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**National Oceanography
Centre, Southampton**
UNIVERSITY OF SOUTHAMPTON AND
NATURAL ENVIRONMENT RESEARCH COUNCIL



Reproductive Ecology of Vestimentifera (Polychaeta: Siboglinidae) from Hydrothermal Vents and Cold Seeps

PhD Dissertation



submitted by

Ana Hilário

to the Graduate School of the National Oceanography Centre, Southampton in partial
fulfillment of the requirements for the degree of Doctor of Philosophy

June 2005



**National Oceanography
Centre, Southampton**
UNIVERSITY OF SOUTHAMPTON AND
NATURAL ENVIRONMENT RESEARCH COUNCIL

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I hereby declare that no part of this thesis has been submitted for a degree to the University of Southampton, or any other University, at any time previously. The material included is the work of the author, except where expressly stated.

Southampton 17 June 2005, Ana Hilário

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Abstract

Faculty of Engineering, Science and Mathematics
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Doctor of Philosophy

REPRODUCTIVE ECOLOGY OF VESTIMENTIFERA (POLYCHAETA: SIBOGLINIDAE) FROM
HYDROTHERMAL VENTS AND COLD SEEPS

by Ana Hilário

Giant siboglinid tubeworms (formerly Vestimentifera) are ecologically important members of deep-sea chemosynthetic communities, including hydrothermal vents and cold seeps. Species of siboglinids are community dominants, primary colonists of new vent sites, and the longest living and fastest growing marine invertebrates. Their mechanisms of propagation, dispersal and genetic exchange have been widely discussed. Direct sperm transfer from males to females has been documented in two species, *Ridgeia piscesae* and *Tevnia jerichonana*, but others were believed to spawn primary oocytes. Brooding of embryos have never been observed in any species. These observations have led to the logical supposition that fertilization must be external in all but two species. Here I report sperm storage at the posterior end of the oviduct in 5 species, including tubeworms from both vents and seeps. I show experimentally that fertilization is internal, that meiosis is completed after eggs are released from the female, and that the dispersal phase includes the entire embryonic period.

The contrasting physical and chemical conditions of hydrothermal vents and cold seeps are reflected in the life history patterns of the different species of vestimentiferans. Image analysis of histological sections show that vestimentiferans from hydrothermal vents have significantly less percentage of trophosome than species from cold seeps. The reproductive condition, however, was found to be irrespective of the surrounding environmental conditions.

Previous studies of the lipid class composition suggested that vestimentiferans store lipids in the gonad. However, because the gonadal tissue has never been dissected and compared with the other tissues of the trunk, it was not possible to determine whether the gonad comprises the only lipid store in vestimentiferans, or if different lipid classes are stored in different tissues. Here I show that two main storage lipids are found in the trunk of female vestimentiferans. Substantial reserves of wax esters are found in the gonad, and triacylglycerols in other tissues of the trunk. Due to the close association between the gonadal tissue and the trophosome, the only way to determine the reproductive effort in vestimentiferan tubeworms has been, to date, the study of histological sections of the trunk of the individual. The results presented here show a linear relationship between the amount of gonad and the concentration of wax esters in the trunk of female vestimentiferans. The determination of the concentration of wax esters is a biochemical technique that allows comparison of the amount of gonadal tissue with the amount of trophosome, and should be considered as a new and simple method for the determination of the reproductive condition in this group.

Acknowledgements

I owe a massive debt of gratitude to my supervisors Paul Tyler and Craig Young for support, encouragement, opportunities and enthusiasm throughout. I am also grateful to Dave Pond at the British Antarctic Survey for help and advice on lipid identification.

I would like to acknowledge Craig Young, Sandra Brooke, Shawn Arellano, Chuck Fisher and Breea Govenar for their help on collecting samples on various cruises. Many big thanks go to Eva Ramirez-Llodra and Jon Copley for the many interesting discussions about chemosynthetic environments and reproduction of invertebrates.

Thanks to Raquel, Lisete, Hugo and Joana for their continuous friendship. A big thank you to Oli and Sinhué for many nights of music and poetry. Finally I would like to thank my family for their support during all these years.

...and to anyone who I've forgotten, it's not personal...it's just me being vague!

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Introduction

The history of the deep-sea biology is littered with generalisations abandoned after the discovery of exceptions to their ‘rules’. One of the most striking revelations in the recent history of deep-sea biology was the discovery of exceptions to the exponential decrease in biomass along a depth gradient in the form of luxuriant animal communities at deep-sea hydrothermal vents.

First discovered along the Galápagos Rift in 1977 (Corliss and Ballard, 1977), these communities are remarkable for their use of geothermal energy as an alternative to solar radiation in the fixation of inorganic carbon. Supported by high levels of *in situ* microbial-mediated chemosynthesis, hydrothermal vents, in contrast to “normal” deep-sea, have very high biomass and relatively low diversity with highly adapted, mainly novel, megafaunal and macrofaunal species (Tunnicliffe, 1991). This discovery led to one of the most active programmes in the deep-sea biology, and the discovery and analysis of hydrothermal vents continues to this day.

The last two decades have led to the investigation of productive ecosystems at cold seeps, which host highly diverse and abundant biota (Carney, 1994). Although the domain of cold seeps has received considerably less attention compared to the investigations of the deep-sea hydrothermal vents, it has been suggested that the diversity is greater at cold seeps and that the interactions between geological and biological systems are more complex at low than at high temperatures (Aharon, 1994; Tunnicliffe et al., 1996). Both hydrothermal vents and cold seeps are driven by the availability of reduced chemicals such as hydrogen sulphide and methane, the main difference being the temperature of emission.

The awareness of the non-biotic natural processes of the deep ocean is recognised as a prerequisite for understanding its ecology (Gage and Tyler, 1991). Because of the perceived extremes in geological, physical and chemical conditions present at hydrothermal vents and cold seeps, which have an effect on the survival of faunal communities, this requirement is even greater.

Until now over 400 species from hydrothermal vents and more than 200 from cold seeps have

been described and an avalanche of studies describing aspects of physiology for a variety of them have been published. The autoecology of vents and cold seeps have been described (Sibuet and Olu, 1998; Van Dover, 2000), and the evolutionary history and fossil record are being interpreted (Tunnicliffe, 1991; Van Dover et al., 2002; Little and Vrijenhoek, 2003). Life history biology, however, has proved to be the least tractable of biological processes, though widely recognised as being of fundamental importance to understand the establishment and maintenance of both vents and seeps populations (Tyler and Young, 1999).

The discovery of hydrothermal vents in 1977 heralded the description of large vestimentiferan tubeworms initially placed as a class in the Phylum Pogonophora (Jones, 1981a) and then elevated to the phylum level as a Phylum Vestimentifera (Jones, 1985). Genetic and embryological evidence have now shown convincingly that these gutless, deep-sea tubeworms are annelids from the class Polychaeta and family Siboglinidae (Young et al., 1996; McHugh, 1997; Rouse and Faulchald, 1997). The vestimentiferan tubeworms came to symbolise hydrothermal vents in the Pacific, and with their discovery at cold seeps in the Gulf of Mexico and elsewhere, became the icon of communities driven by chemosynthetic primary production. Species are known to be the first colonisers of new vents (Shank et al., 1998) as well one of the fastest growing marine invertebrates (Lutz et al., 1994) and one of the longest lived (Fisher et al., 1997).

Because of their ecological importance at vent and seeps, the reproductive and dispersal biology of vestimentiferans is of considerable interest. In this context, the present thesis presents the first complete study of the reproductive ecology of vestimentiferans from deep-sea hydrothermal vents and cold seeps.

Chapter 1 gives an outline of the underlying theory that the remainder of this thesis will draw upon. It summarises the relevant issues of the physical and chemical conditions, and how they relate to the ecology of the communities, found in the different reducing environments of the deep-sea floor. An introduction to the taxonomy and general biology of vestimentiferans is given in the final section of this chapter. Chapter 2 presents the settings of the sampled sites and gives a summary of the biology of all species studied in this project. Chapter 3 examines the anatomy of the female reproductive tract, with special emphasis on the previously unknown spermatheca, and presents a series of *in situ* and *in vitro* experiments that allowed the elucidation of the site of fertilization and the dispersive biology of species from hydrothermal vents and cold seeps. In chapter 4, the intra and interspecific variability of the reproductive condition is discussed based on histology

and image analysis of sections of the trunk of both male and female of four species, two from vents and two from seeps. Chapter 5 examines the lipid partitioning in the trunk of two species from cold seeps and establishes a biochemical method for the determination of the reproductive condition in this taxa. Chapter 6 presents the conclusions of this study by summarising the achievements of the different analysis to the reproductive tract of vestimentiferans, and pointing the direction of future work arising from this project.

Chapter 1

General Background

1.1 Reducing Environments of the Deep-Sea Floor

1.1.1 Hydrothermal vents

Heat deep within the mantle of Earth drives the cyclical process of creation and destruction of the Earth's crust. With two-thirds of that heat being released in the generation and cooling of the ocean crust, hydrothermal circulation represents an important mechanism for cooling newly generated crust. Venting occurs at both divergent plate boundaries, the locus of incremental seafloor spreading also known as spreading centres, and convergent ocean plates where back-arc spreading occurs; back-arc spreading centres form behind island arcs along active plate margins where thick, old ocean crust is undergoing subduction beneath a continental plate moving in the same direction (Tunnicliffe, 1991). Around the spreading centres the ocean floor is highly permeable and cold, dense seawater penetrates the crust.

With depth and lithology, crustal permeability decreases and, ultimately a magma chamber, localized a few kilometres below the ridge crest cannot maintain fractures for water penetration, providing a maximum depth for fluid circulation. The exact number of these chambers, and how the circulating fluid interacts remains poorly understood. During circulation through the upper ocean crust vent fluids are geothermally heated and enriched in energy-yielding reduced compounds. Figure 1.1 schematises hydrothermal circulation at seafloor and processes occurring through it.

Compared to seawater, hydrothermal fluids have a low pH (3–5) and are especially enriched in sulphide (H_2S), hydrogen (H_2), methane (CH_4), manganese (Mn), and other transition metals (iron, copper, zinc, lead, cobalt). Magnesium and oxygen are completely stripped

from vent fluids (Alt, 1995). Vent fluids are also enriched, through degassing of the mantle and crystallising magmas, with volatiles such as ^3He . ^3He is a conservative tracer and has been used to discover and track hydrothermal plumes (Craig and Lupton, 1981). Table 1.1 presents a comparison between hydrothermal fluids and seawater constituents.

Because of the increased buoyancy at high temperatures, fluids rise rapidly to the seabed surface through discharge zones (Alt, 1995). Hydrothermal fluids discharged from vents are rapidly diluted with ambient seawater by factors of 10^4 – 10^5 (Lupton et al., 1985). During dilution, the mixture rises to a height of neutral density and it spreads out laterally to form the effluent layer or neutrally-buoyant plume. Plumes are important as habitat and resource for microorganisms and zooplankton, and as advective mechanisms for chemical fluxes and dispersal stages of vent biota. Plumes are also excellent indicators of vent emissions (Lupton et al., 1985). This cycling water links the overlying ocean, the forming lithosphere, the underlying mantle and the associated biota (Tunnicliffe, 1991).

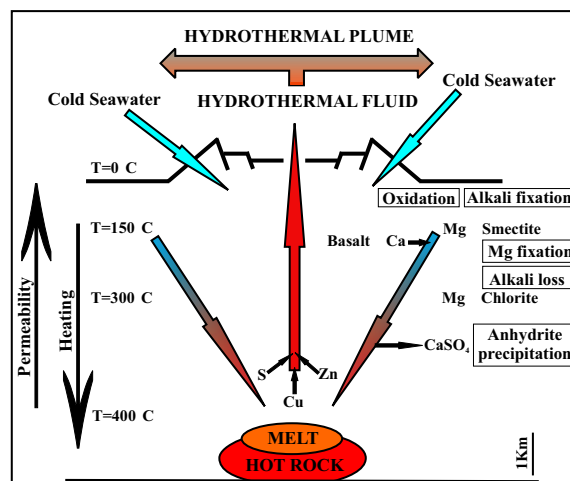


Figure 1.1: Hydrothermal circulation at seafloor and processes occurring through it (after Alt (1995)).

plumes for chemical fluxes and dispersal stages of vent biota. Plumes are also excellent indicators of vent emissions (Lupton et al., 1985). This cycling water links the overlying ocean, the forming lithosphere, the underlying mantle and the associated biota (Tunnicliffe, 1991).

Distribution of hydrothermal vent sites is sparse and uneven (Figure 1.2). The global ridge is about 75000 km long but underlying heat sources are not continuous. At a first-order scale, ridge axes are divided into distinct segments up to 1000 km in length bounded by deep transform faults that offset the spreading axis by 50 km or more. Second-, third-, and fourth-order segmentation with more subtle and less persistent morphological boundaries can further subdivide a first-order ridge segment into discrete units of tens to a few hundred kilometers in length which can have quite different magmatic activity beneath them (Batiza, 1996). Hydrothermal vent fields are the basic unit of hydrothermal activity on ridges axes. Vent fields range in size from metres to hundreds metres across. A single vent field may contain from a few to hundreds of openings that create a mosaic of colonies (Tunnicliffe et al., 2003).

Although most known hydrothermal fields are located on young crust, a growing body of evidence from recent water column and sea-floor studies indicate that lower-temperature

Table 1.1: Chemical composition of a typical 350°C black-smoker fluid compared to seawater, but with number rounded and ranked by degree of enrichment above seawater. (After Van Dover (2000)).

Element	Hydrothermal Fluid	Seawater	Units	Enrichment Factor(minimum)
H ₂ S	3–12	0	mM.Kg ⁻¹	∞
H ₂	0.05–1	0	mM.Kg ⁻¹	∞
CH ₄	25–100	0	μM.Kg ⁻¹	∞
Mn	360–1140	0	μM.Kg ⁻¹	∞
Fe	750–6500	0	μM.Kg ⁻¹	∞
Be	10–40	0	μM.Kg ⁻¹	∞
Zn	40–100	0.01	μM.Kg ⁻¹	4000
Cu	10–40	0.007	μM.Kg ⁻¹	1500
Ag	25–40	0.02	nM.Kg ⁻¹	1250
Pb	10–360	0.01	nM.Kg ⁻¹	1000
Co	20–220	0.03	nM.Kg ⁻¹	650
Si	15–20	0.05	mM.Kg ⁻¹	300
Al	5–20	0.02	μM.Kg ⁻¹	250
Ba	10–40	0.15	μM.Kg ⁻¹	66
Cs	100–200	2	μM.Kg ⁻¹	50
Li	410–1320	25	μM.Kg ⁻¹	16
Rb	10–30	1	μM.Kg ⁻¹	10
CO ₂	5–15	2	nM.Kg ⁻¹	2.5
Ca	10–55	10	nM.Kg ⁻¹	1
Sr	90	85	μM.Kg ⁻¹	1
B	450–560	415	μM.Kg ⁻¹	1
As	30–450	30	nM.Kg ⁻¹	1
Se	1–75	2	nM.Kg ⁻¹	0.5
P	0.5	2	μM.Kg ⁻¹	0.25
Mg	0	50	mM.Kg ⁻¹	0
SO ₄	0–1	30	mM.Kg ⁻¹	0
Alk	(-0.1)–(-1)	2	mM.Kg ⁻¹	0

venting associated with older, tectonized portions of the oceanic crust may be common along much of the mid-ocean ridge spreading network (Kelley et al., 2001). Off-axis hydrothermal activity has been found close to ridges spreading at intermediate (50–90 mm yr⁻¹) or slow rates (10–50 mm yr⁻¹). In slow spreading ridges the fluids are quite cold and alkaline (Von Damm, 2001).

As a result of differences in the styles and temperatures of venting, different processes of mineralisation and evolution of massive sulphide deposits are formed at seafloor hydrothermal vents, ranging from seafloor deposition within conduits to simple column chimneys, to larger, complex structures, to ore bodies that are equal in size to commercially impor-

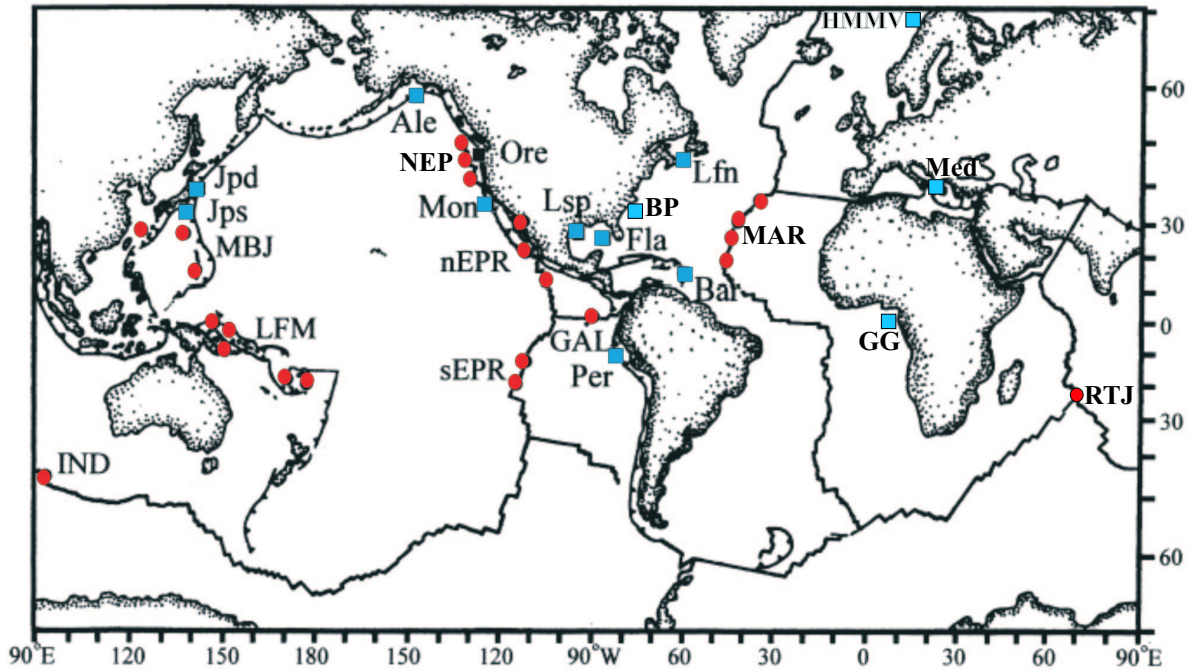


Figure 1.2: Distribution of vents (red dots) and seeps (blue squares) in the deep sea. Several other sites are known but poorly documented. Vents: nEPR, numerous sites from 21°N to 9°N, East Pacific Rise; sEPR, numerous sites from 17°S to 21°S, East Pacific Rise; GAL, Galapagos Rift; GMS, Guyamas Basin; IND, southeast Indian Ridge; LFM, several sites around Fiji, Lau, and Manus Back-Arc Basins; MAR, five sites on Mid-Atlantic Ridge; MBJ, two major sites in Marianus Back-Arc Basin and Okinawa Trough; NEP, numerous sites on Explorer, Juan de Fuca and Gorda Ridges; RTJ, Rodriguez Triple Junction. Seeps: Ale, ;Bar, Barbados Prism; BP, Blake Plateau; Fla, Florida Escarpment; GG, Gulf of Guinea; HMMV, Haakon Mosby Mud Volcano; Jpd, deep Japan Margin; Jps, shallow Japan Margin; Lfn, Laurentian Fan; Lsp, Louisiana and Gulf of Mexico; Med, Mediterranean Sea; Mon, Monterey Bay; Per, Peru Margin; Ore, Oregon Margin. From Tunnicliffe et al. (2003).

tant deposits on land. As actively-forming hydrothermal mineral deposits provide a source of energy for chemosynthesis and a substratum for colonization by adult and larval vent organisms, such differences have implications for the distribution of vent fauna (Sarrazin et al., 1997).

The simplest of sulphide structures is the columnar chimney typical of hydrothermal vents on the East Pacific Rise. These chimneys can form early in the evolution of a hydrothermal site, in the aftermath of a volcanic eruption (Haymon et al., 1993).

Formation of a simple black-smoker chimney begins as hydrothermal fluid mixes with the surrounding cold, alkaline seawater at the vent orifice, causing the metal sulphides to precipitate and form particle-rich “black-smoker” plumes. The first stage of chimney growth is precipitation of a friable, porous anhydrite (CaSO_4) sheath or tube around the

exiting fluids. Horizontal flux of fluids across the chimney wall occurs until the pore space is filled with anhydrite and other copper-iron sulphide mineral (Hannington et al., 1995). As the outer anhydrite walls cool to temperatures below 150°C, the anhydrite starts to dissolve back into seawater again. Most mature chimneys have tortuous plumbing and complex mineralogies with zones of horizontal porosity and diffuse warm-water flow at temperatures and fluxes suitable for exploitation by organisms (Hannington et al., 1995).

Bulbous beehive or wasp-nest-like structures occur as outgrowths on the sides or tops of sulphide chimneys. They are extremely porous and often very friable structures through which high-temperature fluids ($> 300^{\circ}\text{C}$) diffuse (Fouquet et al., 1993). Because of their high surface temperatures, they are typically bare of organisms, and thus their surfaces are often in conspicuous contrast to the animal- or bacteria-covered surfaces of sulphide chimneys on which they occur (Van Dover, 2000).

Flanges are accretionary sulphide structures that are lateral outgrowths from the supporting sulphide mound. They commonly trap inverted pools of buoyant, high-temperature ($\sim 350^{\circ}\text{C}$) hydrothermal fluid on their undersurface (Delaney et al., 1992). Upper surfaces of flanges, where there is a diffuse flow of 10–80°C fluids that have percolated through the flange matrix, often support dense invertebrate and microbial populations. This difference in fluid temperature forms one of the strongest thermal gradients on Earth.

White smokers are those chimneys from which fluids at intermediate temperature (100 – 300°C) are emitted. At these temperatures, silica, anhydrite and barite precipitate as white particles (Hannington et al., 1995). As these particles are insoluble in cold water, they provide lasting stability for large sulphide structures. White smokers chimneys can grow at any stage in the development of a vent field, during early, low-temperature venting, when the fluids are not yet hot enough to carry sufficient metals and sulphur to the seafloor, or alternatively, in a high-temperature system where conductive cooling or subseafloor mixing results in the loss of metals at depth.

Large sulphide mounds (e.g. the 45 m high “Godzilla” structure of the High Rise vent field, Juan de Fuca Ridge; Figure 1.3) may be constructed from the accumulation and infilling of sulphide debris produced by collapsing chimneys. Much of the heat and fluid flux from a large mound is emitted as diffuse flow at temperatures low enough to permit colonisation by organisms (Van Dover, 2000). Differences in sulphide edifice morphologies may have consequences for vent biota: large diameter, low relief mounds at the Galápagos, Explorer Ridge and TAG provide a large and continuous habitat with several sites of high-

temperature venting over the mound structure with areas of diffuse flow between them, while in steep-walled structures at Endeavour Segment, Juan de Fuca Ridge, fauna is restricted to the upper parts, where most venting occurs.

Diffuse venting typically occurs throughout the life of a hydrothermal system. It may be the earliest form of discharge in a new hydrothermal field but commonly occurs at the margin of existing high-temperature upflow where rising hydrothermal fluids mix with cold seawater. Diffuse venting also typically dominates the last stages of activity in a waning hydrothermal system (Hannington et al., 1995). Diffuse flows may issue from surfaces of active mineral deposits (black smokers, white smokers, and complex sulphide mounds) or directly from fissures and cracks in basalt lavas. It is the diffuse, warm-water flows that sustain productive populations of thermophilic microorganisms ($< 115^{\circ}\text{C}$) and dense invertebrate communities (usually $< 40^{\circ}\text{C}$) at deep-sea hydrothermal vents (Van Dover, 2000), but they are not generally associated with extensive mineralisation because the low temperature of the fluids does not allow the transport of significant concentrations of dissolved metals and sulphur (Hannington et al., 1995).

When hot fluids ultimately cease to flow through a sulphide mound, acidic pore fluids are produced during the oxidation of sulphides by cold water and the chimneys matrix becomes soft and unstable. Dense hydrothermal communities die off and are often replaced by a few suspension-feeding brisingid sea-stars or sponges, which take advantage of the richer supply of food delivered on currents modified by local topographic relief (Van Dover, 2000).



Figure 1.3: “Godzilla”, a 45 m high sulphide mound with flanges on the Juan de Fuca Ridge. The submersible ALVIN is drawn to scale. From Robigou et al. (1993).

1.1.2 Seeps of continental margins

Soon after chemoautotrophic communities were discovered at hydrothermal vents, dense communities of animals were encountered at brine seeps along the base of the Florida Escarpment in the Gulf of Mexico, and subsequently in subduction zones along convergent margins of plates where porewaters are squeezed out of sediments. As flow rate is usually slow and fluids have only small temperature anomalies, these sites are called “cold seeps”. To date, cold seeps have been observed in 24 deep-sea areas located in the Atlantic, the Eastern and Western Pacific, and the Mediterranean Sea, on both passive and active margins at depth ranging from 400 to 6000 m (Sibuet and Olu, 1998) (Figure 1.2).

Cold seeps are related to geological processes such as tectonically-induced high-fluid pressures, petroleum or natural gas escape, artesian flow or catastrophic erosion and submarine slides. The distribution of seep fauna has been used in parallel with geological observations to identify tectonic and sedimentologic features, and conduits of fluids. The characteristic seep megafauna also been used as an indicator of the location of methane or sulphide-rich environments (Sibuet and Olu, 1998).

On passive margins seeping is usually associated with oil and gas reservoirs. In some passive margins, like the Louisiana Slope, salt tectonics creates the conduits for the seeping fluids. Ancient salt deposits below the sediments where hydrocarbons and methane have accumulated push upward (because they are less dense), forming deep cracks in the sediments through which gases and petroleum escape (Kennicutt et al., 1985). Fluids may also arise from sulphide-rich brine seeps, as described from the Florida Escarpment, located at the juncture of the escarpment base and the abyssal sediment plain (Paull et al., 1984).

Seepage of methane and hydrocarbons of thermogenic origin was also discovered along a transform fault on Guaymas Basin (Gulf of California). Seepage occurs through shallow pockmarks along the eroding crest of a steep anticline belonging to the transform fault (Simoneit et al., 1990). These cold seeps are located a few kilometers from a hydrothermal vent field and have been colonised by chemosynthetic animals similar to those found at other cold seeps but also by species usually found at hydrothermal vents.

In subduction areas, seeps occur both on well-developed accretionary prisms and along erosive margins to a depth of at least 6000 m (the limit of the deepest research submersibles) (Tunnicliffe et al., 2003).

On accretionary prisms faults develop in the compacting sediment, providing conduits for

trapped fluid which may be of both continental or oceanic origin (Figure 1.4). Compressional forces yield not only active thrust faults near the deformation front, as in Nankai prism (Sibuet and Olu, 1998), but can also yield diapiric structures like mud volcanoes that are created by an influx of water from deep over-pressured zones, as in the Barbados prism (Olu et al., 1997). At some sites the subduction trench is pulling apart, and the associated seismic activity forces out fluids; in these cases, seeping may be less stable.

In erosive margins and landslides seepage occurs on the head scars of large-scale debris slides probably linked to earthquake occurrences as on the Peru margin (Olu et al., 1996), and along canyon walls where sediment piles have been removed by tectonic motions -and faults on seamounts entering subduction zones (Sibuet and Olu, 1998).

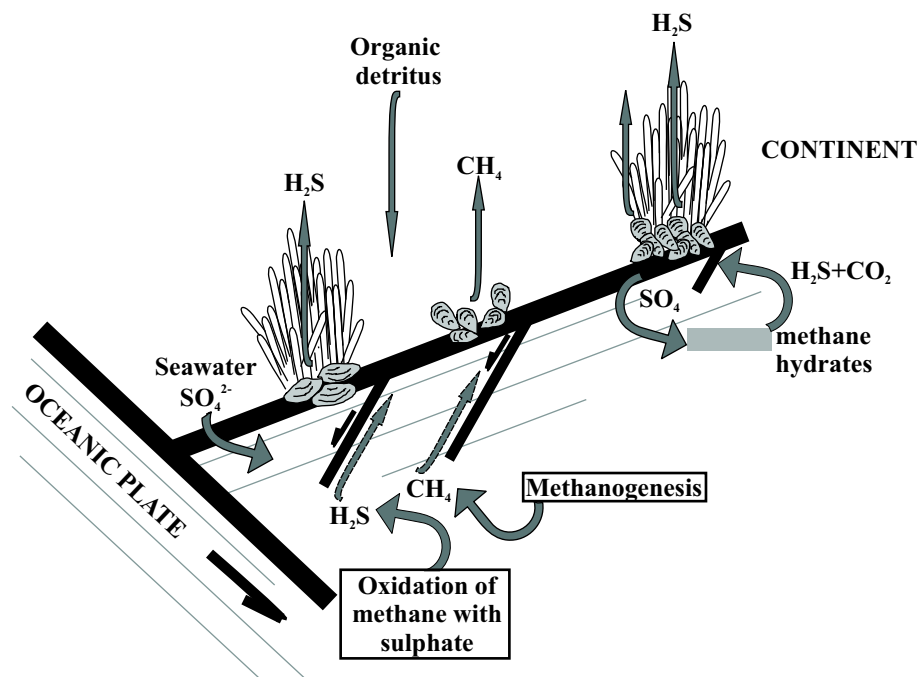


Figure 1.4: Generalized view of seepage of reduced fluids at an active continental margin. The downslipping oceanic plate piles sediment on the continental margin as an accretionary prism. Faults develop in the compacting sediment, providing conduits for trapped fluid which may be of both continental or oceanic origin. From Tunnicliffe et al. (2003).

Cold seep fluid is as varied as the settings. Unlike hydrothermal vent systems, where aerobic sulphide oxidation was identified as the most important potential energy source for chemotrophy, both aerobic and anaerobic methane oxidation are probably the most important chemosynthetic processes at cold seeps. However, in association with organic remains, two anaerobic processes are important to chemosynthesis based on sulphide oxidation. At active margins seeps, sulphide is produced in near-surface sediments as a result of bacterial reduction of sulphate, which utilizes methane or other hydrocarbons as energy sources (Carney, 1994). Reduction of sulphate in sea water also occurs as a terminal metabolic process in the anaerobic degradation of organic remains, providing sulphide for development of microbial mats and invertebrate symbioses. It has been suggested that dissolved sulphide does not escape from near-surface sediments, restricting the utilization of sulphide to animals that extend part of their body into the sediment (clams and lamellibranchiid siboglinids) and to sediment microorganisms. The type of symbiont-containing invertebrate that dominates a site may depend on which compound(s) the symbionts can exploit.

The carbon sources are organic: methane, petroleum, other hydrocarbon gases, or from solid gas hydrate that derive from accumulated sedimentary organic carbon and thus are photosynthetic in origin (Tunncliffe et al., 2003). At several sites, petroleum products are abundant enough to coat collected biota with oil (personal observation).

It has been found that the biological production at cold seeps are related to the intensity of the fluid flow. Direct and indirect measurements of fluid flow on some active seep sites have shown values ranging from 86 to 1765 $\text{lm}^{-2}\text{d}^{-1}$ (Sibuet and Olu, 1998). Large variation of fluid flow rates within a single cold seep area and between different sites have been observed. Like hydrothermal vents (Fustec et al., 1988; Van Dover, 2000), the patchy and ephemeral occurrence of chemosynthetic fauna can be attributed to spatial and temporal variations in the fluid supply (Olu et al., 1996, 1997). Patch size is generally less than 20 m^2 and frequently of about 0.5 to 2 m^2 which means that the fluid emission is restricted to simple conduits. However, some exceptions exist at Sagami Bay, Gulf of Mexico and at the Peru Trench, where clam beds can reach areas of 1000 to 6000 m^2 . It has been suggested that the existence of such large and continuous fields are consistent with regular and diffuse expulsion (Olu et al., 1996). It remains difficult to relate biological production to flow rates together with the chemical composition of the fluids because the biological and geochemical analysis and measurement of fluid flow are seldom undertaken together.

