Biotic and Abiotic Interactions of Deep-Sea Hydrothermal Vent-Endemic Fish on the East Pacific Rise

By

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for

Grampy and Karen

who believed that I could do it,

but didn't get to see me finish;

and for

my Family

who just want me to be happy.

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Abstract

A study of the ecology of fish endemic to hydrothermal vents on the East Pacific Rise was undertaken utilizing a variety of techniques, focusing on the bythitid *Thermichthys hollisi*. Stable isotope and gut content analyses were used to elucidate prey choice and trophic relationships. Otolith chemical signatures were investigated to determine whether this technique could be utilized to examine life history strategy and habitat use. Chemical characteristics of preferred fish habitat and gene expression responses habitat chemistry were explored. Gut contents indicated that *T. hollisi* specimens were actively feeding upon a combination of brachyuran crabs, *Alvinocaris* shrimp, polychaetes, and zoarcid fish with the majority of fish containing evidence of crustacean prey. Carbon, nitrogen, and sulfur stable isotopic measurements support a chemosynthetically based prey source and place *T. hollisi* in the uppermost trophic levels of vent communities. The influence of exposure to hydrothermal fluids was apparent in otoliths from both species of vent fish, most noticeably within the relatively elevated Sr:Ca and depleted Mg:Ca ratios. Otolith chemistry suggested that the zoarcid *Thermarces cerberus* experiences greater direct exposure to diffuse fluids than does *T. hollisi*, which is concurrent with apparent habitat preferences. Isotopic patterns across the span of the otolith suggested that *T. cerberus* spends its entire life within the vent system. In contrast, it appeared that *T. hollisi* exists outside of the influence of hydrothermal activity for some early portion of its life-cycle. Time-lapse photography and *in situ* electrochemistry indicated that *T. hollisi* are preferentially utilizing fish holes where there are elevated temperatures and sulfide levels, and variable oxygen levels in comparison to ambient bottom water. A fragment of Cu, Zn superoxide dismutase was successfully amplified from *T. hollisi* mRNA, but there were no differences in expression levels between tissue types or among individuals within the small sample examined. In general, it appears that *T. hollisi* is both influenced by and may exert a greater influence on hydrothermal vent communities to a greater degree than initially hypothesized.

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Chapter One

An Introduction to Hydrothermal Vents and their associated Fish fauna

1.1 Hydrothermal Vents

Deep-sea hydrothermal vents exist throughout the global ocean at tectonically and volcanically-active mid-ocean spreading centers and subduction zones, where nutrient-rich fluids emanate from the seafloor (Figure 1.1). Vent ecosystems occur in a variety of geological settings and support communities based on chemoautotrophic primary production. Vent biological communities were discovered in 1977 at the Galápagos rift, with the discovery of black smokers and their associated fauna on the East Pacific Rise following shortly after (Lonsdale, 1977; Ballard & Grassle, 1979). This discovery changed the face of deep-sea research, leading to a greater understanding of the heat fluxes, element cycling, geology, chemistry, and biology of the deep ocean.

Hydrothermal fluids are generated when seawater percolates down through the porous oceanic crust, is heated by proximity to magma chambers, and subsequently altered through water-rock reactions. The resultant hydrothermal fluids are typically hot, acidic, enriched in sulfides and a variety of other elements and elemental compounds (strontium, cadmium, iron, lithium) and depleted in magnesium (Edmond et al., 1982; Von Damm, 1995). The hot, buoyant fluids rise up through the crust and are discharged through high-temperature direct flow chimneys (Haymon et al., 1993) or can mix with seawater subsurface and trickle out through cracks or porous substrates as diffuse flow (Edmond et al., 1979). The mixing of super-heated hydrothermal fluids with seawater

causes metal sulfides and minerals to precipitate and form the distinctive and picturesque sulfide chimneys that are the conduits of high-temperature fluids. Lower-temperature diluted or diffuse flow fluids maintain a chemical composition distinct from that of seawater. Chemical species such as hydrogen sulfide and methane within these fluids support the chemosynthetic-based biological communities that are found in conjunction with active venting (Jannasch, 1979; Johnson et al., 1988). Though the presence of hydrothermal fluids is necessary for endemic life at vents, prolonged exposure to components of the fluids may simultaneously be stressful or harmful for the vent macrofauna. Numerous studies have been dedicated to studying the biology of various vent fauna and the diverse physiological and ecological adaptations these fauna have to their environment (Childress & Fisher, 1992; Hourdez & Lallier, 2007).

1.2 East Pacific Rise

This thesis focuses on the resident biological communities of the fast-spreading East Pacific Rise (EPR). Basalt-hosted hydrothermal vents such as those on the EPR are characterized by hot (2-400°C), acidic (pH ~2-5.6), heavy metal and sulfide (3-110mmol/kg H_2S) rich fluids, though the actual fluid composition varies both spatially and temporally (Johnson et al., 1988; Van Dover, 2000; Kelley et al., 2002). The greatest abundance and diversity of vent faunal species is located in lower temperature diffuse flow areas. Diffuse flow fields have cooler fluids (usually lower than 30°C), containing lower concentrations of sulfides and metals than direct flow environments such as black smokers (Kelley et al., 2002). Along with sulfides, other key fluid components are

methane (CH_4), hydrogen (H), and metals such as copper (Cu), iron (Fe), manganese (Mn), and lead (Pb) (Van Dover, 2000; Kelley et al., 2002). The seawater provides organisms with oxygen, while the hydrothermal fluid provides the necessary substrates (H_2S, CH_4) for chemosynthesis. In part, the degree of mixing and exposure to venting fluid in conjunction with individual species physiologies determines whether or not an organism is able to exist in these habitats (Luther et al, 2001).

The focus of this study, the 9°45-50'N segment of the EPR (Figure 1.2), has been studied extensively. Two known eruptive events, one in April of 1991 (Haymon et al., 1991; Haymon et al., 1993; Von Damm et al., 1995) and another in late 2006 (Tolstoy et al., 2006; Soule et al., 2007), have been observed, allowing for a greater understanding of the geological, chemical and biological interactions at vents, as well as providing a unique opportunity to observe biological succession in the vent ecosystem following a catastrophic event. In 1991 the high temperature fluids immediately following the eruption were high in sulfide (41mmol/kg), carbon dioxide (15.5mmol/kg), and methane (0.19mmol/kg) (Von Damm, 2000; Lilley and Olson, 2001). These values decreased as time post-eruption increased, forming a temporally and spatially diverse patchwork of chemistries within the region, yet all vent fluids remained elevated in basic properties relative to non-vent deep-sea environments.

Just as the age of a vent field influences fluid chemical composition, it also affects community composition and abundance. On the EPR, successionally younger biological communities are initially dominated by *Tevnia* tubeworms, but soon become overtaken by *Riftia* tubeworms. Older communities and areas where flow conditions tend to be less

vigorous host bathymodiolid mussels and vesicomyid clams (Shank et al., 1998). All of the above mentioned organisms are primarily if not totally dependent upon a symbiosis with chemoautotrophic symbionts for nutrition. These structural species provide habitat for a suite of other vent-endemic fauna such as limpets, amphipods, crabs, polychaetes, and fish that may or may not directly utilize chemoautotrophic microbes for nutrition, but are ultimately dependent upon this carbon fixation process for sustenance (Govenar et al., 2005).

Chemosynthetic production forms the base of all trophic interactions within the system, and it is unusual for non-vent fauna to take advantage of the high productivity and large biomass present within a vent field. This may in part be due to an inability of non-vent fauna to adapt to and offset the affects of the shifting and potentially harmful chemical environment, including potential prey items that may be unpalatable or unsafe due to their habitat and physiology (Childress & Fisher, 1993; Kicklighter et al., 2004). All vent fauna have some sort of adaptation to meet the challenges present within their habitat, including the presence of high levels of hydrogen sulfide, high concentrations of heavy metals, and low levels of oxygen, all of which can be toxic. Vent fauna are uniquely suited to deal with these challenges, utilizing behavioral, structural, metabolic, and even gene level adaptations to allow them to survive and thrive. For example, the tubeworm *Riftia* has a unique hemoglobin that allows for the simultaneous transfer of hydrogen sulfide for the symbiont and oxygen for the host (Arp & Childress, 1983; Zal et al., 1998). Some organisms have enzymes that are able to function at higher

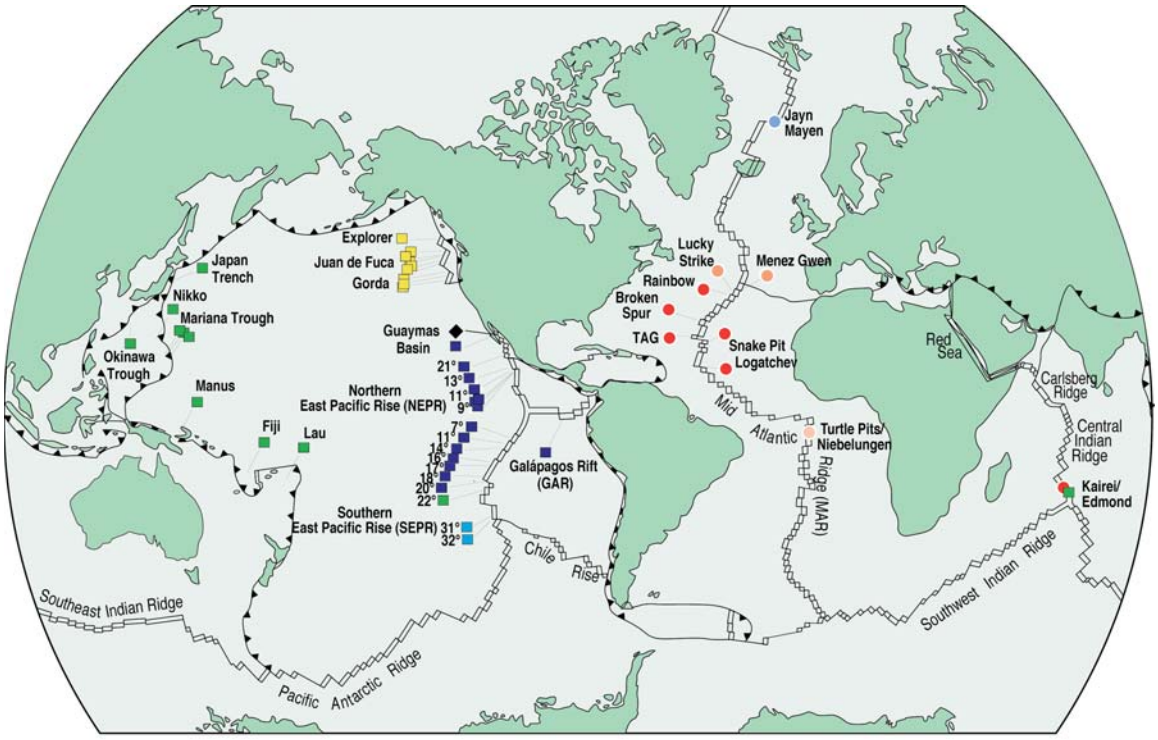

Figure 1.1 Areas of known venting around the world.

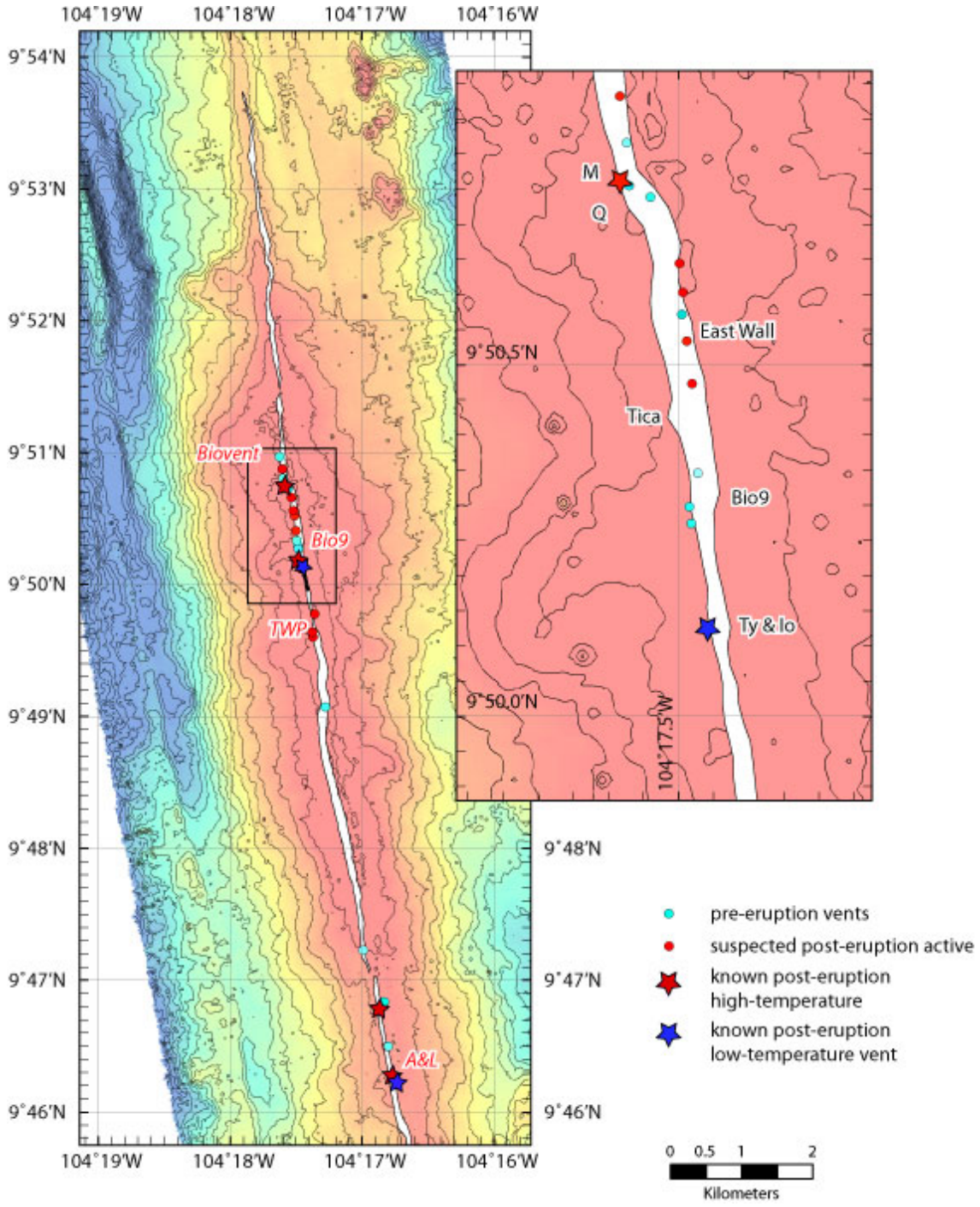

Figure 1.2 9°N segment of the East Pacific Rise with known pre- and post-eruption (2006) vent sites indicated. The enlarged box encompasses the BioGeoTransect.

temperatures than the same enzyme in non-vent fauna (Dahlhoff & Somero, 1991). *Paralvinella grasslei*, a polychaete worm, is thought to shed potential toxins such as sulfides along with its mucous, and numerous other behavioral and physiological adaptations of vent fauna have been examined (Van Dover, 2000). Despite the growing body of knowledge regarding vent-endemic invertebrate adaptations, life histories, and community dynamics, little is known regarding the ecology of fish species who are also important member of these deep-sea communities.

1.3 Vent Vertebrates

To date numerous fish species have been observed in association with hydrothermal vents worldwide (Geistdoerfer, 1996; Tunnicliffe, 1991), though a lack of captured specimens has made comprehensive species identifications difficult to achieve. Desbruyères et al. (2006) list nineteen species of fish from ten families as being associated with hydrothermal venting worldwide, with at least another six species waiting description. Of the nineteen listed, nine are considered endemic to hydrothermal vents. Individual species from the family Zoarcidae dominate both the associated fauna and endemic fauna categories (Biscoito et al., 2002). Along the Mid-Atlantic Ridge, the zoarcid *Pachycara thermophilum* is most commonly observed at vent communities while species such as the morid *Lepidion schmidti* have been seen in proximity to venting areas, but are likely not dependent exclusively upon vent ecosystems for either food or habitat (Geistdoerfer, 1994; Saldanha & Biscoito, 1997). Similarly, on the EPR, a number of species have been observed near vents, including liparids, macrourids, synaphobranchids,

and ophidiids, but only two (with a potential third) are currently considered endemic to the chemosynthetic communities (Desbruyères et al., 2006). These are the zoarcid *Thermarces cerberus* (Rosenblatt & Cohen, 1986) and the bythitid *Thermichthys hollisi* (Cohen et al., 1990). While considerably more research has focused on understanding vent invertebrate communities and adaptations to the environment, there is some general knowledge regarding these two species of vent-endemic fish.

Thermarces cerberus is a member of the family Zoarcidae, commonly called eelpouts. Zoarcid individuals are often rare, though this family has been highly successful at colonizing the deep continental shelves of northern-hemispheric oceans (Anderson, 1994). At vents, *T. cerberus* is typically found in diffuse flow areas colonized by tubeworms or mussels, with numerous individuals residing amongst the tubeworm tubes (Geistdoerfer & Seuront, 1995). A similar species (*Thermarces andersoni* Rosenblatt & Cohen, 1986) has also been described from Pacific vents, but it is unclear whether the two are actually separate species. Numerous small and presumably young zoarcid individuals were observed after both the 1991 and 2006 eruptions (Shank et al., 1998; pers. obs.). *T. cerberus* is known to prey mainly upon limpets and small crustaceans with a preference shown for the large lepetodrilid limpet individuals (Sancho et al., 2005), while Pond et al. (2008) found that *T. cerberus* fatty acid signatures were consistent with a diet composed of vent invertebrates. Exclusion studies suggest that *T. cerberus* may play a role in structuring the invertebrate community composition through their selective predation (Micheli et al., 2002). They are thought to be potentially well-adapted to exposure to warm diffuse fluids as their lactate dehydrogenase has a higher

functional temperature tolerance than that of most other deep-sea fish, including the other vent endemic species along the EPR, *Thermichthys hollisi* (Dahlhoff et al., 1990).

Less is known regarding the ecology of the bythitid *Thermichthys hollisi*. The family Bythitidae encompasses numerous live-bearing fishes and its members inhabit a wide range of environments, from tropical reefs and freshwater caves to the continental slopes and mesopelagic areas, and employ a variety of life strategies (Cohen et al., 1990). Originally described from a single individual from the Galápagos, *T. hollisi* was previously named *Bythites hollisi* (Cohen et al., 1990). Upon further examination of individuals of the genus *Bythites*, it was proposed that *Bythites hollisi* be designated into a new, distinct genus, *Thermichthys*, in reference to their habitat (Neilsen & Cohen, 2002; Neilsen & Cohen, 2005). Speculation regarding *T. hollisi*'s prey preferences is scant and anecdotal within the literature (Ballard & Grassle, 1979; Desbruyères et al., 2006). One early study suggested on the basis of behavioral observations that perhaps the fish employs a symbiosis with chemoautotrophs for nutrition, as was being recognized in multiple invertebrate species at the time (Hessler & Smithey, 1984), while others suggested that they feed upon the microbial community (Ballard & Grassle, 1979; Grassle, 1985). Counter to these hypotheses are additional anecdotes: whereby an observer in a submersible saw a bythitid attempt to consume what may have been a zoarcid (Desbruyères et al., 2006) and another paper claimed they are carnivorous or detritivorous (Geistdoerfer, 1998). The inclusion of a single individual from the Galápagos in a stable isotope study suggests that *T. hollisi* is positioned higher in the trophic network than a species wholly dependent upon chemoautotrophs (Fisher et al.,

1994). Fundamental questions regarding the feeding habits, trophic interactions, and impact upon vent community composition of *T. hollisi* remain unanswered, yet are integral to understanding the whole of vent community ecology.

Members of the family Bythitidae are often viviparous, and *T. hollisi* is no exception; live-bearing broods of up to an estimated 10,000 young (Wourms & Moser, 1991). There is no knowledge of life-history strategy for these fish, and whether young settle at vents or migrate to them is a matter for debate. Despite numerous studies investigating larval dispersal and connectivity of invertebrates at vents (Tyler & Young, 2003; Shank & Halanych, 2007), this area has not been explored for vent vertebrates. Similarly, adaptation of vent vertebrates to conditions experienced within venting communities is not well defined. Geistdoerfer (1996) noted that vertebrates adapted to life at vents do not differ physically from similar non-vent species, leading to speculation that any adaptations must be at a biochemical or genetic level. To date only one study has attempted to explore *T. hollisi* adaptation to vent life at this level. As previously mentioned, an study of enzyme function including one *T. hollisi* individual suggests that they are less well adapted to a high temperature lifestyle (Dahlhoff et al., 1990), which may partially explain why they are typically observed in the periphery of vent fields or in enigmatic aggregations in collapse pits and cracks termed "fish holes", and not directly in active diffuse flow as is *Thermarces cerberus*. Yet these fish are found at venting environments and nowhere else, and what habitat conditions they are experiencing and selecting, as well as their physiological tolerances, responses, and adaptations to their

environment remain areas of exploration in order to elucidate the role of fish within the hydrothermal vent ecosystem.

1.4 Goals of thesis

The ecology of vent invertebrates have been well-researched for the past thirty years, yet despite their potential ecological importance, there is little direct knowledge of the ecology and impact of fishes within hydrothermal vent ecosystems. It is the goal of this thesis to provide fundamental insights into the ecology and adaptation of vent-endemic vertebrate fauna, specifically to explore the function and role of fish that are found in these environments on the East Pacific Rise, with a particular emphasis on the bythitid *Thermichthys hollisi*. This goal is approached from three main directions; a study of the trophic ecology of *T. hollisi* through stable isotope and gut content analysis to establish trophic interactions and impact on vent ecosystem structure; a study of otolith chemistry to provide the first evidence of life history strategies and habitat use in vent-endemic fish; and an exploration of the characterization and expression of stress-inducible genes to get a first look at vertebrate gene-level adaptation to the venting environment.

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Chapter Two

Trophic Ecology of the Hydrothermal Vent Fish *Thermichthys hollisi*

Abstract

Dietary preferences and trophic interactions of the vent fish *Thermichthys hollisi* were explored using a combination of gut content and stable isotope analyses. Gut contents indicated that *T. hollisi* specimens were actively feeding upon a combination of brachyuran crabs, *Alvinocaris* shrimp, polychaetes, and zoarcid fish with the majority of fish containing evidence of various crustacean prey. Carbon, nitrogen, and sulfur stable isotopic measurements of *T. hollisi* tissues and potential prey items support a chemosynthetically based prey source and place *T. hollisi* in the uppermost trophic levels of vent communities. Liver and muscle tissue exhibited significantly different carbon and sulfur isotopic values, which may be indicative of a dietary shift. The implications of *T. hollisi* predation upon vent community composition and structure are discussed.

2.1 Introduction

Energy transfer through an ecosystem as a result of trophic interactions and dynamics is a ubiquitous factor affecting the function of all marine systems. At mid-ocean ridge venting ecosystems, the flow of energy through the biological system is inherently linked to geological and chemical processes, ultimately generated from energy and material from the mantle of the earth itself. Marine ecologists have made it a fundamental goal to understand this flow of energy through ecosystems, and in the case of mid-ocean ridge ecosystems, understanding the complex linkages between planetary processes and biological communities is a goal of national research programs such as Ridge 2000 (www.ridge2000.org). Microbial – invertebrate trophic linkages at mid-ocean ridges have been investigated for thirty years, forming the basis for energetic models that are currently being refined. A fundamental question still remaining is how the energy and mass at these lower levels is transferred to higher-level consumers (e.g.

predatory fish) and the surrounding deep-sea and how these interactions affect the community as a whole.

The influence of species interactions on ecosystem structure and/or function has been documented in numerous environments, ranging from temperate coastal (Bologna, 2007) to tropical (Carpenter, 1988) to polar areas (Barrett & Krasnov, 1996). More specifically, the ability of predators to both directly and indirectly influence the composition and structure of ecosystems has long been acknowledged (the concept of tropho-dynamics in Lindeman, 1942). The classic study of sea otter predation on urchin populations within Pacific kelp forests (Estes et al., 1978) illustrates how the actions of a single predator can impact an entire ecosystem.

In numerous aquatic ecosystems, fish often occupy high trophic levels. Many economically important fish species play a role in the structuring of ecosystems, making an understanding of their role in trophic and ecosystem dynamics and energy transfer valuable for both conservation and management decisions. Diverse habitats such as coral reefs, seamounts, estuaries, and deep-sea environments all contain examples of how predatory fish both directly and indirectly influence their environment (Table 2.1). Not only the presence of predators, but the density and diversity of predatory species may affect the rest of the ecosystem (Guidetti, 2007). The collapse of the North Atlantic cod fishery lead directly to an increase in shrimp and snow crab, and a shift from demersal to pelagic fish species (Savenkoff et al., 2007; Worm & Myers, 2003; Bundy, 2005). This shift in dominant species has been implicated as the driving force behind changes in species composition and abundance of sediment fauna (Quijon & Snelgrove, 2005),

providing another example of how predation can play a major, though perhaps indirect, role in shaping the surrounding ecosystem.

Table 2.1 Examples of direct and indirect effects of fish predation.

