackson ImmunoResearch: 115-175-062, 1:400] and anti-rabbit TRITC (Sigma T3268, 1:200/1:400). Hereafter specimens were incubated for 60 min in phalloidin conjugated with FITC or Alexa Flour 488 (Sigma F3282 or Invitrogen A12379, 0.17 or 0.33 μmol l−1 phalloidin in PBS). Specimens were mounted in 100% Vectashield® containing DAPI (Vector Laboratories inc., California, USA) and stored at −18°C. The specificity of primary antibody binding versus e.g., autofluorescence was tested by omitting one of the primary antibodies, but otherwise treating specimens as described.

Specimens were studied using a Leica TCS SP5 confocal laser scanning microscope (CLSM) (University of Copenhagen, Faculty of Health Science, courtesy of M. Givskov and T. Bjarnsholt). Leica LASAF computer software or Imaris® x64 6.0.0 (Bitplane AG, Zurich, Switzerland) was used to produce projections of z-stacks of CLSM images, while further analyses of z-stack series were performed with Imaris® x64 6.0.0. Computed 2D images of muscles, nerve and cilia with relation to respiration and palps morphology were further optimized with Adobe Photoshop CS5 and Adobe Illustrator (Adobe System Incorporated) for presentation.

Histology

One specimen was embedded in epon and used for histological analysis. Semi-thick 1.2 μm sections were cut on a microtome (EM UC6, Leica, Wetzlar Germany) with a diamond knife (Diatome; Biel, Switzerland). A small amount of Pattex contact adhesive (Pattex Compact; Henkel KGAA, Düsseldorf, Germany) was diluted with a few drops of xylene in an Eppendorf tube and applied to the side of the epon block to make serial sectioning of ribbons possible following the protocol of Henry [46] and Ruthersteiner [47]. Bands of ~20 sections were stained with toluidine blue and mounted in Entellan® (Electron Microscopy Sciences, Pennsylvania, USA). Sections were studied and photographed using light microscopy (BX50 microscope; DP71 camera; Cell® software; Olympus, Japan).

Benzidine staining of haemoglobin

Using a modified version of the benzidine staining method by Knox [48], haemoglobin was stained in four fixed specimens. A 100% saturated benzidine solution was prepared by adding benzidine to distilled water. The solution was stirred for two hours. Fixed specimens were rinsed in running tap water in the same time period. Specimens were subsequently incubated in the filtered benzidine solution for 1 hour, also under stirring. Next, 5% hydrogen peroxide was added drop by drop until blood vessels turned dark blue. Specimens were either mounted in glycerol directly or dehydrated in a series of alcohol acidified with drops of 0.1% acetic acid, where after tissues were cleared in xylene and mounted in D.P.X between two cover slips. Specimens were analyzed and photographed under a light microscope (BX50 microscope; Dp71 camera; Cell® software; Olympus, Japan).

Oxygen consumption measurements

O2 consumption was measured in seawater kept at ~100% atmospheric saturation using intermittent respirometry in accordance with Vismann and Hagerman [49]. The experimental setup was placed in a constant temperature room at 6°C. Each experiment encompassed 3–8 measuring sequences consisting of a flushing period of 10–30 min and a measuring period of 30–45 min. The set-up had a chamber flushing rate of 30 ml min−1 and a shunt water flow of 8 ml min−1, and a shut water flow of 8 ml min−1 past the O2 electrode (E5046, Radiometer Medical ApS, Bronshoj, Denmark).

The O2 electrode was connected to a blood/gas monitor (PHM73, Radiometer Medical ApS, Bronshoj, Denmark) and measuring signals were acquired continuously on a PC via data acquisition software (Labtech Notebook Pro version 12.1, Laboratory Technologies Corporation, USA). Prior to each experiment, the O2 electrode was calibrated in 0% O2 solution (saturated solution of sodium sulphite in borax) and 100% atmospheric O2 solution. The O2 consumption (MO2, μg O2 g−1 h−1) was calculated according to the equation:

\[ MO_2 = \frac{p_{O2}}{100 \times w_w} \times \alpha \times \beta \times \nu \]

where \( p_{O2} \) is the O2 partial pressure at full saturation (kPa), \( w_w \) is wet weight of fixed specimen rinsed for mucus tube (g) (issue fixed in 4% paraformaldehyde and stored in PBS is assumed to have similar weight as unfixed tissue), \( \alpha \) is slope of regression line for O2 decrease (% h−1), \( \beta = O_2 \) solubility (μg O2 l−1 kPa−1) and \( \nu = \) volume of the respiration chamber (l).