As the margin environments are not completely explored, the extent of seepage communities at the deep-sea is unknown. Observations of demographic features, trophic complexity and

high species richness suggest sustained seepage at several sites (Carney, 1994).

1.1.3 Other reducing environments

Although on a smaller and more ephemeral scale, the same chemoautotrophic microbial production as found in hydrothermal vents and cold seeps occurs in windfalls of organic origin. Perhaps the best known is that of large carcasses. The fortuitous discovery of a whale skeleton in 1989 led to descriptions of a community of invertebrates with many features similar to those of vent communities (Smith et al., 1989; Smith and Baco, 2003; Rouse et al., 2004). Microbial degradation generates sulphite from sulphate reduction and putrefaction of the lipid-rich organic material. Closely associated with the whale bones are mats of sulphide-oxidizing bacteria. Wood falls are also known to develop reducing environments. One interesting find is that of a siboglinid tubeworm and a symbiont-containing mussel in the wreck of a cargo ship (Dando et al., 1992). The animals were found among the cargo of beans, sunflower seeds and sisal – all organic-rich and liable to produce sulphide upon decay.

There are several places in the ocean in which oxygen concentrations are low and occasionally are zero. Larger basins occur mostly in marginal areas where organic input is relatively high. Microbial oxidation depletes dissolved oxygen, and hydrogen sulphide can build up in bottom waters and sediments (Tunnicliffe et al., 2003).

The most voluminous reducing habitat in the deep-sea lies below the seafloor in anoxic sediments and crustal rocks. As far as can be determined, this environment is populated only by microorganisms capable of anaerobiosis, although zones of aerobic microbial growth may exist in near-surface rocks. For the most part, deep-living sediment microorganisms are deriving energy and nutrients from fossil organic material, and do not interact with life in the overlying ocean. An exception occurs in the cold-seep environment where metabolic products of deep-living sediment microbes are discharged at the seafloor surface and fuel chemosynthesis (Tunnicliffe et al., 2003). The most dynamic subsurface microbial habitat may be within the crustal rocks at the mid-ocean ridges. The heat-driven circulation of energy-rich fluids within a large volume of porous and permeable rock should be very favorable to microbial growth. The venting of microbial floc that appear in the weeks and months following seafloor eruptions on ridge crests indicates that microbial growth is occurring below the seafloor (Haymon et al., 1993). In addition to the exportation of biomass, subsurface microbial growth at ridge crests may have a significant impact on the

chemical composition of hydrothermal fluid which, in turn, may influence the colonization of vents by free-living microorganisms and invertebrates (Tunnicliffe et al., 1997).

1.1.4 Habitat conditions

For benthic organisms in reducing environments, substratum and supply of reducing substances are intimately linked. Substrata are colonized because they serve as a medium for molecular diffusion or fluid flow or are located in the path of fluid discharge providing access to reducing substances or the products of chemosynthesis. Flow rate has been identified as one of the most important variables influencing the structure of communities around vents and subduction-zone seeps (Olu et al., 1996; Sarrazin et al., 1997). Thus, the availability of appropriate substrata may influence faunal community composition.

In the mid-ocean ridge and back-arc ridge settings of hydrothermalism, hard substratum is the most common benthic habitat, occurring as basaltic rock or sulphide structures. It has been suggested that the presence of hard substrata at vents may limit the presence of some species (Tunnicliffe et al., 1996). The external surfaces of many vent animals, particularly siboglinid tubes, can represent an important substratum for microbial growth and colonization by small metazoans (Tunnicliffe et al., 1997; Sarrazin and Juniper, 1999).

Cold seeps are predominantly soft-bottom environments, but hard substrata exist in the form of carbonate concretions, rocky outcrops, clam shells, siboglinid tubes and even methane hydrates. While there are a considerable variety of hard surfaces at seeps, understanding of their importance to seep-community composition and diversity is very incomplete (Tunnicliffe et al., 2003).

Cold seeps are normally characterised by small temperature anomalies in the bottom waters. Faunal communities at seeps mostly experience ambient seawater temperatures that varies from $< 2^{\circ}\text{C}$ in deep water to 8°C at seeps on the Louisiana Slope (MacDonald et al., 1994).

In hydrothermal vents, temperature varies considerably within and among sites. Organisms inhabiting a single structure can encounter conditions that range from diffuse flow at near ambient temperature ($\approx 2^{\circ}\text{C}$) to vigorously venting smoker fluids ($\approx 350^{\circ}\text{C}$), and major habitat change can occur within species' life spans (Van Dover, 1995). High-temperature sulphide chimneys must be one of the most extreme habitats in the ocean. Although measurements of actual temperatures experienced by animals are very difficult, values of 80°C have been reported for the polychaete *Alvinella pompejana* (Cary et al., 1998). Distinct

macrofaunal assemblages have been associated with the hotter areas of the active sulphide edifices (Sarrazin et al., 1997), and descriptive models of temperature control of species distribution have been proposed. However, temperature and chemical properties of hydrothermal fluids are often highly correlated, rendering it difficult to separate temperature and chemical influences on species distribution (Sarrazin and Juniper, 1999).

Redox conditions in deep-sea reducing habitats range from that of a completely anoxic milieu in which reducing substances accumulate, and which are suitable only for prokaryotic life, to the highly reactive interface of oxic and anoxic environments where aerobic chemosynthesis and dependent animal life flourish. In areas of active flow the fauna lives in the interface between venting and seepage fluid and the overlying ambient water, and is exposed to both pools on time scales of seconds (Johnson et al., 1988). Superimposed on this flickering variability in microhabitat chemistry are significant differences in average longer-term exposures to both pools, which are equally variable and may have a tidal pattern. *In situ* chemical analyzers can describe the chemical habitat of different vent species (Sarrazin and Juniper, 1999; Luther et al., 2001).

Despite the difficulties in generalising about the taxonomic composition of vent and seep communities it is obvious that only those animals that can tolerate the physical and chemical conditions of the vent and seep environment can thrive there, and a variety of physiological and biochemical adaptations are exhibited by the organisms of reducing habitats to this end.

1.1.5 Chemosynthetic primary production

Microbiologists first observed chemosynthesis more than 100 years ago (Winogradsky, 1887, cited in Jannasch (1995)). It is a process that has been microbiologically and biochemically well studied. However, its quantitative role in the carbon cycle of the photosynthetically dominated Earth's surface has never been considered to be significant. The biogeochemical significance of chemosynthesis emerged only upon discovery of deep-sea hydrothermal vent systems, where photosynthetic production of plant organic biomass at the base of the food web is hypothesised to be replaced by chemosynthetic production of microbial organic carbon (Jannasch, 1995).

The presence of hydrogen sulphide in hydrothermal fluids and an abundance of sulphide-oxidizing bacteria were the first clues leading to the hypothesis that faunal communities at hydrothermal vents are sustained by microorganisms that chemosynthesize organic matter

Table 1.2: Microbial metabolic processes in deep-sea reducing habitats

Electron donor	Electron acceptor	Carbon source	Metabolic process
Aerobic conditions			
H ₂	O ₂	CO ₂	H oxidation
HS ⁻ , S ⁰	O ₂	CO ₂	S oxidation
S ₂ O ₃ ²⁻	O ₂	CO ₂	S oxidation
Fe ²⁺	O ₂	CO ₂	Fe oxidation
Mn ²⁺	O ₂	?	Fe oxidation
C-1 compounds	O ₂	CH ₄ , CO ₂ , CO	C-1 oxidation
Org. compounds	O ₂	Org. compounds	Heterotrophic metab.
Anaerobic conditions			
H ₂	CO ₂	CO ₂	Methanogenesis
H ₂	S ⁰ , SO ₄ ²⁻	CO ₂	S ⁰ and SO ₄ ²⁻ reduction
H ₂	NO ³⁻	CO ₂	H oxidation
Org. compounds	NO ³	Org. compounds	Denitrification
CH ₄	SO ₄ ²⁻	?	Methane oxidation
Org. compounds	S ⁰ , SO ₄ ²⁻	Org. compounds	S ⁰ and SO ₄ ²⁻ reduction
Org. compounds	Org. compounds	Org. compounds	Fermentation

from carbon dioxide and mineral nutrients. The microbes catalyse oxidation of hydrogen sulphide and other reducing substances present in vent fluids, and use the chemical energy released to produce adenosine triphosphate (ATP) required for carbon dioxide reduction. Since hydrothermal fluids are formed by reaction of seawater with hot rock, researchers then understood that vent ecosystems are fuelled by geothermal rather than solar energy.

Chemosynthesis is also the primary energy source for microbe-containing faunal communities in cold-seeps although, in this case, the reducing substances are derived from the degradation of sedimentary organic matter and not from the high-temperature reaction of rock with crustal seawater.

Chemosynthetic microbial growth in reducing habitats is coupled to the oxidation of H₂S, CH₄, H₂, Fe²⁺, Mn²⁺ and other substances (Table 1.2). Chemolithoautotrophy is the generation of organic carbon compounds using chemical energy derived from reduction or oxidation of non-organic compounds. In hydrothermal vents aerobic sulphide oxidation is the most important potential energy source for chemolithoautotrophic growth (McCollom and Shock, 1997). Both aerobic and anaerobic methane oxidation are probably more important chemosynthetic processes at cold seeps where methane is initially the most abundant reducing substance in migrating fluids. Sulphide at subduction-zone seeps is produced in near-surface sediments by anaerobic oxidation of methane in migrating fluids, using sulphate as the oxidant. Reduction of sulphate in sea water also occurs as a terminal metabolic

process in anaerobic degradation of organic remains, providing sulphide for development of microbial mats and invertebrates symbioses (Tunnicliffe et al., 2003).

Despite being fuelled by chemosynthesis, reducing environments are closely linked to the photosynthetic ecosystems in the upper layer of the ocean (Figure 1.5). All animals and many microorganisms at vents require dissolved oxygen for their metabolism, which is a by-product of photosynthesis. At seeps, in addition to the requirement of dissolved oxygen for respiration, the methane that powers seep chemosynthesis is derived from photosynthetically-produced organic matter.

A great deal of organic-matter synthesis occurs in symbiotic associations between bacteria and invertebrate hosts, in which the chemosynthetic symbiont convert carbon dioxide into organic matter which nourishes themselves and their hosts. The synthesis of organic-matter by free-living microorganisms appears important in reducing habitats but remains unquantified (Tunnicliffe et al., 2003). Chemosynthesis in biofilms and filamentous mats on mineral and animal surfaces provides food for grazing and deposit-feeding animals.

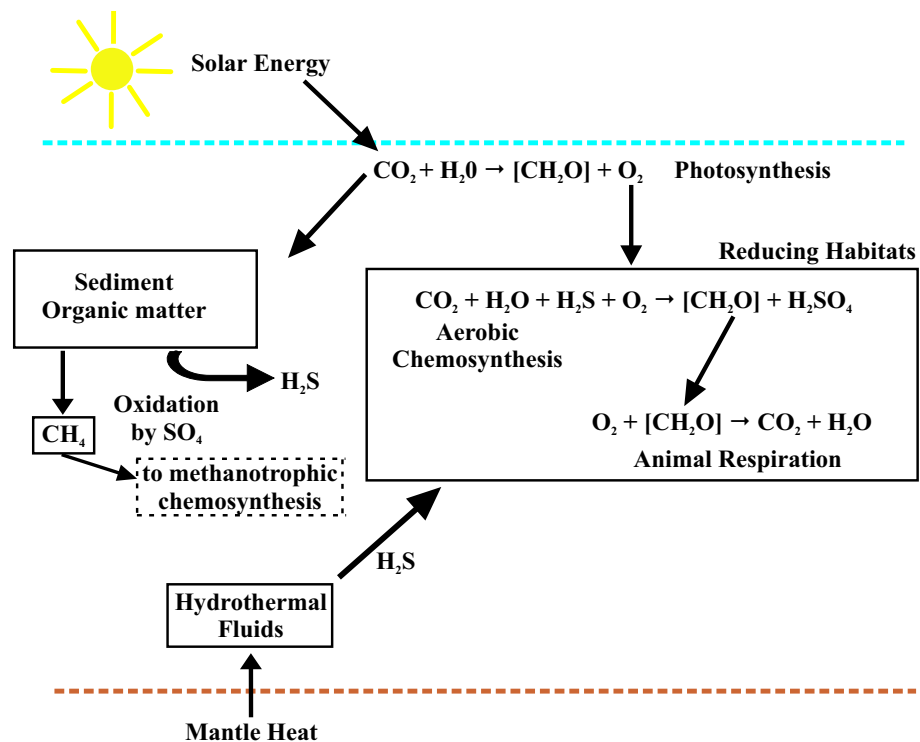


Figure 1.5: Link between chemosynthesis and photosynthesis. Representative energy-consuming (chemosynthesis) and energy-producing (respiration) reactions in reducing habitat metabolism and their relationship to solar and geothermal energy sources. A requirement for dissolved oxygen links aerobic chemosynthesis and animal respiration to photosynthesis in the sunlit surface ocean. Only chemosynthesis based on sulphide oxidation is illustrated. From Tunnicliffe et al. (2003).

1.1.6 The food web

Despite their chemosynthetic base, deep-sea reducing habitats present a trophic structure comparable in many ways to food webs of shallow-water ecosystems. In addition to primary producers, there are a variety of consumer types among invertebrates and fish (i.e., grazers, suspension-feeders, deposit-feeders, predators, parasites, commensals). A generalized scheme of trophic interactions in vent ecosystems is shown in the figure 1.6.

Several compartments for primary production can be recognised including (1) endo- and ectosymbiont production, (2) near bottom and subsurface-derived suspended microbial production, (3) microbial production on inanimate surfaces, (4) plume microbial production, and (5) sinking photosynthetically derived production (Tunnicliffe, 1991).

For most vents and seeps, the major consumer biomass lies in the symbiont host (Tunnicliffe et al., 2003). The role of autotrophic symbionts in the nutrition of their hosts ranges from being the principal source for host species lacking a functional digestive system (e.g., siboglinid tubeworms) to being an additional source to host species with a functional digestive system (e.g., mussels, shrimps).

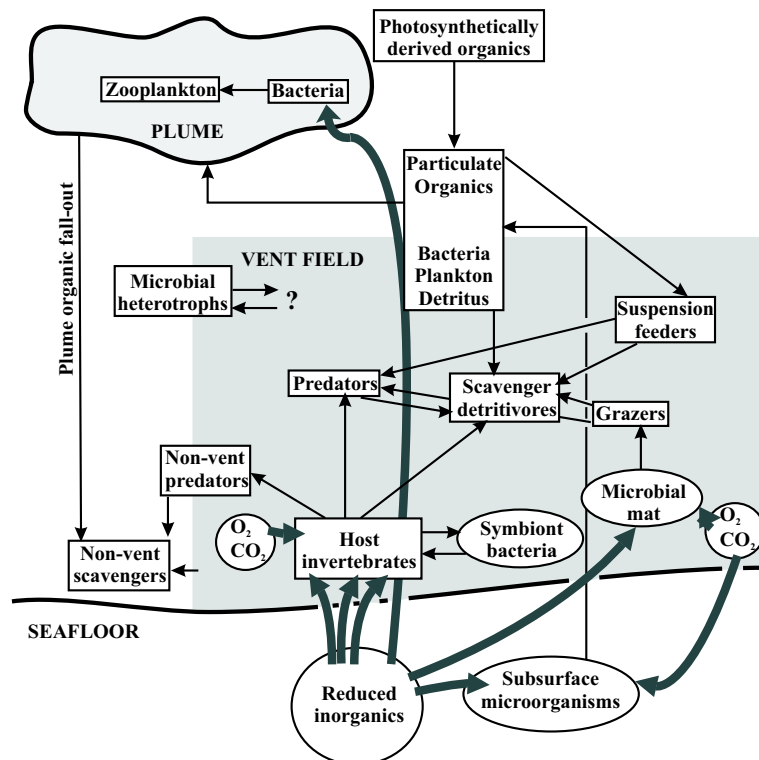


Figure 1.6: Generalized scheme of trophic interactions in vent ecosystems. From Tunnicliffe (1991).

Most of the information available about the trophic structure of deep-sea reducing habitats is based on functional morphology, anecdotal observations of feeding behaviour and analogy to related shallow-water species. Methods that are not always to be trusted, especially in ecosystems where trophic rules may be short-circuited by chemoautotrophic endosymbionts. Biochemical biomarkers such as stable isotope composition can provide temporally integrated signatures of trophic relationships and complement more traditional approaches (Van Dover, 2000; Colaço et al., 2002).

1.1.7 Faunal composition

The physical and chemical conditions of reducing environments select for a small pool of inhabitants compared to the huge diversity in the deep-sea. The adaptations required to partake of the enhanced productivity of these environments have greatly limited the dominant inhabitants to a relatively few groups. While the oasis analogy is used to describe these island of plenty [e.g., Carney (1994)], few taxa have found it beneficial.

Most of the general kinds of animals that populate hydrothermal vents live nowhere else in the deep-sea except in other reducing environments (Hessler and Kaharl, 1995). Of 443 species recorded from vent habitats (Desbruyères and Segonzac, 1997), 82% are apparently endemic (Tunnicliffe et al., 1998). Most significantly, not only the same species, but entire groups of animals are found only at reducing environments. Vent and seep fauna are endemic at high taxonomic levels, including class and order.

This vent assemblage is striking both in that many species are rare or absent elsewhere in the world, and that the most common elements of the deep-sea fauna are not represented (Grassle, 1986). The few species that are also found in the “normal” deep-sea tend to live on the periphery of the vents or occur as occasional visitors.

Several factors affect the composition of a vent community and how and why it changes with time. Geochemical factors as alteration in the flow rates, changes in the concentration of sulphide in the effluent or the type of mineralisation of hydrothermal structures are known to influence the faunal composition. But biotic factors are, perhaps the most important, it is the strength and weakness of interacting species that control much of their fate. During the early stage of the vent community’s life, the species with higher dispersal success exert the strongest influences. For example, in the East Pacific Rise, tubeworms are more successful than mussels at rapid colonisation, but mussels appear to be more successful competitors, and eventually replace the tubeworms (Shank et al., 1998). All these causative forces maybe

operating simultaneously, although with considerable variation (Hessler and Kaharl, 1995).

Vent fauna may have several evolutionary origins (Van Dover et al., 2002). The high degree of endemism, the larger number of taxonomically new higher taxa and ancient taxonomic features suggest that they are survivors of relic lineages that took refuge and radiated in the vent environment. On the other hand, species belonging to families represented elsewhere in the deep-sea suggest more recent invasions. Plate tectonic history can also influence the regional composition of vent fauna (Tunnicliffe et al., 1998).

Because the exploration of cold-seep environments is relatively recent and mostly undertaken for geological purposes, faunal sampling is often not a priority. Thus, there is a lack of knowledge of the composition of the fauna (especially meiofauna and small macrofauna) living in the immediate vicinity of seeps. It appears that at some seeps only symbiont-containing species are present at high-densities, whereas at others the symbiont-containing species are accompanied by non-symbiont-containing species at exceptional densities. This accompanying fauna, composed of species known from more typical deep-sea habitats, is likely to be attracted by the local organic enrichment (Sibuet and Olu, 1998).

Colonization of seeps by species not containing symbionts, endemic or otherwise, contributes to the development of a complex ecosystem. Seeps represent localized perturbations of the vast and well-established soft-bottom benthic environment of the deep-sea. The participation of non-symbiont-containing deep-sea species in seep food webs is an important feature at several sites where extremely high densities of meiofauna, suspension feeders, deposit feeders and carnivorous occur (Carney, 1994).

Compared to hydrothermal vent communities, dominated by a small number of species (seldom more than one species of one genus) and where accompanying species are more scattered, cold seeps show a relatively high species richness. The presumed stability of the seeps habitats might create different selection pressures, providing more opportunity for local diversification and speciation. Currently, there are many more symbiont-containing species known from seeps than from vents (Sibuet and Olu, 1998).

Although few species are shared by cold seep and hydrothermal vent environments, the many similarities among taxonomic groups indicate a strong historical linkage even if there is not an extensive gene flow today.

1.1.8 Life history patterns and dispersal

Study of the reproduction and dispersal of hydrothermal vents and cold seeps species is seriously affected by the remoteness of these habitats and by the fact that sites are visited just for a few hours at time. Additionally, due to weather, funding, and scheduling issues, sites are often visited at the same time each year, or visits are separated by spans of well over a year (Tyler and Young, 1999).

Of the approximately 500 putative species described from hydrothermal vents and cold seeps, very few have been studied primarily for their reproductive biology and the complete life cycle is not known for any of them. From the limited data available at present, it is apparent that the reproductive patterns of vent and seep organism have strong phylogenetic constraints, and that adaptations to vents and seeps are mainly in the nutritional and respiratory physiology of the organism (Tyler and Young, 1999).

In hydrothermal vent organisms, growth is rapid and reproductive maturity is quickly attained, even in small individuals of large species (Shank et al., 1998). These are metabolic and life-history traits favourable to life in transient and insular habitats (Grassle, 1985). Species living at hydrothermal vents are faced with the problem of how to maintain their populations in a habitat that is patchy and ephemeral on time scales as short as decades. When a vent dies, most of the sessile vent fauna dies with it, thus species must be capable of dispersing to a new location before a local vent closes.

Adult migration is clearly not an option for sessile fauna, and the scale of isolation also precludes adult migration for many vent species with limited mobility such as gastropods. However, more motile vagrant species such as crabs and amphipods may achieve it. Therefore larval dispersal seems the most likely means by which vent invertebrates colonise neighbouring and distant vent sites.

Dispersal is a function of the duration of larval life and of the direction and magnitude of prevailing currents (Tyler and Young, 1999; Mullineaux et al., 2002). Given the great distances believed to separate some vent habitats and their nearest neighbours and the large risk to the vent species of dispersing only locally, it would be expected that most vent species should have planktotrophic larvae, whose ability to feed in the plankton allows them to survive for weeks to months and become highly dispersed (Mullineaux and France, 1995). Surprisingly, most of the vent species whose dispersal mode has been investigated have lecithotrophic larvae, previously associated with a more limitative dispersal ability. However, at low deep-sea temperatures, it cannot necessarily be assumed that lecithotrophic

larvae are limited in their dispersal capabilities (Shillito and Manahan, 1994; Marsh et al., 2001).

Currents are expected to play a dominant role in horizontal transport of vent larvae. Near bottom currents are likely to transport larvae from vent to vent along a ridge axis, but if larvae are entrained into the buoyant plume, they may be retained near the source vent, or possibly advected away as a concentrated patch in a different direction and at a different speed than those dispersing directly along the seafloor (Mullineaux and France, 1995).

Most marine invertebrates have a specialized larval settlement stage that responds to environmental signals presumably indicative of a suitable location for survival and successful reproduction. A variety of settlement cues that might be active in simulating metamorphosis in vent and seep invertebrates have been suggested, including temperature, sulphide concentration, bacterial populations, and established adult colonies. To date there has been little experimental tests of these hypotheses.

Dispersal potential of vent organisms has been inferred from the genetic structure of populations with two fundamental models being applied to the genetic data. The “stepping-stone” model applies to organisms with limited dispersal and predicts a high genetic similarity between adjacent populations which is negatively correlated with distance (Wright, 1943). The “island” model applies to species with long-distance dispersal and predicts that all populations contribute to and recruit from a single well-mixed larval pool (Wright, 1931). An additional model of genetic divergence for vent taxa has been proposed: the “ridge-based isolation” model is a hybridisation of island and stepping-stone models (Vrijenhoek, 1997). Vent taxa conform to no single model of gene flow (Van Dover, 2000).

1.2 Vestimentifera (Polychaeta, Siboglinidae)

The varied and complex taxonomic history of Pogonophora and Vestimentifera represents one of the more fascinating tales in animal systematics. The fact that they tend to be found in deep-sea sediments resulted in the first member of this group, *Siboglinum weberi* Caullery, 1914, not being described until early in the 20th century. There are now more than 100 nominal species described (Rouse, 2001), most from abyssal regions, though exceptionally they are found in depth of less than 100 m (Miura et al., 1997).

Siboglinids reached public notice when giant tubeworms, more than a metre long, with bright red tentacular plumes, were photographed at hydrothermal vents along the Galápagos

Ridge in the East Pacific Rise (Corliss and Ballard, 1977). Since then, their systematic position has been the subject of lively discussion between invertebrate zoologists, vent biogeographers and evolutionary biologists.

When first discovered, the giant tubeworm *Riftia pachyptila* (Figure 1.7) was placed in Phylum Pogonophora (Jones, 1981a). Together with two previously known lamellibrachiid species (*Lamellibrachia barhami* Webb and *Lamellibrachia luymesii* van der Land and Nørrevang), made up their own subphylum (Obturata), distinct from the subphylum Perviata, in which all other previously known pogonophorans were placed. Based on morphologic characters, Jones (1985) elevated the Obturata to the phylum level, while the Perviata were retained as the Phylum Pogonophora.



Figure 1.7: *Riftia pachyptila* (image source www.exploretheabyss.com)

Other authors consider that vestimentiferans and pogonophorans might better be classified with closer association to annelids and echiurans. Early development (Young et al., 1996) and juvenile morphological characters (Southward, 1988) have been found to be very similar to those of polychaetes. Amino acids sequences encoded by an elongation factor gene fragment also indicate close phylogenetic relationships between vestimentiferans and the annelid classes Polychaeta and Oligochaeta (Kojima et al., 1993). DNA sequences used to examine evolutionary relationships within vestimentiferans, pogonophorans and a variety of out groups suggests that vestimentiferans are nested within the pogonophorans and they share an evolutionary history with annelids, with the echiurans outside this group (Black et al., 1997).

Rouse and Faulchald (1997) conducted a series of cladistic analysis of polychaetes and showed that Pogonophora (including Vestimentifera) represents a member of a polychaete clade. They argued that since the name Pogonophora was misleading at this level, the name of the group should revert to that of the first family name originally formulated for members of the group, that of Siboglinidae Caullery, 1914. This name change was also proposed by McHugh (1997). For the taxonomy within the Siboglinidae, Rouse (2001) suggested the names Frenulata (=Perviata), Monilifera and Vestimentifera. This suggestion has been endorsed in several recent papers (Rouse, 2001; Schulze, 2003), and is adopted in

this study.

Fourteen species of vestimentiferans, representing 10 genera, have been described. The literature mentions 10 more species, making a total of 24 known species (Table 1.3). Although Vestimentifera inhabit cold seeps on the Atlantic margins, there are no vestimentiferans at the Mid-Atlantic Ridge hydrothermal sites.

Anatomically, there are two “strange” features that characterise adult vestimentiferans, (1) they completely lack a mouth and digestive system and, (2) they have a specialised organ, the trophosome, that houses sulphide-oxidising, chemolithoautotrophic bacteria inside the animal. Much of the anatomy of vestimentiferans can be understood once it is placed in the context of the substrate demands of the symbiotic bacteria on which the gutless worm depends for sustenance. All vestimentiferans are composed of four body regions (Jones, 1988) (Figure 1.8): (1) the obturacular region; (2) the vestimentum; (3) the trunk; and (4) the segmented opisthosoma bearing setae. Vestimentiferans

live their entire postlarval life in a chitinous, cylindrical tube secreted by glands found in the vestimentum and trunk. The tube is sealed basally and attached permanently to the substratum. The only independent movement they show is extension and retraction of the obturacular region (Tunncliffe, 1990).

The primary functions of the obturacular region are to close off the tube when the animal is withdrawn and to act as an exchange organ (Jones, 1988). It consists of a central supporting structure, the obturaculum, and of a series of paired, half-circular branchial lamellae, each of which comprised of fused, and highly vascularised branchial filaments. The result is a gill-like organ with a large surface area for uptake of nutrients and oxygen from the surrounding environment.

Two primary vessels of the vestimentiferans’ closed vascular system circulate blood from the trophosome (in the trunk) through the vestimentum to the plume (via the dorsal blood vessel) and from the plume through the vestimentum back to the trophosome (via the

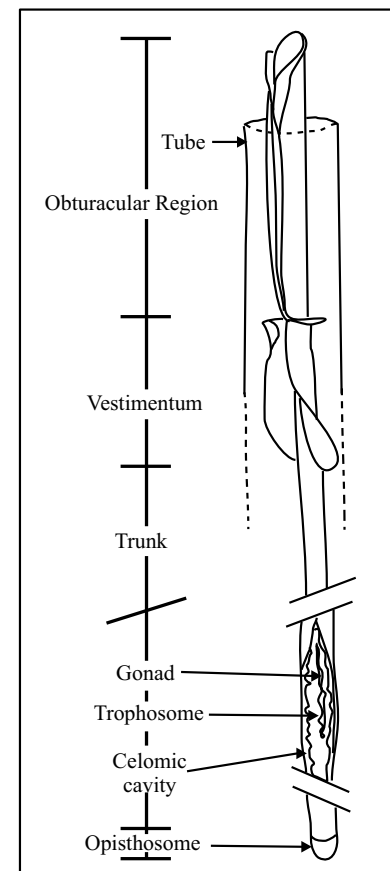


Figure 1.8: Vestimentifera general anatomy. From (Van Dover, 2000)

Table 1.3: Vestimentiferan species described or mentioned in the literature. General area: WP, west Pacific; EP, east Pacific; WA, west Atlantic; EA, east Atlantic. Specific sites: Cle, San Clemente Fault; Edi, Edison Seamount, Papua New Guinea; Fiji, North Fiji Basin; Flo, Florida Escarpment, Gulf of Mexico; Gal, Galápagos Ridge; Gua, Guaymas Basin; Guy, Guyana continental margin; Jav, Java Trench; JdF, Juan de Fuca Ridge; Kag, Kagoshima Bay, Japan; Kan' Kanasuno-se, Japan; Lau, Lau Basin; Lou-l, Louisiana lower continental slope; Lou-u, Louisiana upper continental slope; Man, Manus Basin; Med, Mediterranean off Turkey; Mex, Mid-American Trench off Mexico; Mid, Middle Valley, Juan de Fuca Ridge; Mon, Monterey bay; Nan-T1–3, Nankai Trough, sites of increasing depth; Oki, Okinawa Trough; Ore, Oregon Prism; Sag, Sagami Bay; Uru, Uruguay continental margin; Vig, shipwreck off Vigo, Spain; 9–13°N, 21°N, geographical latitude on East Pacific Rise. Habitat Type: bv, basaltic vent; se, cold seep; sv, sedimented vent; wh, whale bones; wr, shipwreck.