Fish species	habitat	directly affects	indirectly affects	study
Cynocion regulus & *Paralichthys dentatus*	eelgrass beds	shrimp, crabs and smaller fish	benthic species	Bologna, 2007
kelp perch & señorita	kelp forest	mesograzers	kelp invertebrate recruitment	Davenport & Anderson, 2007
Salvelinus fontinalis	boreal stream	invertebrates	algae	Bechara et al., 2007
Paralichthys albigutta & spot	estuary		Terrebillid polychaetes	Gloeckner & Luczkovich, 2008
foraging herbivores (e.g. acanthurids)	coral reef	macroalgae	coral	Ceccarelli et al., 2005
seabream & wrasse	rocky sublittoral	sea urchins		Guidetti, 2007

2.1.1 Fish at Hydrothermal Vents

Despite evidence that organisms such as fish may play a significant role in structuring community composition through predation, higher trophic level interactions have not been extensively explored in deep-sea systems, and in particular, in chemosynthetic vent environments known to support high biomass (but see Sancho et al., 2005; Micheli et al., 2002; MacAvoy et al., 2002). Ecological studies on the East Pacific Rise (EPR) have typically focused on understanding bottom-up controls of community structure through fluid chemistry (Shank et al., 1998; Luther et al., 2001) and species colonization (Mullineaux et al., 2000), with less attention paid to top-down regulation through predation. Yet predation has been hypothesized to play a structural role in these

communities as well. On the EPR, there are two species of vent-endemic fish: the zoarcid *Thermarces cerberus*, and the bythitid *Thermichthys hollisi*. *T. cerberus* is typically found in close association with diffuse fluid flow, among individuals of the vestimentiferan tubeworm *Riftia pachyptila*, or the mussel *Bathymodiolus thermophilus*. In contrast, *T. hollisi* is often observed on the periphery of vent fields and in enigmatic aggregations referred to as "fish holes" with no visible fluid flow or associated fauna (Figure 2.1). The effects of *T. cerberus* predation on the surrounding community are evident within the epifaunal communities nearest to active vents. When *T. cerberus* was excluded, the invertebrate species that showed the greatest increases were those most often preyed upon by the zoarcid, including the gastropods *Cyathermia naticoides* and *Lepetodrilus elevatus*, and the amphipod *Ventiella sulfuris* (Micheli et al., 2002). *T. cerberus* may have effects on community structure through feeding habits that are selective for both species and size (Sancho et al., 2005), with preference shown for the larger limpets. It has been hypothesized that *T. cerberus* exhibits control over invertebrate settlement and recruitment though its selective predation.

Thermichthys hollisi, which lives in a slightly different micro-environment than the *Thermarces cerberus* may exhibit a similar structural impact upon its prey species. Currently, there is an absence of basic ecological knowledge of this species, including the identity of preferred prey species. There are no direct studies of *T. hollisi* feeding preferences, yet they are referenced as being carnivorous or detrivorous (Geistdoerfer, 1998) with anecdotal evidence that they may feed upon other fish (Desbruyères et al., 2006).

This study specifically aims to investigate the feeding ecology of *Thermichthys hollisi* and to better understand what potential role this species may play in structuring vent community dynamics. Gut content analyses and stable isotope measurements were employed in order to accomplish these aims.

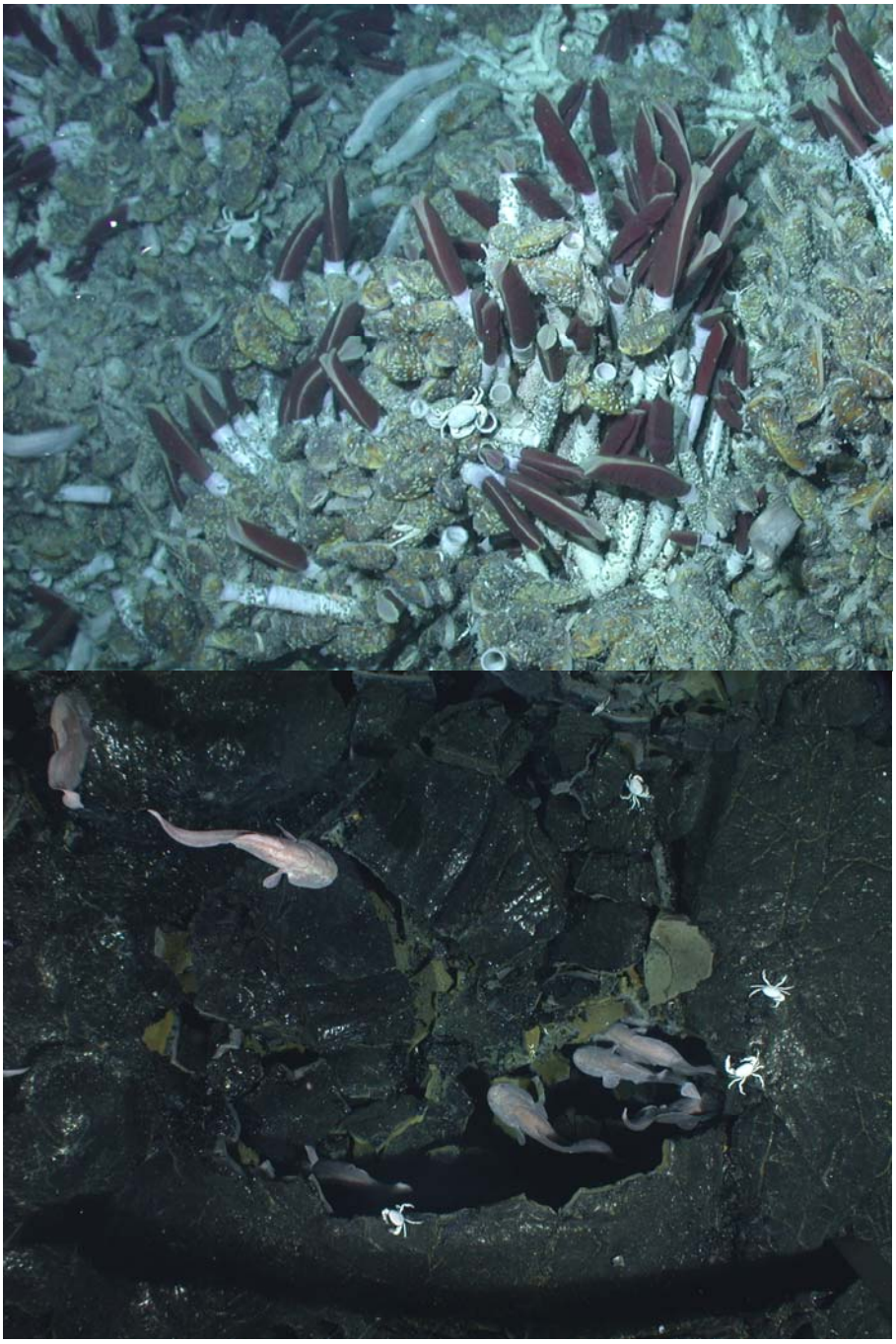


Figure 2.1 *Thermarces cerberus* and *Thermichthys hollisi* in their typical habitat. The zoarcid (upper panel) is commonly associated with tubeworm communities in diffuse flow areas while the bythitid (lower panel) is more often seen in the periphery or in collapse pits.

2.1.2 Gut Content and Stable Isotope Dynamics

Gut content analyses have historically been a relatively simple way to determine the diet of fish. However, there are some caveats that warrant consideration. Opportunistic or sit-and-wait predators, including many deep-sea fish, do not feed regularly. When examining fish that employ such a feeding strategy, the probability of finding an empty gut is high. Herbivorous fish and some benthic feeders consume items that are difficult to identify before they are consumed and particularly difficult after they have been chewed and swallowed (Hadwen et al., 2007). Despite these potential pitfalls, gut content analyses remain an important component in determining the feeding ecology of fishes. When used in combination with stable isotope analyses, feeding habits (both long and short term), and their potential affect upon the surrounding community may be established.

Stable isotopes have been utilized for many years in ecological research (DeNiro & Epstein, 1978; Peterson & Fry, 1987; Hobson, 1999). The relative predictability of fractionation effects, consistent cross-species and cross-environment behaviors and trends of stable isotopes have made them useful in determining trophic relationships, tracking migrations (Clark et al., 2006), identifying feeding grounds, and quantifying dietary contributions (McConnaughey and McRoy, 1979) across many taxa in both terrestrial and aquatic environments (reviewed in Gannes et al., 1998 and Hobson, 1999). In trophic studies, carbon and nitrogen are the most commonly utilized elements, but sulfur isotopes have also proven useful in clarifying trophic relationships and contributions, especially in

reducing environments such as marsh sediments or hydrothermal vents (Yamanaka et al., 2003; MacAvoy et al., 2005).

The ratio of ^{13}C to ^{12}C is established during carbon fixation by primary producers such as plants, algae, and microbial organisms. Different carbon fixation pathways result in different ratios, and these unique signatures are transferred relatively unchanged (~1‰ per trophic level) throughout the trophic network. $\delta^{13}C$ (the ratio of ^{13}C to ^{12}C in a sample relative to that in a standard) can therefore be useful in establishing the base of a food web. Higher level predators such as fish can exhibit intermediate values which may indicate the utilization of multiple carbon sources. For example, Winemiller et al. (2007) were able to distinguish primary production sources (C4 plants, C3 plants, etc.) using carbon isotopes, but were unable to fully resolve which were consumed by marsh invertebrates and fish due to overlapping signatures.

Nitrogen isotopes have been shown to fractionate more substantially during trophic interactions. Light isotopes are preferentially utilized, leading to approximately 3-5‰ enrichment in whole body samples per trophic level (Minigawa & Wada, 1984). Nitrogen tracks proteins well and is often used to indicate potential trophic level. Tissue analyzed, turnover rate, and amino acid composition, and lipid content can each affect the measured isotope ratios and the fractionation rates, and must therefore be considered when analyzing data.

Multiple species of sulfur are found in the marine environment, the most common and biologically relevant in the open ocean being sulfate. Average seawater sulfate has an isotopic value of approximately 21‰ (Rees et al., 1978). Phytoplankton take up and

utilize sulfate resulting in a 0 to -3‰ fractionation. This signature is retained throughout successive trophic levels with no appreciable trophic fractionation (Fry et al., 1983). Due to the homogenous nature of sulfur signatures in the open ocean, $\delta^{34}S$ is of limited use in ecological studies of strictly pelagic species. However, in environments such as near-coastal, estuarine areas or reducing environments such as vents of tidal mud flats, sulfur isotopes have proven useful. The lack of trophic fractionation makes sulfur particularly useful for understanding trophic and feeding dynamics of fauna, and through them, migratory dynamics in environments with variable sulfur signature inputs (MacAvoy et al., 1998).

For all elements, the rate of turnover may have an effect on which target tissues are most useful. MacAvoy et al. (2001) and Tarboush et al. (2006) have found that sulfur turnover in tissues may be slow enough to underestimate dietary shifts, especially if prey species are migratory. In appropriate habitats, where sulfur water signatures vary, sulfur isotopes, in combination with other stable isotopes can be a powerful tool to study trophic influence and through these results, a means to learn about ecological processes such as migration, life history strategies, and nutritional dependence.

2.1.3 Stable Isotope Studies at Hydrothermal Vents

Stable isotope studies have been conducted in hydrothermal vent and cold-seep environments since shortly after their discovery (Rau & Hedges, 1979). These studies have been conducted across ocean basins in dissimilar locations including the Louisiana slope (Brooks et al., 1987), the Galápagos (Fisher et al., 1994), the Juan de Fuca ridge

(Bergquist et al., 2007), the Mid-Atlantic Ridge (Colaco et al., 2002), the Indian Ocean (Van Dover, 2002) the Japan subduction zone (Saino & Ohta, 1989), and the East Pacific Rise (Van Dover & Fry, 1994). General trends found across most of these locales indicate that many vent systems host similar biogeochemical processes (Van Dover & Fry, 1994). The above studies were able to verify the presence of local sources of carbon and nitrogen at the vents, showing that vent organisms are not dependent upon surface primary production (Rau, 1981; Williams et al., 1981; Van Dover & Fry, 1989). Chemoautotrophic microbes, the primary producers at vents, discriminate against ^{13}C (Fisher et al., 1990), thus organisms harboring chemoautotrophic symbionts, such as the tubeworm *Riftia pachyptila* or the mussel *Bathymodiolus thermophilus*, tend to have depleted carbon signatures relative to "typical" or non-chemosynthetic deep-sea fauna. Typical non-vent deep-sea invertebrate stable isotope values have been reported as ranging from -22 to -17‰ for carbon and 11 to 16‰ for nitrogen (Brooks et al., 1987; Van Dover & Fry, 1989). Though non-symbiont-containing vent invertebrates display a large range in carbon isotope values that at times include those of non-vent fauna (Van Dover & Fry, 1989, Fisher et al., 1994), nitrogen values are generally depleted relative to non-vent fauna (Van Dover & Fry, 1989; Van Dover & Fry, 1994). Fisher et al. (1994) hypothesize that vent invertebrates all have a nitrogen value of less than 11‰, making nitrogen a good indicator of vent dependent fauna.

Sulfur isotopes have also been shown to be useful in indicating which species are dependent upon vent "produced" nutrition (MacAvoy et al., 2005). Hydrothermal fluids are in general depleted relative to seawater, with values ranging from -3 to 8‰ (Shanks et

al., 1995). Vent endemic fauna mirror these values and seem to center around 0‰ (Mizota, 1997). Organisms with thiotrophic symbioses generally have tissue sulfur values less than 5‰, indicating their dependence upon the symbiosis, and thus in turn, the depleted sulfide available in the environment (Vetter and Fry, 1998). Dependence upon the primary production of chemosynthesis versus photosynthesis can also be reflected in tissues of scavengers and predators. Carbon, nitrogen, and sulfur tissue isotopic values of fish and invertebrates living near a hydrocarbon seep in the Gulf of Mexico indicated chemosynthetic input and trophic export from the seeps (MacAvoy et al., 2002).

The combination of gut content analyses and stable isotope measurements will allow for an exploration of the dietary preferences and trophic relationships of the vent fish *Thermichthys hollisi*. As presumed top predators, these fish have the potential to significantly affect community structure and composition, making the ecology of *T. hollisi* of importance to understanding hydrothermal vent community ecology and the transfer of energy through this community and to the surrounding deep-sea.

2.2 Methods

2.2.1 Specimen collection and sample sites

Thermichthys hollisi specimens were collected using a thruster powered suction sampler designed by The Deep Submergence Lab at WHOI (Figure 2.2). The sampler consisted of an initial collection chamber attached to a spare thruster with a unidirectional door on the opposite end of the chamber. This door opened into a second chamber where

collected fish were stored for transport to the surface. A large flexible tube ran from the initial collection chamber, ending in a funnel-shaped attachment with a T-handle for the Alvin manipulators to grasp. The thruster was run in one direction to slurp fish through the tube into the initial chamber, and then the direction reversed to push the fish through the door into the final chamber to prevent damaging them during the subsequent collection of additional fish.

The sampler was mounted on the basket of the submersible DSV Alvin during dive 4317 at L vent (9° 46.256 N, 104° 16.776W) on the East Pacific Rise (Figure 2.3). Fish were collected from multiple collapse pits ("fish holes") surrounding the main hydrothermal chimney at the site by approaching a fish hole, stabilizing the submarine, inserting the sampler into the fish hole and turning it on. The fish did not seem to react to the presence of the sub until they were physically touched with the sampler. In total 26 *Thermichthys hollisi* individuals were collected from L vent. Multiple tissue type samples were dissected from each individual and frozen separately. One individual was collected from L vent using a net during dive 4293 and frozen whole. The digestive tract of this individual was subsequently dissected out and examined while still frozen. Invertebrate and *Thermarces cerberus* specimens used in this study were collected from various locations on the EPR (Table 2.2) during multiple cruises in order to account for the range of prey fish may be encountering. Invertebrates were collected using Alvin manipulator grab samples and slurp samples and placed in a biobox for recovery. These specimens were sorted, identified and frozen at -80°C while at sea.

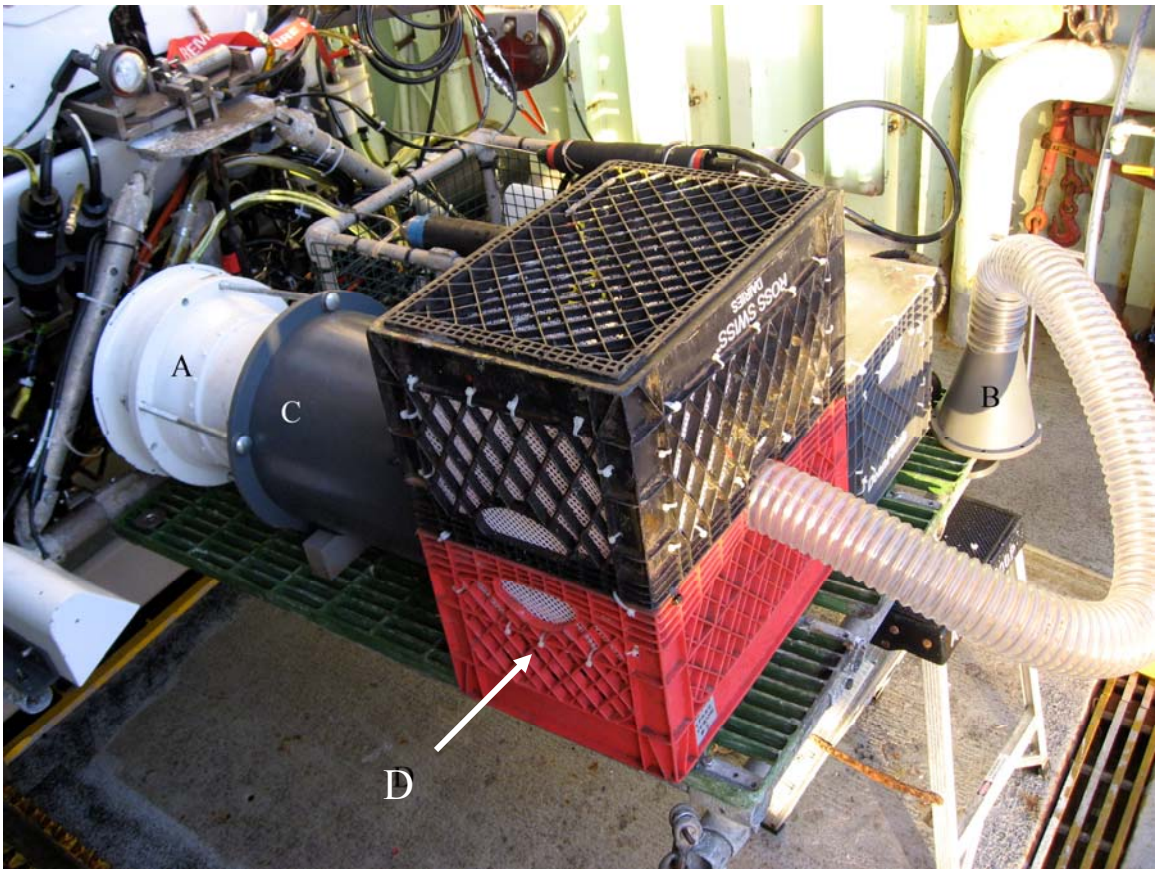


Figure 2.2 The "super-sucker" mounted on the Alvin basket for deployment. A. reversible direction Alvin thruster B. nozzle with T-handle for ease of slurping C. initial collection chamber with trap door into D. final collection and recovery chamber

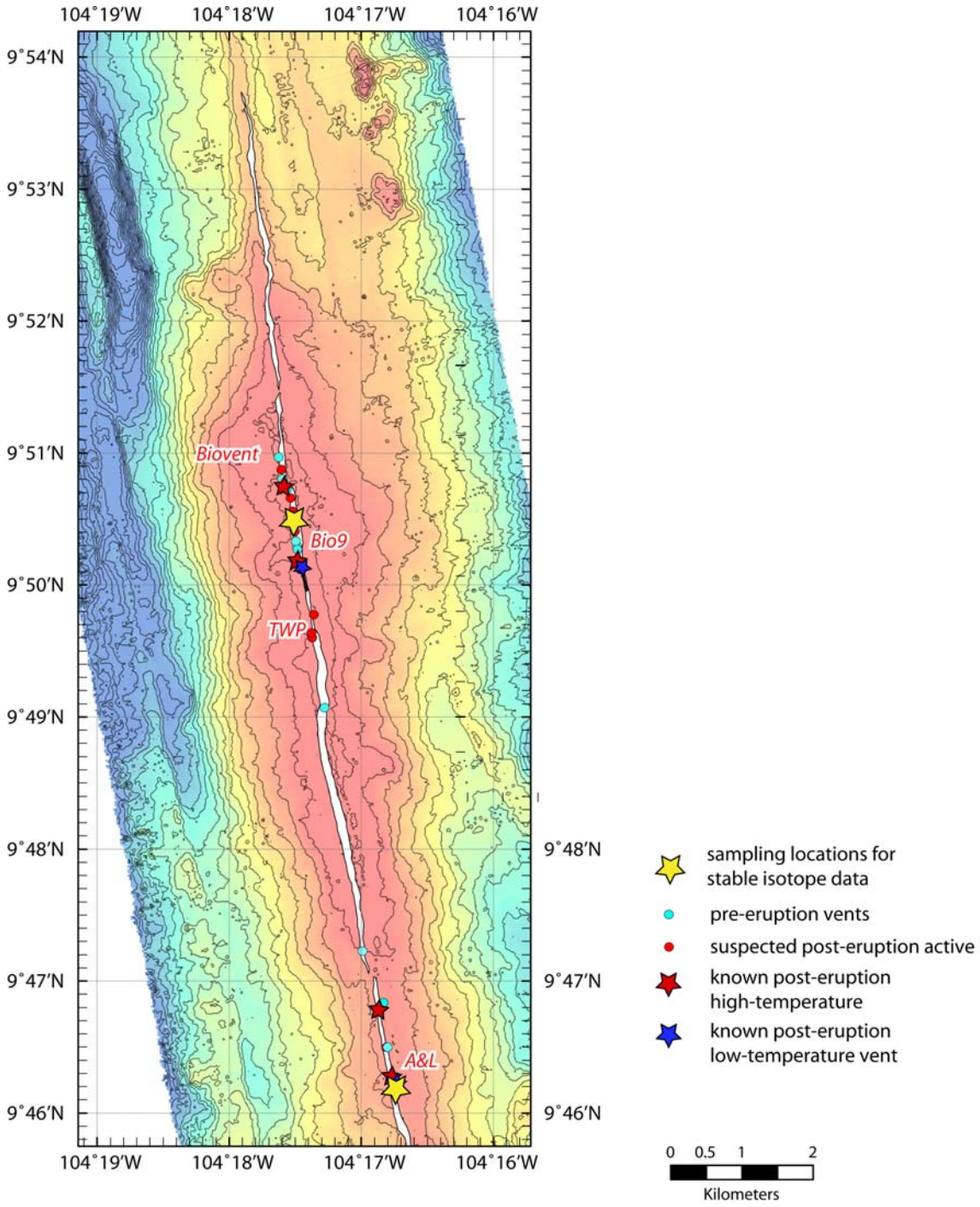


Figure 2.3 9°N segment of the East Pacific Rise with major sampling locations marked by yellow stars.

Table 2.2 Mean isotopic values and locations collected for all species examined.