O2 consumption was measured on O. muciferi inhabiting three sectioned cow bone pieces (B1, B2 and B3) and bones in two cuvettes (C1 and C2). Measurements were either initiated directly
after the annelids protracted subsequently to the disturbance of being moved (C1, C2, B1) or after one night of acclimatization (B2, B3). All measurements were corrected for background respiration. For B2 and B3 the background respiration was measured using the same water and bones (after dissection and 48 hours of acclimation to restore biological activity). For C1, C2 and B1 the background respiration was the mean value of measurements using new water, bones in two cuvettes and three sectioned bone pieces without *O. mucrofloris*. Different respiration chambers were used for sliced bone pieces (volume: 186 ml) and cuvettes (volume: 51 ml).

Microscale O₂ measurements

O₂ concentration profiles were measured in vertical steps of 100 µm with Clark-type O₂ microelectrodes with a guard cathode [50] (OX-10 Unisense A/S, Aarhus, Denmark) mounted on a manually operated micromanipulator (Märzhäuser, Wetzlar, Germany) and connected to a picoammeter (PA2000, Unisense A/S, Aarhus, Denmark) and a strip chart recorder (BD25, Kipp&Zonen, Delft, Netherlands). The microelectrodes had a measuring tip diameter of 10 µm, a stirring sensitivity of <1–2% and a 90% response time of <1 second. Linear calibration of the electrode was done from electrode readings in seawater at 100% atmospheric saturation and in anoxic seawater (by addition of sodium dithionite). The O₂ concentration ([O₂], µmol O₂ m⁻³) at each measuring position was calculated according to the equation;

\[ [O_2] = \frac{x_n}{x_0} * C_{sat} \]

Where \( x_n \) = O₂ reading at depth n (pA), \( x_0 \) = O₂ reading at 100% atmospheric saturation (pA) and \( C_{sat} \) = O₂ concentration at atmospheric O₂ saturation (pA) and Csat = O₂ concentration at atmospheric saturation and in anoxic seawater (by addition of sodium dithionite). The O₂ concentration ([O₂], µmol O₂ 1⁻¹) at each measuring position was calculated according to the equation;

\[ J = D_0 * \frac{dC}{dz} \]

Where dC/dz = the change in O₂ concentration (dC, µmol O₂ cm⁻³) with distance (dz, cm), \( D_0 \) = the molecular diffusion coefficient of O₂ (cm² sec⁻¹). We applied a diffusion coefficient of 1.3382 * 10⁻⁵ cm² sec⁻¹, corrected for experimental temperature and salinity.

Additional microscale O₂ measurements were performed through the mucus tube wall and at the surface of the palps of the individual on WB1. On the mucus tube, the O₂ concentration was measured from outside the tube and half way into the mucus tube wall. Undisturbed measurements could not be obtained within the centre hole of the tube due to contractions of the worm. On the palps, the O₂ concentration was measured by keeping the electrode measuring-tip at a fixed position, while the palps approached the tip position as the annelid protracted after being left undisturbed.

Acknowledgments

We would like to thank Tom Fenchel for guidance and lending of equipment for benzidine staining and live video recordings. We also thank Per Juel Hansen for access to his Olympus BX51 microscope, Dp71 camera and Cell® software. Special thanks are directed to Michael Givskov and Thomas Bjarnsholt for the many hours made available to us on their Leica TCS SP5 confocal laser scanning microscope at the Faculty of Health Science, University of Copenhagen. In addition we thank Tomas Lundløv for ship and ROV operations and Helena Wåhlin for assistance at the Sven Lovén Centre for Marine Sciences – Tjärnö.

Author Contributions

Conceived and designed the experiments: RSH BV MK MM KW. Performed the experiments: RSH MK MM VC KW. Analyzed the data: RSH MM VC KW. Contributed reagents/materials/analysis tools: KW MK BV AG GR TD. Wrote the paper: RSH KW BV.

References