Species	General area	Site(s)	Depth range (m)	Habitat type	Reference(s)
<i>Alaysia sipralis</i>	WP	Lau	1750–1890	bv	Southward (1991)
<i>Alaysia?</i> sp. 1	WP	Sag	800–1450	se	Kojima et al. (2000)
<i>Alaysia?</i> sp. 2	WP	Oki	1400	?	Kojima et al. (2000)
<i>Alaysia?</i> sp. 3	WP	Man	1700–1900	?	Kojima et al. (2000)
<i>Arcovestia ivanovi</i>	WP	Man/Fij	2190	bv	Southward and Galkin (1997); Southward et al. (2002)
<i>Escarpia laminata</i>	WA	Flo/Lou-l	1000–3500	se	Jones (1985); Sibuet and Olu (1998)
<i>Escarpia spicata</i>	EP	Mex/Gua	2000–4000	se/sv/wh	Jones (1985); Sibuet and Olu (1998)
<i>Escarpia</i> sp. 1	WP	Nan-T1	300	se	Kojima et al. (1997)
<i>Escarpia</i> sp. 2	WP	Nan-T2/ Oki/Man	1200–1400	se, sv?	Kojima et al. (1997, 2000)
<i>Lamellibrachia barhami</i>	EP	Mid/Ore/Mon/ Cle	600–2400	se/sv	Webb (1977); Jones (1985); Southward et al. (1996); Sibuet and Olu (1998)
<i>Lamellibrachia columna</i>	WP	Lau	1830–1890	bv	Southward (1991)
<i>Lamellibrachia luymesii</i>	WA	Guy/Lou-u	400–1000	se	van der Land and Nørrevang (1977); Gardiner and Hourdez (2003); Sibuet and Olu (1998)
<i>Lamellibrachia satsuma</i>	WP	Kag/Kan	82–300	se	Kojima et al. (1997); Miura et al. (1997)
<i>Lamellibrachia vitori</i>	WA	Uru	300	se	Mane-Garzón and Montero (1986)
<i>Lamellibrachia</i> sp. 1	WP	Sag/Kan/Nan-T2	300–1300	se	Kojima et al. (1997)
<i>Lamellibrachia</i> sp. 2	WP	Nan-T3	2000	se	Kojima et al. (1997)
<i>Lamellibrachia</i> sp. 3	EA	Vig	1160	wr	Dando et al. (1992)
<i>Lamellibrachia</i> sp. 4	EA	Med	1700–2000	se	Woodside (1997)
<i>Oasisia alvinae</i>	EP	21°N/9–13°N	2500–2600	bv	Jones (1985); Van Dover (2000)
<i>Paraescarpia echinospica</i>	WP	Edi/Jav	1600	se?	Southward et al. (2002)
<i>Ridgeia piscesae</i>	EP	JdF	1570–3250	sv/bv	Jones (1985); Southward and Coates (1989); Tunnicliffe et al. (1998); Van Dover (2000)
<i>Riftia pachyptila</i>	EP	Gua/21°N/9–13°N/Gal	2000–2600	bv	Jones (1981a, 1985); Tunnicliffe et al. (1998); Van Dover (1994, 2000)
<i>Seepiophila jonesi</i>	WA	Lou-u	450–550	se	Gardiner et al. (2001)
<i>Tevnia jerichonana</i>	EP	9–13°N	2500–2600	bv	Jones (1985); Tunnicliffe et al. (1998); Van Dover (2000); Southward (1999)

ventral blood vessel).

The muscular vestimentum assists in holding the animal in the tube and allows it to move up and down. The heart, ciliary field and brain of the worm lie in this region, as do the paired genital pores.

The trunk houses the lobular trophosome and the gonads. The lobes of the trophosome are comprised of peritoneal cells in the outer layer and, internally, they are lined with host cells, or bacteriocytes, each densely packed with bacteria. Several trillion bacteria are found in each gram (wet weight) of trophosome tissue, and the trophosome *in toto* comprises about 15% of the biomass of the organism (Childress et al., 1994). No bacterial cell is more than 10 μm from a blood capillary (Cavanaugh et al., 1981).

Differences in the mechanism of sulphide production at cold seeps compared with hydrothermal vents result in a difference in the availability of sulphide between sites. Because in cold seeps the interstitial fluid is not buoyant and is not flowing rapidly into the water column, it is likely that very little sulphide persists long enough to diffuse up to the level of adult vestimentiferan plumes. Julian et al. (1999) proposed that *Lamellibrachia* sp. [= *Lamellibrachia luymesii* (Gardiner and Hourdez, 2003)] take up sulphide directly from the interstitial water using a long, narrow thin-wall extension of the tube that reaches posteriorly down into the sediment. This extension was named “root” because of the functional and morphological analogy it may have with plant roots. The authors distinguish between the “trunk tube”, which is the portion of the tube anterior to the point of substratum attachment, and the “root tube”, which is the portion of the tube posterior to the point of substratum attachment. Anatomically, this portion of the tubeworm body appears similar to, although much narrower than, the body in the trunk. For example, the body in the root is typically well vascularised and contains both coelomic fluid and trophosome (Julian et al., 1999). A different study has confirmed that *Lamellibrachia luymesii* can take up sulphide across their posterior tube sections at rates sufficient to sustain net inorganic carbon uptake (Freytag et al., 2001). More recently, Cordes et al. (2005) suggested that if sulphate is released by the tubeworm through the root, sulphide generation in the sediment, mainly by hydrocarbon degradation, is sufficient to support moderate-sized aggregations of *Lamellibrachia luymesii* for hundreds of years.

Although species of vestimentiferans vary widely in size, and may be found in widely separated geographic regions, the reproductive biology of this taxon is very conservative (Tyler and Young, 1999). In all species, gametogenesis takes place in gonads that are

surrounded by the lobes of the trophosome. Sexes are separated and eggs are released into the oviduct, in which they pass anteriorly to the gonopores. Although difficult to quantify, fecundity is high in all species and there is no evidence of periodic reproductive output (Tyler and Young, 1999). Spermatogenesis is a complicated process (Gardiner and Jones, 1985; Jones and Gardiner, 1985) with the transference of sperm bundles along the sperm duct.

Although there is evidence of host/symbiont specificity (Southward, 1988; Jones and Gardiner, 1989), symbiotic bacteria have not been found in the eggs of vestimentiferans and it may be that the larva has to acquire new symbionts from the environment at the time of settlement (horizontal transmission).

A lecithotrophic trochophore larvae stage is thought to provide the dispersive phase in the lifecycle of all vestimentiferans (Southward, 1988; Jones and Gardiner, 1989; Southward et al., 1996; Young et al., 1996). Although it is not known whether larvae become planktotrophic during the later part of their planktonic period, Southward (1988) and Jones and Gardiner (1989) showed that the youngest juveniles of *Ridgeia piscesae* have an apparently functional gut. Along the larval development, branchiae develop (later to be resorbed), and a mouth and then the anus are formed.

Jones and Gardiner (1989) proposed that the transmission and establishment of the symbiosis is by uptake of symbionts through the digestive system in early juveniles into endodermal midgut cells, followed by proliferation and transformation of midgut cells into bacteriocytes, and development of the trophosome. A recent study (A. Nussbaumer and M. Bright, personal communication) points to a different pathway: bacteria infect the skin and trigger apoptosis, which facilitates migration into deeper layers until visceral mesoderm is reached. The symbionts are then enclosed in vacuoles and the mesoderm proliferates to form the trophosome.

In many hydrothermal vents and cold seep ecosystems, vestimentiferan tubeworms are among the dominant macrofauna and, due to their obligate endosymbiotic relationship with sulphide-oxidizing chemoautotrophic bacteria, are limited to habitats where hydrogen sulphide is present (Cavanaugh et al., 1981). Across the different systems in which they occur, vestimentiferans occupy a broad environmental gradient from areas where sulphide-rich fluids emanate as relatively vigorous and continuous diffuse vent flow to areas where fluid seep slowly from the seafloor. At one extreme, the hydrothermal vent vestimentiferan *Riftia pachyptila* (from the East Pacific Rise) lives in relatively strong and continuous diffuse

hydrothermal flow where sulphide availability is high and dies when vent flow subsides or sulphide levels decrease due to biotic or abiotic factors (Shank et al., 1998). At the other extreme, *Lamellibrachia luymesii* (from hydrocarbon seeps in the Gulf of Mexico) grows slowly and lives for centuries in environments where exposure to seep fluid is low (Julian et al., 1999; Bergquist et al., 2000).

In both hydrothermal vents and cold seeps, vestimentiferans often form dense, high-biomass aggregations that represent an important component in the nutrient cycles of the vent or seep community. The entangled tubes of vestimentiferan aggregations also create a dense secondary structure that may provide a physical habitat for a wide range of additional species associated with these environments (Southward et al., 1995; Sarrazin and Juniper, 1999; Bergquist et al., 2003b; Tsurumi and Tunnicliffe, 2003).

Chapter 2

Sampling sites and studied species

2.1 Sampling Sites

Samples of the studied species of siboglinid tubeworms were collected from hydrothermal vents in the East Pacific Rise and Juan de Fuca Ridge (Northeast Pacific), and from cold seeps in the Gulf of Mexico.

2.1.1 Endeavour Segment, Juan de Fuca Ridge, Northeast Pacific

The Northeast Pacific has a set of small ridges that is the remnant of a much larger system destroyed in the south-westward migration of the north American Plate. At one time (56Ma and earlier), the East Pacific Rise and the Northeast Pacific Ridge were one continuous submarine ridge system, interrupted only by occasional transform offsets. Around 37 Ma, the North American plate began to interact with the ridge system. Gradually, the continent overrode the ridge, separating the northern triple ridge system from the East Pacific Rise until the present situation was reached, where the East Pacific Rise runs up the Gulf of California to emerge in its terrestrial form as the strike slip San Andreas fault. The San Andreas fault runs through California and resubmerges off Oregon as the Mendocino transform fault running between the East Pacific Rise and the Northeast Pacific Ridge system.

The major components of the Northeast Pacific system are three ridges (Gorda, Juan de Fuca and Explorer), three plates, four transform faults (Mendocino, Blanco, Sovanco and Queen Charlotte) and a series of seamounts (Figure 2.1a).

The Juan de Fuca Ridge is a median rate spreading zone (about 6 cm.yr^{-1}) (Tunnicliffe,

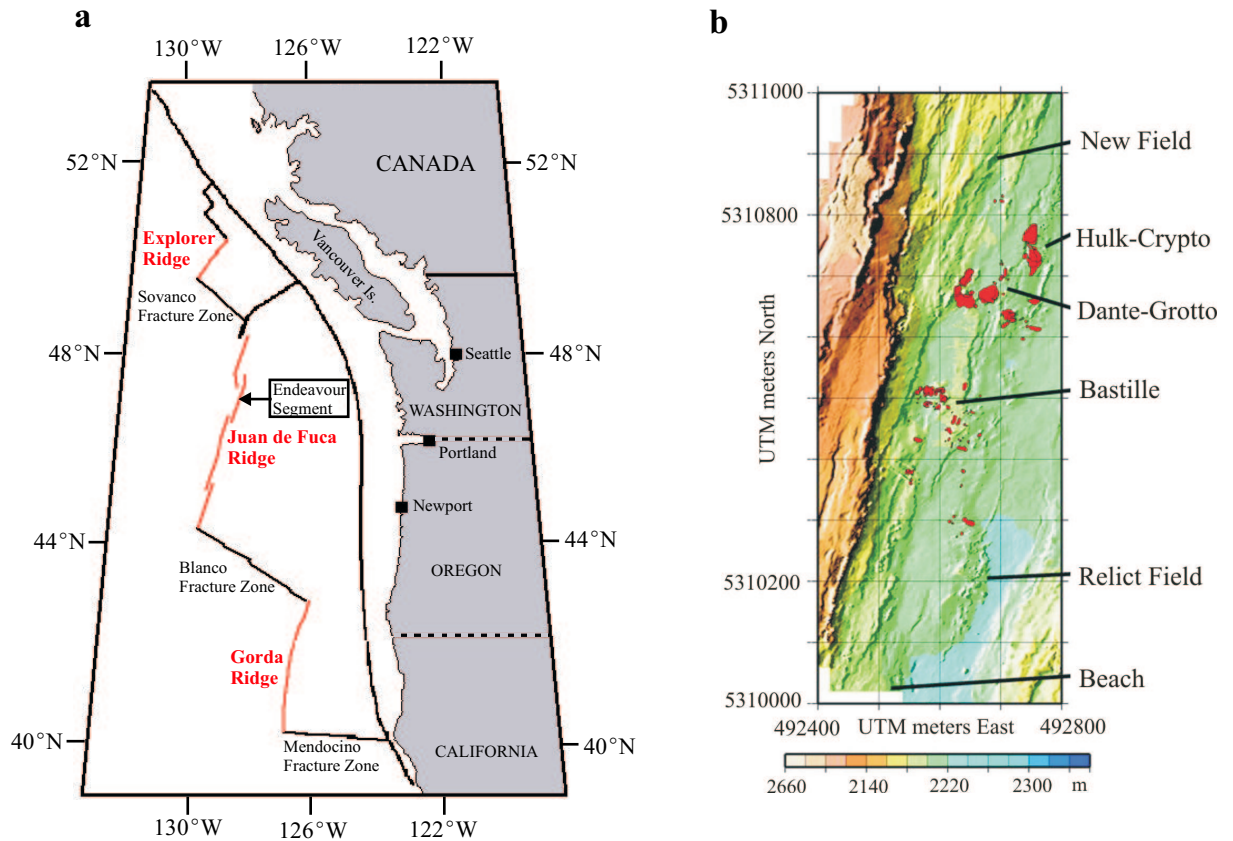


Figure 2.1: (a) Location map of spreading ridges (red) in the NE Pacific. (b) Seafloor bathymetry of Main Endeavour Field. Vent chimney locations are only approximate; errors in location are 10 m and have not been adjusted to more detailed bathymetric map. From Johnson et al. (2002).

1991) that holds a special place in the history of modern geology. It was there that the timescale of ocean-floor magnetic reversals was conceived in 1964 (Cox et al., 1964), and one year later a key paper was published both naming Juan de Fuca Ridge and providing the fundamental model for modern plate tectonic theory (Vine and Wilson, 1965). It is the longest ridge in this system, being comprised of six spreading segments. It is bounded by the Blanco Fracture Zone to the south and by the Sovanco Fracture Zone to the north. Near the northern end of Juan de Fuca Ridge lies Endeavour Segment. The entire segment is approximately 90 km in length and its relief ranges from depth of 2050 m at the crest of the axial valley to 2700 m in the deepest portions at the northern and southern extremities. A narrow axial valley with numerous fissures and scarps supports hydrothermal activity along the western wall of the graben (Karsten et al., 1986). Sulphide mounds are often large and flanges with pooled hot water beneath are common.

The Endeavour Segment has been site of numerous multidisciplinary investigations, and several hydrothermal areas have been described in the region, including the Main Endeav-

our Field.

The Main Endeavour Field includes more than 15 large ($> 1000 \text{ m}^3$), vigorously venting, sulphide structures localised along faults and fissures associated with the actively deforming valley wall (Delaney et al., 1992).

The sulphide structures in the Main Endeavour Field are step-sided, measuring up to 30 m across their base, and several structures rise to heights of 20 m or more. All large, active sulphide structures support growth of multiple, heavily colonised flanges.

Diffuse upward flow through the flanges from the buoyant reservoir supports a dense microbial population within the porous interior. In concert with nutrient flux associated with underflow at the outer edges, diffuse flow also supports a distinctive macrobiological community on the upper surfaces of overhanging flanges. This community consists of small vestimentiferan worms, alvinellid polychaetes and gastropods (Delaney et al., 1992).

Between and above the flanges, the upper slopes of the large, actively venting structures are draped with dense colonies of vestimentiferan tubeworms. Venting temperatures associated with diffuse flow through these areas of larger tubeworms are commonly in the range 8 to 15°C . The uppermost part of each active sulphide structure terminates in multiple chimneys or dead spires. The majority of these chimneys are active black smokers, but some are recently extinct smokers in varying stages of disintegration. The dead spires are neither colonised nor hydrothermally active (Delaney et al., 1992).

The Main Endeavour Field can be divided in two main areas – the Bastille complex to the south and the Dante-Grotto complex to the north (Figure 2.1b). Actively venting structures in the northern portion are higher and more massive than in the southern portion. Maximum venting temperatures of 375°C are associated with the smaller structures in the Bastille complex whereas the highest temperatures found in the more massive structures in the Dante-Grotto complex are consistently 20 to 30°C .

In July 1994 a biological observatory was established in the Main Endeavour Field with an initial emphasis on vent sites located at the Smoke and Mirrors chimney complex and at the Easter Island area, both in the Bastille complex (Juniper et al., 1994). The Smoke and Mirrors site comprises an active sulphide edifice and adjacent diffuse venting in an area approximately 9 m long and 4 m wide. The Easter Island vent site, located along the western margin of the Main Endeavour Field, is hosted by a small, low-walled graben, which appears to be a relic sulphide chimney field. The north end of the graben floor is occupied by a vestimentiferan colony localised along fractures in the underlying pillow

(Juniper et al., 1994).

During reconnaissance dives along the axial valley in 1988, hydrothermal activity was reported 1.3 km north of the Main Endeavour Field (Robigou et al., 1993). The site known as Clam Bed is a small (tens of m²) hydrothermal area with one white smoker.

2.1.2 9°50'N, East Pacific Rise

The ridges that comprise and connect to the East Pacific Rise have been the focus of considerable geophysical research since the 1970's. The variable nature of rates and types of spreading provided localities to test theories of spreading mechanisms. The discovery of the phenomenon of hydrothermal venting was a direct result of this research.

The area between 9° and 10° north latitude on the East Pacific Rise (often simply referred to as “9 North”) has been a focus of mid-ocean ridge studies for almost 20 years. The studies of the hydrothermal systems at this site, and their associated biological communities, began in earnest in 1991.

In this area black-smoker chimneys are scattered in linear fashion along the walls of the narrow axial valley (the eruptive fissure) (Figure 2.2). This kind of narrow, linearly arranged hydrothermal field is characteristic of the East Pacific Rise in general. Haymon et al. (1991) chose this site for a detailed survey of the distribution of hydrothermal, volcanic, and tectonic features, using an unmanned imaging system. When in March–April 1991 Haymon and her colleagues returned to the region with the submersible *Alvin* for detailed interdisciplinary characterization of the mapped ridge axis, a portion of the ridge was covered with fresh basalt that

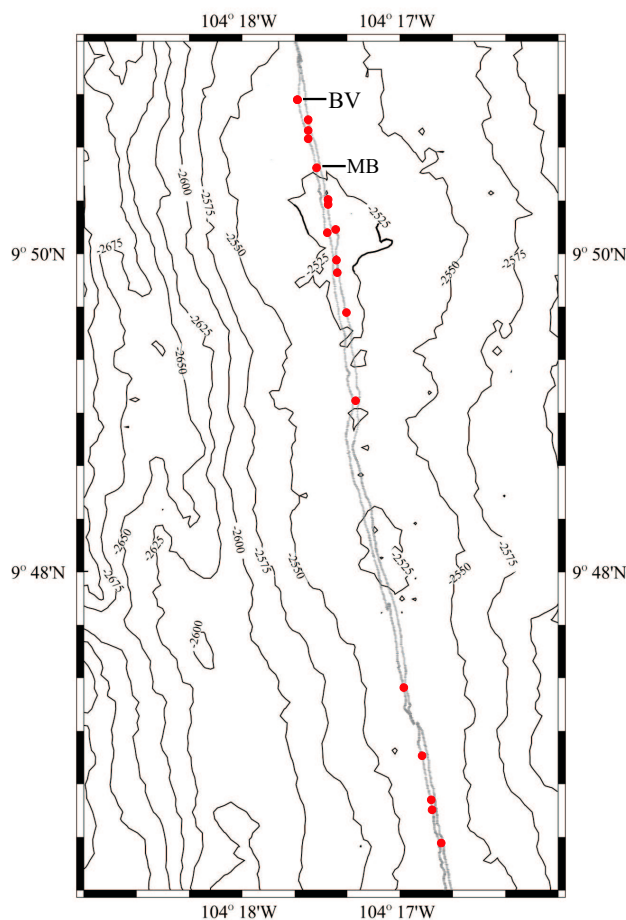


Figure 2.2: Distribution of hydrothermal vents at the 9°N, EPR. After Von Damm (2000). MB: Mussel Bed site; BV: Biovent site.

had wiped out vent communities, and new venting was pervasive. This is often referred to as “time zero” and has provided an unparalleled opportunity to understand the time scales over which changes can occur on the seafloor (Von Damm, 2000). The rapidity of change observed here has truly revolutionized our ideas on processes in the deep sea.

Nascent hydrothermal vents at 9°N were rapidly occupied, beginning with a “bloom” of primarily microbial production coincident with the eruption (Haymon et al., 1993). One year after the eruption, “aggregations” (up to 200 individuals) of *Tevnia jerichonana* had already colonised low-temperature vents, as had dense populations of several species of limpets. Within 32 months of the eruption, several sites were in waning stages of activity or had terminated. Where venting persisted, extensive colonies of mature *Riftia pachyptila* were established (Lutz et al., 1994; Shank et al., 1998). A variety of invertebrates that lived in and among siboglinids tubes were noted.

The first vent mussels were observed nearly after 4 years of the eruption. Both species of tubeworms continued to recruit to persistent vent sites, with largest expansion of communities taking place at specific sites where highest sulphide concentrations were measured (Shank et al., 1998). Within 5 years of the eruption mussels surrounded nearly every tubeworm colony.

2.1.3 Gulf of Mexico

The Gulf of Mexico is a marginal basin of the Atlantic Ocean with a maximum depth of approximately 3600 m. The deep fauna of the gulf is closely related to that of the Atlantic, but some degree of uniqueness is to be expected due to geographic isolation by sills at approximately 1650m in the straights of Yucatan and 800 m in the straights of Florida (Tyler, 2003).

The precursor to today’s Gulf of Mexico first appeared as part of the young Atlantic when North America and Europe began rifting apart. Subsequent southward migration of Gondwana Land resulted in a large connection between the forming Atlantic and the older Pacific to the west. This opening began to form about 175 million years ago and continued to grow until about 100 million years ago.

The ocean between North and South America was partially cut off from the Atlantic and completely cut off from the Pacific by two massive shifts of land. First, Cuba and Hispanola moved in from the Pacific and formed the northern border of the Caribbean. Other continental fragments moved in later but stopped farther west, eventually creating

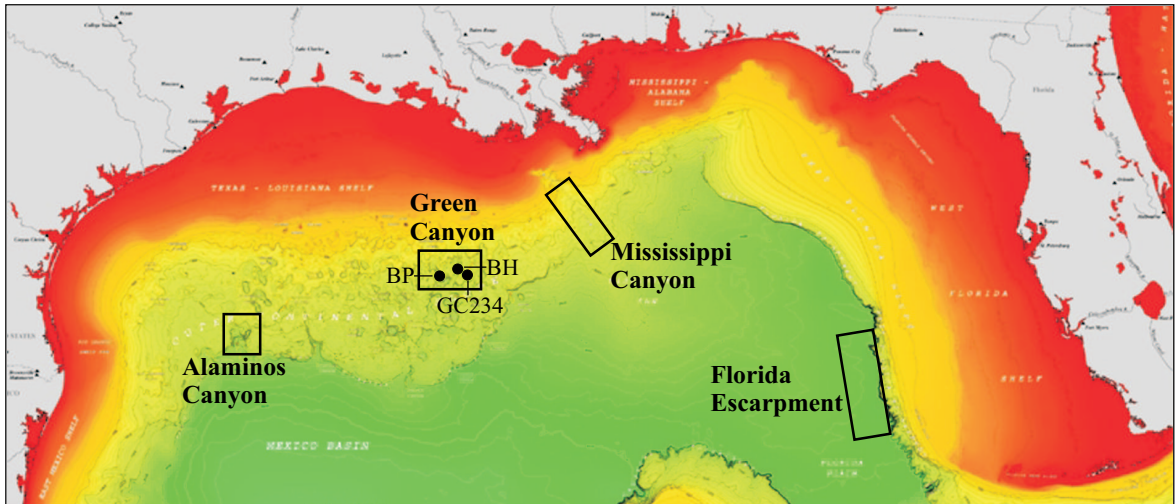


Figure 2.3: Location map of chemosynthetic communities in the Gulf of Mexico and Green Canyon sampling sites. BH: Bush Hill; BP: Brine Pool (image source www.photolib.noaa.gov).

Central America. These closures created the Gulf of Mexico and the adjacent Caribbean marginal basins of the Atlantic, altered gene flow between the oceans, and established the major current patterns that are still prevalent today.

Gulf of Mexico chemosynthetic communities have been documented in three general regions: the upper continental slope (400 – 1000 m off Louisiana), the Alaminos Canyon site on the lower continental slope (2200 m off Louisiana), and the West Florida Escarpment site (3500 m) (Figure 2.3).

Methane and sulphide advection on the continental slope are primarily related to methane discharge from thermogenic petroleum reservoirs perforated by salt domes with secondary venting of biogenic compounds in sediment pore water (Brooks et al., 1987). Venting at the base of the West Florida Escarpment has been attributed to brine discharge from the Florida carbonate platform (Paull et al., 1984).

For this study, samples of *Lamellibrachia luymesii* and *Seepiophila jonesi* were collected from 3 sites in the Green Canyon area in the upper continental slope: Bush Hill (BH), Brine Pool (BP) and lease block GC234 (Figure 2.3).

The oil and gas seep known as Bush Hill (27°46.96'N, 91°30.46'W) is approximately 500 m wide and 40 m high. The oil escaping from Bush Hill forms a slick on the sea surface that is visible from space (MacDonald et al., 1994). It lies over a salt diapir that rises about 40 m above the surrounding sea floor to a minimum water depth of 540 m. The sediment at this site consists of silty-clay and is of considerable thickness (Brooks et al.,

1989). Carbonate outcroppings can be seen, ranging from rubble to prominent boulders. The larger boulders are topped by gorgonians, which, in turn frequently support large euryalid ophiuroids. The mussel *Bathymodiolus childressi* forms discrete beds on both soft sediments and among carbonate outcropping. Both *Lamellibrachia* and *Seepiophila* can be found in this site.

The site called Brine Pool (27°43.44'N, 91°16.75'W) consists of a pool of brine (salinity 121.35 ppm) with an area of approximately 190 m² and 3.5 m deep at a water depth of 650 m (MacDonald et al., 1990). The brine, that fills the pool by welling from below, contains saturating concentrations of microbial methane. It is ringed by a large (540 m²) bed of *Bathymodiolus childressi*. Vestimentiferans can be found in much smaller clusters in the outskirts of the site.

The geology of Green Canyon leasing block 234 (27°44.7'N, 91°13.3'W) has been described by Behrens (1988), who perceived a network of diapir crests that converge to the west of the site as an east-west trending diapir ridge. Substantial quantities of oil are dispersed on the sediment, and stream of gas bubbles are commonly seen escaping from the substratum. The surface topography is uneven with sometimes massive deposits of authigenic carbonate either exposed or covered by a thin layer (less than 10 cm) of soft sediments (Brooks et al., 1989). GC234 lie at a depth of approximately 540 m and is currently known to contain two large communities of chemosynthetic organisms: one that is dominated by mussels, located on the western side of GC234, and other dominated by vestimentiferans on the northeastern side of the lease block. Hydroids, sponges and the bivalve *Acesta bullisi* can be found encrusted in the tubes of vestimentiferans. On the western end of the tubeworm area there is an extensive field of gorgonians.

2.2 Studied species

Specimens of *Riftia pachyptila*, *Ridgeia piscesae* and *Tevnia jerichonana* were collected from hydrothermal vent sites in the Pacific, and specimens of *Lamellibrachia luymesi* and *Seepiophila jonesi* were collected from hydrocarbon seeps in the Gulf of Mexico.

2.2.1 *Ridgeia piscesae* Jones, 1985

Ridgeia: From the English, *ridge* + *-ia*, ending, in reference to the Juan de Fuca Ridge (Jones, 1985).

piscesae: To honour the Canadian submersible *Pisces IV*, her pilots and other crew members + feminine genitive suffix (Jones, 1985).

Ridgeia piscesae shows a great ability to survive and thrive under a wide range of environmental conditions that allows it to exploit many of the different habitats available at the Northeast Pacific vents (Urcuyo et al., 1998, 2003). It has been found at all known hydrothermal vent fields of the Northeast Pacific ridges, including more than 50 vents on the six segments of the Juan de Fuca Ridge (Southward et al., 1996).

Several growth forms, or morphotypes, of this species are found on the Endeavour Segment of the Juan the Fuca Ridge (Southward et al., 1995) and although the different morphotypes were originally thought to represent as many as five different species (Jones, 1985), subsequent molecular work and a morphological reassessment have determined that they all belong to a single species (Southward et al., 1995, 1996; Black et al., 1997). The occurrence of the different morphotypes appears to be correlated with specific microhabitats, defined primarily by vent-fluid conditions (Tunnicliffe and Juniper, 1990; Sarrazin et al., 1997). The “long-skinny” morphotype is normally found associated with sources of diffuse hydrothermal fluids emanating from cracks in the basaltic substratum (Urcuyo et al., 1998). This morphotype can reach over a metre in length, is widespread on the Juan de Fuca Ridge and occurs in very large numbers at many sites (Urcuyo et al., 1998). A short and thicker growth form, the “short-fat” morphotype, grows on high temperature chimneys (Figure 2.4c).

For this study specimens of both morphotypes were collected in the Bastille edifice (Figure 2.1b), in the Easter Island site and in the Clam Bed site (Endeavour Segment, Juan de Fuca Ridge) using the submersible *Alvin* (Table 2.1).

2.2.2 *Riftia pachyptila* Jones, 1981

Riftia: From Danish and Norwegian, *rift* (rent, fissure) + *-ia*, in reference to the Galápagos Rift (Jones, 1981b).

pachyptila: From Greek, *pachys* (thick) + Greek, *ptillon* (feather), in reference to the aspect of the anterior plume of the worm (Jones, 1981b).

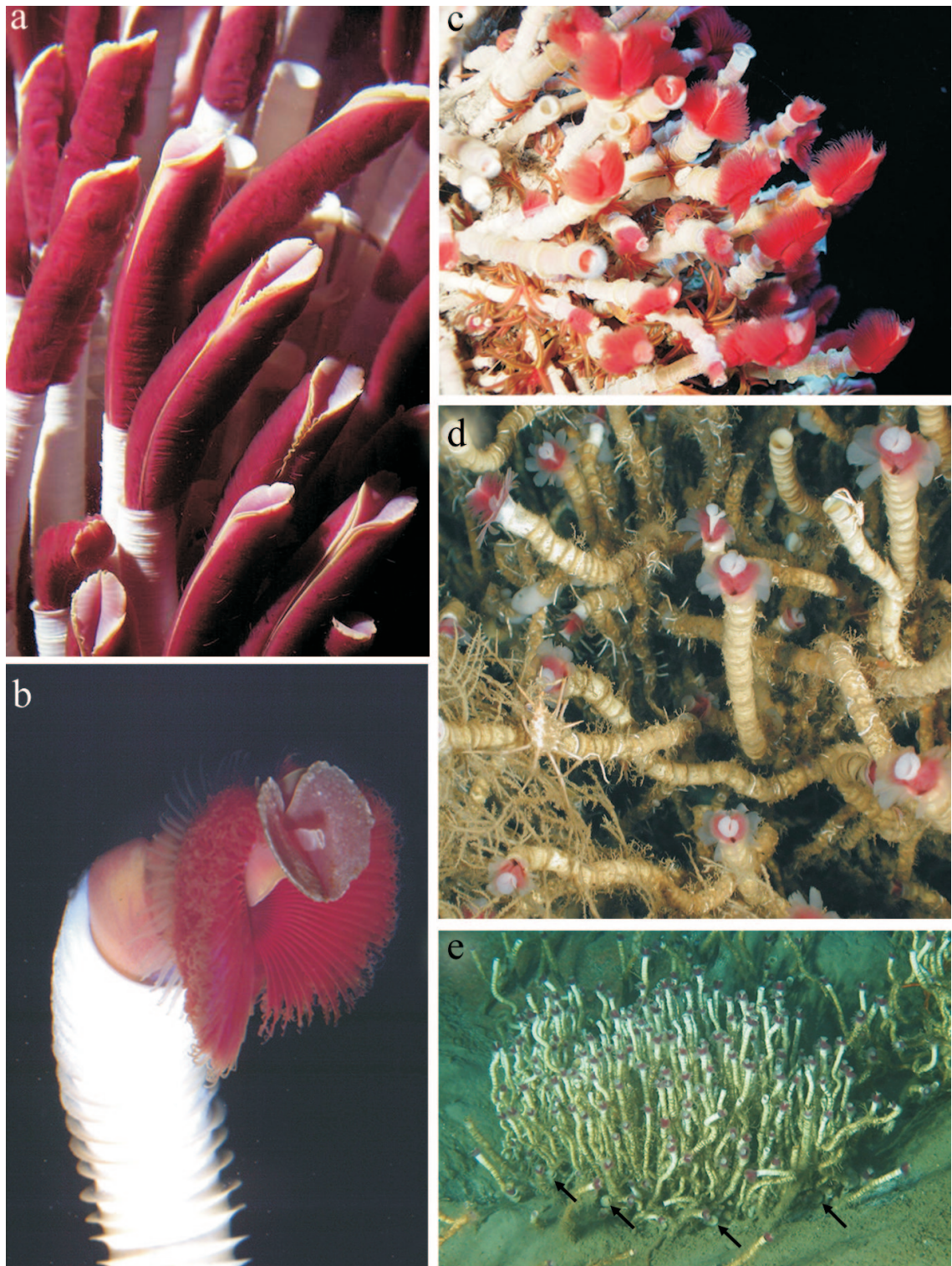


Figure 2.4: (a) *Riftia pachyptila* from the East Pacific Rise; (b) *Tevnia jerichonana* from the East Pacific Rise (image source www.exploretheabyss.com); (c) *Ridgeia piscesae* from Juan de Fuca Ridge (image courtesy Chuck Fisher); (d) *Lamellibrachia luymeri* from the Gulf of Mexico (image courtesy Ian MacDonald); (e) Small bush of *Lamellibrachia luymeri* and *Seepiophila jonesi* in the Gulf of Mexico. Arrows indicate specimens of *Seepiophila jonesi*.