Species	locations collected	n of C/N samples (n of sulfur)	mean $\delta^{13}C$ ‰ (st. dev.)	mean $\delta^{15}N$ ‰ (st. dev.)	mean $\delta^{34}S$ ‰ (st. dev.)
Alvinocaris lusca	L vent	2 (1)	-16.42 (0.07)	10.68 (0.64)	4.94
Alvinella pompejana	L vent, Alvinella Pillar	13 (3)	-10.14 (1.06)	6.11 (0.62)	8.57 (2.14)
Branchinotogluma grasslei	Tica	3 (1)	-11.95 (1.09)	8.05 (0.3)	1.83
Branchinotogluma hessleri	L vent	3	-9.71 (1.15)	8.44 (0.59)	
Bythograea thermydron	East Wall	3 (3)	-14.91 (1.00)	6.95 (0.31)	-2.37 (0.81)
Cyanograea praedator	L vent	1	-9.15	7.99	
copepod	L vent	1	-11.78	8.02	
Cyathermia naticoides	East Wall	2 (1)	-6.00 (5.59)	6.42 (0.98)	
Dahlella calderiensis	L vent	2 (2)	-11.43 (1.99)	7.67 (0.26)	6.17 (0.50)
Eulepotopsis vitrea	Tica	5 (1)	-15.37 (1.52)	7.96 (0.88)	4.5
Munidopsis subsquamosa	East Wall	1	-19.57	12.63	
Hesiolyra bergi	East Wall, L vent, A/L	5 (2)	-10.7 (0.98)	7.09 (1.02)	5.44 (1.37)
Lepetodrilus elevatus	Alvinella Pillar, BM 119, East Wall, L vent, Tica	10 (1)	-15.34 (2.74)	5.89 (2.31)	2.07
Lepetrodrilus ovalis	BM 119	2	-19.47 (0.79)	7.42 (0.12)	
Lepetodrilus pustulosus	BM 119, L vent	5	-15.49 (1.73)	7.93 (1.02)	
Lepidonotopodium fimbriatum	BM 119	1	-11.48	10.00	
Lepidonotopodium williamsae	BM 119	3 (2)	-31.64 (0.13)	4.17 (0.32)	3.65 (0.32)
Nereis sandersi	L vent, Tica	6 (3)	-12.14 (0.81)	8.45 (1.19)	5.7 (1.12)
Oasisia alvinae	East Wall	2	-11.49 (1.48)	8.70 (0.57)	
Paralvinella grasslei	East Wall	4 (2)	-10.64 (1.23)	9.53 (0.84)	6.24 (0.80)
Rhynchopelta concentrica	BM 119, East Wall, Tica	11	-12.84 (2.00)	6.03 (0.88)	
Riftia pachyptila	Alvinella Pillar, East Wall, L vent	8	-12.17 (1.69)	3.09 (0.48)	
Tevnia jerichonana	BM 119, L vent	10 (4)	-9.88 (0.76)	3.10 (1.49)	-2.32 (0.64)
Thermarces cerberus (liver)	East Wall	1 (1)	-10.42	11.91	2.62
Thermarces cerberus (muscle)	East Wall	2 (2)	-9.7 (0.42)	11.62 (1.63)	2.11 (1.15)
Thermichthys hollisi	L vent	15 (15)	-10.72 (0.80)	12.09 (0.97)	2.85 (2.57)

(liver)					
Thermichthys hollisi (muscle)	L vent	14 (15)	-14.69 (1.26)	11.68 (0.84)	0.80 (1.18)
Ventiella sulfuris	East Wall, L vent	9 (2)	-14.19 (4.73)	6.54 (1.74)	9.15 (2.28)

2.2.2 Gut Content Analysis

Digestive systems (stomach and intestines) were excised from the dead fish and preserved whole in 95% EtOH while at sea. Recovery from the seafloor caused some of the fish to lose stomach contents, however, any easily distinguishable prey items that were remaining in the mouth or protruding from the stomach were noted, removed, and frozen whole. Upon return to the shore-based lab the digestive tracts of 23 individuals were dissected and the contents scraped out into a Petri dish. Contents were examined underneath a dissecting microscope (Leica MZ APO), identified to the lowest possible taxonomic level, and photographed. The individual gut contents were subsequently preserved separately from the digestive tract remains in 70% EtOH.

Molecular methods were used to secondarily identify selected gut contents. DNA was extracted from the frozen stomach contents using a Qiagen DNEasy extraction kit according to the manufacturer's instructions. Usable DNA was not recoverable from the preserved contents. The DNA was amplified using degenerate primers (dgLCO1490 and dgHCO2198 Meyer et al., 2005) for the cytochrome oxidase I gene by combining 1µl DNA template, 5µl 5x buffer (Promega Flexi), 5µl 25mM magnesium chloride, 2.5µl 1mM dNTPs, 1.25µl of both forward and reverse primers (10µM concentration), and 0.25µl of Promega Flexi Taq polymerase (5u/µl) in a 25µl reaction. The PCR reaction was run for 3 minutes at 94° followed by either 30 cycles of 1 minute at 94°, 1 minute at

47°, 45 seconds at 72°, or 39 cycles of 1 minute at 94°, 1 minute at 45°, 45 seconds at 72°, with a final extension period of 7 minutes at 72°. PCR products were visualized on an agarose gel stained with ethidium bromide and cleaned up using a Qiagen kit according to the manufacturer's instructions. Amplified fragments were sequenced on an Applied Biosystems 3730XL capillary sequencer at the Josephine Bay Paul Center (Marine Biological Laboratory, Woods Hole, MA). The resulting fragments were analyzed using standard genetic software and compared to sequences in GenBank or sequences from known specimens within the lab.

2.2.3 Stable Isotope Analysis

Tissue samples from both fish and invertebrate samples (muscle, liver, and whole organism) were dried for a minimum of 48 hours at 60°C in a Fisher Scientific drying oven. Whenever possible muscle tissue was utilized for invertebrate specimens, however for smaller species such as amphipods and limpets whole individuals were analyzed. Individuals were pooled when necessary to ensure sufficient tissue for analysis. Fish tissue samples that did not initially appear dry after 48 hours were left in the oven for a minimum of 96 hours to ensure complete desiccation. Samples were analyzed for natural abundance, they were not acid treated, lipid-extracted or otherwise pre-treated. Once dried, the samples were ground to a fine powder using a mortar and pestle and weighed into tin capsules (0.6 – 1.2mg for C/N analysis, 5.8 – 6.2 mg for S analysis). Samples were analyzed at the Colorado Plateau Stable Isotope Laboratory (Flagstaff, AZ) using a Costech ECS 4010 elemental analyzer interfaced to a Delta Plus Advantage mass

spectrometer. Percent C/N or S content as well as delta ratios were reported. Data are presented in standard δ notation relative to a standard. For example $\delta^{13}C = [(R_{sample}/R_{standard}) - 1] * 10^3$ where $R = {}^{13}C/{}^{12}C$. Standards used were Vienna Pee Dee Belemnite for carbon, air for nitrogen and Canyon Diablo Troilite for sulfur.

Fish tissues were lipid normalized according to Kiljunnen's (2006) method. A simple two-source mixing equation (as in MacAvoy et al., 2002) was employed to estimate potential vent/non-vent dietary input. Input was estimated by the equation: $\delta^x E_{predator} - F = (\delta^x E_{vent} * f_{vent}) + (\delta^x E_{ocean} * (1-f_{vent}))$ where $\delta^x E$ is the mean isotopic signature for the element (E) in question, F is a term to correct for trophic enrichment, and f is the fraction of diet made up by a chemoautotrophic source. F for carbon, nitrogen and sulfur was 1, 3, and 0‰ respectively. $\delta^x E_{ocean}$ was represented by mean non-vent fauna measurements of -18.5‰ and 13.5‰ for carbon and nitrogen respectively (Van Dover and Fry, 1989), and 18‰ for sulfur (Mizota, 1997). $\delta^x E_{predator}$ represented mean *T. hollisi* values measured in this study and $\delta^x E_{vent}$ was calculated from mean vent invertebrates values in this study. Analyses were carried out for each isotope separately for muscle and liver tissue values. In order to assess potential dietary contribution with greater resolution, average measured values for major taxonomic categories (crustacean, polychaete, vestimentiferan, limpet, non-vent, and zoarcid) were submitted as potential food sources to the program Isosource (Phillips & Gregg, 2003). 1% increments and a 0.1 tolerance were utilized.

2.3 Results

2.3.1 Gut Content Analyses

Of the 23 fish analyzed, only three of them contained prey items within their upper digestive tract, while all individuals examined contained evidence of prey items within the intestinal portion of the digestive tract. The identifiable prey items fell into four broad taxonomic groups: crustaceans, polychaetes, mollusks, and fish; with the highest percentage of *Themichthys hollisi* individuals containing crustacean remains, followed by polychaetes (Table 2.3). Evidence of polychaete consumption was visible only by the presence of chaetae within the intestinal tract (Figure 2.4). Unfortunately, it was not possible to identify the preserved remains to a lower taxonomic level using molecular techniques. Crustacean remains within the lower intestinal tract were mainly portions of exoskeleton (Figure 2.5). Using molecular techniques, crustacean prey within the stomachs were identified as *Alvinocaris lusca* and brachyuran crabs. Two individuals were found to have consumed *Thermarces cerberus* (Figure 2.6), the other vent fish species frequently observed at East Pacific Rise venting localities.

Table 2.3 Proportion of *Thermicthys hollisi* individuals containing a particular prey type within the digestive tract. Though the ascent to the surface apparently caused a few of the fish to partially empty their stomachs, four of the 23 fish analyzed had prey in their stomach or mouth, and all individuals had evidence of prey in their lower intestines. One lower intestine was not examined because it was lost during transport back to the lab. Additional specimens of both *Alvinocaris lusca* and *Thermarces cerberus* were found in the collection basket, but could not be associated with a specific *T. hollisi* individual.

	prey	present in stomach (n = 23)	present in intestines (n = 22)
percent of fish processed with particular prey item present	crab - brachyuran	4.3	0
	shrimp – *Alvinocaris lusca*	8.6	0
	unid. crustacean	0	40.3
	fish – *Thermarces cerberus*	8.6	0
	polychaete	0	36.4
	non-biological	0	13.6
	mollusk – unid limpet	0	4.5
	unidentifiable	4.3	100

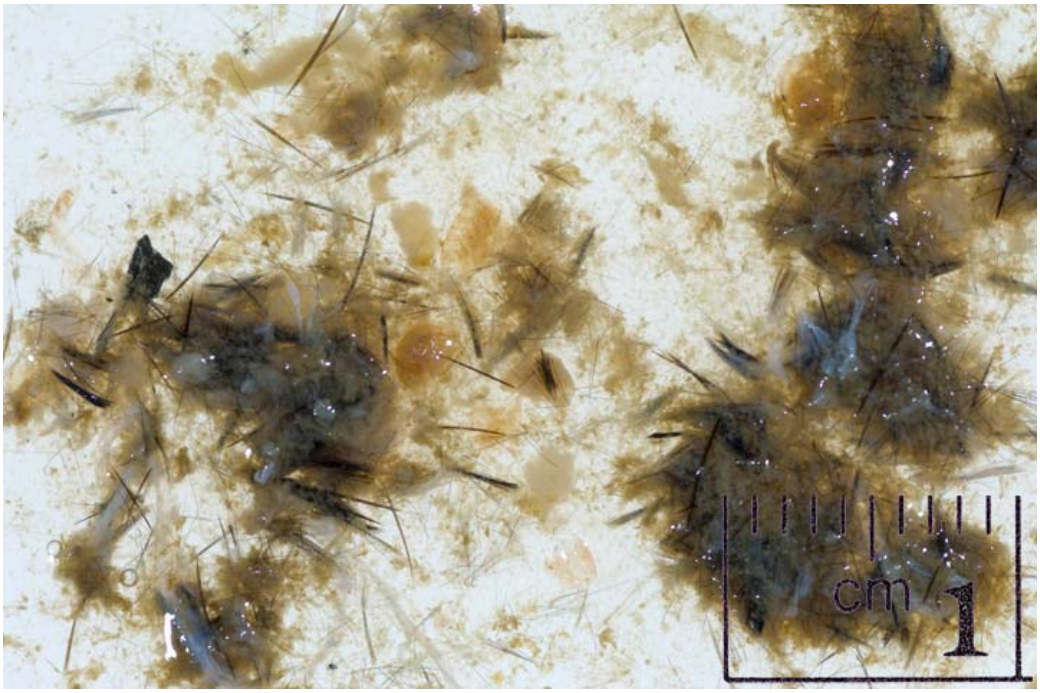


Figure 2.4 Image of polychaete chaetae from lower intestinal tract of *Thermichthys hollisi*.

Figure 2.5 Crustacean exoskeleton from lower intestine of *Thermichthys hollisi*.

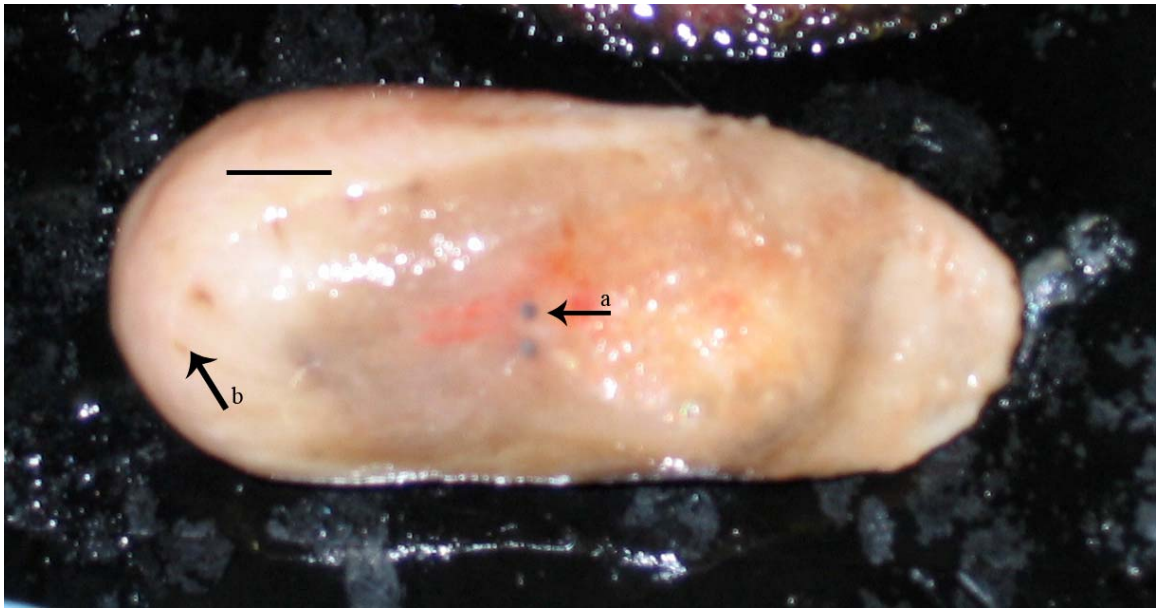


Figure 2.6 *Alvinocaris lusca* (arrow 'a' indicates an eye) and *Thermarces cerberus* (white tissue around outer edge, arrow 'b') from the stomach of *Thermichthys hollisi*. The scale bar is 1cm.

2.3.2 Isotope analyses

Themichthys hollisi $\delta^{13}C$ values ranged from -17 to -9‰ and $\delta^{15}N$ values ranged from 9 to 13‰. The lipid normalization caused an approximate 5‰ shift in $\delta^{13}C$ of the liver values, resulting in significantly ($p<0.01$) different carbon (but not nitrogen) ratios between liver and muscle tissue (Figure 2.7). The measured invertebrate values for both $\delta^{13}C$ and $\delta^{15}N$ fell within the range established by previous studies. Vent invertebrates from L vent only are depleted in nitrogen relative to *T. hollisi* values (Figure 2.8). *T. hollisi* $\delta^{34}S$ values ranged from -1 to 4‰, with vestimentiferan values falling below this and non-vestimentiferan vent invertebrates being relatively enriched (Figure 2.8). There is a significant difference ($p<0.01$) between sulfur signatures of *T. hollisi* liver and muscle tissues. Invertebrate data from sites other than L vent were also collected, as *T. hollisi* are mobile and thus presumably not limited to feeding only in one location. The data represented a minimum of three trophic levels with the lowest occupied by vestimentiferans and *B. hessleri*, the highest by fish and crabs, and the middle by a mix of invertebrate species (Figure 2.9). Both *T. hollisi* nitrogen and carbon data were intermediate in value to the measured vent invertebrates and to previously published values for non-vent, deep-sea invertebrates (Figure 2.9) (Van Dover & Fry, 1989). When potential prey were broken into the broad taxonomic categories determined from gut content analysis, *T. hollisi* liver tissue appeared closest in carbon values to zoarcids, polychaetes and vestimentiferans, while muscle tissue most closely matched the isotopic ratios of limpets and shrimp (Figure 2.10). Even with the addition of non-L vent prey signatures, *T. hollisi* sulfur values remained depleted relative to most invertebrates, with

the exception of the vestimentiferans and brachyuran crabs, and most closely matched the sulfur isotopic signature of zoarcids (Figure 2.10). The invertebrate sulfur signatures were similar to those presented in previous studies (Fry, et al., 1983; Brooks et al., 1987; Kennicutt et al., 1992; Mizota, 1997). The two-source mixing model indicated that chemosynthetically supported carbon sources could make up 64 to 100+% of the diet based on muscle tissue values, and 78 to 100+% based on liver tissue values. The Isosource model was not able to calculate any potential solutions using all three isotopes for the muscle values, but was able to resolve the liver values. The solution indicated that sources with mean isotopic signatures similar to those of zoarcids, crustaceans, and vestimentiferans had the greatest potential contribution to the diet as represented by *T. hollisi* liver values (Figure 2.11).

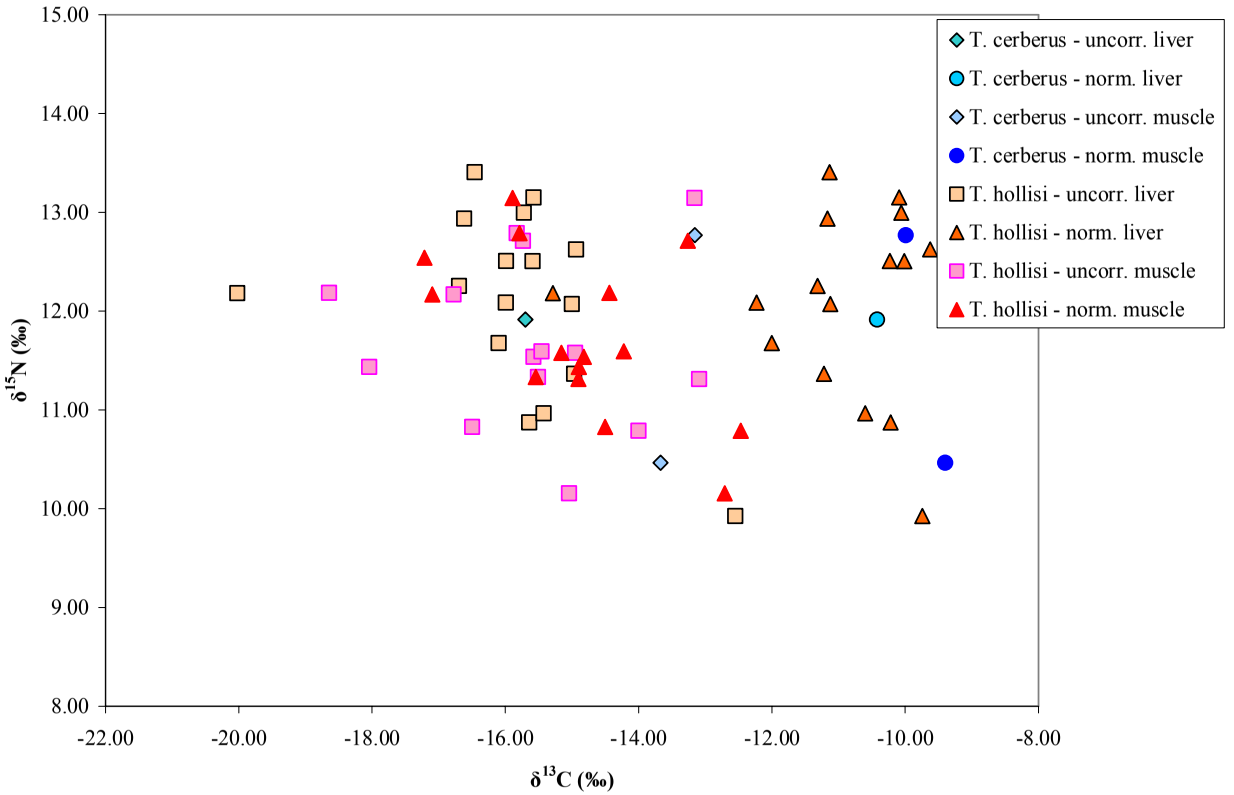
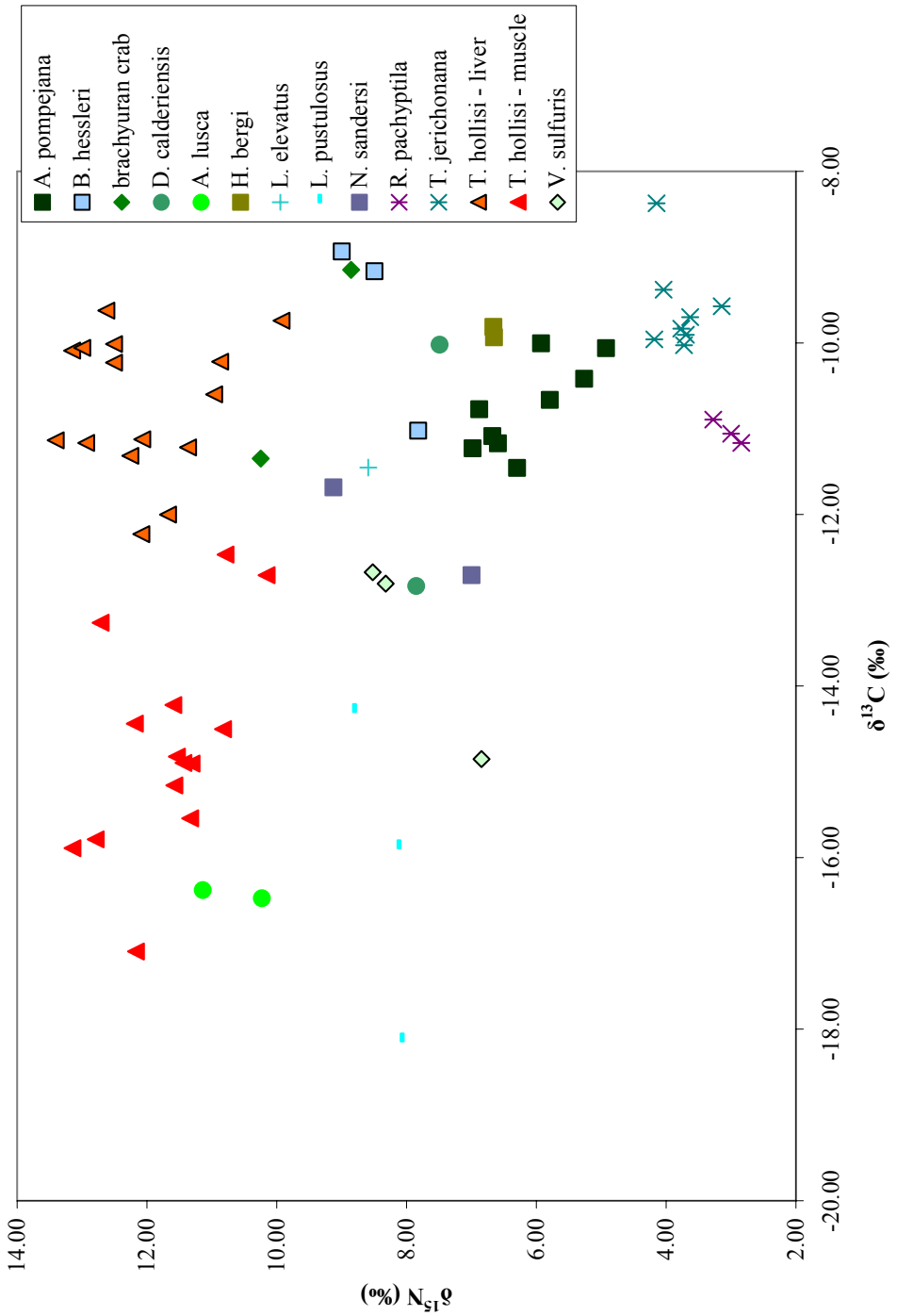
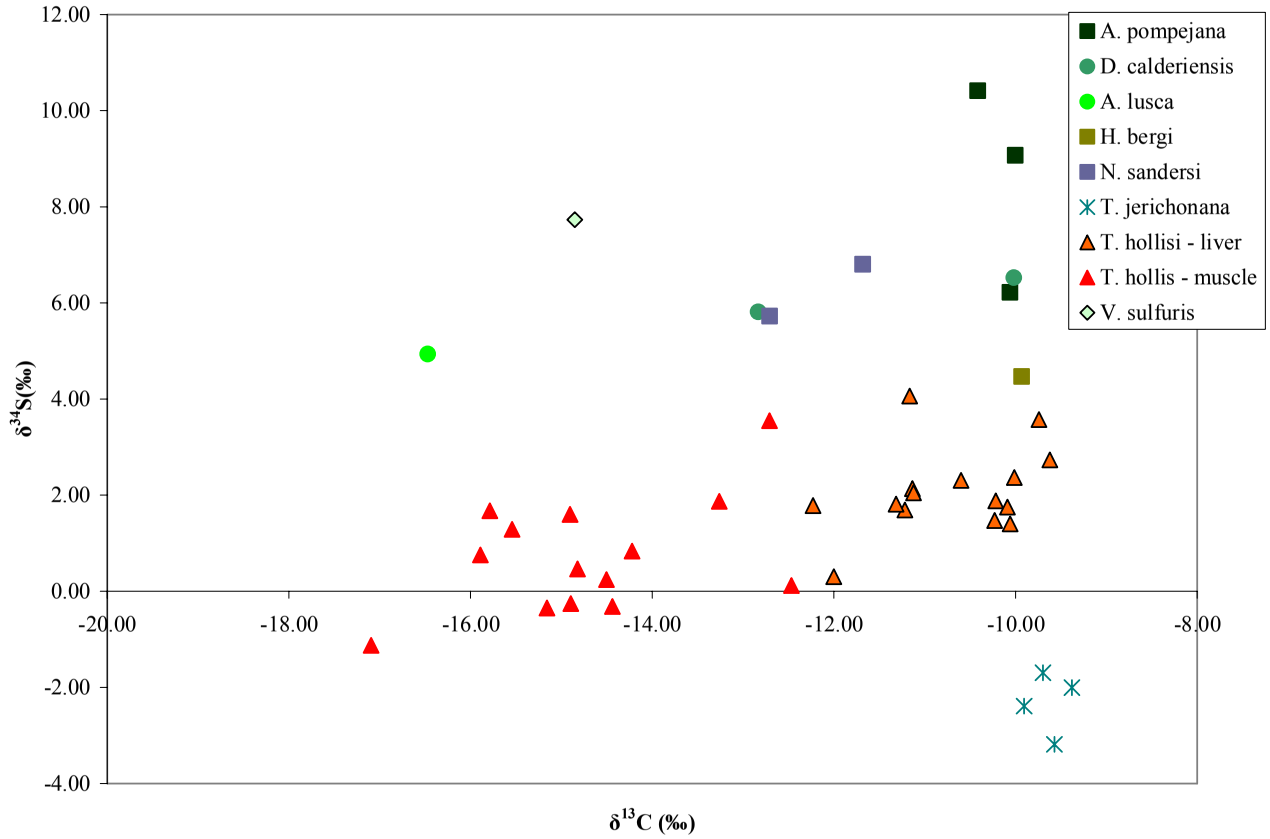


Figure 2.7 Uncorrected carbon isotopic values in comparison to lipid normalized values. Kiljunnen's (2006) lipid normalization caused liver carbon values to shift approximately 5‰ to the right. There is a significant (T test assuming equal variances $p<0.001$) difference between corrected muscle and liver carbon values for *Thermichthys hollisi*.