Although *Lamellibrachia barhami* Webb was the first to be described, much of what is currently known about vestimentiferan biology comes from studies on *Riftia pachyptila*. *Riftia pachyptila* is the best known and most distinctive of animals living around deep-sea hydrothermal vents. It is remarkable for its size, adults can reach 1.5 m in length and 40 mm in diameter (Jones, 1988) (Figure 2.4a). It has also been reported to be one of the fastest growing invertebrates known ($\approx 85 \text{ cm.yr}^{-1}$), and reaches sexual maturity in just one to two years (Lutz et al., 1994; Shank et al., 1998).

Riftia pachyptila dominates the biomass of many hydrothermal vent sites in the Gulf of California (Guyamas Basin) and on the East Pacific Rise and Galápagos Rift (Jones, 1988). The worms typically occur in large clusters in areas of relatively strong and continuous diffuse hydrothermal flow, and high sulphide availability (Fisher et al., 1988). It has been suggested that, by producing an additional connection via tube growth at its posterior end and dissolving its previous attachment to the substratum, *Riftia pachyptila* may be able to modify its relative position continually to maintain access to vent fluids (Gaill et al., 1997).

For this study samples of *Riftia pachyptila* were collected, using the submersible *Alvin*, from the East Pacific Rise (9°N), at the Biovent and Mussel Bed vent sites (Figure 2.2) in December 1999 and May 2000 respectively (Table 2.1).

2.2.3 *Tevnia jerichonana* Jones, 1985

Tevnia: An anagram of *vent* + *-ia*, ending, in reference to hydrothermal vents (Jones, 1985).

jerichonana: From the Latin genitive stem *jerichon* + *-ana*, an adjectival suffix, in reference to the town of Jericho and, by extension, to Joshua's horn, an allusion to the shape of the tube of this vestimentiferan (Jones, 1985).

The small vestimentiferan *Tevnia jerichonana* (Figure 2.4b) is the initial visibly dominant sessile metazoan in vigorous diffuse flow regions at newly opened vents along the East Pacific Rise (Lutz et al., 1994; Shank et al., 1998). This species is then replaced by *Riftia pachyptila*, frequently over a period of less than one year. Shank et al. (1998) suggested that the temporal change in species composition from *Tevnia jerichonana* to *Riftia pachyptila* results from changes in hydrothermal fluid flux. Other authors hypothesized that *Tevnia jerichonana* facilitates settlement of other vestimentiferan species by providing a chemical cue (Mullineaux et al., 2000; Hunt et al., 2004).

Tevnia jerichonana and *Riftia pachyptila* co-occur along the northern and southern East Pacific Rise (Tunnicliffe et al., 1998), but *Tevnia jerichonana* has not been reported from Guaymas Basin or the Galápagos Rift. It is not clear whether its absence in these locations is due to a restricted biogeographic range, incomplete sampling, or the advanced successional stage of those communities (Mullineaux et al., 2000).

Specimens of *Tevnia jerichonana* were collected from the Mussel Bed site in May 2000 (Table 2.1).

2.2.4 *Lamellibrachia luymesii* van der Land and Nørrevang, 1977

Lamellibrachia: In reference to the tentacular crown in which the tentacles are fused to form concentric horseshoe-shaped lamellae (Webb, 1969).

luymesii: To honour the Dutch hydrographic vessel *Luymes* (van der Land and Nørrevang, 1977).

Lamellibrachia luymesii, the most abundant species in the Gulf of Mexico cold seep vestimentiferan communities, grows quite slowly to lengths exceeding two meters and lives in excess 170 to 250 years (Fisher et al., 1997; Bergquist et al., 2002, 2003b; Gardiner and Hourdez, 2003).

As discussed in Chapter 1, little active mixing of seep fluids and bottom water occurs above the water-sediment interface around seep vestimentiferans. As a result, the plumes of adult *Lamellibrachia luymesii* (which may be positioned more than 2 m above the seafloor) do not have access to significant amounts of hydrogen sulphide (MacDonald et al., 1989; Julian et al., 1999; Freytag et al., 2001). Rather, current data indicate that *Lamellibrachia luymesii* can obtain oxygen across its plume and sulphide across buried posterior extensions of its tube (Julian et al., 1999; Freytag et al., 2001).

At hydrocarbon seep sites on the upper Louisiana slope *Lamellibrachia luymesii* and *Seepiophila jonesi* (Figure 2.4d and e) co-occur in aggregations that commonly reach several metres in diameter (MacDonald et al., 1989). As the individuals within an aggregation grow, they extend their tubes upward and outward from the central point of attachment to the substratum, giving the aggregation a distinctive domed or bush-like profile. Aggregations begin with larval vestimentiferans settling in areas of active seepage where the precipitation of carbonates forms the hard substratum they need for recruitment (Behrens, 1988; Fisher et al., 1997). For the first few decades, the level of seepage continues and recruitment

to this aggregation persists as long as sulphide is released from the seafloor (Bergquist et al., 2003a). Over time, the tubeworms continue to grow, simultaneously increasing the standing crop of primary production, habitat patch size and subsurface sulphide demand (Bergquist et al., 2003b). During this period, which may last for hundreds of years, primary production associated with the aggregation is sufficient to maintain a moderately high biomass of associated fauna, and yet the overall toxicity of much of the habitat is benign enough that a wide variety of non-endemic fauna can colonize or forage amongst the aggregations as well (Bergquist et al., 2003b).

2.2.5 *Seepiophila jonesi* Gardiner et al., 2001

Seepiophila: From English *seep* + Greek *philia* (= affection, fondness), in reference to the strong preference of these worms to inhabit hydrocarbon seep communities in the deep sea (Gardiner et al., 2001).

jonesi: The species is named in honour of the late Meredith L. Jones whose studies of vestimentiferan anatomy, morphology and development contributed greatly to our understanding of this enigmatic group of marine worms (Gardiner et al., 2001).

Seepiophila jonesi grows slower than *Lamellibrachia luymesii* when small (<50 cm in length) and shows little evidence of growth when large (Fisher et al., 1997). Typically, it does not exceed 1 m in length and grows so that its plume is much closer to the sediment level than *Lamellibrachia luymesii*. Aggregations consisting entirely of very large *Seepiophila jonesi* have been observed and may be an indication that *Seepiophila jonesi* can live longer than *Lamellibrachia luymesii* (Bergquist et al., 2002). The estimated mean ages and ages range of the two species are very similar within aggregations, which indicates that both species recruit roughly simultaneously to a substratum (Bergquist et al., 2002).

The aperture of the tube of *Seepiophila jonesi* is surrounded by a broadly flaring funnel, which makes the two species easily distinguishable.

Specimens of *Seepiophila jonesi* and *Lamellibrachia luymesii* were collected from Bush Hill, Brine Pool and GC234 (Figure 2.3) sites using the submersible *Johnson Sea Link II* (Table 2.1).

Table 2.1: Site and date of collection, and type of analysis done with each species in this study.

Species	Sampling site	Date	Analysis
<i>Ridgeia piscesae</i>	Easter Island	08/98	Reproductive condition
	Clam Bed	08/98	Reproductive condition
	Bastille	09/99	Reproductive anatomy; Reproductive condition
<i>Riftia pachyptila</i>	Biovent	12/99	Reproductive anatomy; Reproductive condition
	Mussel Bed	05/00	Reproductive anatomy; Reproductive condition
<i>Tevnia jerichonana</i>	Mussel Bed	05/00	Reproductive anatomy
<i>Lamellibrachia luymeri</i>	Bush Hill	10/02	Reproductive anatomy
	Brine Pool	10/02	Reproductive condition
		02/03	Reproductive condition
	GC 234	02/03 11/03 07/04	Reproductive condition Reproductive anatomy; Reproductive condition Lipid Composition
<i>Seepiophila jonesi</i>	Bush Hill	10/02	Reproductive anatomy
	Brine Pool	02/03 07/04	Reproductive condition Lipid composition

Chapter 3

The female reproductive system and dispersive embryogenesis

3.1 Introduction

Species of vestimentiferans are known to be the first colonisers of newly-formed vents (Hessler et al., 1988; Shank et al., 1998), some of the fastest growing marine invertebrates (Lutz et al., 1994) and one of the longest lived (Fisher et al., 1997) (see Section 1.3). Because of their ecological importance at vents and seeps there has been considerable interest in their reproductive and dispersal biology.

In vestimentiferans, small, yolky and slightly buoyant eggs develop into non-feeding trochophore larvae that are thought to disperse in the plankton for up to several weeks (Young et al., 1996; Marsh et al., 2001), facilitating genetic exchange and colonization of newly available seep and vent habitats. It has been estimated that *Riftia pachyptila* can disperse more than 100 km over a 5-week period (Marsh et al., 2001). This estimate is based on the assumption that passive dispersal occurs during a 3-week embryonic period as well as a 2-week period when larvae are capable of independent ciliary movement. However, because the site of fertilization and the location of embryogenesis remain unresolved for all species of vestimentiferans, these dispersal estimates could be as much as 60% too high. Thus, the site of fertilization in vestimentiferans is of great interest and has been the subject of considerable debate.

Free sperm have been observed in the oviduct of *Riftia pachyptila* (Gardiner and Jones, 1985), a free spawning species (Van Dover, 1994). Moreover, transfer of large white sperma-

tozeugmata (sperm masses) from male to female plumes has been documented in *Ridgeia piscesae* (Southward and Coates, 1989; MacDonald et al., 2002) and *Tevnia jerichonana* (Southward, 1999). On the basis of these observations, Southward (1999) has proposed that eggs are fertilized either in the ovisac just before spawning, or externally as the eggs are spawned. Internal fertilization is consistent with the unusual sperm morphology (sperm are elongate and have spiral mitochondria and nuclei) in all known species (Gardiner and Jones, 1985; Cary et al., 1989b), and also with the presence of external ciliary tracts leading away from the male gonopores on the dorsal surface of the vestimentum (Gardiner and Jones, 1993). However, massive spermatzeugmata are known only in *Ridgeia piscesae* (Southward and Coates, 1989; MacDonald et al., 2002) and *Tevnia jerichonana* (Southward, 1999); in other species, sperm appear to be released in smaller flame-shaped bundles that are capable of swimming as coordinated units and that eventually break down in seawater (Gardiner and Jones, 1985; Cary et al., 1989b). The observations of modified sperm and direct sperm transfer are difficult to reconcile with numerous field and laboratory observations of apparent free spawning in *Riftia pachyptila* (Cary et al., 1989a; Van Dover, 1994). However, the clouds of presumed gametes observed in these “spawning events” could consist of zygotes, embryos or even larvae if fertilization is internal.

The female reproductive system of *Riftia pachyptila*, *Ridgeia piscesae*, *Tevnia jerichonana*, *Lamellibrachia luymesii* and *Seepiophila jonesi* was examined using histology. In all five species, a sperm storage region was found at the far posterior end of the reproductive tract. This region will be referred as the spermatheca. *In vitro* fertilization and field experiments show that fertilization is internal, that meiosis is completed after the eggs are released from the female, and that the dispersal phase of vestimentiferans includes the entire embryonic period.

3.2 Material and Methods

Samples of *Riftia pachyptila* and *Tevnia jerichonana* were collected from a depth of 2500 m at hydrothermal vents on the East Pacific Rise (9°50'N). Samples of *Ridgeia piscesae* were collected from the Main Endeavour Field (Endeavour Segment, Juan de Fuca Ridge), and samples of *Lamellibrachia luymesii* and *Seepiophila jonesi* were collected from Bush Hill, GC-234 and Brine Pool (Green Canyon, Gulf of Mexico).

For histological analysis the worms were removed from their natural tubes and preserved in 5% seawater formalin for 48h and subsequently transferred to 70% ethanol. Previous

studies (Jones, 1988; Malakhov et al., 1996) reported that the gonad structure of vestimentiferans is situated wholly within the trunk region, running its entire length. In order to get randomised subsamples of the reproductive structure from each individual, a random stratified sampling design was followed. The trunk region of each individual was divided in 10 equal sections from which one segment of 1 mm was chosen by means of a random numbers table. The segments were slowly dehydrated by transference to 90% propan-2-ol overnight followed by period of 9 hours in 100% propan-2-ol with change of solution every 3 hours to prevent dilution of the alcohol with tissue based-water. Before being impregnated in paraffin wax at 70°C for 12 to 24 hours, the segments were cleared with 100% xylene for 6 to 12 hours depending on the size of the segment. The impregnated tissue was then embedded in wax, sectioned at 5 μm , and stained with Mayer's hematoxylin and eosin. In sections of females where sperm was found a Feulgen reaction for DNA staining procedure was followed for confirmation. Each stained slide was examined using an Olympus BH-2 binocular microscope under magnifications of x4 to x100.

For electron microscopy studies, pieces of gonadal tissue of female *Lamellibrachia luymesii* were immersed for 1.5 hours at room temperature in 2.5% glutaraldehyde buffered with Millonig's 0.4 M phosphate buffer at pH 7.4. Due to exigencies of ship scheduling, tissue was stored in Millonig's 0.4 M phosphate buffer with 0.6 M NaCl for 2 weeks prior to post-fixation and embedment. Tissue was post-fixed for 1 hour at room temperature in 1% osmium tetroxide in 0.1 M phosphate buffer plus 1% NaCl at pH 7.2. Tissue was then dehydrated in ascending concentrations of ethanol to 100%, followed by immersion in acetonitrile for 10 minutes, left overnight in a solution 50:50 of acetonitrile:resin, and embedded in epoxy resin. Thin sections (1 μm) were stained with uranyl acetate and lead citrate, and examined with a Hitachi H7000 transmission electron microscope.

Eggs of some vestimentiferans are stored in an expanded distal ovisac prior to release. Eggs from the ovisac or the distal region of the oviduct in all species studied and on many occasions to assess the gametogenic stage just prior to spawning. Eggs were examined for the presence of visible germinal vesicles using a light microscope immediately after they were removed.

Oocytes were collected from the ovisacs of 7 female *Lamellibrachia luymesii* and incubated without adding sperm to determine if they had already been fertilized internally. New pipettes were used, taking special care to eliminate any possible contamination from extraneous sperm. These primary oocytes were incubated in 0.45 μm filtered seawater for 24 hours at 6°C, after which at least 100 were examined from each individual.

In broadcast spawners with external fertilization, fertilization rates virtually always decline as a function of distance from spawning males (Pennington, 1985; Levitan and Young, 1995). If fertilization in vestimentiferans is external the same relationship would be expected. A field experiment was conducted to determine if distance from a tubeworm bush had any effect on fertilization rate of oocytes removed from the ovisacs of female *Lamellibrachia luymesii*. Eggs were held 25 cm above the bottom in small plastic tubes (2 cm diameter, 2 cm long) capped on both ends with 50 μ m nylon mesh. Three were deployed by submersible in a large bush of tubeworms, and the others were deployed in a straight line with three replicates each at 1.5 m, 3.0 m, 4.5 m, and 6 m downstream. The cultures were left to incubate for 24 hours. Again, special care was taken to sterilize all containers, including the plexiglas box in which eggs were transported to the bottom, to eliminate artefactual fertilization events.

Sperm bundles were removed from 4 male *Lamellibrachia luymesii* and diluted and stored in 0.45 μ m filtered seawater at 6°C. To determine whether the bundles break down in the presence of female tissue, samples of the sperm solutions were placed in 3 microscope slides each of which with different female tissues each (vestimentum, oviduct and spermatheca). Sperm from another male was used as a control.

3.3 Results

3.3.1 General anatomy of the female reproductive tract

The reproductive system of female vestimentiferans consist of a paired system of meandering ducts surrounded by trophosome. While in *Riftia pachyptila*, *Tevnia jerichonana* and *Ridgeia piscesae* it runs through the length of the whole trunk, in *Lamellibrachia luymesii* and *Seepiophila jonesi* it can only be found in the anterior 2/3 of the trunk.

At the posterior end of the vestimentum there is a pair of gonopores opening dorsally into the space formed by the body and the vestimental wings.

The gonopores lead into a short genital duct that has a wall composed of a non-ciliated columnar epithelium. The genital ducts pass postero-ventrally and open in the anterior portion of a large ovisac in *Riftia pachyptila* and *Lamellibrachia luymesii*. In *Tevnia jerichonana*, *Ridgeia piscesae* and *Seepiophila jonesi* there is no ovisac and the genital duct continues posteriorly as the oviduct, which follows a parallel course lateral to the gonocoel.

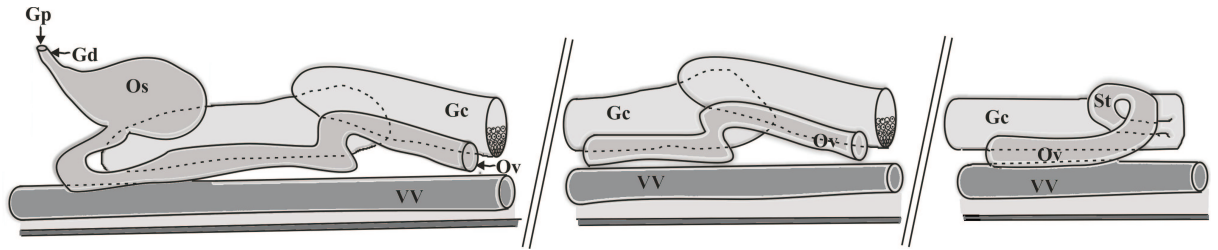


Figure 3.1: Lateral reconstruction of the right side of the female reproductive system of *Riftia pachyptila*. (Gc) Gonocoel; (Gd) Genital duct; (Gp) Gonopore; (Os) Ovisac; (Ov) Oviduct; (St) Spermatheca; (VV) Ventral blood vessel.

The ovisac is situated ventrally to the dorsal blood vessel, has an oval shape, and its wall is formed by ciliated columnar epithelium and a well-defined layer of circular muscles that is thicker in the dorso-lateral portion of the ovisac where the epithelium forms triangular ridges. The presence of an ovisac only in some species might be an indication that this feature developed at different stages during the evolution of siboglinds.

Both in *Riftia pachyptila* and *Lamellibrachia luymesii* the oviduct emerges just anteriorly to the posterior end of the ovisac, still in the trunk overlying the vestimentum. It passes antero-ventrally and medially contouring the anterior end of the gonocoel, and then it turns back to follow a posterior course parallel and dorso-lateral to the ventral blood vessel and ventro-medial to the gonocoel (Figure 3.1).

The gonocoels lead posteriorly from the posterior end of the ovisac to the point where the oviduct opens into it. A sheet of connective tissue separates both gonocoels and each of them from the ventral blood vessel. From this tissue arises a strip of germinal epithelium which grows into the gonocoels filling them with rows of developing oocytes (Figure 3.2). Among the developing oocytes branches of the ventral blood vessel subdivide to form a network of capillaries. The walls of the capillaries are formed by myoepithelial cells, and hematocytes are frequently observed associated with the inner wall of the capillary. Myoepithelial cells are absent in

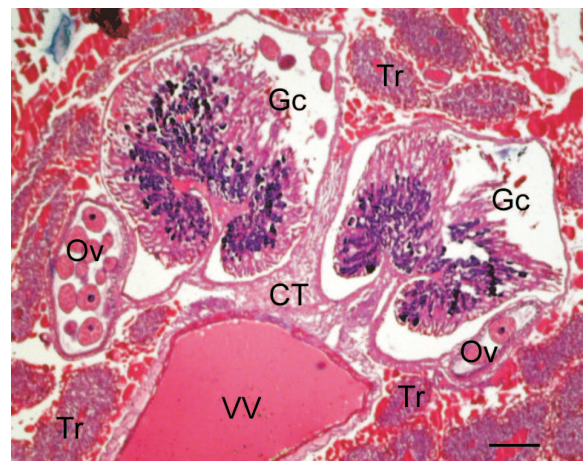


Figure 3.2: Cross section of the reproductive tract of *Riftia pachyptila*. (CT) connective tissue; (Gc) gonocoel; (Gd) gonoduct; (Ov) Oviduct; (Tr) Trophosome; (VV) ventral blood vessel. Scale bar: 200 μm .

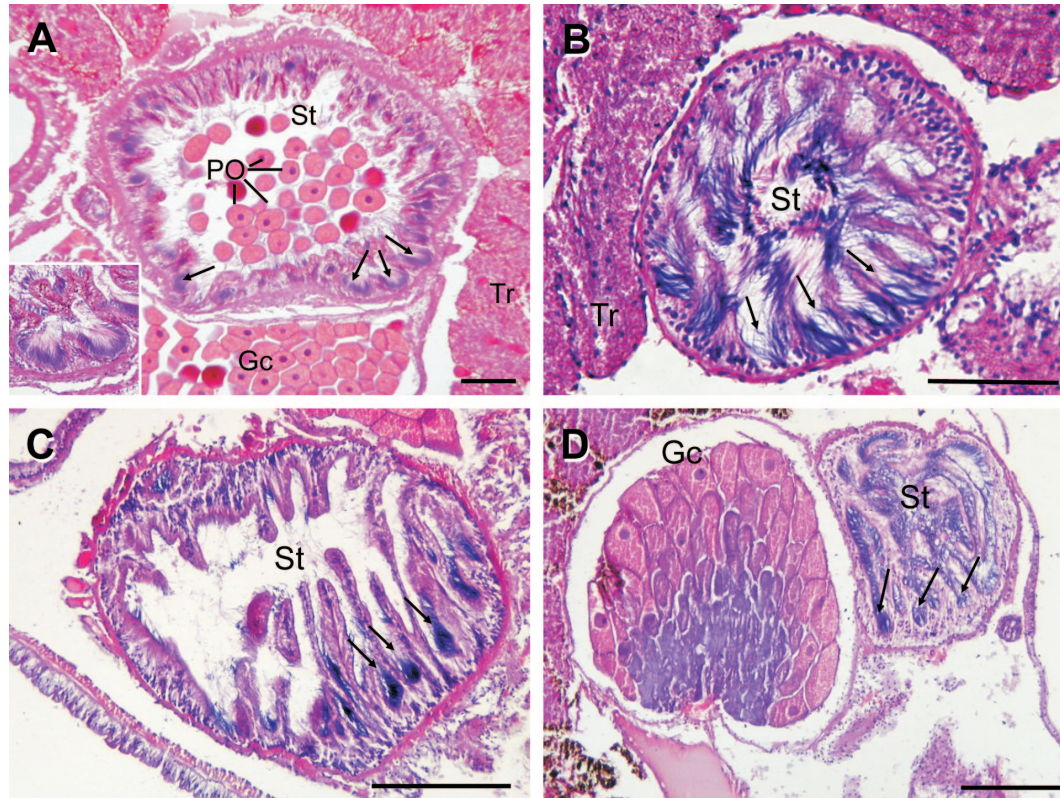


Figure 3.3: Light microscopy of the spermatheca of *Riftia pachyptila* (A), *Tevnia jerichonana* (B), *Lamellibrachia luymeri* (C) and *Seepiophila jonesi* (D). The insert in (A) shows detail of the spermatheca of *Riftia pachyptila* with clusters of spermatozoa. (Gc) gonocoel; (PO) primary oocyte; (St) spermatheca; (Tr) trophosome; (Arrow) clusters of spermatozoa. Scale bar: 200 μ m.

certain regions of the capillary wall allowing direct contact between the oocyte and the vascular lamina of the capillary.

Webb (1977) described structures called “transverses” situated at irregular intervals along the length of the trunk of *Lamellibrachia barhami*, where the gonocoel, the oviduct and the ventral blood vessel transverse from right to left, and where the oviduct opens into the gonocoel by a non-ciliated funnel. These structures were also reported by Malakhov et al. (1996) in *Ridgeia piscesae*. Although transverses were observed in all the species studied, histological sections revealed no openings that would permit the passage of oocytes from the gonocoels to the oviducts. Thus, all eggs must pass through the posterior ends of their respective gonocoels before entering the oviducts.

3.3.2 The spermatheca

Through most of its length, the wall of the oviduct is composed of a ciliated cuboidal epithelium surrounded by a thin layer of circular muscle. However, in all five species

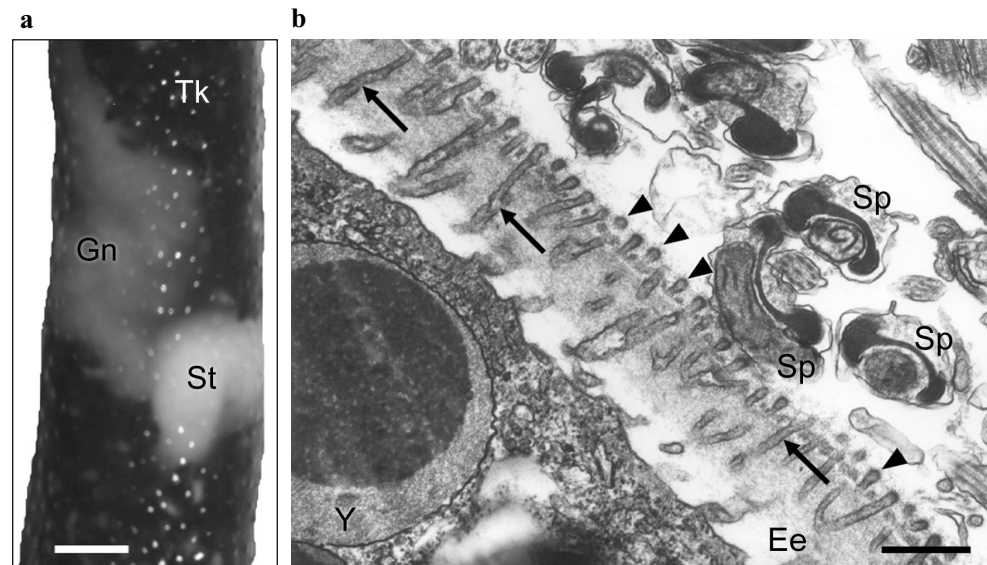


Figure 3.4: Spermatheca of *Lamellibrachia luymesii*. (a) As seen through the body wall. Scale bar: 1 mm. (b) TEM section of the spermatheca showing an oocyte and several spermatozoa in close proximity. (Ee) egg envelope; (Gn) Gonad; (Sp) sperm; (St) spermatheca; (Tk) trunk; (Y) yolk granule; (Arrow head) granule with filamentous glycocalyx; (Arrow) Microvilli. Scale bar: 0.5 μm .

examined, the oviducal epithelium is folded into a series of loops and sacs at the far posterior end of the reproductive tract, where the ovarian gonocoel joins the oviduct. In every female examined, these small sacs contained clusters of spermatozoa, with the heads all aligned towards the wall of the sac (Figure 3.3). These sperm are no longer packaged in the discrete flame-shaped bundles released by the male (Gardiner and Jones, 1985; Cary et al., 1989b).

This region of sperm storage is referred to as the spermatheca. In *Lamellibrachia luymesii* and *Seepiophila jonesi*, the spermatheca appears as a white, hook-shaped structure, easily visible with the naked eye through the body of the worm (Figure 3.4a).

3.3.3 Status of the oocytes at “spawning” and fertilization rates

In all five species examined, oocytes in the spermatheca, the ovisac, and along the length of the oviduct had a large germinal vesicle, suggesting that they were primary oocytes in the first prophase of meiosis. Oocytes are roughly circular in cross section, but some display irregular shape due to packing.

Ultrastructural examination of oocytes collected from the spermatheca shows that the egg envelope is formed of branched microvilli that terminate in a monolayer of granules situated

along the outer surface of the envelope and underlying a filamentous glycocalyx (Figure 3.4b). Sperm heads may be found in direct contact with glycocalyx where they appear to be bound to the eggs (Figure 3.4b). However, no sperm that had penetrated the egg envelope was found.

In no instance were already developing embryos found in the ovisac or in any other region of the oviduct. These observations are completely consistent with other evidence suggesting that female vestimentiferans release primary oocytes (Marsh et al., 2001).

Oocytes dissected from 7 female *Lamellibrachia luymesii* were incubated *in vitro* without adding additional sperm. After 24 hours, a mean of 90.09% (s.d.: 8.45) of the embryos had attained 2-cell to 16-cell embryonic stages (Figure 3.5), showing that a large percentage of oocytes had already been inseminated by the time they reached the ovisac.

In the field experiment with *Lamellibrachia luymesii*, distance from a tubeworm bush (0 m to 6 m) had no significant effect on the fertilization rate of oocytes taken from the ovisac of females, with mean percentages of development ranging from 85 to 95% (Figure 3.6). This strongly suggests that fertilization occurred prior to or

at the time of the experimental deployment. Three outcomes were possible from this experiment: (1) if fertilization is external the fertilization rate would decline with the distance from the bush (red line in figure 3.6); (2) fertilization rates would vary randomly if fertil-

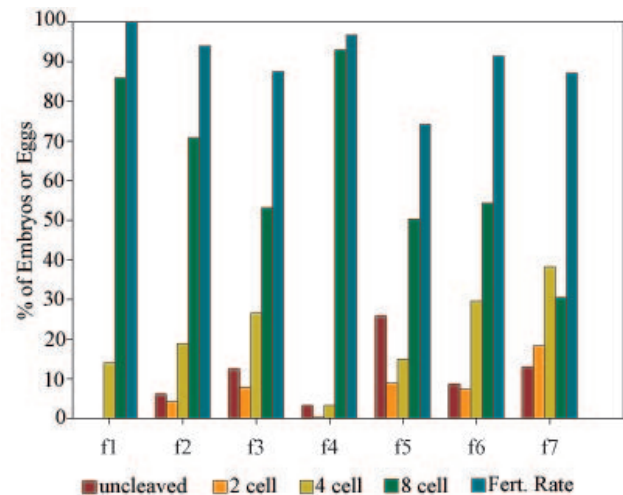


Figure 3.5: Percentage of embryos or eggs, and fertilization rates of 7 females (f1 to f7) of *Lamellibrachia luymesii* after a 24h incubation *in vitro*.

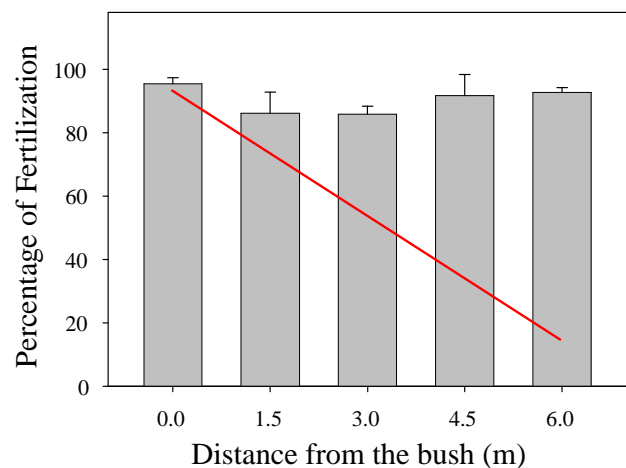


Figure 3.6: Percentage of fertilized eggs (mean + s.d) of *Lamellibrachia luymesii* after a 24 hours *in situ* deployment in mesh-covered tubes at various distances from a large bush of conspecifics. The red line shows the predicted relationship between the two variables for species with external fertilization.

ization is external there is a patchy distribution of sperm in the water column (Levitan and Petersen, 1995); and (3) if fertilization is internal there would be no significant difference in the fertilization rates (Figure 3.6). Similar experiments with freely spawning invertebrates that fertilize externally always show a strong declining relationship between fertilization rate and distance from males (Pennington, 1985; Levitan and Young, 1995).

3.3.4 Sperm bundle longevity

Cary et al. (1989b) reported that after swimming for about 15 minutes, the sperm bundles of *Riftia pachyptila* disaggregate as the individual sperm separated from one another and from their acrosomes, and that the resulting individual sperm were almost immotile. As in *Riftia pachyptila*, the bundles of *Lamellibrachia luymesii* started to swim a few seconds after dilution, but instead of breaking down into individual sperm cells they broke down into smaller bundles that did not disaggregate until 72 hours. However, when placed on a microscope slide and observed under the microscope, these smaller bundles started to disaggregate after 2 to 5 minutes, which could be a result of an increase of the temperature. The smaller sperm bundles disaggregate both in the presence of female tissue and sperm.

In the site GC234 the bivalve *Acesta bullisi* was found attached to the anterior end of the tube of *Lamellibrachia luymesii*, covering both the plume and the vestimentum when the animal is withdrawn (Figure 3.7). *Acesta bullisi* seems to prefer association with female *Lamellibrachia luymesii* than with male, and stable isotope analysis showed that *Acesta bullisi* feed on oocytes of *Lamellibrachia luymesii*, which can provide up to half of the metabolic requirement of the bivalve (J. Järnegren, personal communication).

Oocytes collected from one female *Lamellibrachia luymesii* associated with *Acesta bullisi* developed normally, showing that this female had been inseminated. These results also suggest that in *Lamellibrachia luymesii* sperm are released into the water column and not transferred through the plumes, as it has been suggested for other species of vestimentiferans (Southward and Coates, 1989; Southward, 1999; MacDonald et al., 2002). The plumes of tubeworms associated with *Acesta bullisi* are bathed with circulating seawater



Figure 3.7: *Acesta bullisi* attached to *Lamellibrachia luymesii*. Image courtesy Johanna Järnegren.

due to the filtering activity of the bivalve, this water must carry sperm into the female.

3.4 Discussion

3.4.1 Site of fertilization

The reproductive biology of vestimentiferans has been studied in *Lamellibrachia luymesii* (van der Land and Nørrevang, 1977), *Lamellibrachia barhami* (Webb, 1977) and more superficially in *Riftia pachyptila* (Gardiner and Jones, 1985; Jones and Gardiner, 1985; Gardiner et al., 1992). Although embryos and larvae have never been collected, early development has been described for *Ridgeia piscesae* (Jones and Gardiner, 1989), *Riftia pachyptila* (Marsh et al., 2001) and for *Lamellibrachia* sp. and *Escarpia* sp. from the Gulf of Mexico (Young et al., 1996).

Apparent spawning events have been observed in *Riftia pachyptila* (Van Dover, 1994) and *Lamellibrachia luymesii* (C. M. Young, unpublished observations). However, in none of these observations was it possible to ascertain whether the spawn consisted of unfertilized oocytes, zygotes, developing embryos, bundles of sperm, or free sperm.

Based on morphological criteria of modified sperm, direct sperm transfer and internal fertilization was suggested for *Riftia pachyptila* by Gardiner and Jones (1985), who also mentioned an anecdotal observation of sperm in the genital tract of females. In *Ridgeia piscesae* and *Tevnia jerichonana*, sperm masses have been found attached to the female vestimentum and within the oviduct, strongly suggesting active sperm transfer when the plumes of closely juxtaposed animals brush against each other (Southward and Coates, 1989; Southward, 1999; MacDonald et al., 2002), followed by internal fertilization. These observations also raised the possibility that embryos are brooded.

Except in *Ridgeia piscesae* (Southward and Coates, 1989; MacDonald et al., 2002), it is not known how sperm or sperm bundles are transferred to the female, but it seems likely, based on spawning observations, that sperm bundles may be released into the water column, from which they are either collected by the females or find their way into the female gonopores. The presence of spermatozoa at the posterior end of the worm demonstrates that sperm either swim or are carried deep into the reproductive system.

Webb (1977), in describing the reproductive anatomy of *Lamellibrachia barhami*, noted that the gonocoel of the gonad opens into the gonoduct by non-ciliated funnels situated at

irregular intervals along the length of the trunk. These connections between the gonoduct and the gonocoels were not found in any of the species studied here, which suggests that all primary oocyte released from the ovary must pass through the spermatheca before they enter the gonoduct.

In eggs of other polychaetes, granulated glycocalyxes similar to the one found in oocytes collected from the spermatheca of *Lamellibrachia luymesii* are known to possess sperm receptors (Eckelbarger, 1992). This study suggests that as primary oocytes pass through the spermatheca sperm are annealed to the primary oocytes, which arrest at the first meiotic prophase until they are released by the female.

The breeding pattern of insemination followed by the release of the oocyte with the sperm annealed, rather than brooding of an embryo assures a high level of fertilization without sacrificing dispersal potential. Moreover, sperm storage is an ideal adaptation for an environment where periodic cues for gametogenesis and spawning synchrony are limited (Young, 1999). Indeed, hydrothermal vent polychaetes from many different families are now known to possess specialized sperm storage organs (Zal et al., 1995; Van Dover et al., 1999; Jollivet et al., 2000).

Previous estimates of dispersal times and distances based on lipid stores, larval metabolism and current speeds (Marsh et al., 2001), have assumed external fertilization and embryogenesis. Even though the presumed location of fertilization was erroneous in these studies, the dispersal estimates themselves remain valid, since embryogenesis does not begin until after inseminated oocytes are released into the water column.

A number of female specimens of the five species studied were observed not to contain mature oocytes in the oviduct, but only developing oocytes in the gonocoel. Some of these specimens were also the smallest within their sample and can therefore be considered sexually immature females. The same features were observed in females with tube length and tube internal diameter comparable to those with oviducts filled with oocytes assumed to be individuals that had just undergone spawning. The presence of sperm in the spermatheca of immature females and of females that had just spawned might be an indication that males and females vestimentiferans are not reproductively synchronous.

3.4.2 Impact of female-conditioned water on sperm activity

For many years before the phenomenon was unequivocally demonstrated, investigators suspected that chemical attraction and subsequent taxis of a sperm towards an egg occurred

in animal phyla. Chemotaxis in marine invertebrates was first shown by Dan (1950), in a medusoid cnidarian. It has since been demonstrated in a number of other species of Cnidaria (Miller, 1970, 1978) and other phyla including molluscs (Miller, 1977), arthropods (Clapper and Brown, 1980), echinoderms (Miller, 1985), and tunicates (Miller, 1975). Whether such chemotaxis exists in annelids is inconclusive at present (Williams and Bentley, 2002).

Chemicals derived from the female can have three types of effect upon the swimming of the sperm. First, they can initiate swimming; second, they can increase velocity; and finally, they can cause sperm to swim in a directed manner towards the egg.

In vestimentiferans, sperm is released by the male in masses (Southward and Coates, 1989; Southward, 1999; MacDonald et al., 2002) or in bundles that are capable of swimming as coordinate units (Cary et al., 1989b). The spermatozoa found in the spermatheca of the five species studied were no longer in any of these forms, suggesting that sperm masses and bundles disaggregate into individual sperm still in the water column or inside the female reproductive tract.

Sperm bundles of *Lamellibrachia luymesii* broke down into smaller bundles capable of swimming for 72 hours. In *Riftia pachyptila* the sperm bundles disaggregate into individual, almost immotile, sperm after 15 minutes. Southward and Coates (1989) suggested, based on characteristics of the acrosome, that Cary et al. (1989b) witnessed expulsion of immature sperm, which might explain such a big difference in the longevity of the sperm bundles between the two species.

The results of the incubations of sperm with female tissues (vestimentum, oviduct and spermatheca) and with sperm from another male suggest that these female tissues do not send a chemical signal capable of inducing the disaggregation of the bundles. However, these results should be taken carefully since the effect of the rising temperature might be stronger than a chemical signal emitted by the female. Further work is required to determine if sperm bundles swim downward as cohesive bundles against the ciliary current that carries oocytes upward in the oviduct, or if they are transported by ciliary action or peristalsis. Future experiments should be carried out in order (1) to establish more clearly the point at which spermatozoa become fully active, and (2) to suggest a possible mechanism for the stimulation of forward motility in vestimentiferans.

3.4.3 Consequences of sperm storage

Polychaetes exhibit a remarkable diversity of reproductive attributes (Wilson, 1991) and are often a major component of marine benthic communities, but the entire life history is only known for 3% of described species (Giangrande, 1997). Female sperm storage is known to occur in some of the Capitellidae, Sabellidae and Serpulidae in shallow water, and Alvinellidae and Polynoidae at deep-sea hydrothermal vents (Jollivet et al., 2000), but prevalence of this feature is poorly known. Sperm storage has long been recognised as an important feature in terrestrial ecology, providing a playing-field for sperm competition and female mate choice, but its context and consequences are little understood in marine ecology.

With the proposed fertilization model vestimentiferans can be classified as “spermcast” organisms, a term suggested by Pemberton et al. (2003b) to define organisms that release sperm into the water column but retain eggs by the acting female. In such organisms a female is likely to be subjected to large amounts of sperm from a few close males, with smaller quantities of sperm arriving from distant sources. These considerations are of great importance as gene flow in sessile communities is largely restricted to the gamete and larval stages.

Allozyme and genetic data have revealed little population differentiation among *Riftia pachyptila* across 4000 km of the East Pacific Rise spreading center (Vrijenhoek, 1997). Hydrothermal vent sites are typically linearly arrayed along spreading centre ridge axes, with vent plumes that rise upwards through the water column, and near-bottom currents that flow along the axis of the valley. Propagule dispersal modelling of vent organisms based on this water current regime cannot completely explain the amount of genetic homogeneity observed in vent vestimentiferans (Kim and Mullineaux, 1998). Similarly, heterozygotic deficiency observed in vestimentiferans from cold seeps in the Gulf of the Mexico has not been totally explained (McMullin, 2003). A deficiency in heterozygotes can be explained by a number of reasons. The existence of structure within a defined ‘population’ can generate an apparent heterozygote deficiency by violating the Hardy Weinberg assumption of random mating within the population. Other violations of the assumption of random mating, such as mating of related individuals, assortative mating, and self fertilization, will also cause a decrease in heterozygosity (Hastings, 1997).

Previous studies of population genetics of vestimentiferans do not support population structure within collections, sites or geographical regions (McMullin, 2003). Vestimentiferans

are dioecious, and there is no evidence of self fertilization in this group. Adult vestimentiferans have no opportunity for assortative mating, and until now assortative mating between gametes seemed very unlikely due to the probable low density of gametes in the water column. However, with evidence of storage and accumulation of sperm inside the female reproductive tract it is possible to assume non-random mating between gametes which may explain the genetic homogeneity observed in species from hydrothermal vents and cold seeps.

In organisms where the females store sperm but the fusion of the two gametes does not occur until the female releases them in the water column, postmating barriers to fertilization can exist at a variety of levels. Sperm must successfully enter the female and be transported to the spermatheca. They must retain adequate mobility until they are attached to the oocyte, activated, and enter the oocyte in the water column. A failure at any of these steps prevents fertilization. To promote assortative fertilization, there must be some degree of heritable specificity in the male and female components of the above processes. Thus, the relevant barriers are those that are associated with naturally occurring, normal variations in the population, such that the male effectively signals the female to keep his sperm alive and to utilize it in fertilization (Markow, 1997). These variations can be quantitative (e.g. the amount of sperm released in each spawning) and/or qualitative (e.g. size and/or shape of the sperm and/or oocytes) or biochemical (sperm viability or its reduction may result from biochemical interactions between the sperm and the females reproductive tract).

From the five species studied, *Lamellibrachia luymesii* and *Seepiophila jonesi* have a more tortuous spermatheca which allows a better separation of spermatozoa from different mates. These two species co-occur in aggregations that often have in excess 1000 individuals, raising the question whether a mismatch between sperm morphology and that of the female storage organ can avoid the cross-fertilization of the two species and consequent production of unviable hybrids.

Chapter 4

Intra and interspecific variability of the reproductive condition

4.1 Introduction

To understand fully the functioning of an ecosystem, it is imperative to have a sound knowledge of the reproductive adaptations of its components (Giangrande et al., 1994). The reproductive pattern of an organism plays a major role in the dynamics of the population and the biogeography and continuity of the species. The theoretical optimal life-history adaptation will optimise lifetime reproductive success by maximizing the allocation of resources to growth, survival and reproduction from birth to death (Ramirez-Llodra, 2002). Because the total energy that an organism can assimilate and allocate to reproduction is limited, the allocation of energy to reproduction will be associated with a decrease in somatic investment.

Hydrothermal vents and cold seeps produce local environments that are rich in chemical energy and that are often inhabited by dense aggregations of species harbouring chemoautotrophic bacterial symbionts. The patchy spatial distribution of vent and seep fluid at the seafloor is biologically manifested in the clustering of symbiont-bearing animals around sources of vent and seep fluid and the complete absence of these animals from the substratum only metres away (Olu et al., 1997; Tunnicliffe et al., 1997). Due to the apparent ephemeral nature of hydrothermal vents, a life history that involves fast growth to reproductive size has obvious advantages to fauna strictly dependent on the vent effluent for nourishment (Tunnicliffe et al., 1990). The cold seep environment is much more stable

than the hydrothermal vent environment and may provide an habitat rich in sulphide for centuries.

The seep vestimentiferans *Lamellibrachia luymesii* and *Seepiophila jonesi* have slow growth rates (3 cm.yr^{-1} and $< 3 \text{ cm.yr}^{-1}$, respectively) compared to that of the vent vestimentiferans *Tevnia jerichonana* (30 cm.yr^{-1}), *Riftia pachyptila* ($\sim 85 \text{ cm.yr}^{-1}$) (Lutz et al., 1994). The longevity and low growth rates of the cold-seep vestimentiferans (Fisher et al., 1997; Bergquist et al., 2000) reflect life history patterns very different from their hydrothermal vent relatives. In the case of the seep vestimentiferans, the energy source for their chemoautotrophic symbionts is stable and constant over long periods. However, sulphide is not as abundant in sediment round the seep vestimentiferans as it is in hydrothermal fluid that bathes vent vestimentiferan habitats. Slow growth and long life span are therefore good adaptations for seep vestimentiferans.

From the data available at present, it is apparent that the reproductive patterns of vent and seep organisms have strong phylogenetic constraints (Tyler and Young, 1999), and that adaptations to vents and seeps are mainly in the nutritional and respiratory physiology of the organism. Although the general reproductive pattern may be conservative, aspects of the life history must have evolved to ensure that the reproductive propagule can locate and colonise newly-available seep and vent habitats.

Because time-series samples from vents and cold seeps are difficult to obtain, few accounts of reproduction and development of vent and cold seep fauna have been published [reviewed by Tyler and Young (1999)]. The present study describes aspects of the gametogenic biology and trophosome condition of four species of vestimentiferans from cold seeps in the Gulf of Mexico and from hydrothermal vents in the Pacific. It also examines how gonad condition varies with a measure of growth and with trophosome condition.

4.2 Material and Methods

4.2.1 Collection and laboratory treatment of samples

The reproductive condition was studied for *Ridgeia piscesae* using samples collected in the Endeavour Segment of the Juan de Fuca Ridge in 1998 and 1999, *Riftia pachyptila* collected in the 9°N EPR in 1999 and 2000, *Lamellibrachia luymesii* and *Seepiophila jonesi* from the Gulf of Mexico collected in 2002 and 2003 (Table 4.1).

Table 4.1: Sites, depth (m) and dates of collection and size of samples.

Species	Collection Site	Location	Depth (m)	Date	Sample size
<i>Ridgeia piscesae</i>	Easter Island E1	47°56.86'N, 129°05.93'W	2199	08/98	15
	Easter Island t#15	47°56.93'N, 129°05.97'W	2198	08/98	20
	Clam Bed	47°57.70'N, 129°05.51'W	3260	08/98	17
	Bastille	47°56.87'N, 129°06.04'W	2200	09/99	42
<i>Riftia pachyptila</i>	Biovent	09°50.44'N, 104°17.62'W	2505	12/99	33
	Mussel Bed	09°50.18'N, 104°17.50'W	2501	05/00	10
<i>Lamellibrachia luymesii</i>	Brine Pool	27°43.44'N, 91°16.75'W	650	10/02	16
				02/03	37
	GC 234	27°44.70'N, 91°13.30'W	540	02/03	12
				11/03	23
<i>Seepiophila jonesi</i>	Brine Pool	27°43.44'N, 91°16.75'W	650	02/03	16

Immediately after reaching the surface after collection, the animals were removed from their tubes and preserved in 5% formaldehyde (seawater) for 48h and subsequently transferred to 70% ethanol.

In the laboratory, the width of the vestimentum (VW) was measured with a calliper to the nearest 0.1 mm as an independent measure of size. This measure was chosen since, owing to the nature of the sampling method, retrieval of whole worms of *Ridgeia piscesae*, *Lamellibrachia luymesii* and *Seepiophila jonesi* could not be relied upon and thus total length and weight measurements were ruled out. The width of the vestimentum has previously been shown to reflect the allometric growth of *Riftia pachyptila* (Thiébaud et al., 2002).

The procedure described in Section 3.2 was used to obtain randomised subsamples of the reproductive structure of each individual. Using a Leica MZ8 stereomicroscope, one section from each of the ten segments of each individual was digitised with a Nikon 990 camera mounted on the stereomicroscope.

After calibration with a 100 μm slide graticule, the gonad area, the trophosome area and the somatic tissue area were calculated using a Jandel Scientific's SigmaScan-Pro software (version 4.01). Microsoft Excel 2002 was used to convert the gonad, the trophosome and the somatic tissue areas to percentage from each section. The percentage of gonad from each section from each individual was averaged to produce a mean individual gonadosomatic index (GSI), used as measure of reproductive condition. Mean individual percentage of trophosome (%T) and mean individual percentage of somatic tissue (%ST) for each specimen were calculated in a similar way.

4.2.2 Statistical analysis

Goodness-of-fit χ^2 tests were performed to investigate the sex ratio of the different sample stations. Mann-Whitney tests were carried out to investigate any differences in VW, %T, %ST and GSI values of males and females from each sample. In both tests the null hypothesis was that there is no difference between the number of females and males at the 95% confidence level.

Owing to the sampling method used in the present study a normal distribution of sampled organisms could not be relied upon, thus variation of mean individual GSI, mean individual %T, mean individual %ST and VW between samples was determined using the non-parametric Kruskal-Wallis one way ANOVA on ranks test, or Mann-Whitney tests when there were no more than 2 samples to compare. The null hypothesis was that there was no significant difference among the treatment groups at the 95% confidence level. If a difference was found, a Dunn's all pairwise multiple comparison test was performed to isolate the group or groups that differ from the others.

Spearman's rank correlation procedure was performed to investigate the relationship between the four variables. Correlation, rather than regression, was performed since there was no distinction between independent and dependent variables, only the degree of association between the pairs of variables wanting to be elucidated (Zar, 1996).

All graphs were plotted using Jandel Scientific's SigmaPlot software (version 8.0) and statistical analysis were performed using Jandel Scientific's SigmaStat software (version 3.0).

4.3 Results

4.3.1 *Ridgeia piscesae*

In *Riftia pachyptila* and *Lamellibrachia luymesii* adult males may be distinguished from females by the presence of a pair of well-developed ciliated grooves running the entire length of the dorsal face of the vestimental region (van der Land and Nørrevang, 1977; Gardiner and Jones, 1993). However, in *Ridgeia piscesae* both sexes present these ciliated grooves, and although they are shorter and narrower in females than in males (Southward and Coates, 1989) in the present study this was not used as a means of sexing the individuals, microscopic observation of the gonads being preferred.

Results of χ^2 tests investigating the sex ratio indicate that at 95% confidence level there is no statistically significant difference between the number of females and males in all the samples except in the Clam Bed collection, where the number of males is significantly higher than the number of females (Easter Island E1: $\chi^2 = 1.667$; Easter Island t#15: $\chi^2 = 0.200$; Clam Bed: $\chi^2 = 4.764$; Bastille: $\chi^2 = 1.52$; $\chi^2_{(0.05,1)} = 3.841$; Table 4.2).

Table 4.2: Number of females and males of *Ridgeia piscesae*.

Sample Station	Female	Male
Easter Island E1	5	10
Easter Island t#15	9	11
Clam Bed	4	13
Bastille	25	17

The results from the Mann-Whitney tests performed to investigate any difference in the vestimentum width between females and males from each station indicate that, at the 95% confidence level, there are no statistically significant differences in the vestimentum width of males and females from Easter Island E1, Clam Bed and Bastille (Table 4.3). In Easter Island t#15, the vestimentum of the males was found to be significantly wider than that of the females (Table 4.3; Figure 4.1).

Individuals collected in Bastille and Easter Island E1 seem to have smaller values of VW than those collected in Easter Island t#15 and Clam Bed (Figure 4.1). These differences were tested statistically using the non-parametric test Kruskal-Wallis one way ANOVA on ranks. As a statistically significant difference was found among the sample stations (Table 4.3), a Dunn's all pairwise multiple comparison test was performed to isolate the station or stations that differ from the others. Statistically significant differences were found between the two Easter Island stations, between Easter Island t#15 and Bastille, between Clam Bed and Easter Island E1, and between Clam Bed and Bastille. No differences in the vestimentum width were found between Clam Bed and Easter Island t#15, and between Easter Island E1 and Bastille (Figure 4.2a).

When females and males of each sample station were plotted separately a statistically significant difference in the vestimentum width was found among all samples (Table 4.3). Males collected in Easter Island E1 and Bastille have wider vestimentum than those collected in Easter Island t#15 and Clam Bed (Figures 4.1c and 4.2c). In females no differences were found between the two stations of Easter Island, nor between any of these two and Clam Bed, Easter Island E1 and Bastille are also not significantly different. Finally, the females from Bastille have a wider vestimentum than those from Easter Island t#15 and Clam Bed (Figures 4.1b and 4.2b).

Table 4.3: Results of the statistical tests performed to investigate the differences in vestimentum width (VW), percentage of somatic tissue (%ST), percentage of trophosome (%T) and gonadosomatic index (GSI) in *Ridgeia piscesae* (* indicates significant P values).

VW between sample sites	H = 69.083; P < 0.001*
VW between sample sites (female)	H = 21.366; P < 0.001*
VW between sample sites (male)	H = 37.478; P < 0.001*
VW female against male	T = 1946.000; P = 0.469
%ST between sample sites	H = 56.009; P < 0.001*
%ST between sample sites (female)	H = 24.667; P < 0.001*
%ST between sample sites (male)	H = 29.040; P < 0.001*
%ST female against male	T = 1703.000; P = 0.010*
%T between sample sites	H = 52.624; P < 0.001*
%T between sample sites (female)	H = 24.421; P < 0.001*
%T between sample sites (male)	H = 26.689; P < 0.001*
%T female against male	T = 2442.000; P = 0.002*
GSI between sample sites	H = 11.922; P = 0.008*
GSI between sample sites (female)	H = 6.631; P = 0.085
GSI between sample sites (male)	H = 4.683; P = 0.197
GSI female against male	T = 2248.000; P = 0.120
Sample sites:	EI E1 EI t#15 Clam Bed Bastille
VW female against male	T = 32.000 T = 64.500 T = 50.000 T = 334.500 P = 0.358 P = 0.023* P = 0.126 P = 0.126
%ST female against male	T = 44.000 T = 76.000 T = 35.000 T = 474.000 P = 0.668 P = 0.171 P = 0.955 P = 0.006*
%T female against male	T = 37.000 T = 113.000 T = 40.000 T = 260.000 P = 0.759 P = 0.171 P = 0.692 P = 0.007*
GSI female against male	T = 51.000 T = 103.000 T = 28.000 T = 347.000 P = 0.198 P = 0.543 P = 0.396 P = 0.645

The values of mean individual percentage of somatic tissue (%ST) were found to be significantly different between sample sites (Table 4.3). Specimens collected in Clam Bed and Easter Island t#15 have higher values than those collected in Easter Island E1 and Bastille (Figures 4.1a and 4.2a). Statistically significant differences between sample sites were also found when females and males were plotted separately (Table 4.3). Figures 4.2b and 4.2c show at which sample sites differences were found.

The %ST was found to be higher in males than in females in the sample from Bastille (Table 4.3). No differences in the %ST were found between males and females in any of the other samples (Table 4.3).

Statistically significant differences were found between the values of mean individual percentage of trophosome (%T) (Table 4.3). The collections from Clam Bed and Easter Island t#15 have smaller values than those from Easter Island E1 and Bastille (Figures 4.1a and

4.2a).

When females and males of each sample station were plotted separately a difference in the mean individual percentage of trophosome (%T) was found among all samples (Table 4.3). The differences were found between the same pairs of sample sites as for the mean individual percentage of somatic tissue (Figures 4.2 b and 4.2 c). These results suggest a negative correlation of %T with %ST. At the 95% confidence level, a negative correlation was found ($r = -0.989$, $P < 0.050$, $n = 94$). Between %T and VW a positive correlation

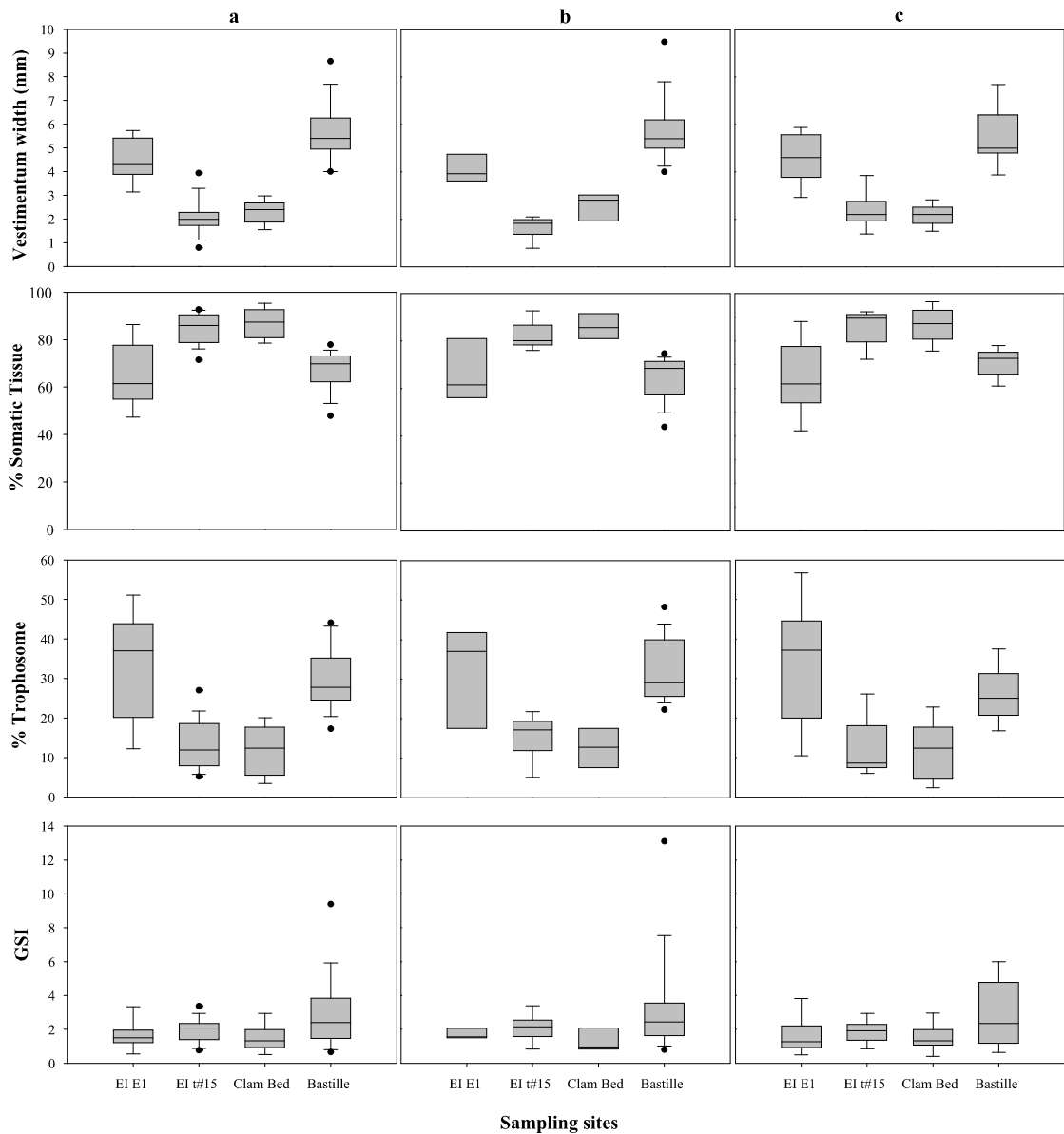


Figure 4.1: Vestimentum width, percentage of somatic tissue, percentage of trophosome and gonadosomatic index of *Ridgeia piscesae*. Bottom and top boundaries of the box indicate the 25% and 75% percentile, line within the box marks the median, and error bars represent the 10% and 90% percentile. Dots indicate the 5% and 95% percentile. a) Female and male plotted together; b) Female; c) Male.