A. pompejana
B. hessleri
brachyuran crab
D. calderiensis
A. lusca
H. bergi
L. elevatus
L. pustulosus
N. sandersi
R. pachyptila
T. jerichonana
T. hollisi - liver
T. hollisi - muscle
V. sulfuris
14.00
12.00
10.00
8.00
6.00
4.00
2.00
-20.00
-18.00
-16.00
-14.00
-12.00
-10.00
-8.00
δ15N (‰)
δ13C (‰)

2.8a



2.8b

Figure 2.8 Stable isotope values for fauna collected only from L vent. Carbon and nitrogen values are in the upper panel (2.8a) while carbon and sulfur values are in the lower panel (2.8b). *Thermichthys hollisi* muscle values were more depleted than liver values for both carbon and sulfur, indicating potentially different food sources over short and long-term time scales.

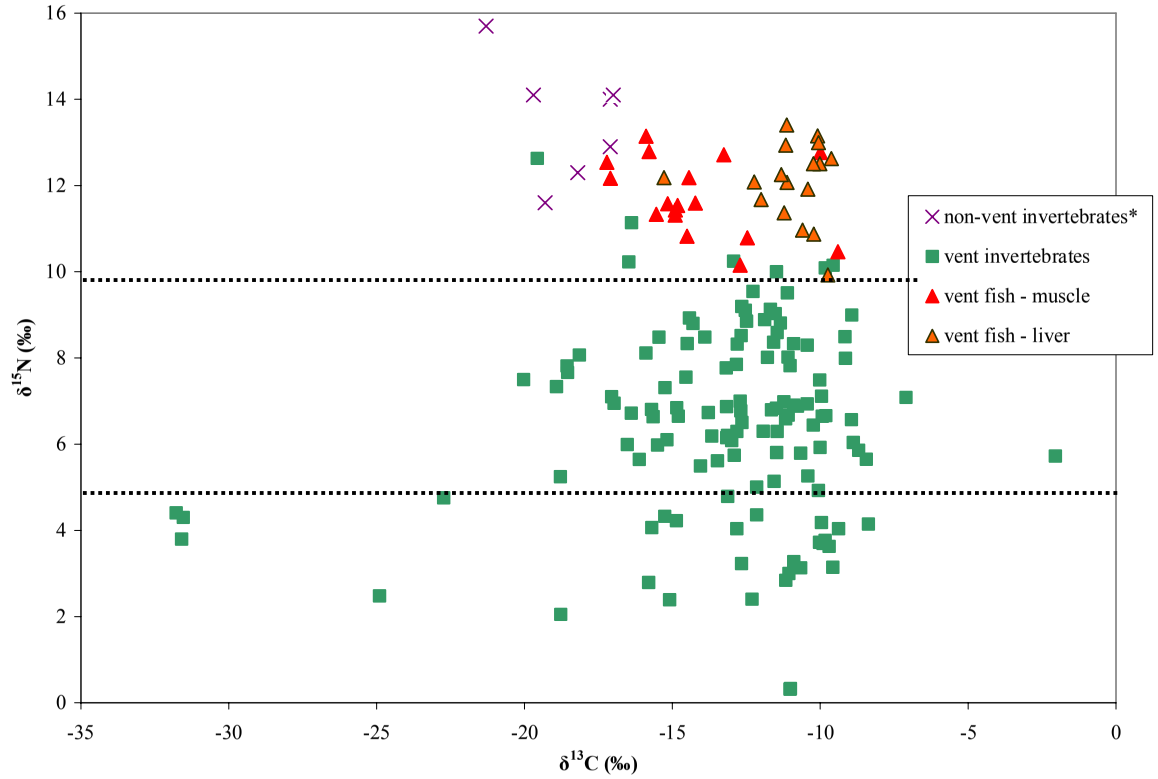
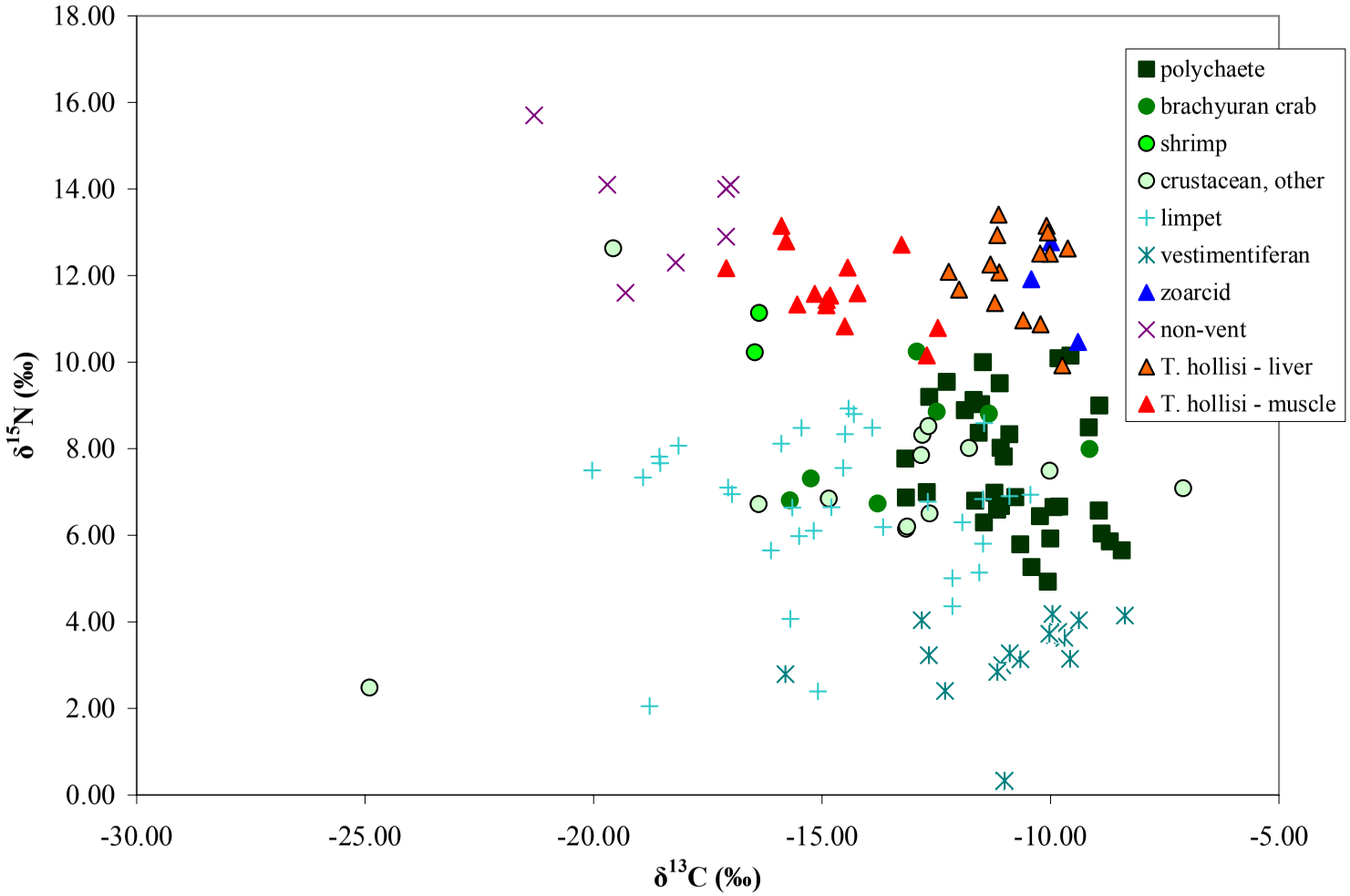
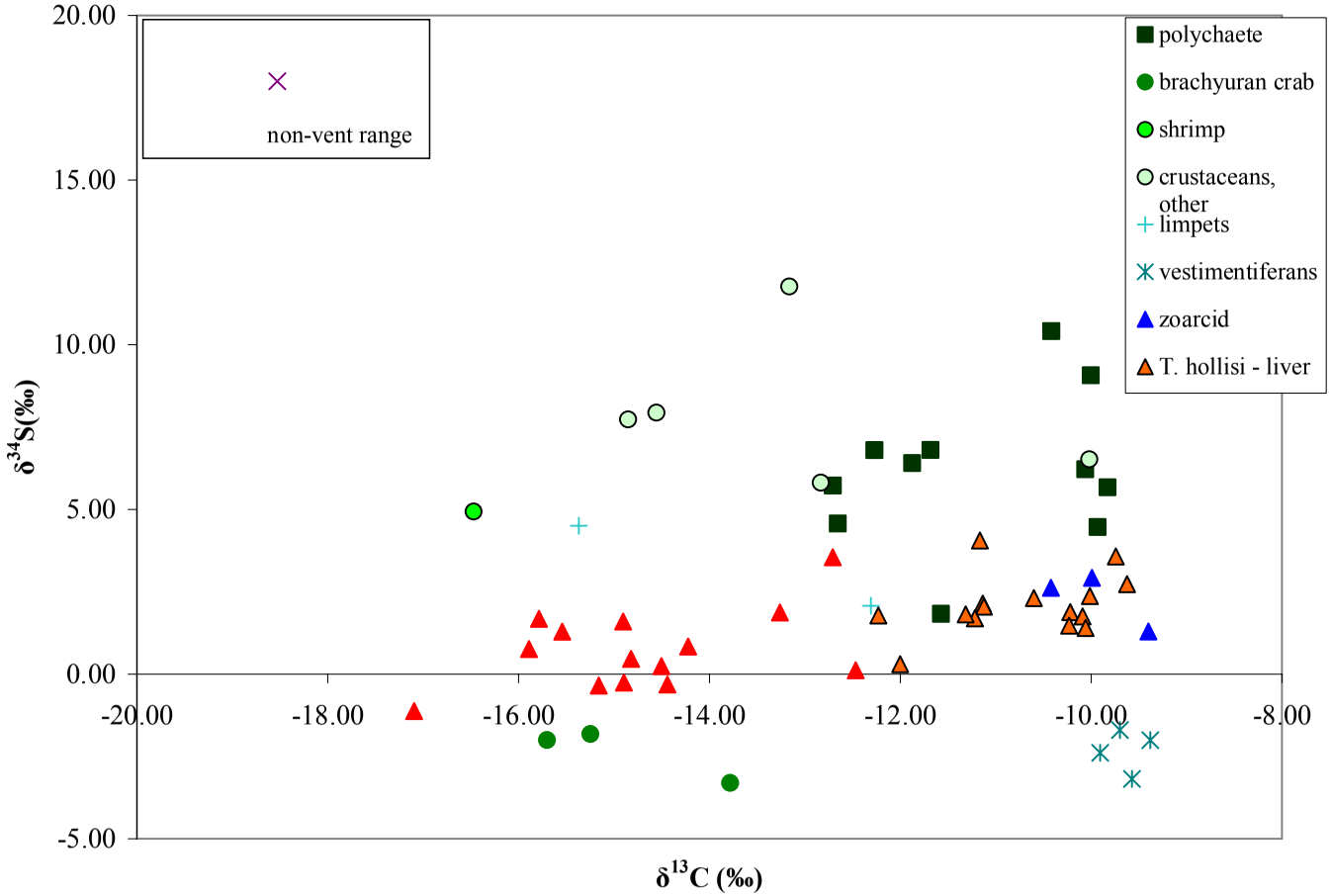


Figure 2.9 Carbon versus nitrogen values for all fauna analyzed. Vent fish values range from -17 to -9‰ ($\delta^{13}C$) and 9 to 13‰ ($\delta^{15}N$) and fall between those of vent and non-vent invertebrates. There appears to be at least three trophic levels (roughly indicated by dotted lines) represented in these samples, with the fish and one species of crab occupying the highest level and vestimentiferans and the polynoid *B. hessleri* the lowest.


18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
δ15N (‰)
-30.00
-25.00
-20.00
-15.00
-10.00
-5.00
δ13C (‰)
polychaete
brachyuran crab
shrimp
crustacean, other
limpet
vestimentiferan
zoarcid
non-vent
T. hollisi - liver
T. hollisi - muscle

2.10a



2.10b

Figure 2.10 – Carbon and nitrogen values (2.10a) and sulfur values (2.10b) for all samples analyzed, grouped by gross taxonomic categories. Non-vent invertebrate values are from Van Dover and Fry (1989).

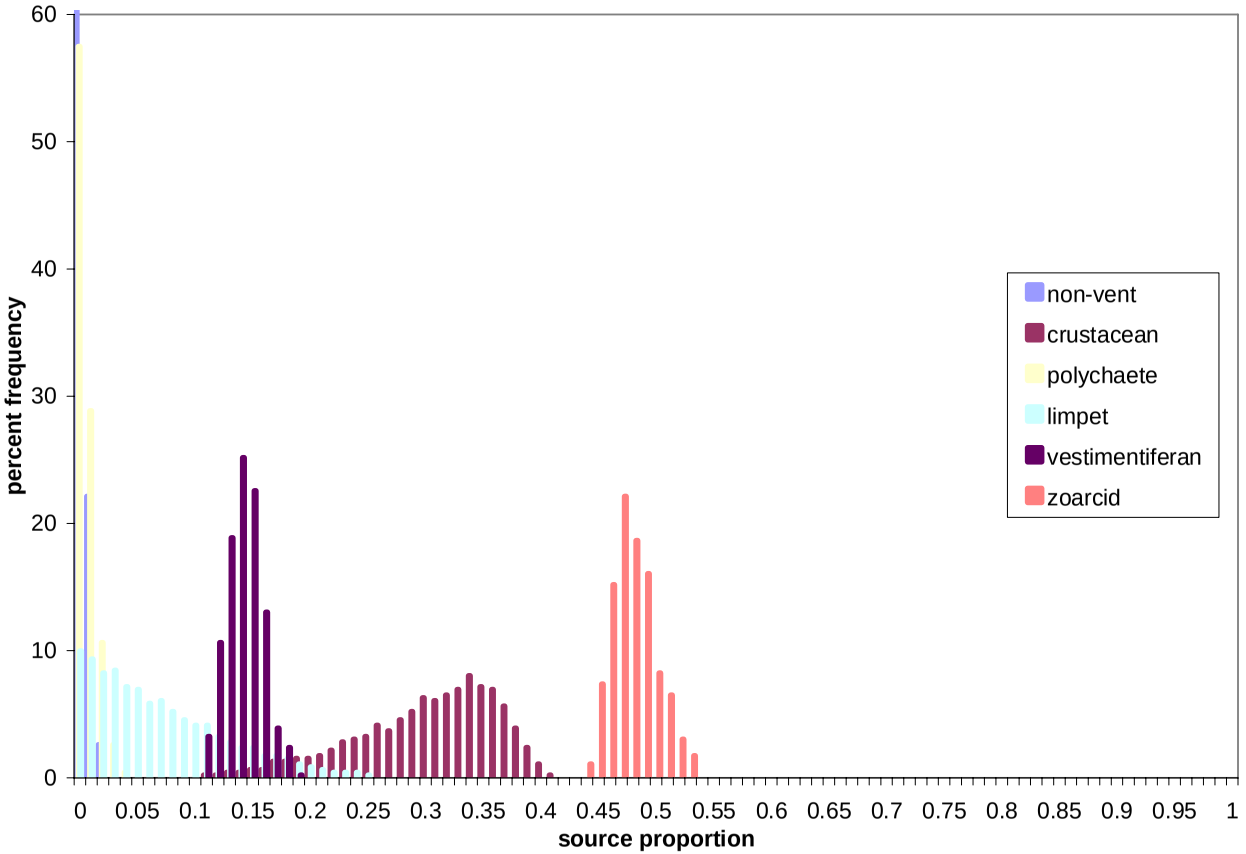


Figure 2.11 Range of potential importance of sources towards creating the isotopic mixture represented by liver tissue as generated by the Isosource (Phillips & Gregg, 2003) program. Using a mass balance mixing model, zoarcids (or something with a similar isotopic signature) have the greatest potential contribution to the diet of *Thermichthys hollisi* as represented by liver carbon, nitrogen and sulfur isotope values. The program was run at 1% increments with a 0.1‰ tolerance.

2.4 Discussion

The gut content analysis supports the hypothesis that *Thermichthys hollisi* prey upon species typically associated with hydrothermal vent environments, including the zoarcid *Thermarces cerberus*, the shrimp *Alvinocaris lusca*, and the crab *Bythograea thermydron*. The accompanying isotope data support this conclusion and allow for a more comprehensive look at *T. hollisi* prey preference and trophic ecology. The range of $\delta^{13}C$ values for *T. hollisi* fall within areas that are covered by both vent and non-vent carbon sources, indicating that the fish may be utilizing both vent and non-vent carbon sources. This result is not entirely unexpected given that the fish is a large mobile predator that tends to be found more in the periphery of vent fields than directly in diffuse or direct flow areas.

As carbon values typically change little between trophic levels, it would appear that *T. hollisi* is utilizing more vent-produced carbon sources than non-vent. However, it must be remembered that interpretation of carbon isotope values can be influenced by tissue analyzed. $\delta^{13}C$ values can be especially influenced by lipid content, as lipids have been found to be $\delta^{13}C$ depleted, thus differences in lipid content between individuals and tissue types can lead to variation in $\delta^{13}C$ values that are greater than the expected 1‰ difference between trophic levels, resulting in potential misinterpretations of trophic relationships. Various "lipid-correction" methods have been employed to counteract this, including both empirical and analytical methods. Lipid extraction methods typically utilize organic solvents such as chloroform/methanol to physically remove lipids from the

tissue to be analyzed. These methods cause a change in $\delta^{15}N$ as well as $\delta^{13}C$, and thus separate carbon and nitrogen analyses must be performed on both extracted and non-extracted tissues respectively (Sweeting et al., 2006). Murry et al. (2006) suggested that though lipid-extraction methods change both the carbon and nitrogen values and shift the placement of the food web, the extraction process does not alter interpretation of community structure. Lipid extraction methods can be disadvantageous to undertake in that they are often costly, time consuming, and require greater amounts of tissue than may be available (Sweeting et al., 2006). These disadvantages can be avoided by applying theoretical corrections (lipid-normalizations) based on C:N ratios measured within the tissues. Kiljunen et al. (2006) review the problems and processes associated with both empirical and theoretical lipid-corrections and offer a revised model for arithmetic lipid-normalization that is proposed to be valid across multiple fish taxa, but can be fine-tuned by testing against lipid-extraction data for a more species(or tissue)-specific model. This study also suggested that lipid-normalization should be applied to aquatic fish but not invertebrates for use in mixing models (Kiljunen et al., 2006). The application of this revised lipid-normalization technique to the *T. hollisi* tissues resulted in a shift of liver carbon values approximately 5‰ heavier, and created a significant difference between average *T. hollisi* muscle and liver carbon values. This difference may represent changes in long-term versus short-term feeding habits. Perga and Gerdeaux (2005) hypothesized that fish liver tissue, due to its constant turnover, would be a better estimate of recent food consumption than muscle, whereas muscle would provide a more accurate long-term estimate. In the course of their study, they found

evidence that seasonal dietary shifts may be better reflected in liver tissue. Both carbon and sulfur signatures showed significant differences between the two tissue types analyzed, which may reflect differences in diet or differences in tissue turnover. It is possible that the differences in muscle and liver tissue values could represent differences in long-term versus short-term feeding trends, as was hypothesized by Perga and Gerdeaux (2005).

It is typical to see only one size class (mean length of those collected was 33cm with a standard deviation of 3cm) of *Thermichthys hollisi* in the venting areas (pers. obs.), leading one to hypothesize that the species may be migrating and feeding away from vents for part of their life cycle, explaining in part the different signatures evidenced by the muscle and liver tissues. It can also be hypothesized that *T. hollisi* is altering prey preference in response to prey availability. At the time of collection, L vent was home to numerous brachyuran crabs (*Bythograea thermydron*) (pers. obs.), potentially making the crabs an easy prey target for *T. hollisi*.

Regardless of these differences in carbon values between different tissues, both the gut content analyses and the carbon and nitrogen isotopic data indicated that *Thermichthys hollisi* is getting at least part (and more likely the majority) of its nutrition from chemosynthetically supported carbon sources. This conclusion is strongly supported by the sulfur isotope data. Tissue sulfur isotopic values are generally derived almost exclusively from dietary intake and do not typically fractionate between trophic levels, thus one would expect a similar value between prey and predator (Fry et al., 1983; Kennicutt et al., 1992). There are relatively few sulfur data for non-vent species within

the study region, but species relying on photosynthetically-fixed carbon sources would be expected to have $\delta^{34}S$ values ranging from approximately 16 to 20‰, whereas those at vents trend toward 0‰ (Mizota, 1997). Sulfur values for *T. hollisi* are surprisingly depleted, falling below the proposed 5‰ limit delineating chemoautotropic organisms (Vetter & Fry, 1998). Of the data measured in this study, only the vestimentiferan *Tevnia jerichonana* and the crab *Bythograea thermydron* are more depleted than the fish. Invertebrate measurements are consistent with previous studies (Fry, et al., 1983; Brooks et al., 1987; Kennicutt et al., 1992; Mizota, 1997). The depleted (near 0‰) sulfur signatures of the fish tissues indicate that they are consuming prey items that are associated with venting environments. The simple two-source mixing model for the sulfur data alone indicates a dietary fraction of 88% from a chemosynthetic source, additionally supporting the conclusion that *T. hollisi* is feeding almost entirely upon vent-endemic fauna. However, the sulfur signatures of *T. hollisi* tissues do not exactly match any of the vent invertebrates measured, indicating a need for additional sulfur isotope data from vent fauna to further clarify which prey sources provide the greatest dietary contribution.

The gut content analyses and the nitrogen data support the hypothesis that *Thermichthys hollisi* are high trophic level predators within the vent ecosystem, extending the existing knowledge of energy transfer within the system. As a large predator, *T. hollisi* has the potential to affect the community composition of their environment. Recorded observations of *T. hollisi* do not indicate that the species is particularly widespread, but where they are located they are typically abundant, and thus

may be affecting localized, but not widespread community dynamics and composition through their feeding habits. In particular, the present study shows that the zoarcid *Thermarces cerberus* substantially contributes to the diet of *T. hollisi*. It can be hypothesized that *T. hollisi* directly affects the density of zoarcids and thus indirectly affects gastropod populations through the removal of *T. cerberus* within a localized area. Manipulative experiments would be useful to determine the effect of *T. hollisi* predation (or lack thereof) on the localized community composition and structure. The information garnered within the scope of this study allows for a more comprehensive view of trophic networks and the flow of energy within the venting system on the East Pacific Rise (Figure 2.12), particularly expanding the identity of upper trophic level predators.

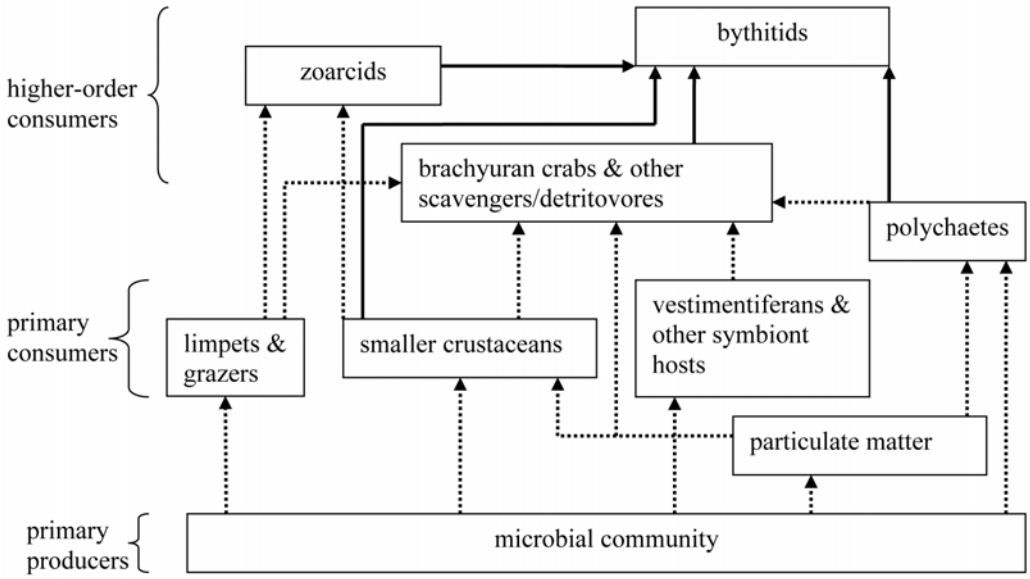


Figure 2.12 Hypothesized transfer of energy through trophic levels at venting communities on the East Pacific Rise. Solid arrows represent data from this study, dotted lines are inferred from this and previous work. (Modified from Bergquist et al., 2007).

Summary

This study offers the first direct evidence of *Thermichthys hollisi* prey choice and dependence upon the chemosynthetically supported vent communities for nutrition; and supports the hypothesis that this species of fish is an upper trophic level predator within the venting environments on the East Pacific Rise. As such they have the potential to both directly and indirectly affect the surrounding invertebrate community population density, composition, and structure.

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Chapter Three

Otolith Isotope Chemistry of Hydrothermal Vent Fish: Insights into Habitat Use and Life History Strategy

Abstract

Laser-ablation ICPMS was used to analyze otolith isotope chemistry of hydrothermal vent-endemic fish for the first time. Strontium, barium, lithium and magnesium were measured with the greatest accuracy and showed significant differences between both species of vent fish (*Thermarces cerberus* and *Thermichthys hollisi*) and non-vent specimens analyzed. The influence of exposure to hydrothermal fluid is apparent in otoliths from both species of vent fish, most noticeably within the relatively elevated Sr:Ca and depleted Mg:Ca ratios. Otolith chemistry suggests that *T. cerberus* experiences greater direct exposure to diffuse fluids than does *T. hollisi*, which is consistent with apparent habitat preferences. Isotopic patterns across the span of the otolith suggest that *T. cerberus* spends its entire life within the vent system. In contrast it appears that *T. hollisi* exists outside of the influence of hydrothermal activity for some early portion of its life-cycle.

3.1 Introduction

Since the discovery of hydrothermal vents and their associated biological communities, biological studies have largely focused on understanding the physiology, evolution, adaptation, larval dispersal, trophic relationships and connectivity of the vent-endemic fauna (Childress & Fisher, 1992; Vrijenhoek, 1997; Tyler & Young, 1999). An area of particular interest that has proven challenging to study is the clarification of life history strategies employed by vent fauna, beyond the basic categorization of larval type and the inferred dispersal and gene flow. Research in these areas has focused on vent invertebrates, with no prior studies dedicated to studying the life history of the vent-endemic vertebrate fauna on the East Pacific Rise (EPR). Two species of fish are considered endemic to vents on the EPR, the zoarcid *Thermarces cerberus* (Rosenblatt & Cohen, 1986) and the bythitid *Thermichthys hollisi* (Cohen et al., 1990). Both *T. cerberus* and *T. hollisi* are considered dependent upon vent communities for nutrition

(Micheli et al., 2002; Sancho et al, 2005; Buckman & Shank, in prep). As top predators within the system, vent fish have the ability to influence species composition and abundance of lower trophic levels. Despite this ecological importance, little is known regarding their life history. *T. hollisi* typically occupies the periphery of vent fields, particularly in "fish holes" (collapse pits or cracks in the basalt where fish aggregate), but there are no recorded observations of different size classes. It is common to observe multiple sizes (and presumably ages) of the zoarcid *Thermarces cerberus* (Shank et al., 1998), suggesting that as a species *T. cerberus* spends its entire life cycle within the vent field while *T. hollisi* may migrate outside the vent field for some portion of its existence.

Difficulties in observing deep-sea fish *in situ*, as well as challenges to capturing them in relevant numbers, make ecological studies challenging and elusive. When unable to observe an organism directly to learn about its biology and life strategy, tools such as tags, tracers, and/or population genetic techniques can be employed to approximate life histories and migrations. The study of otolith (fish ear bone) chemical composition is an effective alternative approach to direct observation in order to elucidate the life histories of deep-sea fish.

Otoliths are paired calcium carbonate structures found within the semicircular canals of all teleost fish, where they assist in balance and/or hearing. There are typically three pairs of otoliths per fish, the largest and most commonly utilized is the sagittal pair. Two main attributes make them useful as recorders of fish habitats conditions. First, calcium carbonate is routinely and regularly deposited onto the otolith. The regularity of otolith deposition, as well as its inability to be reworked or resorbed (Campana, 1999),

accounts for the annuli (rings) that are commonly used for age determination within fish (Campana and Nielsen, 1985; Jones, 1986). Second, the composition of the accreted otolith reflects the fluid chemistry of the fish's habitat. Stable isotopes and trace elements within the fish's environment are also regularly incorporated into the otolith. The ability of otoliths to incorporate these isotopes and trace metals in a relatively predictable manner has lead to an area of research dedicated to examining otolith chemistry in order to reconstruct the physical and chemical environments experienced by fish throughout their lifetime, and has proven most useful for fish that utilize chemically distinct water masses (Elsdon and Gillanders, 2002; Brazner et al., 2004).

In principle, environmental water chemistry should be correlated with otolith chemistry, but in reality the incorporation of elements into the otolith and the interpretation of the resulting otolith chemistry are less straightforward. Elements that are less sensitive to physiological regulation are better candidates for otolith studies, and the uptake and deposition of trace elements including strontium (Sr), barium (Ba), manganese (Mn), zinc (Zn), lead (Pb), iron (Fe), lithium (Li), cadmium (Cd) and nickel (Ni) appear to be regulated more by environmental availability than biological activity (Campana, 1999). Ba, Sr, and Mn otolith concentrations in particular appear to be dictated more by habitat water chemistry than environmental or physiological factors such as salinity or fish growth rates (Martin & Thorrold, 2005; Walther & Thorrold, 2006); though the linkages between fish environment and otolith composition are complex and not fully understood.

Venting environments, in contrast to the surrounding deep-sea, have highly variable fluid chemical composition and flux that can influence the peripheral habitats. Though fluid chemistry varies both temporally and spatially, a number of elements often considered in otolith studies (Sr, Ba, Mn) are typically enriched in hydrothermal fluids relative to non-vent seawater values (Table 1). Fish associated with these chemosynthetic habitats may incorporate hydrothermally enriched concentrations of elements into their otoliths, making otoliths a useful tool for gaining insights into vent fish life history and interaction with venting habitats during their lifespan. Due to the high variability in vent fluid flow over short temporal and spatial scales at hydrothermally active areas, it is likely that only broad scale patterns may be visible within the otolith. Finer-scale observations, on the level of individual vent sites and local temporal variability, may be too ephemeral or short-lived to be resolved within the otolith. However, it may be possible to distinguish between fish that utilize different micro-habitats within the same vent field. Given that *Thermarces cerberus* is known to live directly within diffuse flow, while *Thermichthys hollisi* tends to inhabit areas less directly influenced by hydrothermal flow, the chemical composition of *T. hollisi* otoliths could be expected to exhibit elemental values intermediate to those of *T. cerberus* and a "typical" deep-sea fish.

Table 3.1 Examples of element concentrations in East Pacific Rise and Galápagos hydrothermal fluids as well as in seawater samples.

Area	vent	sample year	T °C	Li µmol kg^{-1}	Mg mmol kg^{-1}	Ca mmol kg^{-1}	Mn µmol kg^{-1}	Cu µmol kg^{-1}	Sr µmol kg^{-1}	Cd nmol kg^{-1}	Ba µmol kg^{-1}	rare earth element	H_2S mmol kg^{-1}
	typical smoker [a]		350	410-1320	0	10-55	360-1140	10-40	90		10-40	+ Eu anomaly [h]	
	average seawater [a]			25	50	10	0	0.007	85		0.15	-Ce anomaly [h]	0
EPR	northern transect [b]	1991-2000	22-33.3		46.3-50.5		11.5-78.9						.003-.901
	middle transect [b]	1991-2000	10.5-34.7		46.9-50.4		7-48.8						0.127-1.91
	southern transect [b]	1991-2000	11.9-55		0-50.5		0.7-192						0.001-11.24
	9°29-40'N [c]	1991		268-1290		12.2-46.9	314-854		34.4-100				5.9-9.9
	9°46-54'N [c]	1991	403	20-98.1		2.1	109-283		20				110
	11°N [c]	1984	347	484-884		10.6-35.2	742-925		38-135				4.4-12.2
	13°N [c]	1982;1984	317-380	591-688		44.6-55	1000-2930	168-182					
	21°N [c]	1979;81;85	273-355	891-1322		11.7-20.8	669-1000		65-97		8-16		6.6-8.4
	OBS [d]	1983?		891	0	0.0156	960	35	81	155	7		7.3
	northern transect [b]	1991-2000	664-381		0		172-1190						6.2-26
	middle transect [b]	1997-2000	352-355		0		1080-1410						

	southern transect [b]	1992-2000	160-351		0		192-690						11.2-20.7
	Biovent [e]	1996			1.36-2.83				42.2-44.9				
	M vent [e]	1996			3.23-42.23			93.7-124.2					
	P vent [e]	1996			3.25-19.2				93.8-104.7				
	Bio9' [e]	1996			1.36-22.18			89.2-91.8					
	A vent [e]	1996			4.03-40.1				86.5-97.6				
	L vent [e]	1996			1.61-3.63				147.9-155.9				
	K vent [f]	2004	203		8.2	22.3	0.191	0.1					
	K vent [f]	2004	203		27.31	17.55	0.109	0.1					
	Bio9" [f]	2004	383		5.3	11.9	0.483	50.33					
	Bio9" [f]	2004	383		8.5	11.9	0.461	45.79					
	Tica [f]	2004	344		5.2	11.5	0.377	8.45					
	Tica [f]	2004	344		7.2	11.1	0.369	7.45					
	Biovent [f]	2004	331		25.3	10.1	0.101	8.45					
	Biovent [f]	2004	331		4.3	9.5	0.18	12.34					
Galapagos	Rosebud [g]	2002, 2005	14-18	36.9-48	49.88-51.54	2.2-5							
	Garden of Eden [g]	2005	17	44	50.53		4.8						0.018
	New Site [g]	2005	8	37.1	51.66		2						0
	ALR vent [g]	2002	10	31.1	51.77		0.6						0
	Galapagos [c]	1977	13	689-1142		24.6-40.2	360-1140		87		17.2-42.6		
	seawater [g]	2002	2	25.1	52.65		0.1						0
	seawater [d]			26	52.7	0.0102	0.001	0.007	87	1			0
	seawater (9°50'N) [b]	1.8		52.2		0.0005							
	seawater [e]	1996			52.2				85				

	seawater [f]	2004	2		54.8	9.95	0.00001	<0.1					

a. Van Dover, 2000 b. Von Damm & Lilley, 2004 c. Von Damm, 1995 d. Edmond & Von Damm, 1985 e. Ravizza et al., 2001 f. Rouxel et al., 2008 g. Pester et al., 2008 h. Klinkhammer et al., 1994 Blank boxes indicate the data were not measured or reported as part of the study.

The goal of this study is to explore (chemical) habitat preferences over the lifespan of vent-endemic fish by using otolith chemical composition in order to elucidate habitat usage and life history of the organisms. Initially we examine the limitations and assumptions of otolith isotope studies in the application to variable environments (in this case vents). In other words, is this technique sensitive enough to detect variability within the venting environment and are there differences between vent and non-vent fish? We then examine *Thermichthys hollisi* and *Thermarces cerberus* life history strategies through the chemical analysis of the otolith over the fishes' life span.

Hypotheses:

- Hydrothermal fluid exposure will be reflected within the otoliths of vent fish as an increased concentration of hydrothermally enriched elements in comparison to non-vent fish.
- *Thermarces cerberus*, due to its habitat preference, will exhibit a greater concentration of hydrothermal elements than *Thermichthys hollisi*.
- *Thermichthys hollisi*, based on observed sizes of fish associated with vents, does not spend its entire life cycle within the influence of hydrothermal systems.

3.2 Methods

3.2.1 Otolith processing

Individuals of *Thermichthys hollisi* and *Thermarces cerberus* were collected as described in the previous chapter. The fish were frozen at sea with the otoliths intact inside the fish until processing in the laboratory could occur. Twelve individuals of *T. hollisi* were partially defrosted in warm water, the top portion of the head removed, and both sagittal otoliths (hereafter otolith will only be referring to the processed sagittae) were removed from each individual (Figure 3.1). The otolith pairs were rinsed in Milli-Q water and left to dry overnight. *T. hollisi* otoliths (one per individual) were mounted in wax and horizontally sectioned on a Buehler Isomet low speed diamond-blade saw with three spacers. The resulting ~24μm wide thick section was mounted on a slide using superglue. The otoliths were polished using decreasing grit lapping paper (240 grit, 30 micron and 3 micron) until it was clear the core had been reached (Figure 3.2). Each otolith was then soaked in Milli-Q water until it lifted off of the slide, flipped, mounted in glue, and polished on the opposite side to achieve as thin a section as possible without compromising the structure of the otolith. Non-vent, deep-sea fish otoliths (2 individuals of *Coryphaenoides acrolepis*; 1 individual of *Lycodes diapterus*; and 1 individual of *Nezumia stelgidopelis* from the Pacific slope) were kindly provided by W. Wakefield and V. Simon (NOAA Fisheries, NWFSC). A double sagittal grind was performed on the non-vent and *T. cerberus* otoliths, without prior sectioning, as otolith structure was readily visible under these conditions. Under class 100 clean room conditions the polished otoliths were cleaned by rinsing with ultra-pure 5% nitric acid, and sonicating

for five minutes in ultra-pure Milli-Q water, and then triple rinsing with ultra-pure water. The otoliths were allowed to dry for a minimum of one hour in a laminar flow hood and were mounted onto a clean slide (eight otoliths per slide) using double-sided sticky tape and stored until analysis on the mass spectrometer.

3.2.2 ICPMS Analysis

The chemical composition of the otoliths was analyzed on a ThermoFinningan Element2 single collector inductively-coupled plasma mass spectrometer linked to a New Wave Research UP213 deep-UV YAG laser ablation system. The laser software was utilized to ablate a raster in the core of each otolith using a 70 um width 5 Hz laser beam at 70% power. Each otolith was subsequently ablated in 100um width lines sequentially aligned from the edge of the core to the edge of the otolith. Ablation lines were designed to follow the arrangement of otolith deposition rings as well as possible (Figure 3.3). A suite of elements (^{7}Li, ^{25}Mg, ^{48}Ca, ^{55}Mn, ^{63}Cu, ^{88}Sr, ^{114}Cd, ^{138}Ba, ^{139}La, ^{140}Ce, ^{142}Nd, ^{152}Sm, ^{153}Eu, ^{158}Gd, and ^{208}Pb) were measured at low resolution utilizing a 5% mass window. Blank and standard solutions (40ppm FEBS-1 – Sturgeon et al., 2005 and NIES-022 – Yoshinaga et al., 2000) were run at regular intervals between ablations lines. Data were reported as mean intensity averages. Blank values were linearly interpolated between blank measurements and subtracted from the raw intensity averages to remove background interference. Instrument mass bias was corrected for by standardizing the values to a reference solution (FEBS-1) as per the method outlined in Rosenthal et al. (1999). The Ba:Ca correction factor was used to correct the rare earth elements.

Detection limits were calculated as 3 times the standard deviation of the mean of the blank solutions and are reported as a percentage of the average sample intensity. They are as follows: Li 14.08, Mg 5.94, Ca 0.05, Mn 48.17, Cu 65.85, Sr 0.02, Cd 46.5, Ba 0.23, La 31.42, Ce 31.1, Nd 67.57, Sm 33.73, Eu 31.81, Gd 51.52, Pb 62.25. Relative standard deviations, a measure of external precision were as follows: Li 2.56, Mg 5.09, Mn 43.3, Cu 66.79, Sr 0.64, Cd 26.66, Ba 1.18, La 42.97, Ce 54.17, Nd 111.8, Sm 25.34, Eu 23.59, Gd 114.37, Pb 55.18. Core versus non-core values were compared among groups and statistical analyses were performed using Systat ver. 10.

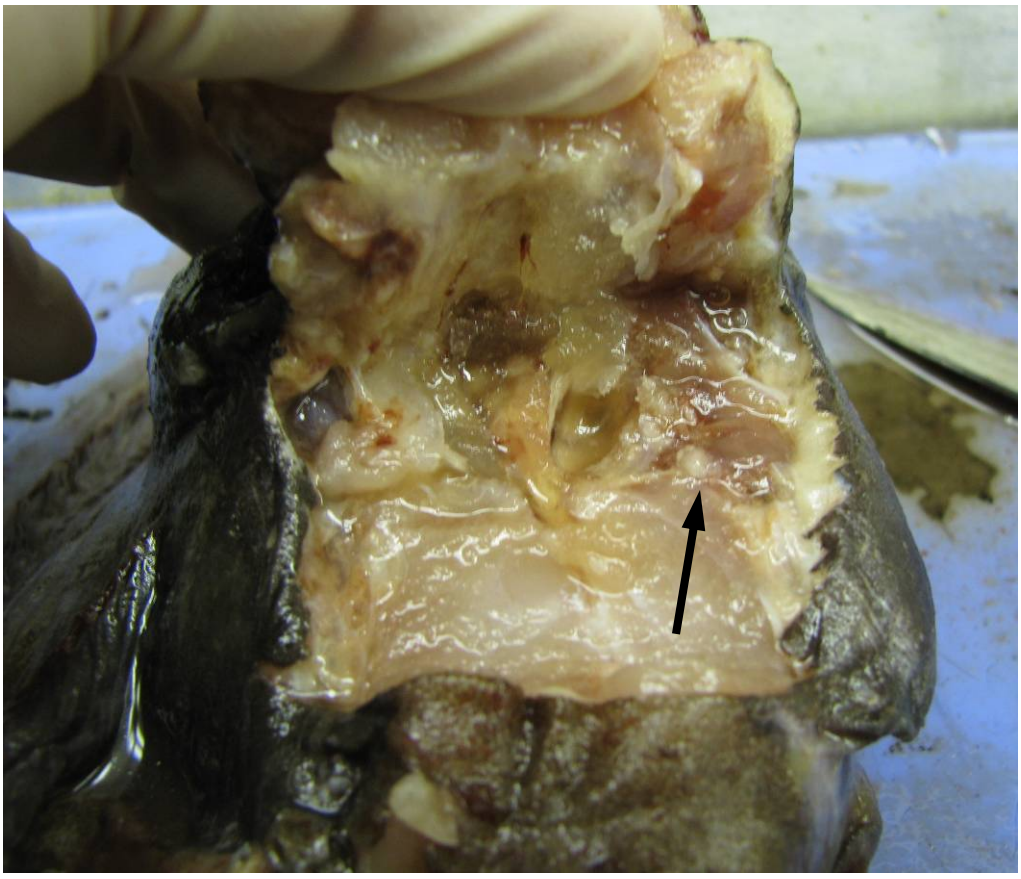

Figure 3.1 Cranial cavity of *Thermichthys hollisi*. The arrow points to a sagittal otolith.

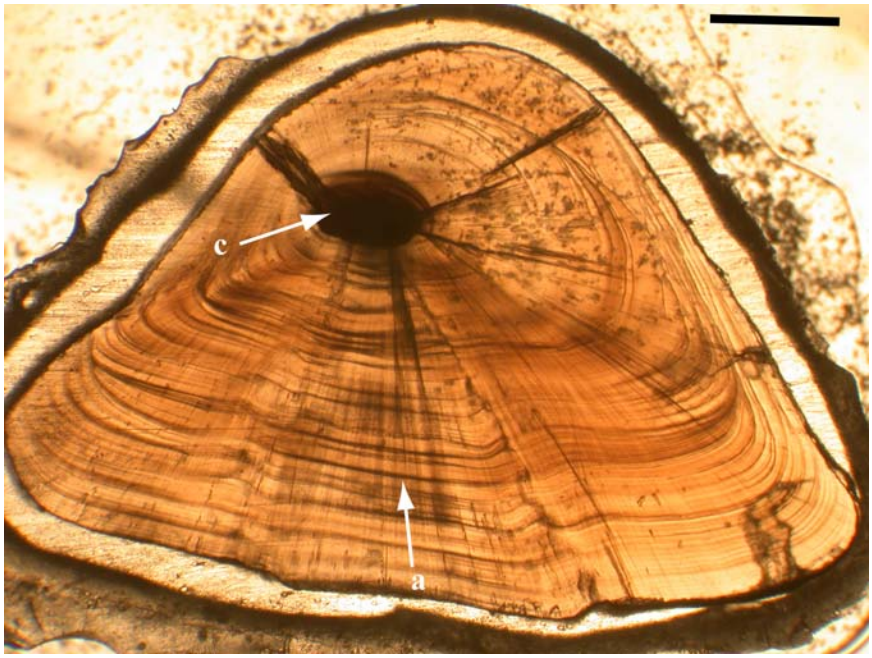

Figure 3.2 Example of a polished *Thermichthys hollisi* otolith, exhibiting clear structure of the core (c) and annuli (a). Scale bar is approximately 1mm.

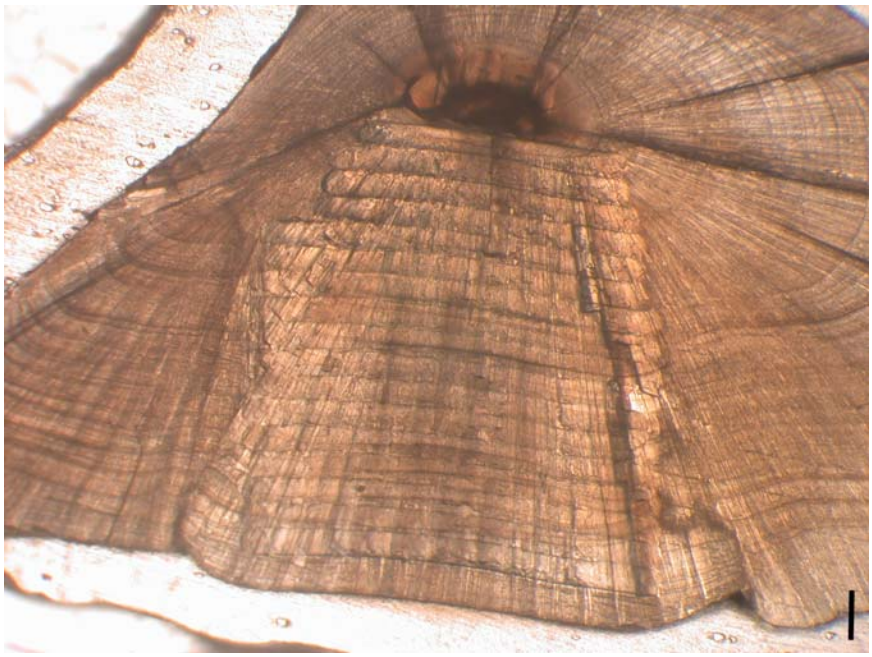

Figure 3.3 Ablated section of *Thermichthys hollisi* otolith. Scale bar is 200µm.

3.3 Results

In total 12 individuals of *Thermichthys hollisi*, two individuals of *Thermarces cerberus*, and four unrelated non-vent fish were analyzed; hereafter referred to as "bythitid", "zoarcid", and "non-vent" respectively. Li, Mg, Sr, and Ba measurements were above detection limits for 100% of the samples taken from all fish (Figure 3.4). Bythitid Li:Ca ranged from 1.700 to 11.700 $\mu mol.mol^{-1}$ with a mean lifetime average of 3.406 $\mu mol.mol^{-1}$, while zoarcid measurements ranged from 6.037 to 9.645 $\mu mol.mol^{-1}$ with a mean lifetime average of 6.805 $\mu mol.mol^{-1}$, and non-vent fish Li:Ca ranged from 4.965 to 15.501 $\mu mol.mol^{-1}$ with a mean lifetime average of 10.005 $\mu mol.mol^{-1}$. Mg:Ca levels ranged from 3.101 to 144.615 $\mu mol.mol^{-1}$ (mean 11.372) for bythitids, 9.313 to 30.466 $\mu mol.mol^{-1}$ (mean 13.056) for zoarcids, and 24.527 to 158.180 $\mu mol.mol^{-1}$ (mean 55.623) for non-vent fish. Sr:Ca ranged from 2500.636 to 10488.313 $\mu mol.mol^{-1}$ (mean 6496) for bythitids, 4119.770 to 14660.969 $\mu mol.mol^{-1}$ (mean 9999.228) for zoarcids, and 1924.316 to 6364.109 $\mu mol.mol^{-1}$ (mean 3443.361) for non-vent fish. Ba:Ca ranged from0.954 to 5.553 $\mu mol.mol^{-1}$ (mean 2.255) for bythitids, 8.134 to 31.012 $\mu mol.mol^{-1}$ (mean 17.563) for zoarcids, and 1.093 to 8.406 $\mu mol.mol^{-1}$ (mean 4.165) for non-vent fish. Between 50 and 90% of the bythitid measurements (Figure 3.5) were above detection limits for Cd:Ca (mean 0.004 $\mu mol.mol^{-1}$), La:Ca (mean 0.001 $\mu mol.mol^{-1}$), Ce:Ca (mean 0.001 $\mu mol.mol^{-1}$), Sm:Ca (mean 0.001 $\mu mol.mol^{-1}$), and Eu:Ca (mean 0.001 $\mu mol.mol^{-1}$). For zoarcids, between 50 and 90% of the measurements (Figure 3.5) were above detection limits for Cd (mean Cd:Ca 0.006 $\mu mol.mol^{-1}$), La (mean La:Ca 0.002 $\mu mol.mol^{-1}$), and Ce (mean Ce:Ca 0.001 $\mu mol.mol^{-1}$); while Sm (mean Sm:Ca

0.005 μmol.mol^{-1}) and Eu (mean Eu:Ca 0.007 μmol.mol^{-1}) were above detection limits for all zoarcid samples. For non-vent fish, Mn (mean Mn:Ca 1.580 μmol.mol^{-1}), Sm (mean Sm:Ca 0.002 μmol.mol^{-1}), and Eu (mean Eu:Ca 0.002 μmol.mol^{-1}) were above detection limits for between 50 to 90% of the samples (Figure 3.6).