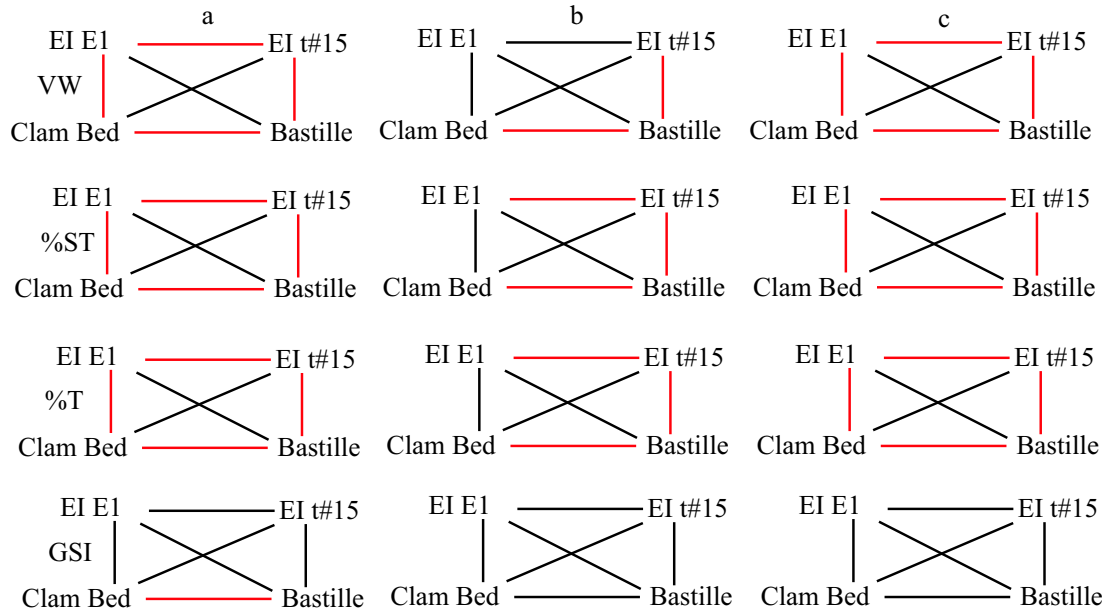


Figure 4.2: Results of the Dunn's all pairwise tests performed to investigate differences between samples sites of *Ridgeia piscesae*. Red lines represent significant differences between two sample sites. a) Female and male plotted together; b) Female; c) Male.

was found ($r = 0.589$, $P = 0.003$, $n = 94$). However, due to the small coefficient of correlation this relationship should be interpreted cautiously.

The %T was found to be higher in females than in males in the sample from Bastille (Table 4.3). No differences in the %T were found between males and females in any of the other samples (Table 4.3).

The result of the Kruskal-Wallis test performed to investigate the differences between the GSI values of *Ridgeia piscesae* from the different sampling stations show a statistically significant difference among all the samples (Table 4.3). Specimens from Bastille have higher values of GSI than those from Clam Bed (Figures 4.1a and 4.2a). No other significant differences were found between any other pair of samples. However, when females and males are plotted separately there were no significant differences between any of the samples (Table 4.3; Figures 4.1b, 4.1c, 4.2b and 4.2c).

No difference in the gonadosomatic index was found between females and males in all the samples (Table 4.3).

Using the Spearman's rank correlation procedure a significant relationship was found between GSI and the width of the vestimentum ($r = 0.307$, $P = 0.003$, $n = 94$). However, due to the small coefficient of correlation this relationship should be interpreted cautiously. No significant relationship was found between GSI and %ST or between GSI and %T.

When all the samples were pooled, males were found to have greater %ST and smaller %T than females. No significant differences were found in the GSI nor in the VW (Table 4.3).

4.3.2 *Riftia pachyptila*

The results of the χ^2 tests performed to investigate the difference in the number of females and males from each sample station show that, at the 95% confidence level H_0 should be accepted, i.e., the sex ratio is 1:1 (Biovent: $\chi^2 = 0.273$; Mussel Bed: $\chi^2 = 0.000$; $\chi^2_{(0.05,1)} = 3.841$; Table 4.4).

Table 4.4: Number of females and males of *Riftia pachyptila*.

Sample Station	Female	Male
Biovent	18	15
Mussel Bed	5	5

The results from the Mann-Whitney tests performed to investigate any difference in the vestimentum width between females and males from each station indicate that there are no statistically significant differences in the VW of males and females of *Riftia pachyptila* (Table 4.5).

Vestimentum width (VW), mean individual percentage of trophosome (%T) and mean individual gonadosomatic index (GSI) of males and females from both stations are plotted in Figure 4.3. No statistically significant differences were found in the VW and GSI values between the two sites. However, specimens from the Biovent site have higher %ST and lower %T than those from Mussel Bed. The same results were obtained when females and males were plotted separately (Table 4.5).

Differences in the %T and GSI between males and females were found to be statistically significant in both samples (Table 4.5). Females have higher %T and lower GSI than males (Figure 4.3a and 4.3b).

Using the Spearman's rank correlation procedure it was found that there is a positive relationship between VW and GSI ($r = 0.370$, $P = 0.002$). Negative relationships were found between %ST and %T ($r = -0.440$, $P = 0.006$), between %ST and GSI ($r = -0.491$, $P = 0.002$) and between %T and GSI ($r = -0.493$, $P = 0.002$). However, due to the small coefficient of correlation these relationships should be interpreted cautiously.

Table 4.5: Results of the statistical tests performed to investigate the differences in vestimentum width (VW), percentage of somatic tissue (%ST), percentage of trophosome (%T) and gonadosomatic index (GSI) in *Riftia pachyptila* (* indicates significant P values).

VW between sample sites	T = 220.500; P = 1.000	
VW between sample sites (female)	T = 59.500; P = 1.000	
VW between sample sites (male)	T = 54.500; P = 0.896	
VW female against male	T = 435.500; P = 0.922	
%ST between sample sites	T = 95.100; P < 0.001*	
%ST between sample sites (female)	T = 22.000; P = 0.005*	
%ST between sample sites (male)	T = 26.000; P = 0.023*	
%ST female against male	T = 372.000; P = 0.100	
%T between sample sites	T = 350.000; P = 0.001*	
%T between sample sites (female)	T = 104.000; P = 0.001*	
%T between sample sites (male)	T = 81.000; P = 0.015*	
%T female against male	T = 333.000; P = 0.010*	
GSI between sample sites	T = 244.000; P = 0.499	
GSI between sample sites (female)	T = 55.000; P = 0.737	
GSI between sample sites (male)	T = 63.000; P = 0.383	
GSI female against male	T = 612.000; P < 0.001*	
Sample sites:	Biovent	Mussel Bed
VW female against male	T = 243.000 P = 0.971	T = 27.500 P = 0.917
%ST female against male	T = 207.000 P = 0.086	T = 34.000 P = 0.222
%T female against male	T = 178.000 P = 0.006*	T = 41.000 P = 0.008*
GSI female against male	T = 345.000 P = 0.001*	T = 15.000 P = 0.008*

4.3.3 *Lamellibrachia luymesii*

Lamellibrachia luymesii samples were collected from the GC234 site in February 2003 (GC234-1) and November 2003 (GC234-2), and from the Brine Pool site in October 2002 (BP-1) and February 2003 (BP-2) (Table 4.1).

Results of χ^2 tests investigating the sex ratio indicate that at 95% confidence level there is no statistically significant difference between the number of females and males in all the samples (GC234-1: $\chi^2 = 1.333$; GC234-2: $\chi^2 = 1.087$; BP-1: $\chi^2 = 2.25$; BP-2: $\chi^2 = 0.027$; $\chi^2_{(0.05,1)} = 3.841$; Table 4.6).

Table 4.6: Number of females and males of *Lamellibrachia luymesii*.

Sample	Female	Male
GC234-1	8	4
GC234-2	9	14
BP-1	5	11
BP-2	18	19

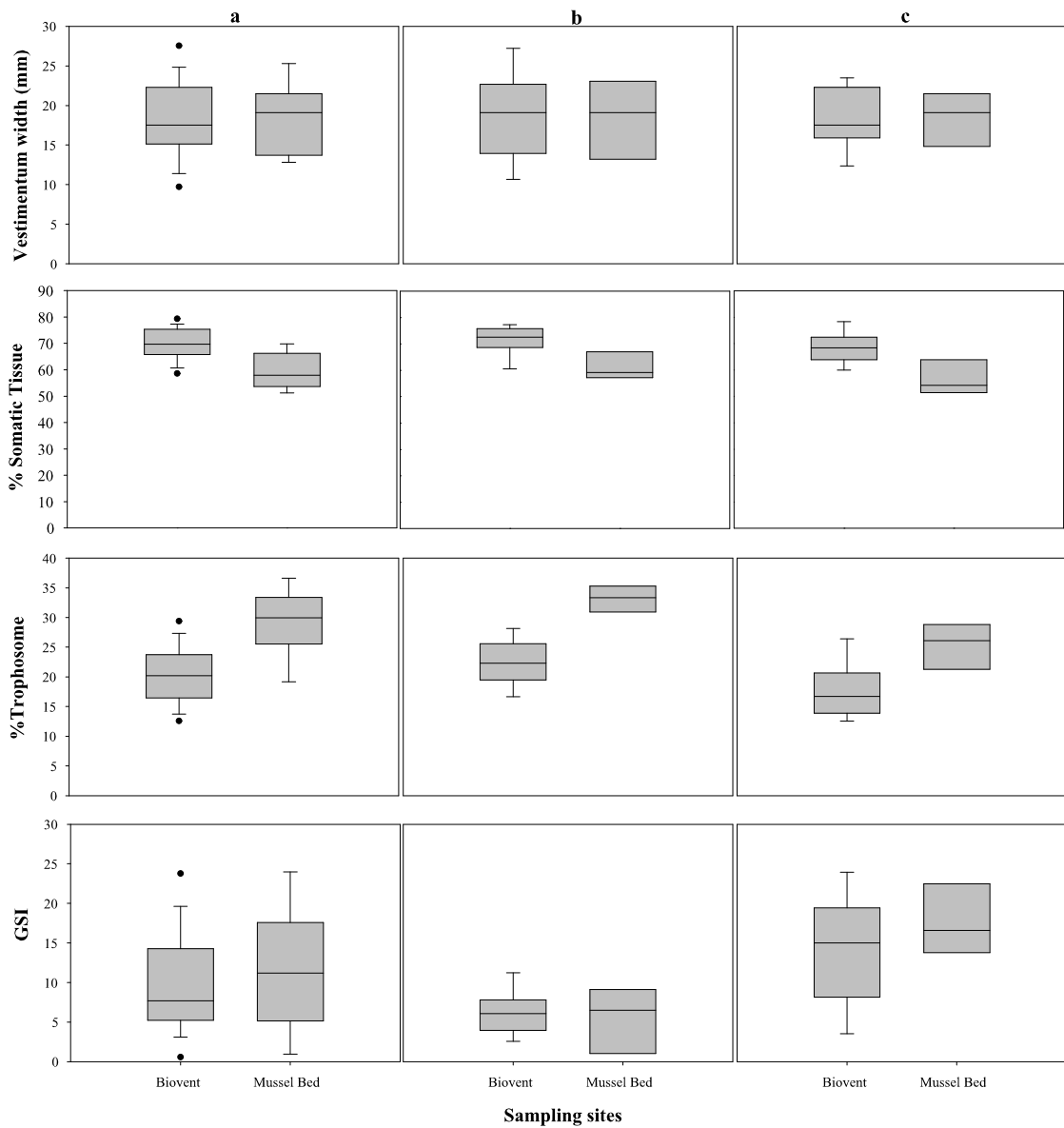


Figure 4.3: Vestimentum width, percentage of somatic tissue, percentage of trophosome and gonadosomatic index of *Riftia pachyptila*. Bottom and top boundaries of the box indicate the 25% and 75% percentile, line within the box marks the median, and error bars represent the 10% and 90% percentile. Dots indicate the 5% and 95% percentile. a) Female and male plotted together; b) Female; c) Male.

The results from the Mann-Whitney tests performed to investigate any difference in the vestimentum width between females and males from each sample indicate that there are no statistically significant differences in any of the samples (Table 4.7).

When samples from the same site were plotted together it appears that the specimens from GC234 are larger than those from Brine Pool (Figure 4.4a). This difference was tested statistically using a Mann-Whitney test and it was found to be significant (Table 4.7).

Table 4.7: Results of the statistical tests performed to investigate the differences in vestimentum width (VW), percentage of somatic tissue (%ST)percentage of trophosome (%T) and gonadosomatic index (GSI) in *Lamellibrachia luymesii* (* indicates significant P values).

VW between sample sites	T = 1869.000; P = 0.008*			
VW between sample sites (female)	T = 391.500; P = 0.245			
VW between sample sites (male)	T = 554.000; P = 0.017*			
VW female against male	T = 1918.500; P = 0.247			
%ST between sample sites	T = 1185.000; P = 0.002*			
%ST between sample sites (female)	T = 342.000; P = 0.870			
%ST between sample sites (male)	T = 216.000; P < 0.001*			
%ST female against male	T = 2137.000; P = 0.003*			
%T between sample sites	T = 2045.000; P < 0.001*			
%T between sample sites (female)	T = 391.000; P = 0.251			
%T between sample sites (male)	T = 657.000; P < 0.001*			
%T female against male	T = 1900.000; P = 0.317			
GSI between sample sites	T = 1349.000; P = 0.076			
GSI between sample sites (female)	T = 298.000; P = 0.171			
GSI between sample sites (male)	T = 390.000; P = 0.282			
GSI female against male	T = 1365.000; P < 0.001*			
VW between all samples	H = 26.558; P < 0.001*			
VW between all samples (female)	H = 7.407; P = 0.060			
VW between all samples (male)	T = 22.542; P < 0.001*			
%ST between all samples	H = 11.811; P = 0.008*			
%ST between all samples (female)	H = 6.201; P = 0.102			
%ST between all samples (male)	T = 23.470; P < 0.001*			
%T between all samples	H = 23.420; P < 0.001*			
%T between all samples (female)	H = 7.107; P = 0.069			
%T between all samples (male)	T = 27.036; P < 0.001*			
GSI between all samples	H = 8.780; P = 0.032*			
GSI between all samples (female)	H = 6.129; P = 0.106			
GSI between all samples (male)	H = 8.153; P = 0.043*			
Sample sites:	GC234		BP	
VW female against male	T = 360.000; P = 0.077		T = 693.000; P = 0.199	
%ST female against male	T = 416.000; P = 0.001*		T = 686.000; P = 0.247	
%T female against male	T = 258.000; P = 0.117		T = 792.000; P = 0.002*	
GSI female against male	T = 253.000; P = 0.083		T = 448.000; P = 0.002*	
Samples	GC234-1	GC234-2	BP-1	BP-2
VW female against male	T = 18.000 P = 0.214	T = 105.000 P = 0.900	T = 48.000 P = 0.571	T = 394.000 P = 0.118
%ST female against male	T = 12.000 P = 0.016*	T = 159.000 P = 0.001*	T = 35.000 P = 0.428	T = 403.000 P = 0.066*
%T female against male	T = 34.000 P = 0.214	T = 75.000 P = 0.041*	T = 52.000 P = 0.308	T = 447.000 P = 0.001*
GSI female against male	T = 24.000 P = 0.808	T = 78.000 P = 0.063	T = 39.000 P = 0.734	T = 212.000 P, 0.001*

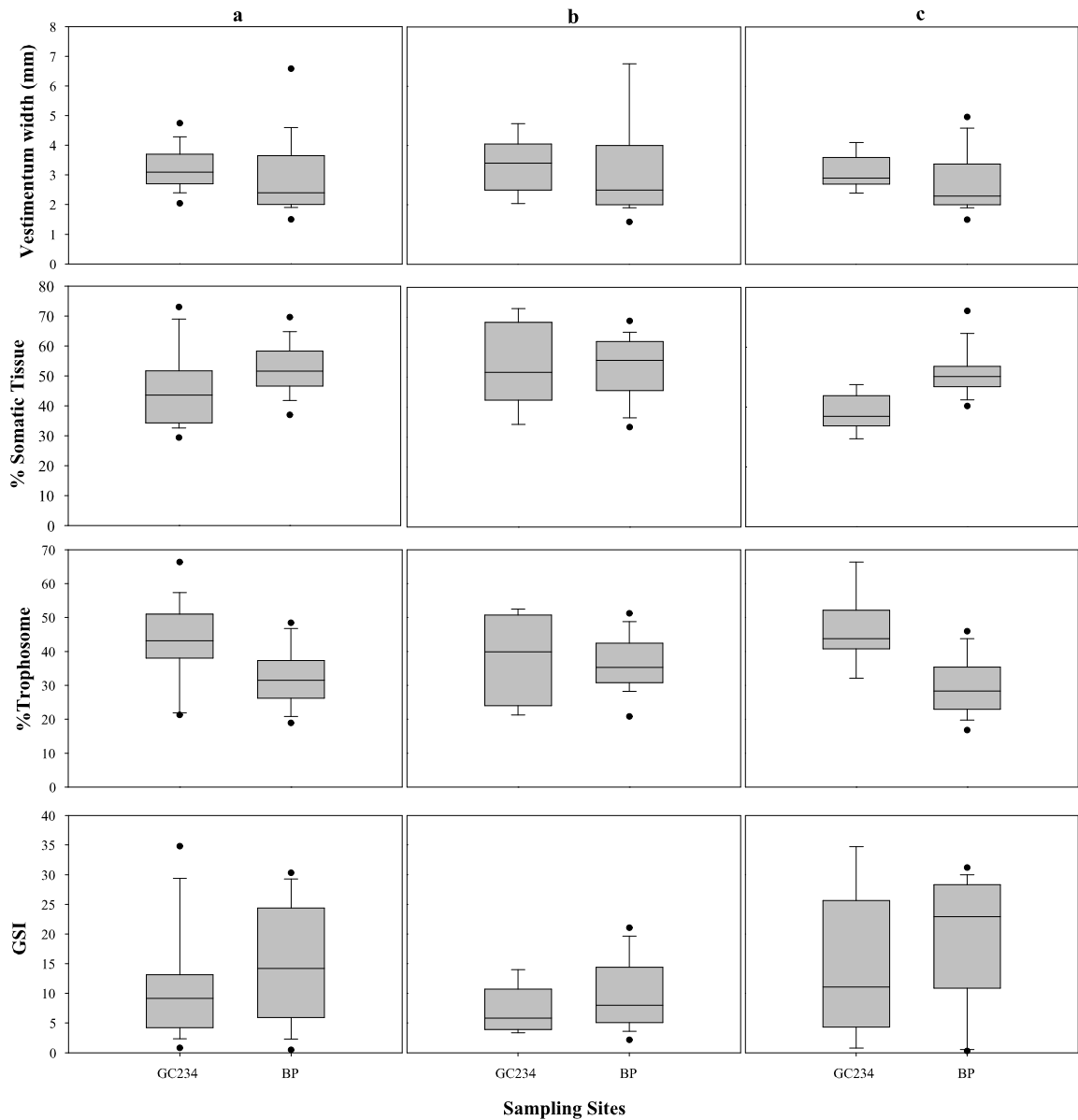


Figure 4.4: Vestimentum width, percentage of somatic tissue, percentage of trophosome and gonado-somatic index of *Lamellibrachia luymesii* from GC234 and Brine Pool. Bottom and top boundaries of the box indicate the 25% and 75% percentile, line within the box marks the median, and error bars represent the 10% and 90% percentile. Dots indicate the 5% and 95% percentile. a) Female and male plotted together; b) Female; c) Male.

A Kruskal-Wallis one way ANOVA on ranks test was used to investigate any difference in the vestimentum width among the four samples of *Lamellibrachia luymesii*. As a statistically significant difference was found (Table 4.7), a Dunn's all pairwise multiple comparison test was performed to isolate the sample or samples that differ from the others. Specimens from BP-2 have significantly narrower VW than those from all the other samples (Figures 4.5a and 4.6a).

When females and males were plotted separately, the results of the Mann-Whitney tests

show that there is no statistically significant difference in the vestimentum width among females of both sites, and that males from GC234 have longer VW than those from Brine Pool (Table 4.7; Figure 4.4c). The results from the Kruskal-Wallis one way ANOVA on ranks tests to investigate differences in the VW of females and males among all the samples show that there is no differences between the females, and that among the males there is a significant difference (Table 4.7). Males from BP-2 have narrower VW than males from any of the other samples (Figures 4.5c and 4.6c).

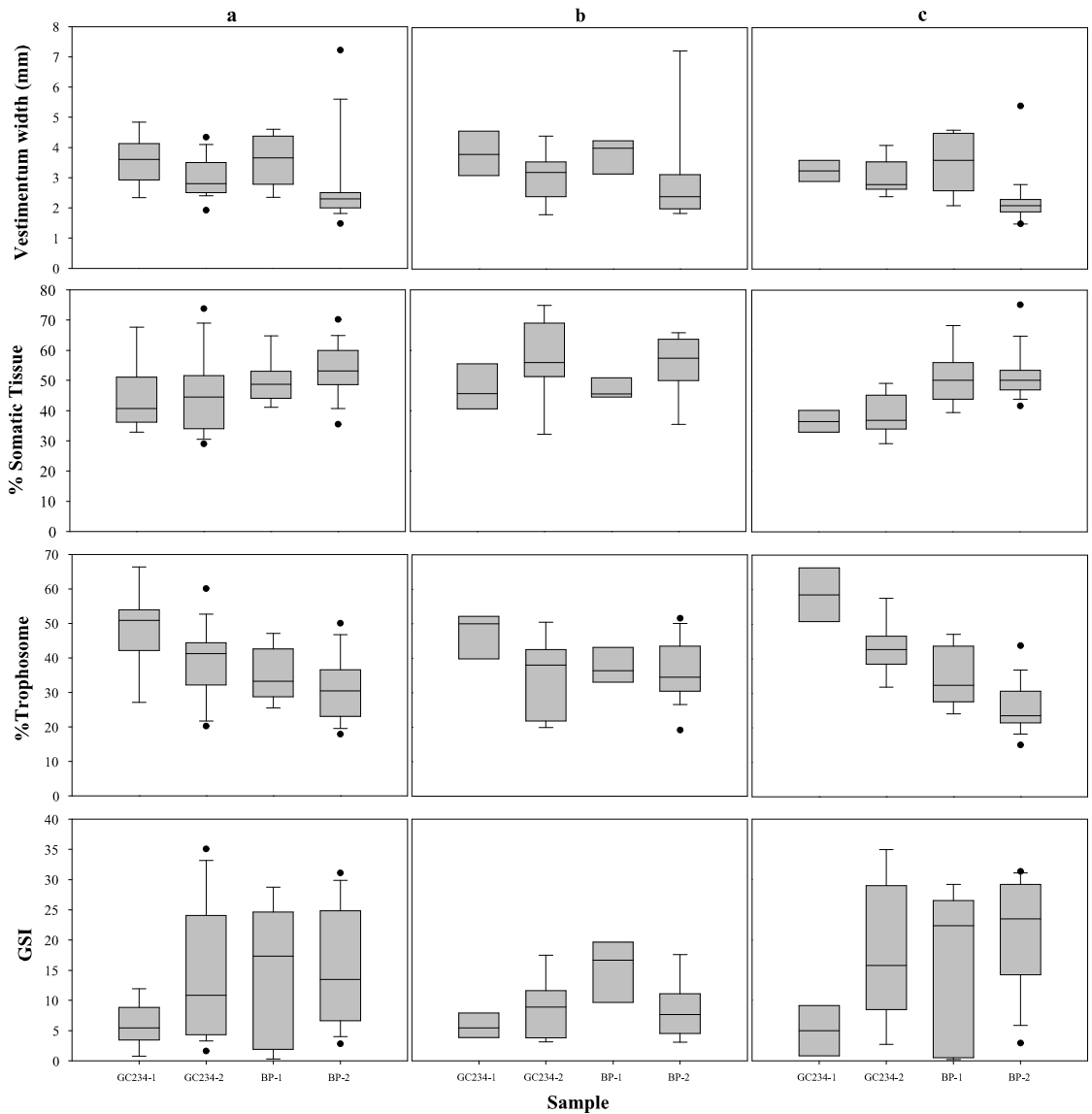


Figure 4.5: Vestimentum width, percentage of somatic tissue, percentage of trophosome and gonadosomatic index of *Lamellibrachia luymeri* from all the samples. Bottom and top boundaries of the box indicate the 25% and 75% percentile, line within the box marks the median, and error bars represent the 10% and 90% percentile. Dots indicate the 5% and 95% percentile. a) Female and male plotted together; b) Female; c) Male.

Specimens from BP have larger %ST than those from GC234 (Table 4.7; Figure 4.4a). A statistically significant difference was also found between all samples (Table 4.7). The results of the Dunn's all pairwise multiple comparison test performed to isolate the sample or samples that differ from the others (Figure 4.6a) and figure 4.5a show that specimens from BP-2 have smaller values of %ST than those from GC234-1 and GC234-2.

When females and males were plotted separately, it was found that the %ST of females does not vary between sites, and that males from BP have higher %ST than those from GC234 (Table 4.7; Figure 4.4). The results from the Kruskal-Wallis one way ANOVA on ranks tests show that there is no statistically significant difference in the %ST of females among all the samples, and that the differences in the median values of %ST of males is greater than it would be expected by chance (Table 4.7). Figure 4.6c shows between which pairs of samples differences were found.

Females from GC234 have larger %ST than those from Brine Pool (Table 4.7; Figures 4.4b and 4.4c). Within each site it was found that females have higher %ST than males in all the samples except BP-1 (Table 4.7; Figures 4.5b and 4.5c).

Specimens from GC234 have larger %T than those from Brine Pool (Table 4.7; Figure 4.4a). A Kruskal-Wallis one way ANOVA on ranks test was used to investigate any difference in the %T between the four samples of *Lamellibrachia luymesii*. As a statistically significant

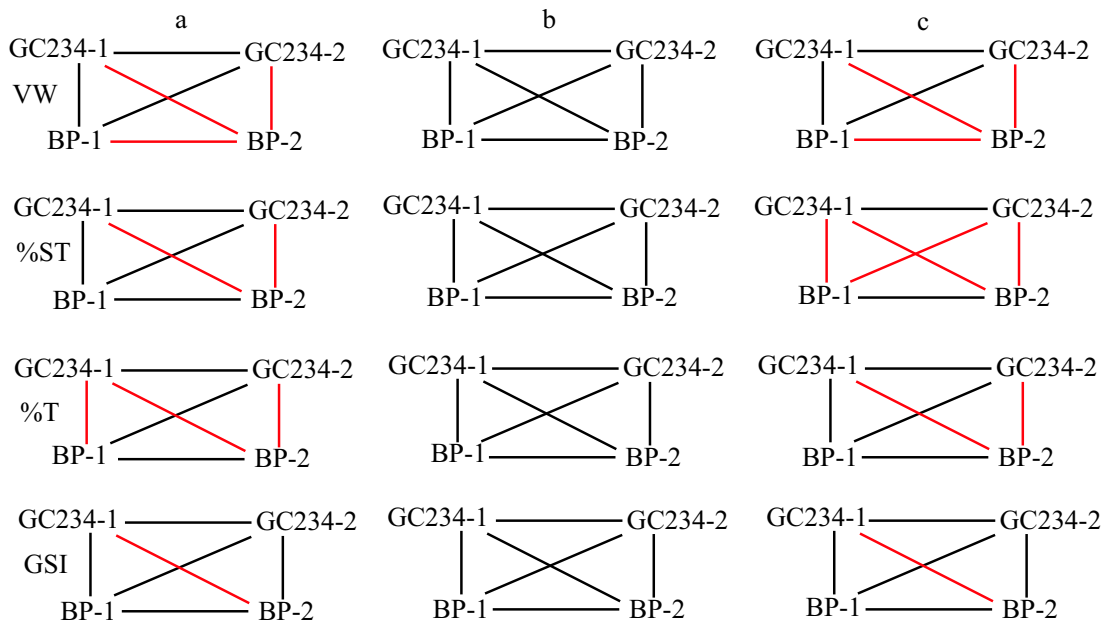


Figure 4.6: Results of the Dunn's all pairwise tests performed to investigate differences between samples sites of *Lamellibrachia luymesii*. Red lines represent significant differences between two sample sites. a) Female and male plotted together; b) Female; c) Male.

difference was found among the sample stations (Table 4.7), a Dunn's all pairwise multiple comparison test was performed to isolate the sample or samples that differ from the others. Specimens from GC234-1 have higher %T than those from both the collections from the Brine Pool, and specimens from GC234-2 have higher %T than those from BP-2 (Figures 4.5a and 4.6a).

When females and males were plotted separately, it was found that the %T of females does not differ significantly between sites, and that the %T of males collected from GC234 is higher than that from males collected in the Brine Pool (Table 4.7; Figures 4.4c and 4.6c).

The results from the Kruskal-Wallis one way ANOVA on ranks tests show that there is no statistically significant difference in the %T of females among all the samples, and that the differences in the median values of %T of males is greater than it would be expected by chance (Table 4.7). The results of a Dunn's all pairwise multiple comparison test and figure 4.5c show that males from GC234-1 and GC234-2 have higher %T than those from BP-2. No statistically significant differences were found among any of the other combinations (Figure 4.6c).

Mann-Whitney tests were performed to investigate the differences in %T between females and males from all the samples. It was found that females have lower %T than males in GC234-2, and higher in BP-2 (Table 4.7; Figure 4.5). No statistically significant difference was found in GC234-1, nor in BP-1. When the samples from the two collections of each site were plotted together no significant difference was found in GC234; in Brine Pool females are found to have higher %T than males (Table 4.7; Figure 4.4).

The data shown in figure 4.4a suggest that individuals from the Brine Pool have higher GSI values than the individuals from GC234. However, no statistically significant difference was found (Table 4.7). When all the samples were plotted individually the results from the Kruskal-Wallis one way ANOVA on ranks test show that there is a significant difference among in the GSI values among the samples (Table 4.7). A Dunn's all pairwise multiple comparison test was performed to isolate the sample or samples that differ from the others, and it was found that the specimens from GC234-1 have a significantly lower GSI than those from BP-2 (Figures 4.5a and 4.6a).

When females and males were plotted separately, it was found that the GSI of females and males does not differ significantly between sites (Table 4.7; Figure 4.4a and b). The results from the Kruskal-Wallis one way ANOVA on ranks tests show that there is no statistically significant difference in the GSI of females among all the samples, and that the differences

in the median values of GSI of males is greater than it would be expected by chance (Table 4.7). The results of a Dunn's all pairwise multiple comparison test and figure 4.5c show that males from GC234-1 have lower GSI than those collected from BP-2. No statistically significant differences were found among any of the other samples (Figure 4.6c).

Results from the Mann-Whitney tests performed to investigate the difference in the GSI values between males and females show that except in BP-2, where males have higher GSI than females, no significant differences were found (Table 4.7). When the samples from the two collections of each site were plotted together no significant difference in GSI values between females and males was found in GC234, and in Brine Pool males were found to have higher GSI than females (Table 4.7; Figure 4.4).

When all the individuals were plotted together a positive relationship was found between %T and VW ($r = 0.300$, $P = 0.005$, $n = 88$). Negative relationships were found between VW and %ST ($r = -0.332$, $P = 0.002$, $n = 88$), between %ST and %T ($r = -0.580$, $P = 0.001$, $n = 88$), between %ST and GSI ($r = -0.407$, $P = 0.001$, $n = 88$), and between %T and GSI ($r = -0.439$, $P = 0.001$, $n = 88$). However, although significantly different, it should be noted that the correlation coefficients (r) are relatively small in all the cases.

When all the samples were pooled males were found to have higher GSI and lower %ST than females (Table 4.3; Figures 4.8b and 4.8c).

4.3.4 *Seepiophila jonesi*

Only one sample of *Seepiophila jonesi* was analyzed in this study. The sample, with 6 females and 10 males, was collected in the Brine Pool site in February 2003 (Table 4.1).

Results of a χ^2 test to investigate the sex ratio indicate that at the 95% confidence level there is no statistically significant difference between the number of females and males ($\chi^2 = 1$, $\chi^2_{(0.05,1)} = 3.841$).

Vestimentum width (VW), mean individual percentage of trophosome (%T) and mean individual gonadosomatic index (GSI) of females and males are plotted in figure 4.7.