Otoliths from individual fish within a species group revealed additional patterns. Li:Ca ratios are visibly different between the three groups (Figure 3.7) with zoarcids exhibiting values intermediate to the other two groups. The two zoarcids examined showed similar patterns over their lifetime for most elements, most noticeably strontium (Figure 3.8), cadmium (Figure 3.9), barium (Figure 3.10), and magnesium (Figure 3.11). Manganese (Figure 3.12), copper and lead did not show distinct differences between between groups. Copper and lead measurements were mostly below detection limits and exhibited large RSDs, and thus will not be considered further. With the exception of Sm:Ca and Eu:Ca, most non-vent fish rare earth measurements were below detection limits, while for the bythitids and zoarcids only Nd:Ca and Gd:Ca were below detection limits for the majority of the samples. For both Sm:Ca and Eu:Ca, zoarcids exhibited the highest ratios and bythitids the lowest, with differences between all groups (Figure 3.13).

The values within the core of bythitid otoliths are noticeably elevated for multiple elements. A comparison of mean core values between groups using a one way ANOVA with a Bonferroni post-hoc test (significance level 0.05) applied indicated that bythitid core values for barium are indistinguishable from that of non-vent fish, but are significantly different from core values of zoarcids. However, mean barium values for all non-core measurements differed significantly between all groups. Similarly, Li:Ca and

Sr:Ca core bythitid values are similar to that of non-vent fish, but significantly different from zoarcids (zoarcids also differ from non-vent), whereas there are differences between all groups for non-core values. For Mg:Ca, bythitid core values did not differ from those of zoarcid or non-vent, but zoarcids were significantly different from non-vent. For non-core Mg:Ca values, bythitids were not able to be distinguished from zoarcids, but both zoarcids and bythitids differed from non-vent. Repeated-measures ANOVA indicated a significant (multivariate Wilks' Lambda significance level $p < 0.05$) increase in strontium across the span of the entire otolith for bythitids, but other than the distinct core signatures, there are no significant cross-otolith patterns for Li, Mg, or Ba.

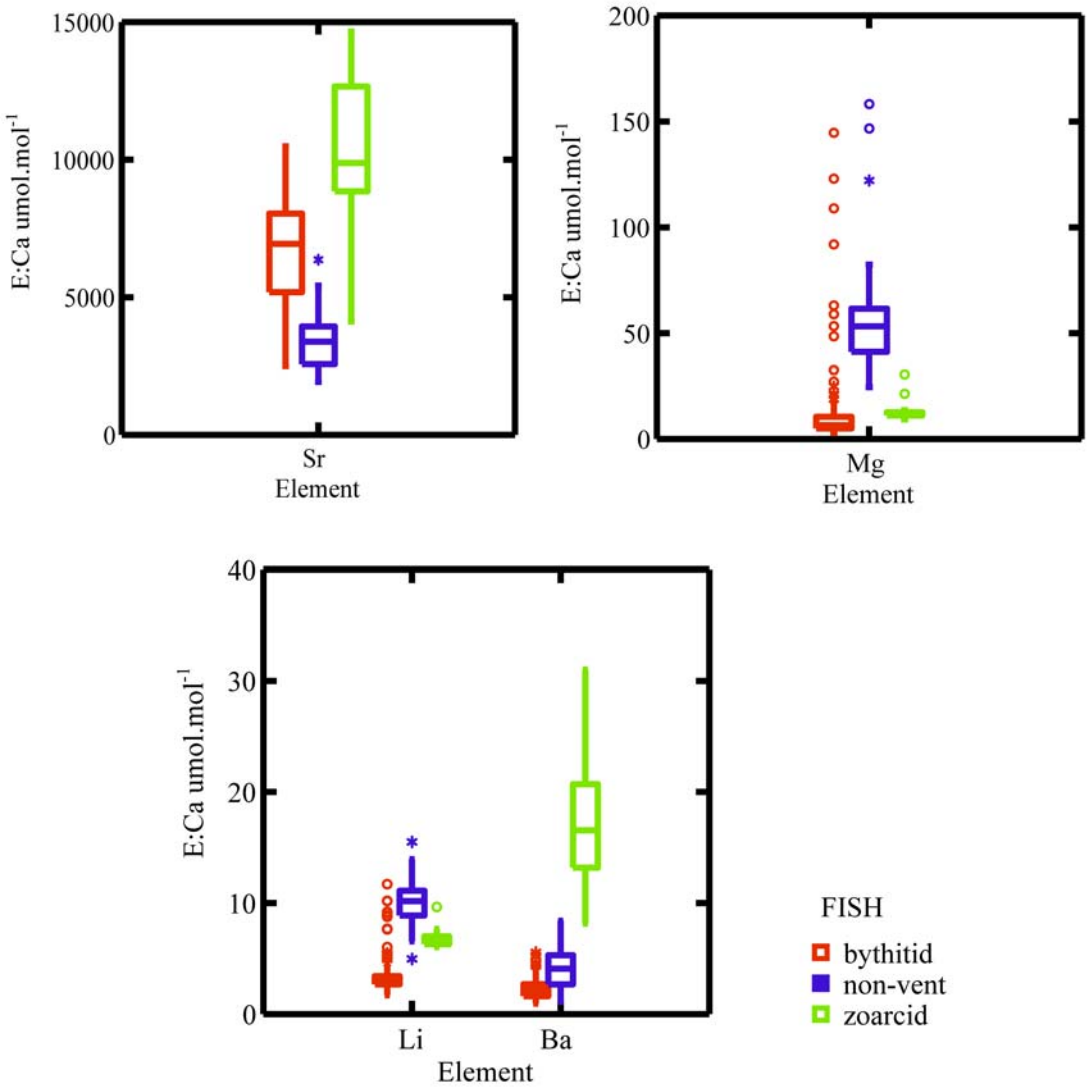

Figure 3.4 Box plot of elements found in 100% of samples taken (Li, Ba, Sr, and Mg), indicating the median and range of values observed. The box indicates the range incorporating 50% of the values. Asterisks indicate values in between the inner and outer fence and open circles represent far outside values.

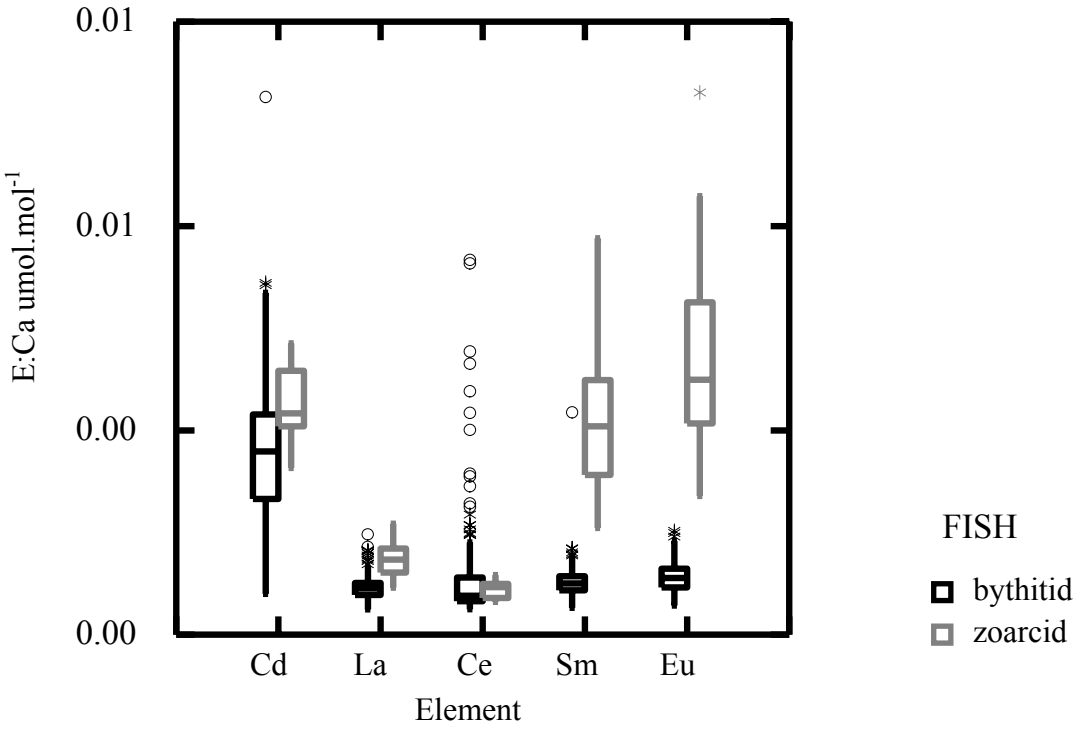

Figure 3.5 Median and range of values for elements present in 50-90% of bythitid and zoarcid samples.

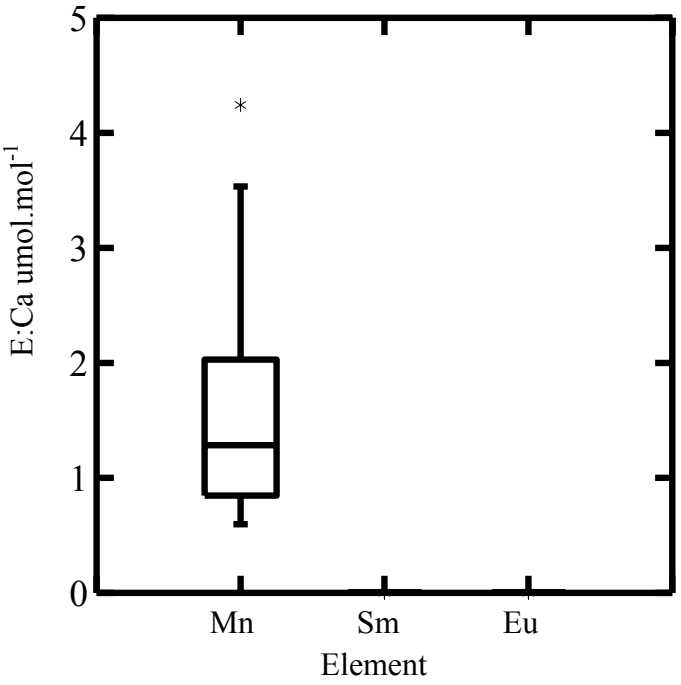

Figure 3.6 Median and range of elements present in 50-90% of non-vent otolith samples. Values for Sm:Ca and Eu:Ca are not zero but are less than 0.005 umol mol^{-1}.

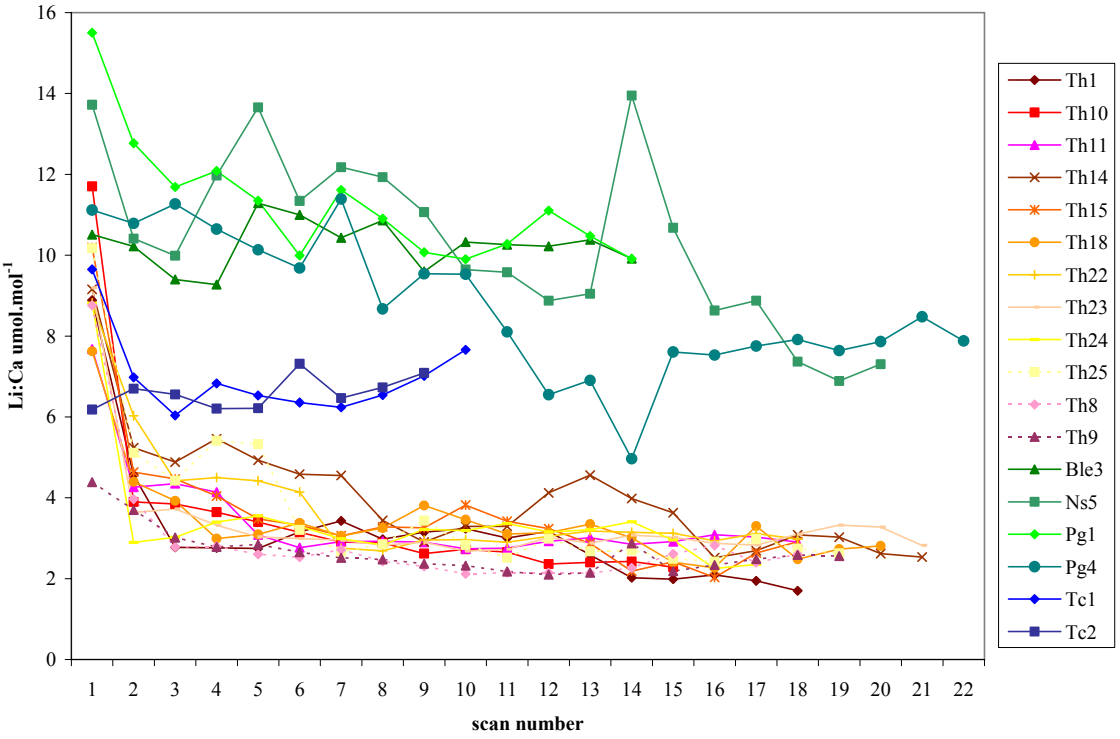

Figure 3.7 Li:Ca measurements of all otoliths. For this and all further graphs the core is represented by scan 1 with increasing scan numbers from the core to the edge of the otolith reflecting the increasing age of the fish. Any values below detection limits are graphed as "0". "Th" designates *Thermichthys hollisi*, "Tc" designates *Thermarces cerberus*, "Ble" is black eelpout, "Pg" is Pacific grenadier, and "Ns" designates *Nezumia stelgidopelis*. For Li:Ca, non-vent fish have the highest Li:Ca levels, bythitids lowest and zoarcids intermediate. Bythitid otolith core measurements are elevated to similar levels as exhibited by the non-vent fish analyzed.

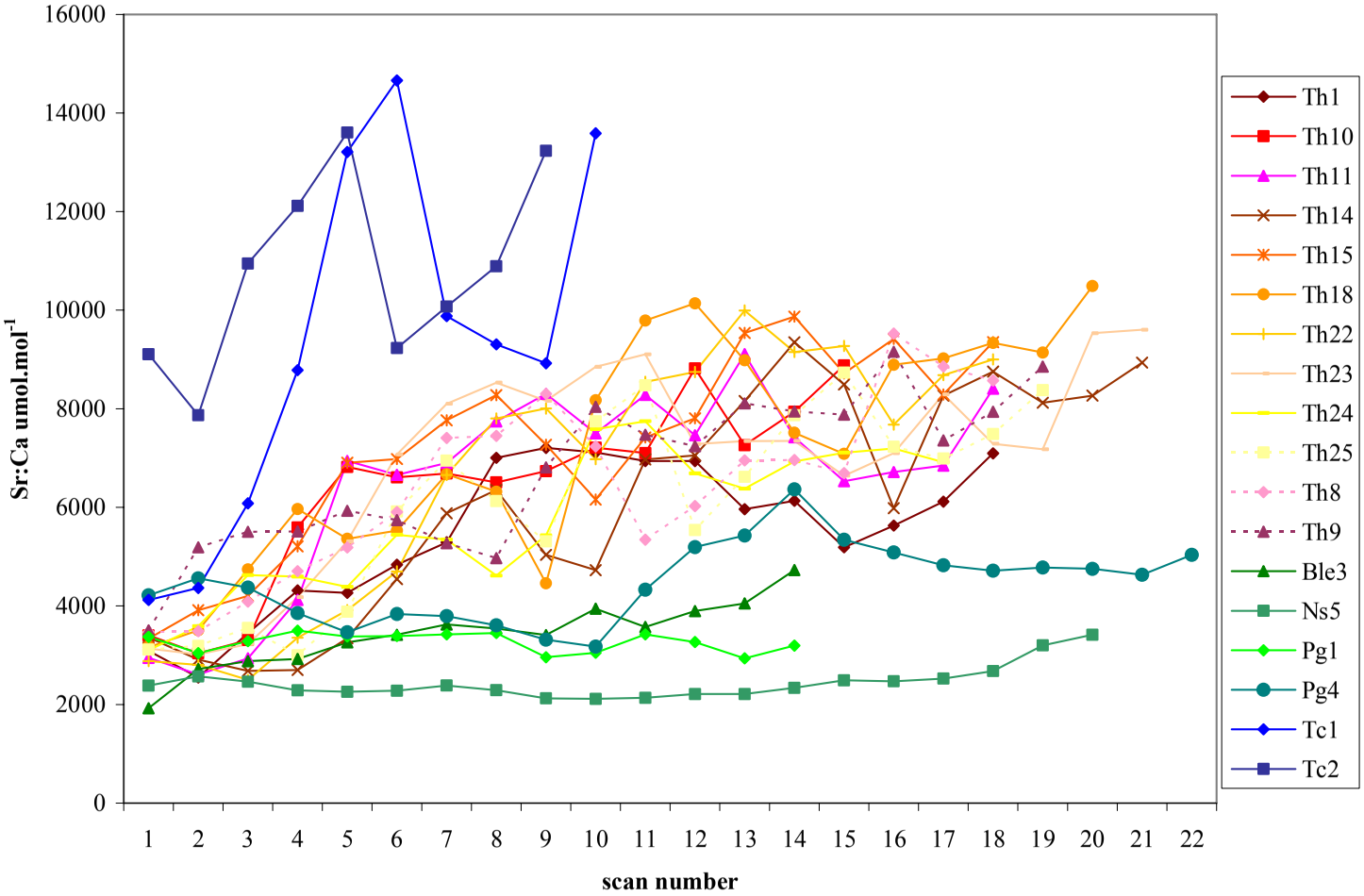

Figure 3.8 Sr:Ca over the lifetime of fish. Sr:Ca is unusually high in zoarcids and increases in concentration over the lifetime of the bythitids. Non-vent levels are within the range of previously published values.

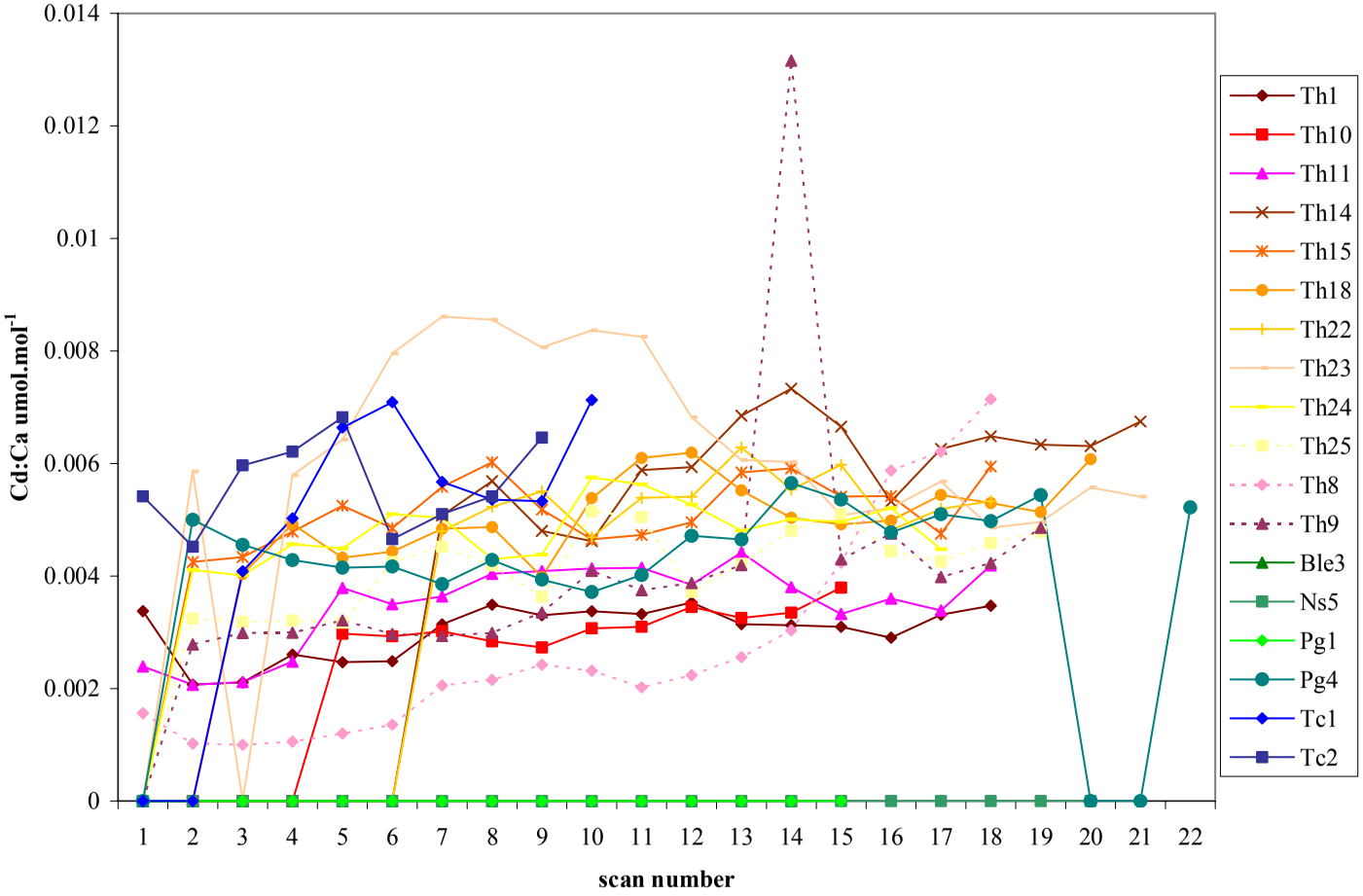

Figure 3.9 Cd:Ca values are similar for zoarcids and bythitids. One individual of the non-vent fish also exhibited similar values while the three other non-vent fish Cd:Ca levels were below detection limits.

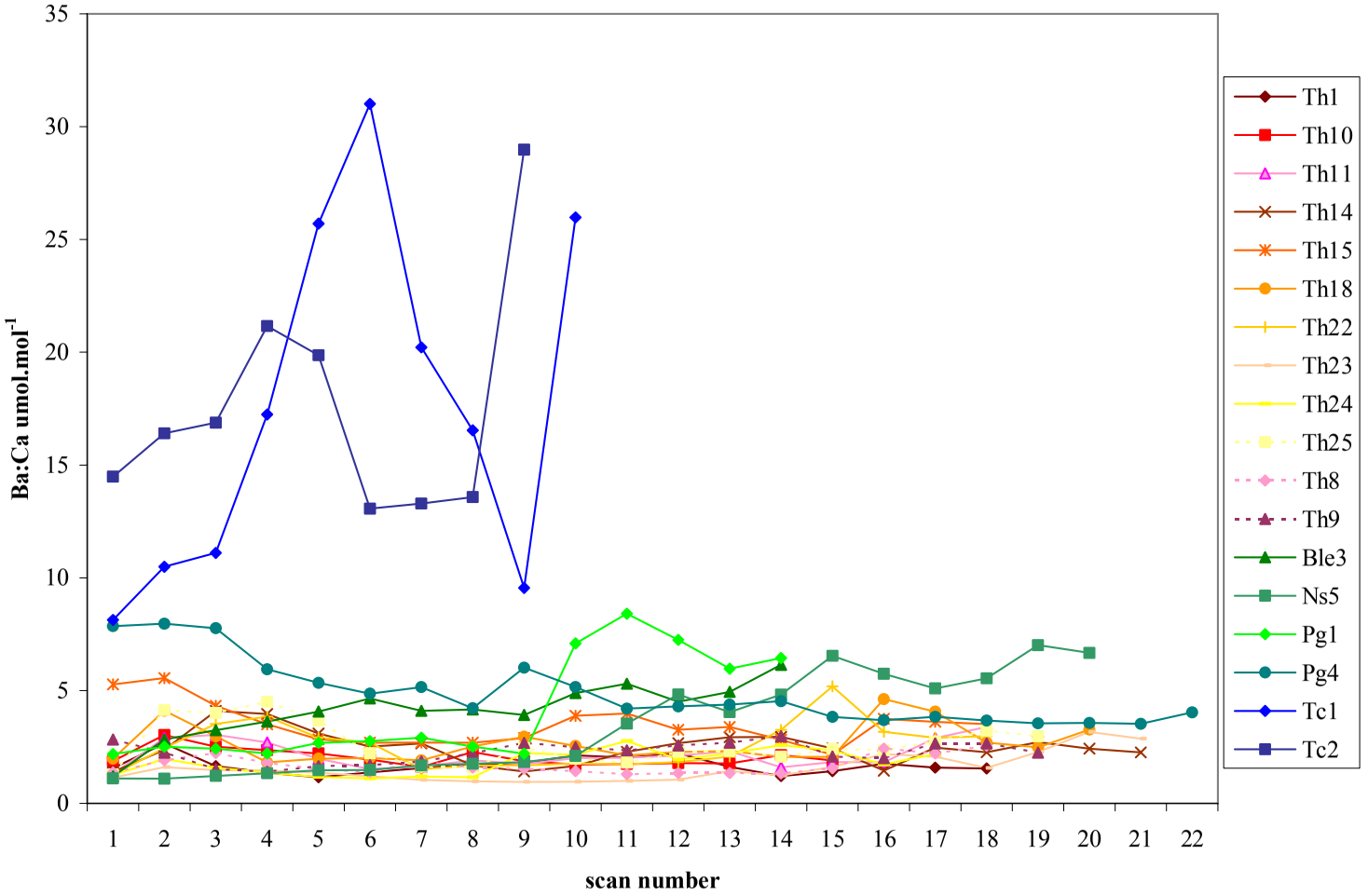

Figure 3.10 Ba:Ca ratios. Zoarcids exhibits distinctly higher values than the similar values of bythitids and non-vent fish.