The results of the Mann-Whitney tests performed to investigate any difference between females and males show that females have lower GSI than males ($T = 27.000$, $P = 0.011$). No statistically significant difference between females and males was found in the other studied variables (VW: $T = 45.000$, $P = 0.551$; %ST: $T = 57.000$, $P = 0.551$ %T: $T = 68.000$, $P = 0.074$).

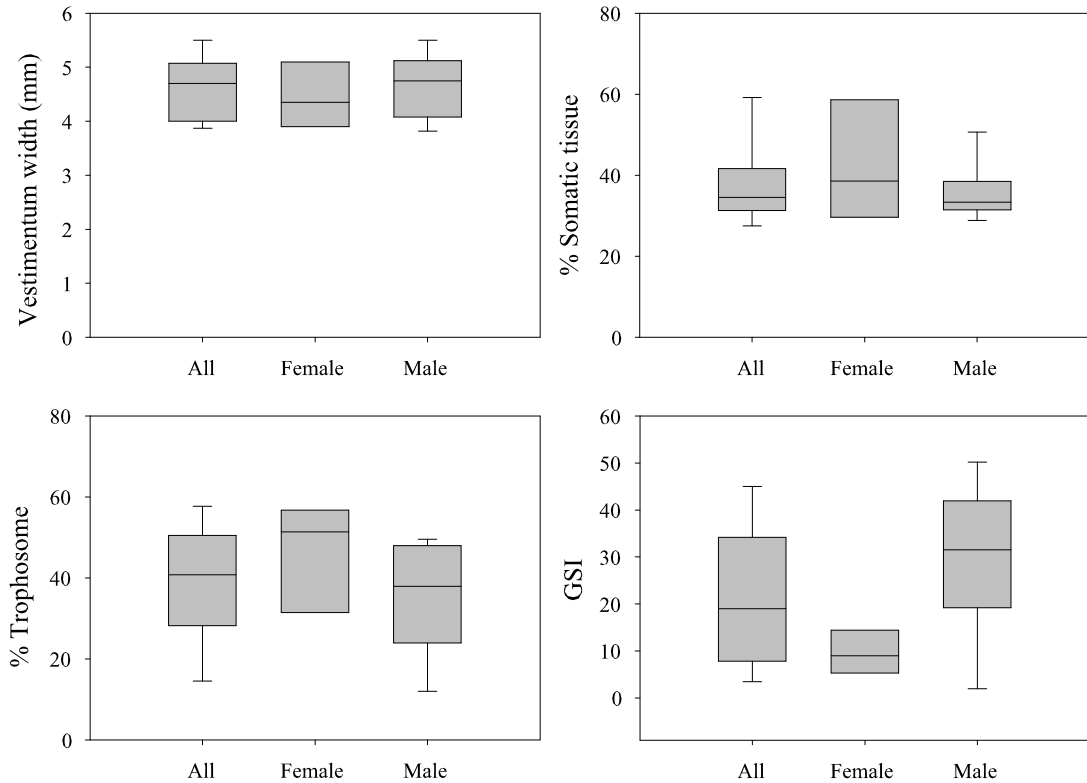


Figure 4.7: Vestimentum width, percentage of somatic tissue, percentage of trophosome and gonado-somatic index of *Seepiophila jonesi*.

When all the specimens were plotted together, the results of the Spearman's correlation procedure show a negative relationship between %T and GSI ($r = -0.674$, $P = 0.004$, $n = 16$). No significant relationship was found between any other pair of variables.

4.3.5 Interspecific variability

Species from the cold seep sites in the Gulf of Mexico have higher %T and lower values of %ST and GSI than the species from hydrothermal vents (Figure 4.8). These differences were tested statistically using the non-parametric test Kruskal-Wallis one way ANOVA on ranks. As statistically significant differences were found (%ST: $H = 134.046$, $P < 0.001$; %T: $H = 58.381$, $P < 0.001$; GSI: $H = 82.275$, $P < 0.001$), a Dunn's all pairwise multiple comparison test was performed for both variables in order to separate any species that differ from the others. No significant differences in %ST and %T were found between *Lamellibrachia luymesii* and *Seepiophila jonesi*, nor between *Ridgeia piscesae* and *Riftia pachyptila*. The two species from cold seeps were found to have lower %ST and higher %T

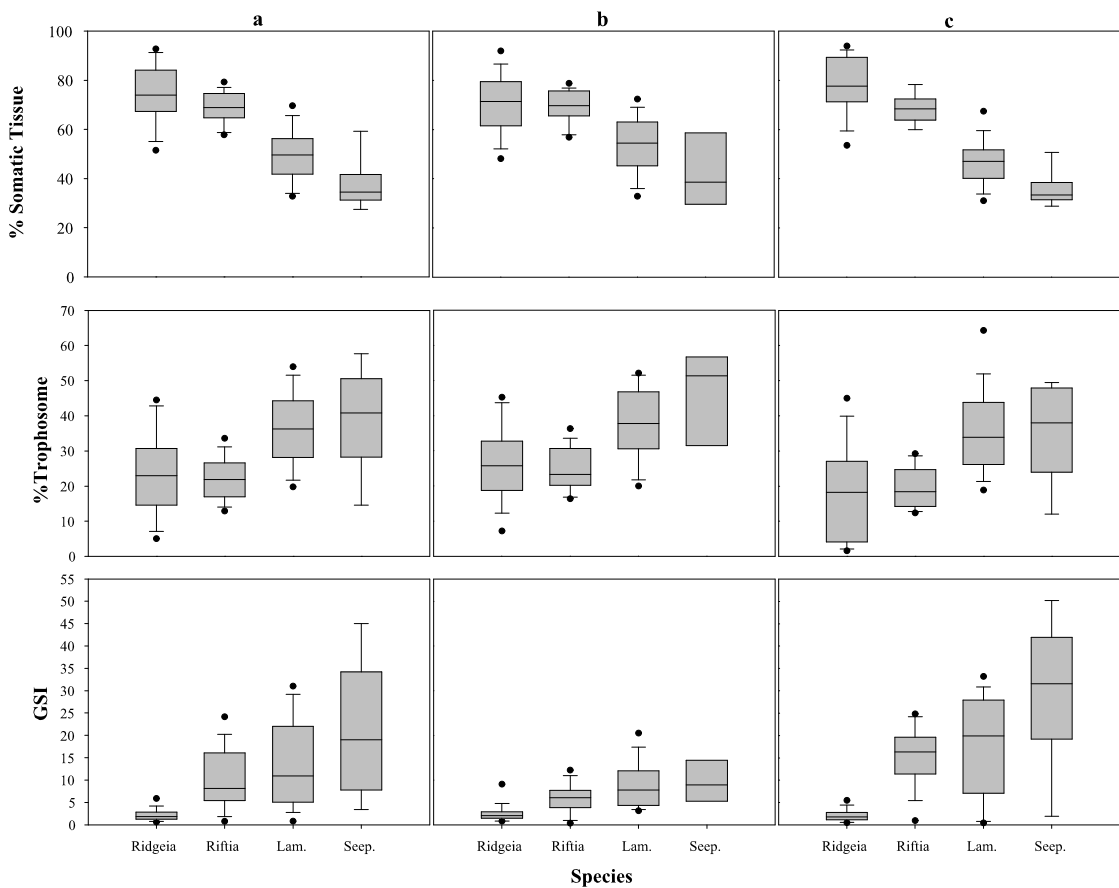


Figure 4.8: Percentage of somatic tissue, percentage of trophosome and gonadosomatic tissue of all the studied species. Bottom and top boundaries of the box indicate the 25% and 75% percentile, line within the box marks the median, and error bars represent the 10% and 90% percentile. Dots indicate the 5% and 95% percentile. a) Female and male plotted together; b) Female; c) Male.

than the two species from hydrothermal vents. The results of the Dunn's test for GSI show that *Lamellibrachia luymesii*, *Seepiophila jonesi* and *Riftia pachyptila* have higher GSI than *Ridgeia piscesae*. No statistically significant differences were found among any of the other pair of species (Figure 4.9).

When females and males were plotted separately (Figure 4.8b and c) statistically significant differences were found among the same pairs of species as when they were plotted together.

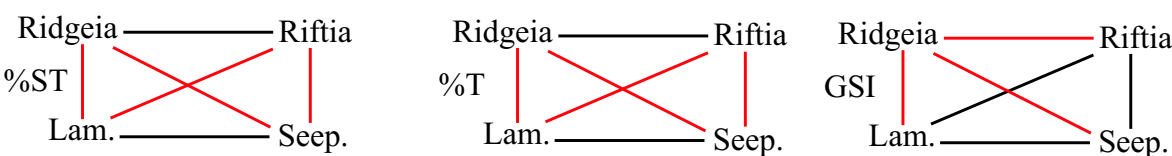


Figure 4.9: Results of the Dunn's all pairwise tests performed to investigate differences between all species. Red lines represent significant differences between two species.

4.4 Discussion and Summary

4.4.1 Reproductive strategy

Although in one of the samples of *Ridgeia piscesae* a sex bias was found, it can be considered that there is no significant differences in the proportion of males and females in vestimentiferan populations. Any sex bias would reduce the number of reproductively-effective individuals, meaning that some reproductive effort in the population would be wasted, thus 1:1 sex ratios and similar growth rates are expected in males and females of sedentary colonising organisms.

In the four species a number of specimens were observed not to have mature gametes in the sections studied. Males, identified by the presence of the dorsal ciliary ridge in the gonoducts (Webb, 1977; van der Land and Nørrevang, 1977), exhibited an apparently empty gonad, and females possessed only early stages of oocytes development. Some of these specimens also presented the smallest vestimentum width within their sample and may therefore be considered sexually immature. Although no measure of the vestimentum width was given, the maturation in *Ridgeia piscesae* was reported to occur in specimens with tube diameter as small as 2.5 mm (Tunncliffe, 1991). In the present study, individuals lacking mature gametes were observed to fit in a wide range of vestimentum width values, which could indicate differences in size at first maturity. However, a more likely explanation for the wide range of vestimentum widths found in specimens with no mature gametes is that they are sexually mature individuals that had just undergone spawning. This suggestion is supported by the relatively small variation of the gonadosomatic index between specimens from different samples, since differences were only found between the specimens of *Ridgeia piscesae* from Bastille and the other samples, and between the specimens of *Lamellibrachia luymesi* from GC234-1 and BP-2.

All early stages of oogenesis were observed within the gonad of all species. This concurs with what had been observed in previous studies of the reproductive biology of *Riftia pachyptila* (Gardiner et al., 1992) and is evidence that oogenesis is a continuous process in mature vestimentiferans.

Both of the above lines of evidence suggest that vestimentiferans exhibit iteroparity, but lack population synchrony in gonadal development. Environments such as hydrothermal vents and cold seeps, where there is a continuous energy supply introduced in the food web by the chemoautotrophic bacteria, can support the asynchronous or quasi-continuous pro-

duction of eggs. Nevertheless, previous studies of population structure of *Riftia pachyptila*, *Lamellibrachia luymesii* and *Seepiophila jonesi* (Thiébaud et al., 2002; Bergquist et al., 2002, 2003a), found a discrete pattern of recruitment, contrasting with the continuous reproductive output that would be expected from a population with an asynchronous reproductive pattern. The ecology of the larval phase may explain this contradiction. The non-feeding trochophore larvae of vestimentiferans are thought to disperse in the plankton for up to several weeks (Young et al., 1996; Marsh et al., 2001). Although the production of larvae might be continuous at population scale, the recruitment could be affected by environmental factors found by the larvae while in the water column and at the time of settlement. In the Gulf of Mexico, the growth model of *Lamellibrachia luymesii* and *Seepiophila jonesi* aggregations involves the settlement of young larvae on exposed carbonate over 20 to 40 years. Progressive burial of the carbonate prevents the settlement of more worms (Fisher et al., 1997), causing the discrete recruitment found by Bergquist et al. (2002, 2003a). The same recruitment model has been suggested for *Escarpia southwardae* from cold seeps off the western coast of Africa (Andersen et al., 2004). Thiébaud et al. (2002) suggested that successive recruitment events of *Riftia pachyptila* in the East Pacific Rise (9°N and 13°N) occur at a high frequency, ranging from 8 to 10 days. This short period can explain the apparent paradox between a continuous reproductive output and a discontinuous recruitment.

Andersen et al. (2004) found that females of *Escarpia southwardae* from the West African cold seeps are significantly larger than males and suggested that males might have a slower growth either because they direct a greater metabolic effort towards reproduction or because the females need to reach a greater body size to sustain oocyte production. In this study, however, females were found to be larger than males only in one sample of *Ridgeia piscesae*. Except in three samples of *Lamellibrachia luymesii*, the mean individual percentage of somatic tissue was also not found to be higher in females.

Invertebrate species that release both eggs and sperm tend to invest equally in male and female gamete production (Levitan, 1998). The observed low levels of fertilization in many of these species imply that equal investment is a function of sperm limitation (Levitan and Petersen, 1995). As discussed in Chapter 3, vestimentiferans can be considered spermcast organisms (organisms that release sperm into the water column but retain eggs by the acting female). Sperm collection and storage can decrease the likelihood of sperm limitation and yield much higher fertilization rates (Yund and McCartney, 1994; Pemberton et al., 2003a). For species that fertilize internally, it is commonly assumed that all eggs are

fertilized (Thorson, 1950). Although below 100%, the rates of fertilization in female vestimentiferans are relatively high, averages of 75% and 88% were found in *Riftia pachyptila* and *Lamellibrachia luymesii* respectively (Hilário et al., 2005). When sperm are “delivered” to eggs internally, sperm are likely to occur in high numbers, fertilization is likely with relatively little female investment, competition among males is probable, and sex dimorphism (in sex allocation and secondary characteristics) is expected (Levitan and Petersen, 1995), which may explain the higher gonadosomatic index of males found in three of the four studied species of vestimentiferans.

4.4.2 Habitat constraints to development

Previous studies suggested that the volume of trophosome in *Ridgeia piscesae* is highly variable and dependent upon venting conditions (Tunnicliffe, 1991). A decrease in volume of trophosome was observed as a response to decreases in vent fluid flow and consequent decreases in supply of H_2S (Tunnicliffe and Juniper, 1990). The data presented here show significant differences in the mean individual percentage of trophosome between species from hydrothermal vents and cold seeps, with cold seep species showing greater values than hydrothermal vent species. Because cold seep species obtain sulphide across buried extensions of their tube (Julian et al., 1999; Freytag et al., 2001) and not across their plume, they have access to more constant flux of sulphide than species from vents, which can explain the differences in the percentage of trophosome between the species from the two habitats.

Species from hydrothermal vents have higher percentage of somatic tissue than species from cold seeps, which, together with the differences in the growth rates (Lutz et al., 1994; Fisher et al., 1997; Bergquist et al., 2000), is an indication that species from hydrothermal vents allocate relatively more energy to growth than species from cold seeps.

It has been hypothesised that, in an unpredictable environment, selection favours early maturity, high fecundity and large reproductive effort (Grassle and Grassle, 1974). Desbruyères and Laubier (1983) suggested that vent fauna could be expected to display all these life-history characteristics, thus it would be reasonable to expect higher gonadosomatic indices in the species from hydrothermal vents than in those from cold seeps. However, because the percentage of trophosome does not vary between species from hydrothermal vents, and because *Riftia pachyptila* from hydrothermal vents on the East Pacific Rise, *Lamellibrachia luymesii* and *Seepiophila jonesi* from cold seeps in the Gulf of Mexico show

similar gonadosomatic index it seems reasonable to infer that the reproductive condition in vestimentiferans is independent of the surrounding environmental conditions.

The above lines of evidence are supported by the results obtained within species.

Ridgeia piscesae

Several morphotypes of *Ridgeia piscesae* are known to live in the Juan de Fuca Ridge (Southward et al., 1995, 1996; Black et al., 1997). In the present study two different morphotypes were used: the “long-skinny” morphotype collected in Bastille and Easter Island E1, and the “short-fat” morphotype collected in Clam Bed and Easter Island t#15. Because of that, the differences found in the vestimentum width between samples were expected. Previous studies suggested that the occurrence of different morphotypes is correlated with vent-fluid conditions (Tunnicliffe and Juniper, 1990; Sarrazin et al., 1997). While the “long-skinny” morphotype is associated with sources of diffuse flow, the “short-fat” morphotype grows on high temperature chimneys (Urcuyo et al., 1998). The data presented show that the “short-fat” morphotype have a greater percentage of trophosome than the “long-skinny” growth form, which is in accordance with the studies cited above.

Specimens from one of the samples of the “short-fat” morphotype were found to have greater gonadosomatic index than those from one of the samples of the “long-skinny” morphotype. However, the gonadosomatic index is not correlated with the percentage of trophosome, nor with the percentage of somatic tissue, and only a very weak correlation was found between the former and the vestimentum width.

It has been observed that growth in *Ridgeia piscesae* occurs as “episodic spurts” followed by “resting periods” (Urcuyo et al., 1998). Thus, it may be suggested that this species reproduces between the periods of growth and that the reproductive effort may account, in part, for the variable growth rates observed in *Ridgeia piscesae* (Tunnicliffe, 1990; Urcuyo et al., 1998).

Riftia pachyptila

The lower mean individual percentage of trophosome found in the sample from Biovent may indicate a reduction of fluid flow at this site leading to the atrophy of the trophosome. DeBevoise and Taghon (1988) used ratios of RNA and DNA to investigate variations in vestimentiferan growth rates over small spatial scales (2–50 m). While the DNA content

of an individual is essentially constant, the RNA content in a variety of species is positively correlated with growth rate. Despite large within-site variation in RNA:DNA ratios, there are significant between-site differences attributed to differences in the quality of the environment (DeBevoise and Taghon, 1988).

In this case, although both samples have the same vestimentum width, it is possible to assume that the individuals from Biovent are older, and that their growth has been slowed down by the reduction of fluid flow. Because vestimentiferans strictly depend on the trophosome for nourishment, it seems reasonable to assume that maintenance of the trophosome is more important for the animal survival than growth, and that the immediate response to a decrease in the fluid flow is a reduction of the growth. A reduction in the percentage of trophosome does not occur until the animal is subject to a low level of sulphide for a prolonged time.

Since no difference was found in the gonadosomatic index of the two samples it seems reasonable to assume that although the metabolic effort towards maintenance and growth depends on the environmental conditions, the reproductive condition does not change at least until the animal is subject to a very prolonged reduction or total lack of sulphide.

Lamellibrachia luymeri

Growth and condition of vestimentiferans from the Gulf of the Mexico have been found to vary on multiple spatial scales including between sites separated by 70 km, between aggregations separated by tens to hundreds of meters within a site and between areas separated by tens of centimeters within a single aggregation (Bergquist et al., 2003a). Such multi-scale environmental heterogeneity is also reflected in the highly variable vestimentiferan abundances found among different aggregations (Bergquist et al., 2002). The strong seepage activity in GC234 supports one of the largest communities of vestimentiferans in the Gulf of Mexico. Around the Brine Pool, seepage is weaker and only small clusters of vestimentiferans are found (see Chapter 2). Although within-site variation is found, specimens from GC234 present greater percentage of trophosome than those from Brine Pool, which might reflect the differences in the fluid quantity and quality. Size effects can be discounted because although specimens from BP-2 were smaller than those from the rest of the samples, the percentage of trophosome between BP-2 and BP-1 is not different and, more importantly, only a very weak relationship was found between the two variables.

The gonadosomatic index is not significantly different between the two sites. There is no

within-site variation and between-site variation was only found between one sample of each site. Only weak correlations were found between the gonadosomatic index and the three other studied variables, which suggests that the reproductive condition of *Lamellibrachia luymesii* is independent of both growth and proportion of other tissues.

Bergquist et al. (2003a) found that juvenile *Lamellibrachia luymesii* have greater growth rates than adult specimens. It would be expected that if a greater metabolic effort is put into growth, maintenance and/or reproduction would be sacrificed. In this study no negative consistent relationship between vestimentum width and any of the other three variables was found. Thus, it may be suggested that the samples were composed mostly by adult individuals.

Because the aggregations where the samples were taken from were not marked it cannot be guaranteed that the different samples from the same site were from the same aggregation. For this reason this study does not take in account temporal variation. The very long lifespan of seep vestimentiferans will ultimately require the use of long-term, repeated monitoring to understand the response of vestimentiferans to environmental variability. However, with the data presented here it seems reasonable to assume that, from the four studied variables, the gonadosomatic index is the least sensitive to changes in the environment conditions.

4.4.3 Summary

Deep-sea hydrothermal vents are generally unstable environments, the physiology and biochemistry of the vent organisms being adapted not only to extreme conditions of the venting fluids and ambient seawater, but also to rapid fluctuations between these extremes. Theories of habitat stability predict that vent organisms are highly opportunistic, with high recruitment numbers, rapid growth, early reproduction and high adult mortality (Tunnicliffe and Juniper, 1990). Vestimentiferans from hydrothermal vents have been shown to exhibit great capacities of colonisation (Southward et al., 1996; Shank et al., 1998) and it has been suggested that a pool of larvae is present in the water overlying the ridge, ready to settle when they reach a suitable site (Southward et al., 1996).

On the other hand, a slow growth rate and a long life span are characteristic of vestimentiferan species from cold seeps (Fisher et al., 1997; Bergquist et al., 2000). Bergquist et al. (2002) suggested that this long life span is a consequence of the recruitment dynamic of these species. Combined effects of spatial limitation and temporal constraint of vestimen-

tiferan settlement sites in cold seeps would tend to increase juvenile mortality rates relative to adults (Bergquist et al., 2002). Such forces would favour those individuals that can effectively capture resources and hold them for long periods of time over which reproduction may occur many times.

The physical and chemical conditions of the two different ecosystems in which vestimentiferans occur are reflected not only in the growth rates of the species inhabiting in them, but also in the proportion of trophosome and other somatic tissues. Vestimentiferans from hydrothermal vents show significantly less percentage of trophosome than species from cold seeps suggesting a greater metabolic effort towards growth in hydrothermal vent species and a greater metabolic effort towards maintenance in species from cold seeps. The reproductive condition was found to be independent of both trophosomal proportion and growth, suggesting that reproduction is the primary function of vestimentiferan species from cold seeps and hydrothermal vents.

Colonization of new available seep and vent habitats is done by a non-feeding trochophore larvae that disperse in the plankton for up to several weeks (Young et al., 1996; Marsh et al., 2001). Larvae are supplied by unbiased sex ratio populations of vestimentiferans exhibiting continuous spawning and lacking synchrony in the spawning periods of the individuals. Recruitment is discontinuous and depends on the environmental factor found by the larvae while in the water column and at the time of settlement.

Due to sperm storage in the female reproductive tract, the high rates of fertilization observed in vestimentiferans (Hilário et al., 2005) can be achieved with relatively little female investment. The higher gonadosomatic indices found in males from both hydrothermal vents and cold seeps may be a result of sperm competition.

Chapter 5

Lipid partitioning in *Seepiophila jonesi* and *Lamellibrachia luymesii*

5.1 Introduction

5.1.1 An introduction to lipids

Lipids occur throughout the living world, from microorganisms to higher plants and animals. They are biological molecules that are soluble in organic solvents and have four general biological functions: 1) they are structural elements of cell membranes, 2) being highly reduced forms of carbon they form metabolic energy stores, 3) lipids are derivatives of some vitamins and hormones, and 4) they form bile acids (Berg et al., 2002). The building blocks of lipids are fatty acids.

Fatty acids have the general formula $\text{CH}_3(\text{CH}_2)_n\text{COOH}$. The CH_3 ‘end’ of the molecule is known as the methyl terminus and the COOH grouping as the carboxyl terminus. The simplest fatty acids are referred to as saturated fatty acids. They have no unsaturated bonds and cannot be altered by hydrogenation or halogenation. When double bonds are present, fatty acids are said to be unsaturated; monounsaturated (MUFA) if only one double bond is present, and polyunsaturated (PUFA) if they have two or more double bonds generally separated by a single methylene group (methylene-interrupted fatty acids). The length of the carbon chain and the position of the double bonds vary between fatty acids and is used in their nomenclature.

The conventional notation a:b(n-c), where “a” is the carbon chain length, “b” is the total number of double bonds in the chain, “n” signifies the number of carbon atoms, “c” from the

methyl terminus, gives a full description of the chemical structure of the fatty acid. There are alternative methods of notation whereby the double bond position is given starting from the carboxyl terminus. This situation is denoted by the symbol Δ , so 18:1(n-7), for example, would be 18:1 Δ 11. In this notation all double bonds positions are given and it is generally used to denote fatty acids where double bonds are not methylene interrupted (NMID fatty acids, e.g. 20:2 Δ 5,11). The physico-chemical properties of fatty acids, and of lipids derived from them, are markedly dependent on chain length and degree of saturation. Short and unsaturated fatty acids have significantly lower melting points than longer, saturated fatty acids, a characteristic that may be relevant in invertebrates living under hydrothermal conditions. Fatty acids are generally found esterified to other molecules. Where elevated levels of free fatty acids are reported in tissues, they are usually artifacts from cell damage and associated lipase activity.

Fatty acids are either obtained from the diet or synthesised *de novo* from carbohydrates or amino-acids. Both breakdown and synthesis of fatty acids occurs in steps of loss/addition of two carbon units. For these reasons, fatty acids in biological systems usually contain an even numbers of carbon atoms, typically between 14 and 24. The general pathway of *de novo* synthesis of fatty acids is similar for all organisms. Although individual fatty acid synthetases vary greatly in structure, their metabolism function is broadly the same. For a detailed description of fatty acid synthesis/degradation see Berg et al. (2002).

Lipids are conventionally separated into two groups, polar or neutral lipids, depending on their solubility in organic solvents. Structural lipids are polar while storage lipids are neutral. The fatty acid composition of storage lipids is more variable than that of structural lipids reflecting, largely, the composition of the diet.

Structural lipids

Structural lipids, or membrane lipids must act as a barrier between two environments and be able to exclude water. A lipid molecule combining a hydrophilic group and a hydrophobic moiety is ideally suited to this. The three major kinds of membrane lipids are phospholipids, glycolipids and sterols.

Membrane lipids need to be sufficiently fluid to allow transport of ions and small molecules. Membrane fluidity is controlled to a large extent by fatty acid composition. Saturated fatty acyl residues have straight chains that fit together favouring a rigid state at high pressures and low temperatures. Unsaturated fatty acids with *cis* double bonds have a bend in their

chains, interfering with the ordered packing of fatty acyl moieties and thereby increasing the membrane fluidity (Berg et al., 2002).

Energy storage lipids

Lipids are much more efficient energy stores than the highly hydrated proteins and carbohydrates, because they are much reduced and, being non-polar are stored in an almost anhydrous form, which obviates the expenditure of energy on osmoregulation that would be required with carbohydrates or hydrate proteins.

Triacylglycerols are the major long-term energy storage lipid in terrestrial organisms. In marine organisms, however, alkildiacylglycerols and cholesteryl and wax esters are also important food stores (Sargent and Whittle, 1981). Triacylglycerols and alkildiacylglycerols are derived from glycerol esterified or ether-linked to fatty acids or alcohols. Cholesteryl and wax esters comprise a sterol or a fatty alcohol molecule esterified to a fatty acid. Storage lipids are broken down first by enzyme hydrolysis by lipases to release free fatty acids. The β -oxidation of fatty acids yields large amounts of energy in the form of ATP.

Adaptations of lipids in deep-sea organisms

The low temperatures and high pressures experienced by marine organisms in the deep-sea require adaptations of their lipids in order that they function normally. The maintenance of membrane fluidity is a good example of this; at high pressures and low temperatures lipid membranes containing saturated fatty acids become less fluid as a result of their higher melting points and more ordered packing.

Deep-sea prokaryotes are able to vary the chain length and saturation of their fatty acid components to accommodate high pressures and lower temperatures in order to maintain membrane fluidity. It has also been shown that some species of the *Vibrio* genus alter their PUFA levels with environmental conditions: increasing PUFA with decreasing temperature (Delong and Yayanos, 1986). Fish and other deep-sea organisms are adapted to incorporate dietary PUFA into their membranes (Bell et al., 1986).

Besides being excellent energy storage molecules, neutral lipids such as triglycerides, and especially wax esters, are less dense than water and may be used by benthic organisms with lecithotrophic development to aid dispersal. Macrourid fish have been shown to incorporate an oil globule into their eggs that makes them buoyant and allows wide dispersal (Merrett,

1986).

5.1.2 Lipid composition of hydrothermal vent and cold seep fauna

Fatty acids compositions have considerable potential for use as biomarkers, since they can have characteristic distributions in nature. As a general rule, major classes of natural lipids can be broadly divided into bacterial and eukaryotic types; bacterial lipids have monounsaturated fatty acids and short carbon chains, while eukaryotic lipids have polyunsaturated fatty acids and long carbon chains. Fatty acid compositions are also used to evaluate the role of phytodetritus in vent ecosystems, since plant and plant-derived fatty acids are assumed to be distinctive, having long carbon chains (typically $n = 20, 22$) and being polyunsaturated (5 or 6 double bonds), with double bonds at the $n-3$ or $n-6$ positions.

The majority of the studies of the lipid composition of hydrothermal vent and cold seep fauna is concerned with determining the quantitative significance of photosynthetically-derived material for the nutrition versus that of bacterial primary production (Pond et al., 1997b,c, 2000a,b, 2002; MacAvoy et al., 2002).

Lipid characteristics of vent organisms are determined empirically, and patterns of distribution of various lipid classes are interpreted based on knowledge of the natural history of the animal. Vestimentiferans, that depend exclusively on symbiotic bacteria for their nutrition, have high abundance of $n-7$ monounsaturated fatty acids (characteristic of bacterial lipids) in both polar and neutral lipids (Rieley et al., 1995). Small amounts of NMID fatty acids are present and only trace amounts of branched fatty acids are present. Long-chain polyunsaturated fatty acids, characteristic of marine photosynthetic food chains, are entirely absent in vestimentiferan tissues. By contrast, the same authors found that mussels that rely on some combination of symbiont nutrition and filter-feeding have large concentrations of NMID fatty acids. These NMID fatty acids apparently replace single-methylene-interrupted polyunsaturated (SMIP) fatty acids (derived from marine algae) that are observed in similarly large abundances in coastal and estuarine bivalves (Rieley et al., 1995). Predominance of NMID fatty acids has also been reported in mussels from cold seeps (Fang et al., 1993). As in tubeworms, monounsaturated fatty acids with $n-7$ double bonds are abundant in mollusks dependent on bacterial endosymbionts for their nutrition.

The ‘essential fatty acids’ which, in terrestrial animals are, $18:2:n-6$ and $18:3n-3$ originate in photosynthetic organisms, mainly plants and algae, and cannot be formed *de novo* by

most animals, which lack the $\Delta 12$ and $\Delta 15$ fatty acids desaturases necessary for their biosynthesis from 18:1n-9 (see review by Sargent et al. (1995), on which the following is based). Most terrestrial animals have an absolute dietary requirement for these PUFAs to enable them to form 20:4n-6, 20:5n-3 and 22:6n-3, which have essential and fundamental roles in animal cell membrane structure and function. Most terrestrial animals can convert 18:3n3 to 20:5n-3 and thence to 22:6n-3, and they can also convert 18:2n-6 to 20:4n-6 by a series of linked fatty acid desaturation and chain-elongation reactions which are complex, particularly so for the formation of 22:6n-3. Freshwater animals are generally similar to terrestrial animals in that they too can convert dietary 18:3n-3 to 20:5n-3, and subsequently to 22:6n-3. They can also convert 18:2n-6 to 20:4n-6. However, marine animals can only convert dietary C18 PUFAs to C20 and thence to C22 PUFAs to a strictly limited extent, if at all. This probably reflects the luxury in marine animal diets of 20:5n-3 and 22:6n-3, which are formed abundantly at the base of the marine food chain by photosynthetic, single-cell eukaryotes, mainly diatoms rich in 20:5n-3 and a range of flagellates rich in 22:6n-3.