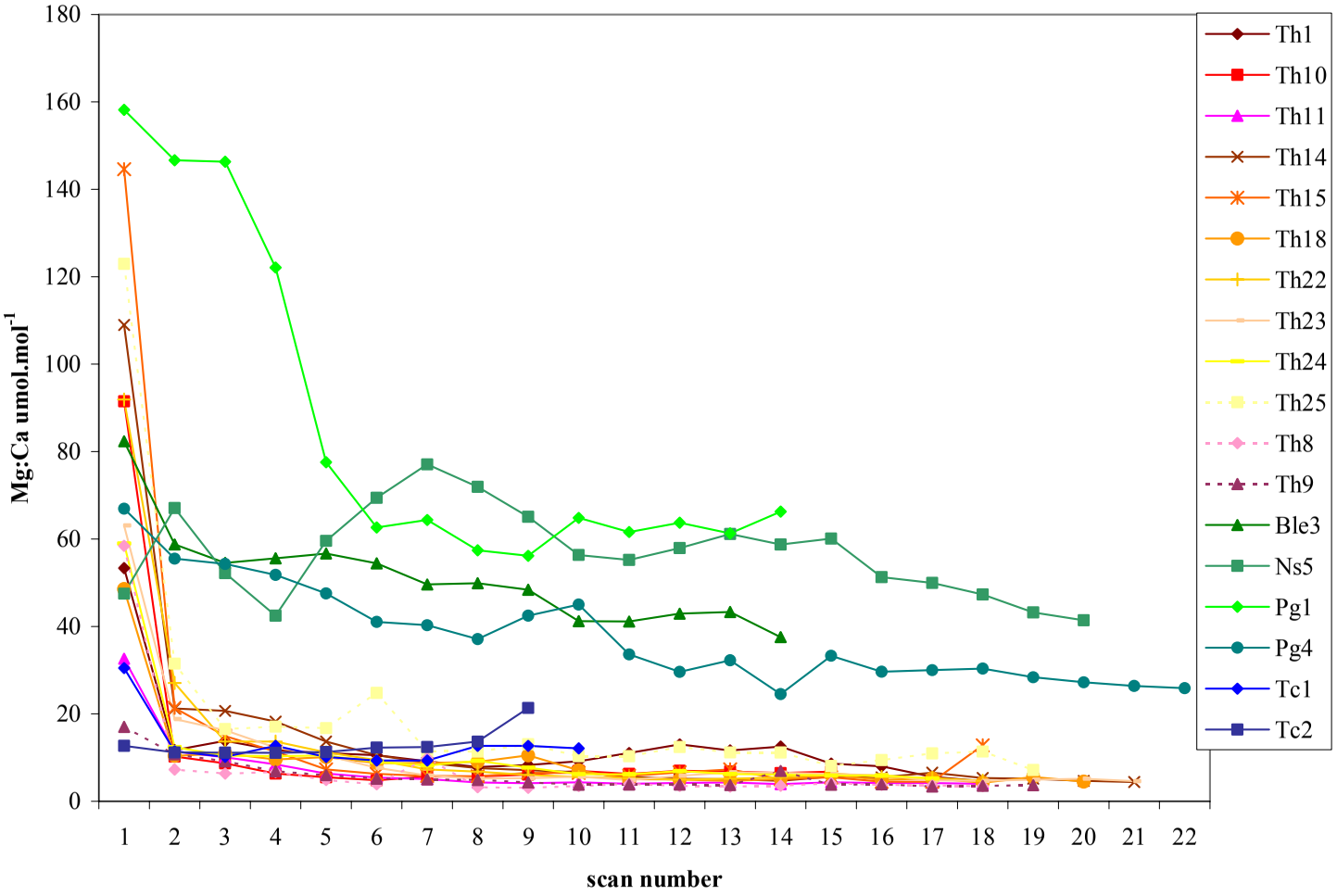

Figure 3.11 Mg:Ca (µmol mol^{-1}) are highest for non-vent fish and the core of bythitids. Mean zoarcid and bythitid non-core values are statistically similar.

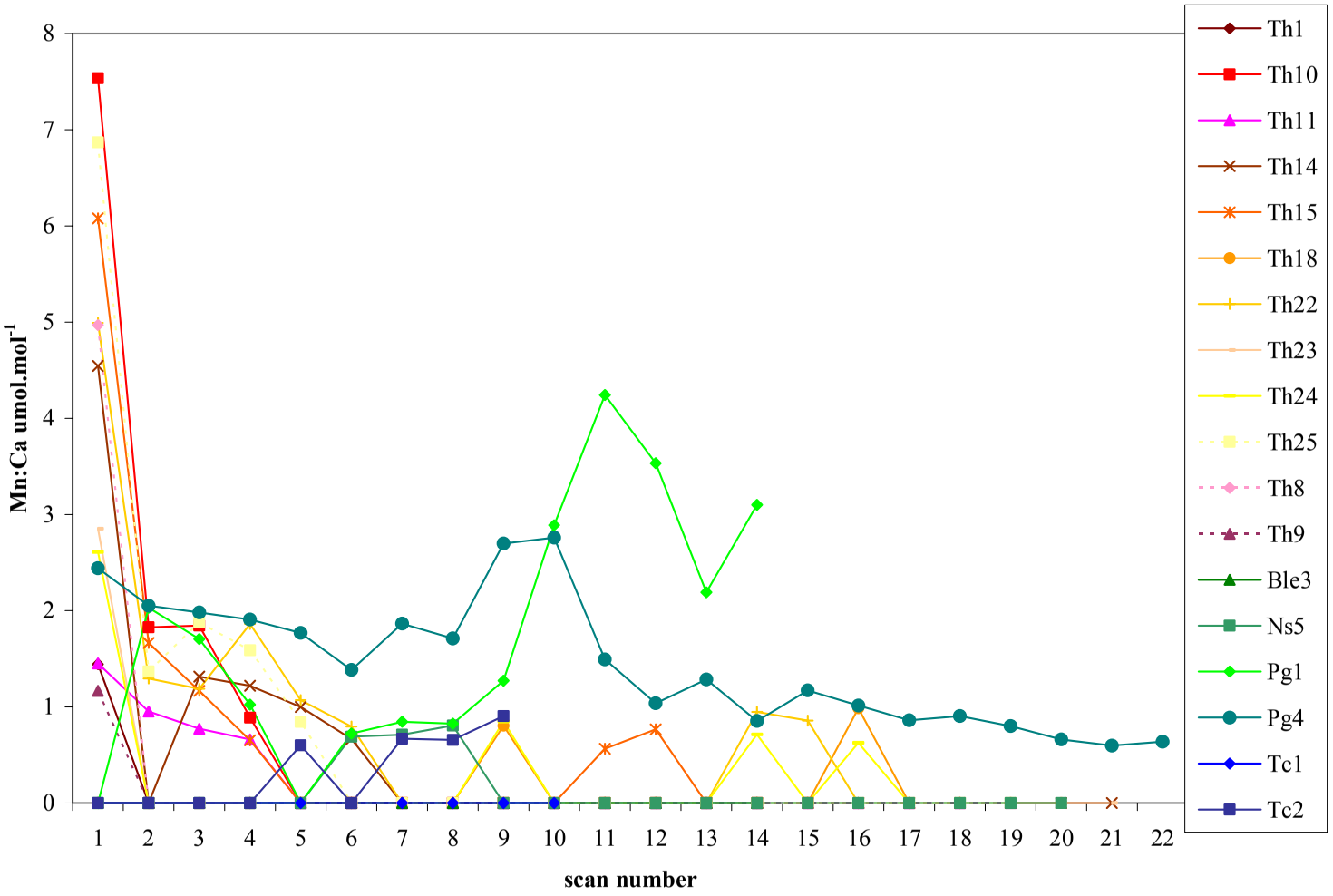

Figure 3.12 Mn:Ca values for most measurements were below detection limits. The core values for bythitids are the highest measurements, followed by the two pacific grenadiers.

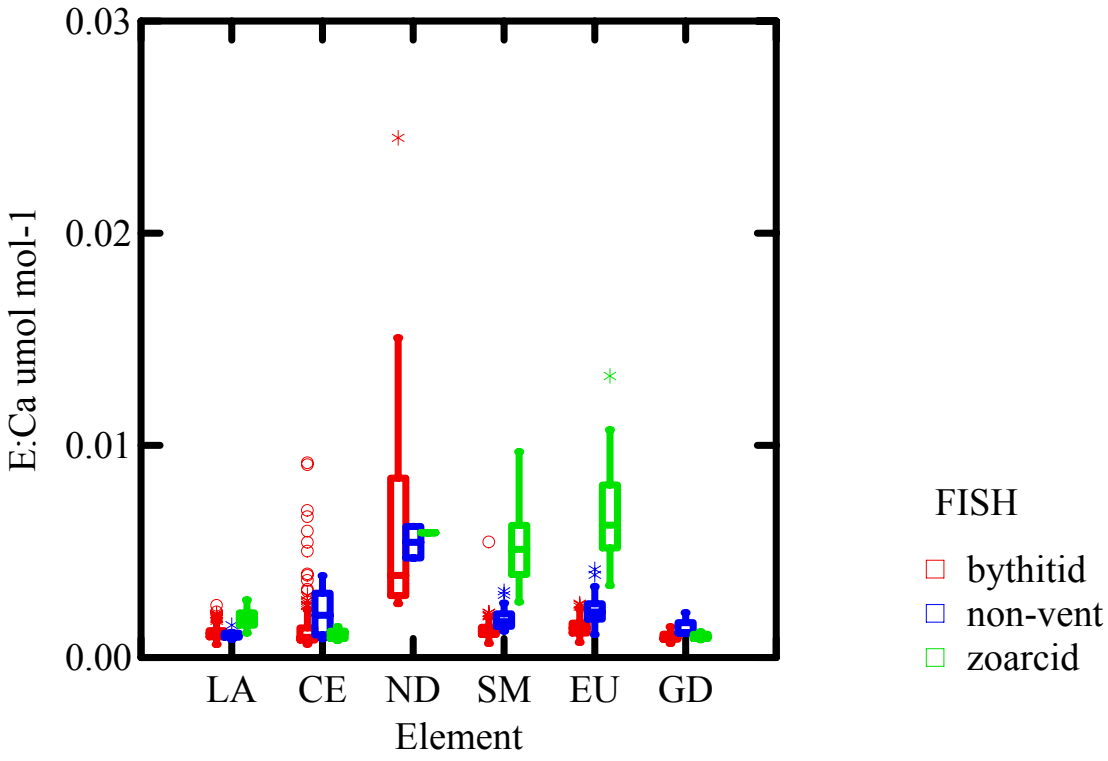

Figure 3.13 Median and range of rare earth elements for all measurements above detection limits. There are significant differences among all groups for Sm:Ca and Eu:Ca.

3.4 Discussion

Numerous studies have indicated the utility of otoliths to impart information regarding the habitat characteristics of fish species. Alkaline and alkaline earth elements (Sr, Li, Ba, Mg) have received the most attention as they are present in higher concentrations than trace metals such as cadmium, copper, or lead, and are not subject to the same physiological regulation as other metabolically active elements (Campana, 1999). Though there is inherently some regulation of element concentrations as they are passed from seawater to blood plasma to endolymph to otolith, and factors such as salinity, temperature, or element interactions may affect element uptake and incorporation, there is still a useful (though not perfect) correlation between the environmental habitat chemistry and elements recorded within the otolith. To date, no studies have explored otolith chemistry within venting environments, where the unique fluid chemistry may provide insights into both fish life history, and how the elements are incorporated into the otolith. Though the vent fish examined live within lower temperature (<30°C) diffuse flow areas where seawater has mixed with the hydrothermal fluid, the habitat chemistry is still measurably different from that of ambient temperature, non vent-influenced areas (Table 3.1).

It was hypothesized that elements elevated within hydrothermal fluids (Mn, Sr, Cd) would show similar elevation within vent fish otoliths in comparison to typical otolith concentrations; and that the two species of vent fish examined may indicate different patterns due to their different microhabitat preferences. As we have only observed one size class of the bythitid *Thermichthys hollisi* at the vents, it was also

hypothesized that this species of fish may be spending part of its life cycle away from the vent systems, and that this migration would be detectable within the otolith. The results of this study indicated that the chemical composition of the hydrothermal fluids is influencing the otolith chemical composition of vent fish, and that the degree of influence may be dependent upon fish preferred habitat.

When hydrothermal fluids interact with seawater, most of the enriched metals precipitate as metal sulfides. This can take the form of black "smoke" and sulfide chimneys in the case of high temperature, focused flow areas. In diffuse flow areas, most of the entrainment of seawater happens subsurface, and the chemically-reduce fluid emerging is depleted relative to end member fluids. Diffuse flow environments also tend to be both spatially and temporally variable, across a spectrum of scales. Despite these caveats, the diffuse flow environments where both species of vent fish are found contain detectable differences in temperature, oxygen, sulfide, and metal concentrations from ambient seawater. These differences appear to be reflected within the otoliths, and may even be indicative of habitat preferences between the two vent fish. This is most readily evident within the Mg:Ca measurements. End-member hydrothermal fluids are considered to have a magnesium concentration of zero (Edmond et al., 1979). In diffuse fluid environments where the fish are found, magnesium concentration is greater than zero, but still lower (typically <50 mmol kg^{-1}) than what is found in unaltered bottom water (Von Damm & Lilley, 2004). This is reflected within the vent fish otoliths, which show lower values than those of the non-vent fish analyzed. In comparison to other biogenic carbonates, the Mg:Ca values in this study are relatively low. Campana (1999)

list marine fish otoliths as having a mean Mg:Ca ratios of approximately 117 μmol mol^{-1}, while most values in the present study are below 100 μmol mol^{-1}. Mollusc shells and corals tend to have even higher Mg:Ca, often above 1 mmol mol^{-1} (Carré et al., 2006; Shirai et al., 2008) with the calcitic portion of a deep-sea oyster shell reaching values of 22.5 mmol mol^{-1} (Wisshak et al., 2009); although Rio et al. (1992) found the shells of the hydrothermal vent bivalves *Calyptogena magnifica* and *Bathymodiolus thermophilus* to be depleted in Mg in comparison with littoral mollusc shells. The lower (typically <40 μmol mol^{-1}) Mg:Ca values of the vent fish otoliths examined are most plausibly due to lowered Mg content of hydrothermal fluids within their environment.

Zoarcids and bythitids show greater mean levels of strontium, an element that is variable, but can be enriched in vent fluids relative to seawater, than do non-vent fish. The zoarcid values are higher than those of the bythitid, perhaps reflecting their greater environmental exposure to Sr or the higher temperatures found within their habitat. Both vent fish Sr:Ca values are remarkably high in comparison to values from other biogenic aragonites. For example, Chesapeake Bay spotted seatrout otolith Sr:Ca ranged from 1970-2070 μmol mol^{-1} (Dorval et al., 2007), shells from *Chione subrugosa* had values as high as 3600 μmol mol^{-1} (Carré et al., 2006) and *Acropora nobilis* coral skeletons were even higher, ranging up to 9500 μmol mol^{-1} (Shirai et al., 2008), yet the zoarcid values are still substantially higher. Sr:Ca incorporation can be influenced not only by the Sr:Ca concentration of the organisms environment, but also by environmental temperature (Bath et al., 2000; Elsdon and Gillanders, 2002; Richardson et al., 2004) and, at least for molluscs, growth rate (Stecher et al., 1996; Carré et al., 2006). Contrary to the results in

the present study, Rio et al. (1992) found hydrothermal vent bivalve shells to be depleted in Sr relative to shallow water shells. While the elvated strontium concentration within hydrothermal fluids (in 1996 fluid samples, L vent had the highest Sr concentrations (Ravizza et al., 2001)) may contribute to the unusually high vent fish Sr:Ca values, the influence of other aspects such as temperature may be equally important; and the combination of factors leading to the high Sr:Ca ratios in vent fish otoliths is not resolved.

Like strontium, barium is highest (with a wide range of values) in zoarcids, yet bythitid otoliths are relatively depleted (with a narrow window of values). Bythitid values are close to the mean marine Ba:Ca otolith ratio (2.84 μmol mol^{-1}) proposed by Campana (1999), but other otolith and mollusc shell Ba:Ca ratios have been measured within the range encompassed by the zoarcids (Stecher et al., 1996; Swan et al., 2006; Carré et al., 2006; Dorval et al., 2007). Ba:Ca is thought to be influenced by environmental Ba:Ca concentrations (Campana, 1999; Bath et al., 2000), and perhaps by salinity (Martin and Thorrold, 2005); thus the difference between zoarcid and bythitid values may again reflect their different exposure to hydrothermal fluids through habitat preference. It is interesting to note the similarity of the strontium and barium patterns between the two zoarcids. The strikingly similar patterns suggest that (at least for exposure to these two elements) that otolith chemistry may be a good predictor of habitat chemistry and that these two zoarcids have experienced the same conditions throughout their individual lives. If this is indeed the case, one can hypothesize that zoarcids may

show fidelity to their particular site (e.g. East Wall vs. Bio9) once they have settled and perhaps are living in cohorts, leading to potential genetic structure between vents.

Lithium also exhibits differences between the three groups, with non-vent fish exhibiting the highest values and bythitids the lowest, with zoarcids intermediate. This result was unexpected, as lithium is enriched in end-member hydrothermal fluids. The behavior of lithium during fluid mixing and the range of values within diffuse fluids is not well defined and though lithium has been measured in prior otolith studies, its behavior during incorporation into the otolith is also not well understood. It has been suggested that lithium incorporation into otoliths may be increased when Li:Ca environmental ratios are low (Milton and Chenery, 2001), yet the relationship between lithium in the environment and lithium within biogenic aragonite is not clear. As there are distinct differences between the different fish types examined, this element and its behavior and utility as an environmental recorder bears further study.

The remaining elements do not appear as useful in recording exposure to vent fluids, as they are not consistently present in measurable levels within the otolith. The non-vent fish, with the exception of one seemingly anomalous individual, have quite low cadmium values, while those of the bythitids and zoarcids are higher. Cd:Ca is highest in zoarcids, though the behavior of this element warrants further exploration. Within the bythitids, Cd:Ca values are high when Mn:Ca values are low and vice-versa. The rare earth elements are in general higher within the vent fish; however, the lack of consistent Nd and Gd data within this study make it difficult to assess whether they display the Eu

anomaly that is indicative of rare earth element concentrations in hydrothermal fluids (Klinkhammer et al., 1994).

Of interest to the elucidation of life history strategy of vent fish are the marked differences in the core concentrations of lithium, magnesium, manganese, strontium, and cadmium within the bythitids. Li:Ca, Mg:Ca, and Mn:Ca values are all distinctly higher within the core of the bythitid otolith than in the rest of the otolith. Li:Ca and Mg:Ca core measurements are within the range of the measured non-vent fish values, while bythitid core Mn:Ca values are even higher than those of the non-vent fish. Conversely, the core measurements for Sr:Ca and Cd:Ca are depleted relative to the rest of the otolith, and in the case of Sr, gradually increase for the entire lifespan of the fish. The bythitid Sr and Cd core measurements are again similar to the values displayed by the non-vent fish. The similarity of bythitid core measurements to those of non-vent fish suggests that *Thermichthys hollisi* spends the initial portion of its life away from hydrothermal vents, in an environment similar to that experienced by non-vent deep-sea and slope species. This hypothesis is consistent with the lack of size structure within observed bythitid populations on the East Pacific Rise. *T. hollisi* is known to be viviparous, and if young bythitids are indeed born away from the vents, the migration of gravid females may represent a previously unknown export of carbon away from the ecosystem.

3.5 Summary and Future Directions

This study provides the first ever examination of otolith chemistry of hydrothermal vent-endemic fish. The chemical composition of hydrothermal fluids

within the fishes' habitat appears to influence the chemical composition of the otoliths. This differences between vent and non-vent otoliths are most noticeable when examining Sr:Ca, Ba:Ca, and Mg:Ca ratios. In general, it appears that *Thermarces cerberus* is experiencing greater exposure to hydrothermal fluids than *Thermichthys hollisi*, as evidenced by higher element:Ca ratios. This is not unexpected due to the habitat preferences exhibited by the different species, with *T. cerberus* most often observed among diffuse flow communities and *T. hollisi* having a seemingly more peripheral habitat. The similar patterns of Ba:Ca and Sr:Ca signatures in the individuals examined suggest that *T. cerberus* may live in cohorts that experience the same habitat conditions throughout their lifetime. An examination of additional zoarcid otoliths from a variety of vent sites would help to support this hypothesis. Interestingly, otolith chemistry provides the first evidence suggesting that *T. hollisi* does not begin its life within the venting environment, but rather spends some portion of its early life in non-vent environments similar to those experienced by typical deep-water or shelf-dwelling species, and then migrates back to the ridge system. This extent of this hypothesized migration may affect dispersal, resulting in distinct genetic population structures between regions where the fish are found.

Further examination of strontium isotopes, in particular δ^{87}Sr of *T. cerberus* otoliths, may elucidate the origins of the unusually high Sr:Ca ratios, and the relative contribution of vent generated strontium to the otolith composition. Strontium isotopes are often utilized as paleothermometers, and further investigation into the influence of temperature on the Sr:Ca otolith values within vent fish is warranted. Hart and Blusztjan

(1998) utilized Sr:Ca within a hydrothermal vent clam shell to reconstruct a temperature history within which there appeared evidenced of increased temperature during eruptive events. It may be possible to reconstruct similar temperature histories using otolith Sr:Ca, provided a suitable exchange coefficient can be determined. In combination with aging studies, these data may indicate the feasibility of vent fish otoliths to track eruptive events or dramatic chemical changes of hydrothermal fluid chemical composition, particularly if fish from spatially distinct sites show similar spikes in Sr:Ca. Higher resolution analyses of the otolith can be undertaken to discern more complex patterns within the otolith and may prove useful in understanding how hydrothermal fluid exposure translates into elemental incorporation.

Chapter 3 References

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Chapter Four

A preliminary exploration of gene expression in the hydrothermal vent fish *Thermichthys hollisi*

Abstract

Time-lapse photography and *in situ* electrochemistry were utilized to characterize *Thermichthys hollisi* habitats. An exploration of the expression of genes that may be responsive to changing and stressful habitat conditions was undertaken. Data indicated that *T. hollisi* are preferentially utilizing fish holes where there are elevated temperatures and sulfide levels, and depleted oxygen levels in comparison to ambient bottom water. A fragment of Cu, Zn superoxide dismutase was successfully amplified from *T. hollisi* mRNA, but there were no differences in expression levels between tissue types or among individuals for the small sample set examined. Recommendations for future deep-sea gene expression studies are given.

4.1 Introduction

Hydrothermal vents on the East Pacific Rise are characterized by the production and emission of hot, acidic, sulfide- and metal-rich hydrothermal fluids. These fluids are enriched in hydrogen sulfide and metals such as copper, iron, cadmium, manganese, and zinc, and are depleted in oxygen in comparison to the surrounding deep water (Von Damm, 1995). Life at hydrothermal vents is able to exist due to the presence of chemical species such as hydrogen sulfide, methane and iron within the vent fluids. Reduced chemicals provide the energy for chemoautotrophic microbial communities to fix carbon, forming the basis of the trophic network within the vent ecosystem (Fisher et al., 1990). Yet the presence and composition of hydrothermal fluids also poses numerous challenges to the metazoan communities that exist in these environments (Childress & Fisher 1992).

Hydrogen sulfide, necessary for the metabolism of chemoautotrophs at vents, is a potent toxin for other organisms. It interfers with cytochrome c oxidase function, thereby

disrupting aerobic respiration, and reduces oxygen transport by reacting with hemoglobin (reviewed in Grieshaber & Völkel, 1998). High concentrations of heavy metals within the environment can be detrimental to an organism if they build up within the tissues. Their presence can facilitate the production of reactive oxygen species (ROS) and free radicals both in the water column and within the organisms themselves. Though ROS are a natural by-product of aerobic metabolism, they can be detrimental to organism health if they are produced faster than they are eliminated, leading to oxidative stress. ROS production can be facilitated by exposure to heavy metals, and if exposure is not reduced, ROS can damage DNA, destroy cell integrity, impair enzyme function, and lead to the production of additional ROS (Di Giulio et al., 1989; Aruoma, 1998; Valko et al., 2005). Hypoxia, a lack of sufficient dissolved oxygen, can lead to additional stress for an organism, and in the extreme may lead to a shut-down of aerobic respiration (Grieshaber et al., 1994). Additionally, vent fauna must be able to cope with elevated and fluctuating temperatures within their chaotic habitat, which may affect enzyme function and efficiency (Dahlhoff & Somero, 1991).