20:4n-6, 20:5n-3 and 22:6n-3 are all present in hydrothermal vents (Fullarton et al., 1995; Allen, 1998; Allen et al., 1998; Pond et al., 2002), in which it is presumed they have the same roles in cellular membrane structure and function as in other marine animals. Alvinocaridid shrimps from hydrothermal sites along the Mid-Atlantic Ridge assimilate substantial amounts of photosynthetically derived 20:5n-3 and, specially 22:6n-3 during their dispersive planktotrophic larval phase (Pond et al., 2000a). However, in other vent invertebrates, including vestimentiferans the size difference between the first stage settled larvae and the adults implies that careful conservation of PUFA would be necessary, so an input of photosynthetic PUFAs by this route is excluded (Pond et al., 2002). Although other processes have been suggested, the source of PUFAs in hydrothermal vent animals from Pacific vent sites is still unknown (Pond et al., 2002). Moreover, in the case of vestimentiferans which are non-motile and have a poorly developed neural system, it is conceivable that vestimentiferans would have a reduced requirement for (n-3) and (n-6)PUFA. The requirement for these fatty acids may be for reproduction, especially the production of motile sperm (Fullarton et al., 1995).

The ratio of neutral glycolipids to polar phospholipids can serve as an index of physiological condition in vent invertebrates. Pranal et al. (1997), in a comparative study of a littoral mussel and two species of bathymodiolid mussels from Lau and N-Fiji back-arc basin hydrothermal vents showed that the levels of neutral lipids were higher in the gills of vent mussels and suggested that this reflects the important metabolic role of gills in

symbiotic mussels. Neutral lipid (and hence stored energy) levels were higher in all vent mussel tissues and it was proposed that this might allow the animals to withstand periods of hydrothermal quiescence. Consequently, the ratio of triglyceride to phospholipid was used as a stress index to compare the condition of mussels from different vent sites: low values were indicative of poor condition of mussels and correlated with low bacterial biomass estimations.

Pond et al. (1997a) and Allen et al. (2001) reported the presence of high concentrations of wax esters in postlarvae of alvinocaridid shrimps collected in the water column above hydrothermal vents on the Mid-Atlantic Ridge. Wax ester reserves are characteristic of organisms that inhabit environments subject to large fluctuations in food availability, enabling the organisms to survive long intervals without feeding (Lee and Hirota, 1973). Planktotrophic larval vent shrimp thus benefit from large lipid reserves, allowing the potential to disperse long distances in food-poor environment of the non-vent deep sea.

5.1.3 The present study

Previous studies of the lipid class composition have shown differences in the composition of anterior (obturaculum and vestimentum) and posterior (trunk and opisthosome) regions of *Ridgeia piscesae* (Allen, 1998). Higher concentrations of wax esters and triacylglycerols were found in the posterior region (Allen, 1998), and wax ester-rich eggs have been reported in *Lamellibrachia luymesii* (Young et al., 1996) and *Riftia pachyptila* (Marsh et al., 2001), suggesting that vestimentiferans store energy storage lipids in the gonads. However, because the gonadal tissue has never been dissected and compared with the other tissues of the trunk it was not possible to determine whether the gonad comprises the only lipid store in vestimentiferans, or if different lipid classes are stored in different tissues.

The work presented here examines the lipid composition of the trunk of adult *Seepiophila jonesi* and *Lamellibrachia luymesii* in order to address the following questions: (1) Are there differences in the partitioning of lipids between the gonad and other tissues of the trunk of vestimentiferans? (2) Does lipid composition vary with the amount of gonad? (3) If it does, is it possible to establish a chemical method to determine the reproductive condition in this taxa?

5.2 Material and Methods

Samples of *Lamellibrachia luymesii* and *Seepiophila jonesi* were collected in GC234 and Brine Pool, respectively, in July of 2004 using the submersible *Johnson Sea-Link II* (Table 5.1). On reaching the surface the worms were removed from their tubes. Males were distinguished from

females by the presence of a pair of well-developed ciliated grooves running the entire length of the dorsal face of the vestimental region (van der Land and Nørrevang, 1977; Gardiner and Jones, 1993). The vestimentum width of each female was measured with a calliper to the nearest 0.1mm as an independent variable of growth, and the first centimetre of the trunk, not underlying the vestimental wings, was cut and stored at -20°C . On land the samples were stored at -80°C until further analysis.

In the laboratory, while the samples were still frozen, the ovary was separated from the other tissues. Dry weight of both components was measured after lyophilization for 24h. The samples were then homogenized in chloroform-methanol (2:1, vol/vol) before being filtered through a prewashed (chloroform-methanol [2:1, vol/vol]) Whatman no. 1 paper filter. Total lipid was extracted by following the method of Folch et al. (1957) and dried under nitrogen. Total lipid was weighed and dissolved in 5 ml of chloroform.

The lipid class composition was determined for the ovary and the other tissues separately and for the whole trunk by combining the ovary and the other tissues. In the first case 600 μl of the total lipid solution were dried under nitrogen in a pre-weighed vial and then dissolved in chloroform to a concentration of 10 $\text{mg}\cdot\text{ml}^{-1}$. To determine the lipid class composition of the whole trunk the same procedure was followed combining 300 μl of both ovary and other tissues total lipid solutions.

Separation of lipid classes was performed by thin layer chromatography. The samples were applied as discrete spots 1 cm from the bottom of silicagel plates that were previously washed in the eluent. The plates were developed in Hexane-Diethyl ether-Acetic acid (90:10:1 by vol.) until the solvent front was about 1.5 cm from the top, and then dried at room temperature in a vacuum desiccator. These proportions of solvents were chosen because they permitted the best resolution between the wax esters and the solvent front.

When dried, the plates were sprayed with a solution of 3% (w/v) copper acetate in 8%

Table 5.1: Sampling sites and sample sizes of *Lamellibrachia luymesii* and *Seepiophila jonesi*.

Species	Site	Sample size
<i>S. jonesi</i>	Brine Pool	14
<i>L. luymesii</i>	GC234	6

(v/v) orthophosphoric acid and dried in a vacuum desiccator. The lipids were made visible as black deposits by heating the plates at 160°C for 12 minutes.

Lipid classes were quantified by photodensitometry with a “Shimadzu Dual-Wavelength Thin-Layer Chromato Scanner CS-930” using a cod roe homogenate as standard.

5.3 Results

In *Seepiophila jonesi*, the percentage of ovary of dry weight of the worm ranged from 1.2% and 49.2%, with an average of 25.6%. The amount of ovary was not found to be correlated with the vestimentum width ($r = 0.142$; $P = 0.627$; $n = 14$).

Table 5.2 shows the mean and standard deviation of the concentration of total lipids (mg.mg^{-1} of dry weight) in the different tissues of female *Seepiophila jonesi*. Results of a Mann-Whitney test performed to investigate the differences in the concentration of total lipids between the ovary and the other tissues of the trunk indicate that the difference in the mean values is

greater than it would be expected by chance ($T = 40.000$; $P = 0.008$, $\alpha = 0.05$). These results suggest a positive relationship between the amount of ovary and the concentration of total lipid in the individual. The results of a Spearman correlation procedure show that the two variables are positively correlated ($r = 0.815$; $P < 0.050$) (Figure 5.1).

Table 5.2: Mean and standard deviation of the concentration (mg.mg^{-1}) of total lipids in the different tissues of *Seepiophila jonesi*.

Tissue	Total Lipids (mg.mg^{-1})
Ovary	0.360 ± 0.022
Other Tissues	0.220 ± 0.082
Whole Trunk	0.224 ± 0.050

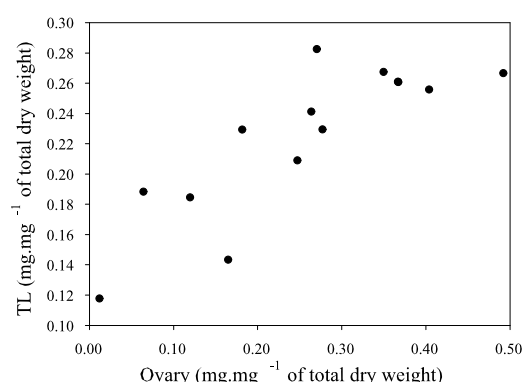


Figure 5.1: Amount of ovary against total lipids (TL) concentration in the trunk of *Seepiophila jonesi*.

Charred TLC plates and corresponding chromatograms show seven different lipid classes (Figures 5.2 and 5.3). Polar lipids (PL), triacylglycerols (TAG) and wax esters (WE), which are presented at relatively high concentrations in all the tissues, can be considered major components and will be treated individually. Sterols (St), free fatty acids (FFA) and two unidentified groups (Und1 and Und2) are present in very low concentrations and will be pooled together (Table 5.3 and Figure 5.4).

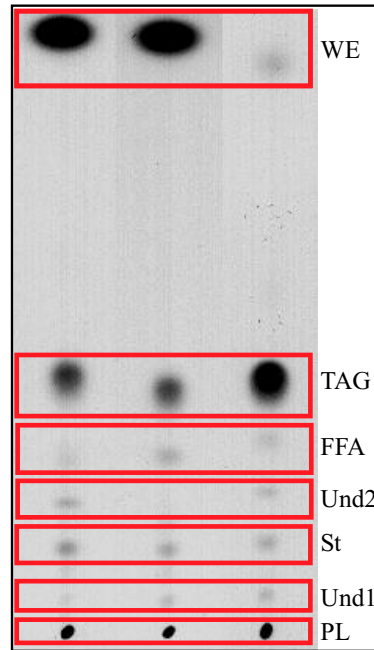


Figure 5.2: Chromatography bands of (from left to right) whole trunk, ovary, and other tissues of female *Seepiophila jonesi*. (FFA) Free fatty acids; (PL) Polar lipids; (St) Sterols; (TAG) Triacylglycerols; (Und1) Unidentified group; (Und2) Unidentified group; (WE) Wax esters.

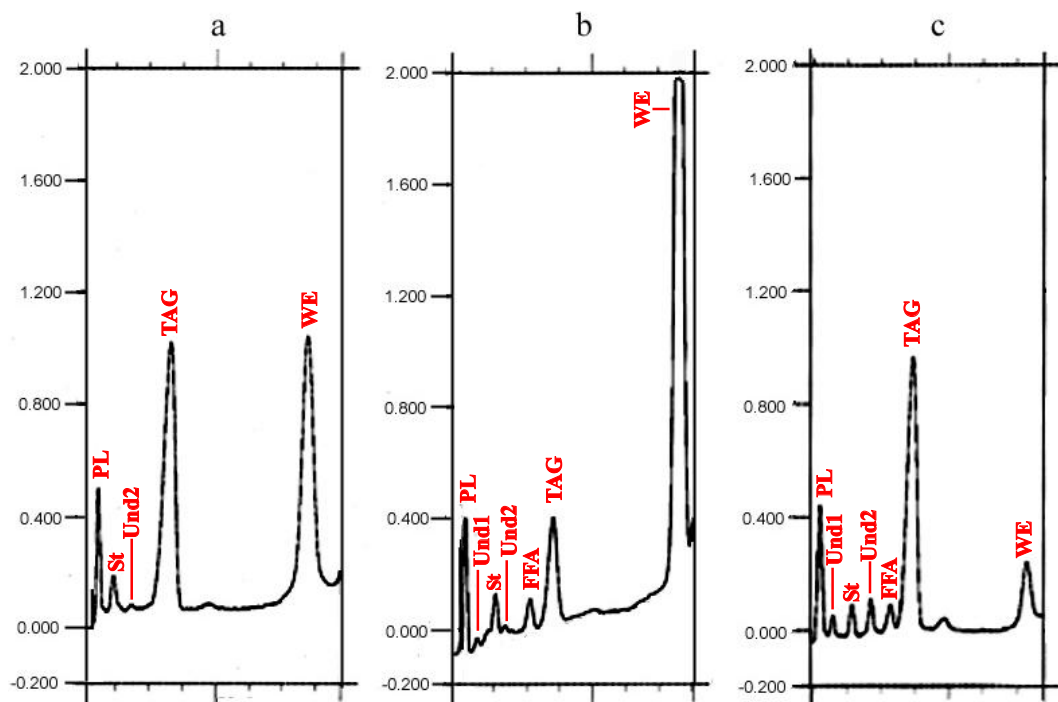


Figure 5.3: Chromatograms of (a) whole trunk , (b) ovary and (c) other tissues of female *Seepiophila jonesi*. (FFA) Free fatty acids; (PL) Polar lipids; (St) Sterols; (TAG) Triacylglycerols; (Und1) Unidentified group; (Und2) Unidentified group; (WE) Wax esters.

Figure 5.4 and Table 5.3 suggest that the ovary presents higher concentration of wax esters and lower concentration of triacylglycerols than the other tissues. The results of a Mann-Whitney test used to compare the two tissues show that the differences are statistically significant (WE: $T = 40.000$; $P = 0.008$, $\alpha = 0.05$; TAG: $T = 40.000$; $P = 0.008$, $\alpha = 0.05$). Both the concentration of wax esters and the concentration of triacylglycerols were found to be linearly related

with the amount of ovary in the worm (WE: $F = 127.27$, $P < 0.001$, $n = 14$; $\alpha = 0.05$; TAG: $F = 30.916$, $P < 0.0001$, $n = 14$; $\alpha = 0.05$; Figure 5.5). From the two variables, the percentage of wax esters is the one that better predicts the amount of gonad in female *Seepiophila jonesi*.

Table 5.3: Mean and standard deviation of the different lipid class concentration ($\text{mg} \cdot \text{mg}^{-1}$ of dry weight) in *Seepiophila jonesi*.

Lipid Class:	Polar lipids	Triacylglycerols	Wax esters	Other
Ovary	0.016 ± 0.002	0.053 ± 0.009	0.258 ± 0.018	0.029 ± 0.015
Other Tissues	0.019 ± 0.008	0.135 ± 0.056	0.035 ± 0.013	0.020 ± 0.009
Whole Trunk	0.019 ± 0.003	0.060 ± 0.034	0.112 ± 0.033	0.027 ± 0.013

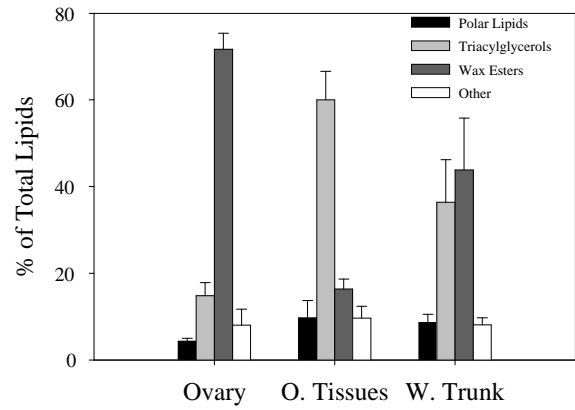


Figure 5.4: Mean and standard deviation of the different lipid classes in female *Seepiophila jonesi* (% of total lipid).

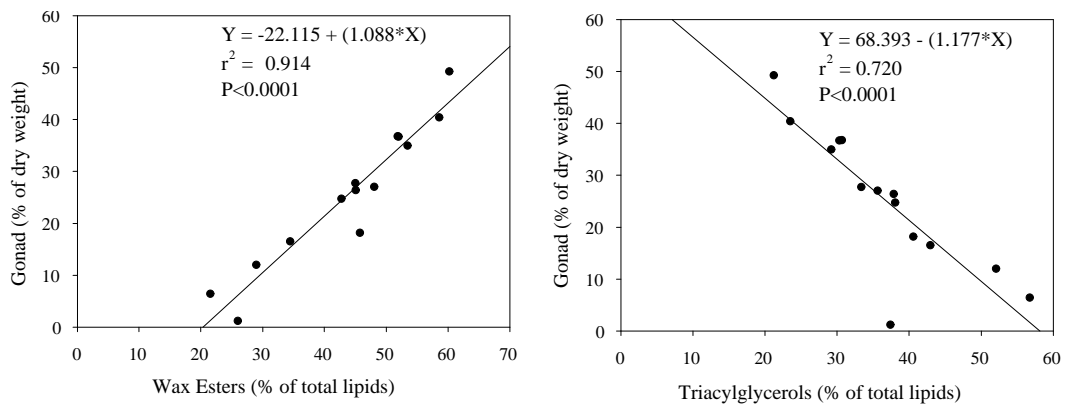


Figure 5.5: Relationship between the amount of gonad and the percentage of wax esters and triacylglycerols in female *Seepiophila jonesi*.

No gonadal tissue was found in any of the individuals of *Lamellibrachia luymesii* dissected. Table 5.4 shows the average and standard deviation of the concentration (mg.mg^{-1} of total dry weight) of total lipids and different lipid classes in female *Lamellibrachia luymesii* and female *Seepiophila jonesi* after dissection of the ovary. The results of a Mann-Whitney test performed to investigate the differences in the concentration of total lipids show that there is no significant difference between the two species ($T = 39.000$; $P = 0.126$). However, on the lipid class level, statistically significant differences were found between the concentration of triacylglycerols ($T = 43.000$; $P = 0.017$) and “other groups” ($T = 18.000$; $P = 0.030$).

Table 5.4: Average and standard deviation of concentration (% of total lipid and mg.mg^{-1} of total dry weight) of total lipid and different lipid classes in the whole trunk of *Lamellibrachia luymesii* and in the “other tissues” of *Seepiophila jonesi*.

Species	Total lipids	Polar lipids	Triacylglycerols	Wax esters	Other
<i>L. luymesii</i>	0.107 ± 0.012	0.018 ± 0.007	0.036 ± 0.006	0.018 ± 0.009	0.032 ± 0.0047
<i>S. jonesi</i>	0.220 ± 0.082	0.019 ± 0.008	0.135 ± 0.056	0.035 ± 0.013	0.020 ± 0.009

5.4 Discussion and Summary

5.4.1 A new method to measure the reproductive condition of female vestimentiferans

The reproductive system of female vestimentiferans consists of a paired system of ducts surrounded by trophosome extending posteriorly from the anterior part of the trunk. Some species have an ovisac situated on the portion of the trunk underlying the vestimentum, and in all species a sperm storage organ, the spermatheca, can be found at the far posterior end of the reproductive tract (Chapter 3). A longitudinal strip of germinal epithelium is present throughout the length of the gonocoels, which run parallel to the oviducts, thus, except at the ovisac and spermatheca, the composition of gonadal tissue is constant through all the gonad length. Because dissection of the entire gonad from trophosome is extremely difficult in all species of vestimentiferans we suggest that the amount of ovary found in the first centimetre of the trunk not underlying the vestimental wings can be used as representative of the reproductive condition of the individual.

The results presented here show a linear relationship between the amount of gonad and the concentration of wax esters in the trunk of female *Seepiophila jonesi*. This relationship, although expected, has never been demonstrated in any species of vestimentiferan before.

The equation

$$G = -22.125 + 1.088WE$$

where G is the amount of gonad (% of total dry weight) and WE the concentration of wax esters (% of total lipids) is specific for *Seepiophila jonesi* and different terms are expected for different species. However, the study of the lipid class composition in general, and the determination of the concentration of wax esters in the trunk in particular, should be considered as a new method to determine the reproductive condition in this taxa.

To date there were no studies on the reproductive condition of vestimentiferans, in part because of sampling constraints inherent to hydrothermal vents and cold seeps (Tyler and Young, 1999) and in part due to the lack of a simple approach to it. In comparison with the image analysis of histological sections of the trunk, the determination of the lipid class composition is much less time consuming both on the initial preparation of the samples on board of the ship and in the laboratory procedures.

5.4.2 Lipid storage and its ecological implications

The total lipid concentrations of the trunk of *Seepiophila jonesi* and *Lamellibrachia luymesii* were considerably higher than those found for *Ridgeia piscesae* from Juan de Fuca Ridge (Fullarton et al., 1995; Allen, 1998) and *Riftia pachyptila* (Rieley et al., 1995). While in the present study only females were considered, Rieley et al. (1995) and Allen (1998) do not differentiate between male and female individuals, which may explain the differences found. Fullarton et al. (1995) studied the trunk and vestimentum of juvenile individuals, also without differentiating between male and female, and of one adult female.

In the present study, free fatty acids comprised less than 10% of total lipid in all tissues, suggesting that samples were in good state of preservation (Jeckel et al., 1989).

The results presented here show that female *Seepiophila jonesi* store triacylglycerols in the trunk, in other tissue than the ovary, and that wax esters are stored in the gonad. In all species of vestimentiferans, the cavity of the trunk houses the reproductive system and the trophosome, which comprises four different types of cells: (1) bacteriocytes, that are densely packed with bacteria; (2) unspecialised non-bacteriocytes; (3) muscle cells; (4) peritoneal cells (Gardiner and Jones, 1993) and *in toto* the trophosome comprises about 15% of the biomass of the organism (Cavanaugh et al., 1981) but no lipid accumulations have been described in any of the cell types of the trophosome (Gardiner and Jones, 1993). Although prokaryotic organisms usually do not accumulate triacylglycerols, under certain

conditions, marine bacteria have been shown to store lipids in the form of triacylglycerols (Alvarez et al., 1997), so it is conceivable that the triacylglycerols found in the trunk of vestimentiferans represent lipids from bacterial endosymbionts.

The storage of lipids may support the survival of the bacteria under adverse conditions and enable them to respond rapidly when favourable conditions are restored (Dawes, 1990), an adaptation that would be most favourable in the extremely unstable environment of hydrothermal vents (Van Dover, 2000) and in the highly variable microhabitats found within vestimentiferans aggregations in cold seeps (Bergquist et al., 2003b).

The fact that no gonads were found in any of the specimens of *Lamellibrachia luymesii* studied is reflected in the small concentrations of wax esters. The major difference found between the two species were in the amount of triacylglycerols and other lipid classes, including sterols, free fatty acids and two undetermined groups. Low concentrations of triacylglycerols may be a result of a decrease in the nutrient delivery to the bacteria, leading to the consumption of the storages of triacylglycerols.

Larvae of hydrothermal vent fauna are known to colonize rapidly new vent sites separated by tens to hundreds of kilometres (Lutz et al., 1994; Mullineaux et al., 2000) however, the mechanisms by which these larvae disperse and recruit are not fully understood. Larval lifespan is determined in part by the rate of energy used during development and the initial amount of energy available in the egg.

In vestimentiferans, embryos and lecithotrophic larvae are thought to disperse in the plankton for up to several weeks (Young et al., 1996; Marsh et al., 2001; Hilário et al., 2005). It has been suggested that the cold ambient bottom waters associated with the deep ocean (2 to 3°C) could lower metabolic and developmental rates, enabling the pelagic larvae of vent organisms to remain in the plankton for prolonged periods (Lutz et al., 1984; Marsh et al., 2001; Pradillon et al., 2001). However, the presence of wax esters (used exclusively for energy storage in cold-water invertebrates (Hagen and Kattner, 1998; Kattner and Hagen, 1998; Evanson et al., 2000)) is a recognised adaptation to environments subject to marked fluctuations in food availability and enable organisms to survive considerable periods without feeding (Lee and Hirota, 1973). The substantial wax ester reserves observed in the ovary of *Seepiophila jonesi* indicate that embryos and larvae of vestimentiferans are adapted to a pelagic lifestyle, enabling them to maintain a prolonged planktrophic existence and thus facilitating widespread dispersal in their search for new vent and seep sites. Indeed, Marsh et al. (2001) have shown that the dispersal potential of *Riftia pachyptila* is

not limited by the physiological performance of the embryos and larvae, but by transport limitations imposed by current regimes.

5.4.3 Summary

Due to the close association between the gonadal tissue and the trophosome, the only way to determine the reproductive condition in vestimentiferan tubeworms has been, to date, the study of histological sections of the trunk of the individual. The results presented establish a biochemical technique to determine the amount of gonadal tissue in the trunk compared to the amount of trophosome, which represent a new method for the determination of the reproductive condition in this group.

Two main lipid storages were found in the trunk of female vestimentiferans. Substantial reserves of wax esters are found in the gonad, and triacylglycerols in other tissues of the trunk. More studies are required to determine which cells or tissue contain triacylglycerols. However, it seems conceivable that they represent lipids from bacterial endosymbionts. Previous studies (Alvarez et al., 1997) have shown that under certain conditions marine bacteria can store lipids in the form of triacylglycerols, and that these can support the survival of the bacteria under adverse environmental conditions (Dawes, 1990).

The wax esters found in the gonadal tissue indicate that embryos and larvae of vestimentiferans are well adapted to a pelagic lifestyle that enables them to disperse in the plankton for several weeks (Marsh et al., 2001), facilitating widespread dispersal in their search for new vent and seep sites.

Chapter 6

Summary and Conclusions

With the discovery of hydrothermal vents in the eastern Pacific in the late 1970s (Corliss and Ballard, 1977), came the recognition that complex ecosystems can be supported using energy that is not solar. Geothermal processes supply reduced chemical species that, through microbial mediation, provide chemical energy for production of organic carbon (Jannash, 1995). Dense biomass, unusual symbioses and novel systematic relations were some of the first characteristics of vent communities to capture the attention of biologists (Tunnicliffe et al., 1998). Further exploration to the sea-floor led to the discovery of other reducing environments, such as cold seeps on continental margins, which foster communities that share some characters with hydrothermal vents (Sibuet and Olu, 1998). Because of the difficulty of sampling in the deep sea in general, and at hydrothermal vents and cold seeps in particular, most of the biological processes in these habitats are incompletely or poorly understood.

The full understanding of an ecosystem involves a sound knowledge of the reproductive adaptations of its components (Giangrande et al., 1994) and it was in this context that this project was developed. The present study examined the reproductive ecology of Vestimentifera (Siboglinidae: Polychaeta) from deep-sea hydrothermal vents and cold seeps. The Vestimentifera form some of the most dramatic images of both hydrothermal vent and cold seep environments. Adult worms in this group are characterized by the absence of a functional digestive system. The trunk region of the adult contains the trophosome, that harbours symbiotic bacteria responsible for chemosynthesis, and the gonad, surrounded by lobes of the trophosome.

The first step to a deeper understanding of the reproductive biology of vestimentiferans was the histological examination of the female reproductive tract (Chapter 3). The study

of five different species from hydrothermal vents in the Pacific, and from seeps in the Gulf of Mexico, revealed the presence of a sperm storage region at the posterior end of the reproductive tract. The observation of a spermatheca was difficult to reconcile with the external fertilization scenario assumed for most vestimentiferans species (Cary et al., 1989a; Van Dover, 1994) and *in situ* and *in vitro* fertilization were designed to solve this apparent paradox.

Estimates of dispersal times and distances based on lipid stores, larval metabolism and current speeds suggested that larvae can disperse more than 100km over a 5-week period (Marsh et al., 2001). However, because the site of fertilization remained unresolved for all species of vestimentiferans, the estimates could be as much as 60% too high. *In vitro* fertilization and field experiments demonstrated that insemination is internal and that the dispersal phase of vestimentiferans includes the entire embryonic period (Chapter 3). Although the previous studies of larvae dispersal potential have erroneously assumed external fertilization and embryogenesis, the estimates themselves remain valid, since embryogenesis does not begin until after inseminated oocytes are released into the water column.

Female sperm storage is known to occur in some families of shallow water polychaetes and in two families in deep-sea hydrothermal vents (Jollivet et al., 2000), but prevalence of this feature is poorly known. Sperm storage provides a playing-field for sperm competition, and although its consequences are not fully understood, it allows the assumption of non-random mating between the gametes, which may explain the genetic homogeneity observed in species from hydrothermal vents and cold seeps. Sperm storage, and subsequent sperm competition can also be responsible different sex allocation in males and females (Levitan and Petersen, 1995). In vestimentiferans, image analysis of sections of the trunk showed that males have higher gonodosomatic index than females (Chapter 4).

The dynamics of a population and the biogeography and continuity of a species is highly related to its reproductive pattern. A optimal life-history pattern will optimise lifetime reproductive success by maximizing the allocation of resources to growth, survival and reproduction from birth to death (Ramirez-Llodra, 2002). The total energy an organism can assimilate and allocate is limited, thus the allocation of energy to reproduction is associated with a decrease in somatic investment. Previous studies have shown that the physical and chemical condition of the two different ecosystems in which vestimentiferans occur are reflected in the growth rates of the species inhabiting them (Lutz et al., 1994; Fisher et al., 1997). This study (Chapter 4) presents evidence that species from cold seeps put a greater metabolic effort towards maintenance, whilst species from hydrothermal vents

invest more in growth. The reproductive condition was found to be independent of both trophosome proportion and growth, and no differences were found in the gonadosomatic index between species from vents and seeps, suggesting that the reproductive condition is independent of the surrounding environmental conditions, and that reproduction is the primary function of vestimentiferan species from cold seeps and hydrothermal vents.

Histological examination of the ovary showed that oogenesis is a continuous process in mature individuals, and suggests that vestimentiferans exhibit iteroparity, but lack population synchrony in gonadal development. These concur with what had been found in previous studies of the reproductive biology of *Riftia pachyptila* (Gardiner et al., 1992).

The last stage of this project consisted of developing of a biochemical technique to determine the reproductive condition in female vestimentiferans (Chapter 5). To date, due to the close association between the gonadal tissue and the trophosome, the determination of the reproductive condition has only been possible by means of image analysis of histological sections of the trunk. Although accurate, this method is extremely time consuming both on the initial preparation of the samples on board of the ship, and in the laboratory procedures. The study of the lipid composition of the trunk showed a linear relationship between the amount of ovary and the concentration of wax esters in the trunk. The initial study of the reproductive tract allows one to assume that the amount of ovary found in first centimetre of the trunk not underlying the vestimental wings reflects the reproductive condition of the individual thus, only dissection and analysis of this portion of the trunk is necessary.

In summary, this project presents the most complete study, to date, of the reproductive biology of vestimentiferans from vents and seeps. Classic tools for reproductive analysis, like biometry and histology were complemented with field experiments and biochemical analysis. New anatomical features were discovered; their role in the reproductive ecology in general, and fertilization and dispersal in particular was discussed. Life history patterns and allocation of energy towards reproduction were examined and related to the environmental conditions of deep-sea hydrothermal vents and cold seeps. A new method for the determination of the reproductive condition was established.

In terms of future work, the following points seem worthwhile to pursue as part of further investigations:

- Determine if sperm bundles swim downward as cohesive bundles against the ciliary current that carries oocytes upward in the oviduct, or if they are transported by ciliary action or peristalsis. The injection of a solution of sperm over a clump of vestimentiferans might be a way to approach this problem. If sperm is transported by peristalsis, the females are expected to respond to the presence of sperm by rapid upward movements. This could be done either *in situ* or *in vivo*, the last one being ideal since it would be possible to mark the sperm and follow their progress inside the female reproductive tract.
- Although electron microscopy has shown unequivocally that sperm cells attach to the oocyte in the spermatheca, a more detailed study of the morphology of the egg envelope is required to understand the nature of this bond and its implications in the fertilization success.
- Determine the terms of the linear equation relating the amount of wax esters to the amount of ovary for other species, and develop a similar chemical method for the determination of the reproductive condition of males. This method should be tested and applied, if possible, to other groups of polychaetes.

The total understanding of the response of vestimentiferans to environmental variability will ultimately require the use of long-term, repeated monitoring. As deep-sea technologies develop to meet requirements to measure physico-chemical properties and to assess spatial and temporal variety, scientific understanding will continue to grow.

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