Vent-endemic fauna must be able to either limit their exposure to potential toxins within hydrothermal fluids, or possess adaptations to ameliorate the effects of exposure to these chemicals. Adaptations can be behavioral (at the simplest, swimming or crawling to a more amenable habitat), structural/physical (tubes or mucous providing a barrier), or at a cellular level (possessing novel proteins or enzymes tolerant of a range of conditions) (Grieshaber and Völkel, 1998; Hagerman, 1998; Van Dover, 2000). Numerous studies have been conducted regarding how vent fauna tolerate the range of conditions and

substances that they are exposed to (Arp & Childress, 1983; Powell & Somero, 1986; Dahlhoff & Somero, 1991). Of particular interest due to the habitat characteristics of venting environments are stress-response genes, those that encode for proteins responsible for the elimination of excess metals, hydrogen sulfide, ROS within the cells, and that are frequently up-regulated in response to negatively changing water conditions. These genes include those that code for hypoxia inducible factor, heat-shock proteins, metallothioneins, and antioxidants such as superoxide dismutase, glutathione peroxidase, catalase, and glutathione reductase. Metallothioneins (MT) are low molecular weight, cysteine-rich proteins found in most eukaryotes that are involved in metal handling, homeostasis and detoxification (Tanguy et al., 2003; Capasso et al., 2003; Laurie, 2004). They have been studied in hydrothermal vent mussels from the Mid-Atlantic Ridge and East Pacific Rise (Hardivillier et al., 2004; Bebbiano et al., 2005; Hardivillier et al., 2006). Antioxidant enzyme activity is of particular interest at vents due to the enriched metal concentration within the hydrothermal fluids, and has been examined in a range of invertebrates, including Alvinellid polychaetes, mussels and shrimp (Bebbiano et al., 2005; Marie et al., 2006; Company et al., 2006; Gonzalez-Rey et al., 2007; Company et al., 2007; Shin et al., 2009). Vent invertebrates are known to bioaccumulate metals present within hydrothermal fluids in their tissues (Rousse et al, 1998; Kádár, 2005; Demina & Galkin, 2008). Upper level predators such as vent-endemic fish must also be adapted to deal with not only habitat variability and stressors, but also the potential bioaccumulation, especially of heavy metals, that can occur through consumption of vent invertebrates. To date, there have been few studies centered on the vent-endemic

vertebrates despite their importance in structuring the community through predation (Micheli et al., 2002; Sancho et al., 2005), and even fewer investigating metazoan gene expression responses to varying environmental conditions (Denis, et al., 2002; Faure, et al., 2005; Sato et al., 2005), though interest in this aspect of vent ecology continues to grow as scientists attempt to predict how species around the world will change in response to pollution and changing habitat conditions and to better understand how these genes may be related to health and diseases of all species (Shin et al., 2009). This knowledge is equally important to the understanding of how vent systems function on the EPR, as well as how they relate to similar communities around the world.

This study seeks to describe *Thermichthys hollisi* habitat usage and characteristics and undertakes a preliminary investigation of the expression of stress response genes within this species.

4.2 Methods

4.2.1 Fish Collection and Preservation

Thermichthys hollisi individuals were collected from L vent using a thruster-powered suction sampler mounted on the submersible Alvin as previously described. The first fish was captured at approximately 1530 GMT, and weights were released for ascent to the surface at 2020 GMT. The bythitids were alive for the majority of the ascent (lasting approximately two hours); death was estimated to occur within the last 500m of ascent, presumably from complications due to changes in pressure. Immediately upon the sub's recovery to the ship, the fish were transferred to chilled seawater and

stored in the walk-in cold room until dissection. Time between initial capture and the onset of dissection was at least eight hours. Subsamples of liver, muscle and gill tissue were excised from each fish and preserved in RNALater (Ambion) as per the manufacturer's instructions. Preserved samples were then frozen at -80°C for transport to the shore-based lab and long-term storage.

4.2.2 Electrochemical Measurements

Physico-chemical parameters of *Thermichthys hollisi* habitat were measured using *in situ* voltammetry using solid state gold-amalgam (Au/Hg) working microelectrodes. Briefly, these electrodes consisted of a 100-µm gold wire housed inside polyethylether ketone (PEEK) tubing and plated with mercury (Brendel and Luther 1995; Luther et al. 1999). They were operated within the DSV *Alvin* through the use of Analytical Instrument Systems, Inc. (AIS) DLK-SUB analyzer (AIS-ISEA I) connected to a laptop computer (Nuzzio et al. 2002). A solid state reference (Ag/AgCl) electrode and counter (Pt) electrode were attached to the *Alvin* basket in ambient seawater, while the working (Au/Hg) electrodes and temperature probe were housed inside a Delrin or titanium wand, along with a thermocouple (Luther et al. 2001a; Luther et al. 2001b). *In situ* electrochemical scans were collected in a program of up to ten to twenty individual scans lasting 1.5-3 minutes and later analyzed onboard ship by member of G. Luther's lab (U. Delaware). Cyclic voltammetry, at a scan rate of 2000 mV s^{-1}, was used for all measurements. Each scan process consisted of an electrode cleaning step with a holding potential of -1.0 V for five seconds, a conditioning step where the initial potential -0.05 V

was held for two seconds, and the measurement step where the electrochemical scan was collected from -0.05 V to -1.8 V to -0.05 V. Detection of multiple analytes included O_2 (detection limit, denoted as DL = ~ 3-5 μM), H_2S (DL = 0.2 μM), and other S(-II) species such as polysulfides (S_x^{2-}, DL = 0.2 μM) and thiosulfate ($S_2O_3^{2-}$, DL = ~ 30 μM) (Brendel and Luther 1995; Luther et al. 2001b; Luther et al. 2008; Mullaugh et al. 2008). When measurements around organisms were not collected, cleaning scans were conducted to prevent electrode fouling and to maintain the integrity of the electrode surface. Measurements were taken during Alvin dives 4310, 4313 and 4317 at the L vent site. *In situ* temperature, oxygen, and free sulfide ($H_2S + HS^-$) measurements were collected at multiple locations where fish were observed (specifically collapsed lava surfaces or "fish holes" and aggregations at cracks in the basalt) (Figure 4.1). Samples were subsequently categorized as "within" (tip of wand below surface of basalt or as far into crack as possible), "edge" (on the lip of a basalt collapse pit), or "adjacent" (bare basalt within 1m of fish hole with no associated fauna) to sampled fish holes.

4.2.3 Time-Lapse Photography

The RatCam, a down-looking time-lapse digital-still camera obtained from the MISO facility (WHOI), was deployed for five days at the main fish hole at L vent. Strobes attached to the side of the frame are angled to illuminate the field of view of the camera and are powered by a 24 volt Subsea battery (Deep-Sea Power and Light) (Figure 4.2). Images were acquired at approximately 3.5 minute intervals (exp 1/60sec, f-5.3). The RatCam system was acoustically guided and released to the seafloor using a wire

from the ship, and once on bottom, positioned over the fish hole using the submersible DSV Alvin (Jan 28, 2007 dive 4313) and recovered at the end of deployment by releasing the drop weights (Feb 2, 2007 dive 4317). A Vemco™ temperature probe was placed in the "fish hole" for concurrent temperature measurements; however it was unable to be recovered.

The abundance and location of fish in each image were analyzed by hand, and fish were classified as either "within" or "outside" of the central collapse pit. Fish were categorized as "within" the hole if all or part of their body appeared below the level of the basalt crust (Figure 4.3). Fish that may have been associated with diffuse flow, but not swimming within the central hole were classified as "outside".

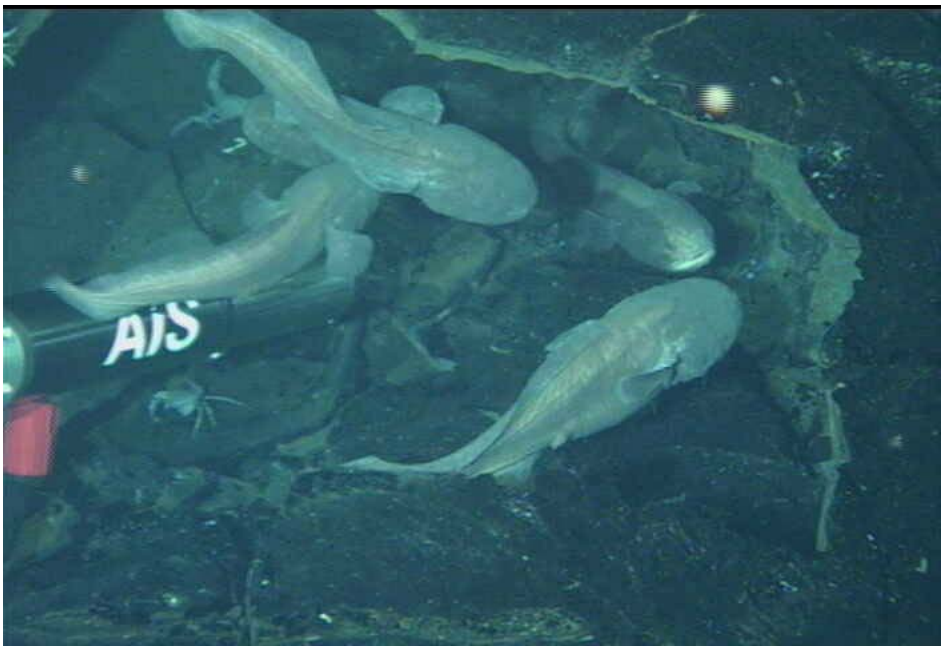


Figure 4.1 *In situ* electrochemical measurements being taken within a fish hole.

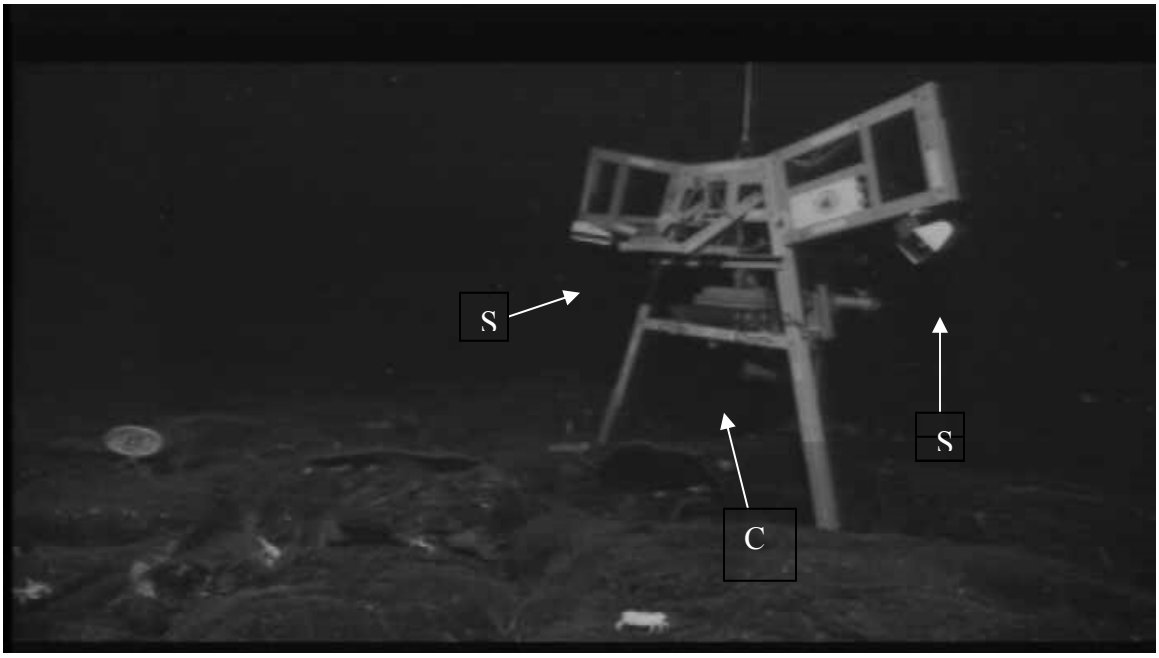


Figure 4.2 RatCam deployed on the seafloor over a fish hole. The strobes (S) and camera (C) are indicated by arrows.

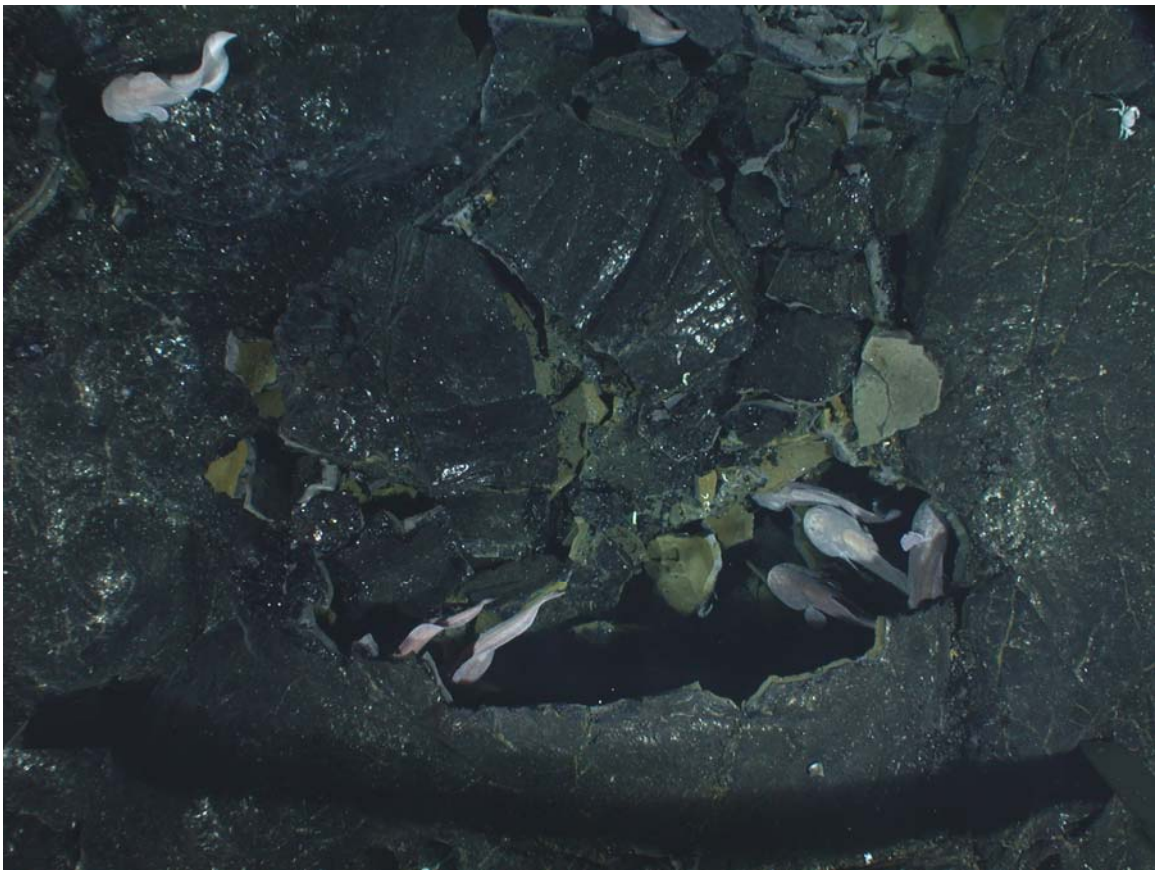


Figure 4.3 Example of image taken by the RatCam. In this image, with a field of view of 3.1 x 2.3 meters, there are six fish counted within the fish hole and two outside of it.

4.2.4 RNA Extraction and RT-PCR

Total RNA was extracted from RNALater preserved gill, liver and muscle *T. hollisi* tissue using a Quiagen RNEasy kit according to the manufacturer's instructions for animal tissue using a rotor-stator homogenizer. Extractions were subsequently treated with an RNase-free DNase (Qiagen), which was heat inactivated at 75°C for 5 minutes. RNA extraction success was tested by running 1µL total RNA on a Nanodrop spectrophotometer (ThermoScientific), with results indicating that all samples contained nucleic acids; 260:280 ratios were within the range expected for RNA. 3µL of total RNA was reverse transcribed using iScript Select cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions using the included oligo-dt primers. No-RT controls were also generated and amplified using Th18f & Th18r primers under the semi-quantitative PCR conditions outlined below in order to ensure no DNA contamination.

Degenerate primers for multiple stress response genes were constructed from known teleost sequences downloaded from GenBank, or from published primer sets and tested using either genomic *T. hollisi* DNA extractions, liver cDNA or both under a variety of pcr conditions (Table 4.1). The pcr products were sequenced on an ABI capillary sequencer at the Josephine Bay Paul Center (Marine Biological Laboratory, Woods Hole, MA), analyzed using standard sequencing software, and compared to sequences in GenBank to determine if the correct gene had been amplified. Of the primers tested we were only able to successfully obtain fragments matching Cu, Zn-SOD sequences contained in GenBank. The successful primers are as follows (5' to 3'): CuZnSOD_1f ACT TCA ACC CYC AYR RYA AR and CuZnSOD_1r WCC TTC

TCR TGR ATC ACC AT. A 236bp pcr product was obtained by combining 2µL template, 5mM MgCl, 1x buffer, 0.1mM dNTPs, 0.5µM each of forward and reverse primer and 2.5 units Promega Flexi Taq in a 25µL reaction. A touchdown PCR was utilized as follows: an initial denaturation of 2 minutes at 94°C was followed by 14 cycles of 45 secs at 94°C, 45 secs of a -0.5°C decrease per cycle beginning at 55°C, and 1 min at 72°C; then 26 cycles of 45 secs at 94°C, 45 secs at 48°C and 1 min at 72°C with a final extension period of 5 min at 72°C. Standard 18h (AGG GTT CGA TTC CGG AGA GGG AGC CTG AGA AA) and 18k (CCC GTG TTG AGT CAA ATT AAG CCG CAG GC) primers (Hillis & Dixon, 1991) were utilized to obtain a 632bp fragment of the 18s small ribosomal subunit under the following pcr conditions: 94°C initial 2 min denaturation followed by 30 cycles of 94°C for 1 minute, 50°C for 45 seconds, 72°C for one minute with a final 72°C extension for 5 minutes. *Thermichthys hollisi* specific SOD and 18s primers were designed from the resultant sequences and are presented in Table 4.2. 2ul of cDNA was used for all subsequent PCR reactions. Semi-quantitative PCR was performed in order to compare expression levels of Cu, Zn-SOD between tissues (liver, gill, and muscle) within the same individual and between individuals from the same location using the same components as above. An initial 2min denaturization at 94°C was followed by 35 cycles of 45 sec at 94°C, 30 sec at 56°C, and 45 sec at 72°C with a final extension period of 2 minutes at 72°C. The pcr products were imaged on a 1.5% agarose gel stained with ethidium bromide and quantified using Kodak Imager software. All no-RT and negative controls were negative, indicating no DNA contamination. SOD levels were normalized to that of 18s with the assumption that 18s

is expressed equally across all tissue types and are reported as relative fluorescent units. The SOD fragment was compared to other teleost nucleotide and amino acid sequences available from GenBank, and neighbor-joining trees (distance: total character difference, bootstrap x100) for both nucleotide and amino acids sequences were constructed using PAUP (ver. 4, Sinauer Associates), with an oyster (*Crassostrea ariakensis*) sequence utilized as an outgroup taxon.

Table 4.1 All primers tested. Forward primers are indicated by (F) preceding the primer sequence. Closest or most common matches are indicated, though it should be noted that most blast matches were less than 150bp sequence length and non-specific. Italicized matches are from unidirectional sequences (only one primer of the pair successfully sequenced) or single occurrence BLAST (Basic Local Alignment Search Tool, NCBI) search matches. Normal text indicates BLAST matches that occurred more than once.

Predicted Gene Region	Primer sequence 5' – 3'	Results
Metallothionein 1/A	Met 1 2f (F) TCCATCTGGCTTTCTCTCGT	no amplification
	met1 1f (F) CCCTTGGGTCCATTGTCTC	random clones
	met 1 3f (F) GCATCACCTGAGAACATGGA	*putative CPSF-domain protein*
	met 1 4r TTTTGCAGGAGCAGTTTGTG	*NADH dehydrogenase subunit*
	met 1 5r KAAMTKMATTTATTTCVACATYG	myosin light chain
	met A 1f (F) TGGGTAGCCATATTTGAATGA	*Wee1-like protein locus*
	metA 2f (F) ATCCTGCAAGTGCTCCAACT	*isotocin and vasotocin locus*
	metA 3r AGTTGGAGCACTTGCAGGAT	*major histocompatability complex 2*
	met A 4r AGGGAATGGACTGCATTGTG	poor sequence quality
	MT1.1f (F) MTGCAMKTGCACWAAYYGMY	
	MT1.3f (F) GCGTGAAAGGGTCATGTTTT	
	Mt1.2r RYCRCARGWMTTSCCYWTRC	
	Mt1.4f (F) AWKASTGGRMHHYGYAAYWG	
Metallothionein 2/B	Met 2 1f (F) TGCACCTACTCACGAGGACA	random clones
	met 2 2f (F) AAAAGTGGGACCTGCAACTG	myosin light chain
	met 2 3r CAAGKAASTGYATTTATTTCAA	*putatative neurotransmitter*
	met B 1f (F) ATGGATCCKTGTGAATGCTCT	

	met B 2f (F) TCCTGCAAGTGCTCAAACTG	*receptor*
	met B 3f (F) TCTCTCTCATGCTGGCTTCA	*Sphingosine-1-*
	met B 4r AGCAACCTGATGGGACAAAA	*phosphate receptor 2*
	met B 5r AGGGAATCGACTGCATTGTC	HLA-B associated
	met2.1f (F) WRASTGGRRBYTGYMYTG	transcript 5
	met2.2r CAMWGGYKCCATYMCRMGRK	*cyclic nucleotide gated*
		channel alphas
		EDG-S receptor
		protein
		poor sequence quality
generic metallothionein	MetX1 (F)[a] ATGGAYCCITGYGARTGYWSIAARAC	no amplification
	MetX2[a] TTYTGIACRCTRTGIWSIACRACRGTYAY	random clones
	MetX3[a] (F) GARTGYWSIAARACIGGIWSITGYAA	zinc fingers
	MetX4[a] ACRTTYCCITTYTGIACRCTRTGIW	*protease*
	FishMTFW[b] TGCYACCTGCAAGTGYACCAA	poor sequence quality
	Metx.1r CRCAKGWMTTSCCYTTRC	
Superoxide dismutase	CuZnSOD1_f (F) ACTTCAACCCYCAYRRYAAR	random clones
	CuZnSOD1_r WCCTTCTCRTGRATCACCAT	Cu, Zn SOD
	UniSOD1[c] (F) CAYGGHTTCCATRTCCA	*protein phosphatase 2*
	UniSOD1r [c] ATGCCRATVACDCCRCAGGCC	*regulatory subunit B*
Glutathione S transferase	GSTpi1 f (F) AAGAYGGTGACCTGGTSCTG	guanine nucleotide
	GSTpi1 r CCRTTGATGGGCAGTTTCTT	binding protein
	GST theta (F) YATGMTSTACCTGGCTGASA	
	GST theta CAYGCCTTGAGYTTGGGTCT	
Hypoxia inducible factor alpha	Hif1a f (F) GYHTSGGBCTDRCWCAGWTY	no amplification
	Hif1a r YTGHGTYTCMASCCAMACAAS	random clones
	HIF4f [d] (F) GTSCTSCACTGYACNGG	
	HIF7r [d] CATNGCGAASAGCTTCTC	
Heat Shock Protein 70	HSP70f (F) GGCACCACCTACTCCTGTGT	no amplification
	HSP70r TTCCCTCCGTCTGAAATCAC	

a. Cousinou et al., 1999 b. Cho et al., 2008 c. Cho et al., 2006 d. Powell & Hahn, 2002

Table 4.2 *Thermichthys hollisi* specific primers used for semi-quantitative RT-PCR.

Target Gene	primer name	5' – 3' sequence
Cu, Zn SOD	ThSOD_f	AGACCTGGGGAATGTGACTG
Cu, Zn SOD	ThSOD_r	TCTGCCGATGATGGAGTAGG
18s	Th18f	CCGCAGCTAGGAATAATGGA
18s	Th18r	GATCGCTAGTTGGCATCGTT

4.3 Results

4.3.1 Electrochemical measurements

Fish hole chemical measurements indicated that these environments are chemically distinct from the surrounding ambient water. Within fish holes, the measured temperatures (Figure 4.4) ranged from 6-15°C (mean 10.54 ± 2.3°C). Free sulfide (Figure 4.5) ranged from non-detectable to 53.5 μM (mean 25.2 ± 8.4 μM) and oxygen (Figure 4.6) ranged from non-detectable to 159 μM (mean 35.8 ± 23.1 μM). Electrochemical measurements taken on bare basalt outside of a fish hole averaged 4.2 ± 6.3°C, non-detectable sulfide, and 55.8 ± 35.1 μM dissolved oxygen, with all measurements for the "edge" of fish hole intermediate to those "within" and "adjacent". ANOVA with Bonferronni post-hoc test indicated significant differences ($p<0.001$) between "within" and "edge"; and "within" and "adjacent"; but not between "edge" and "adjacent" measurements for both temperature and free sulfide. Due to the high variation within oxygen values, there were no significant differences found between the different sampling areas. With the exception of one set of scans, oxygen appears lower within fish holes than outside of them.

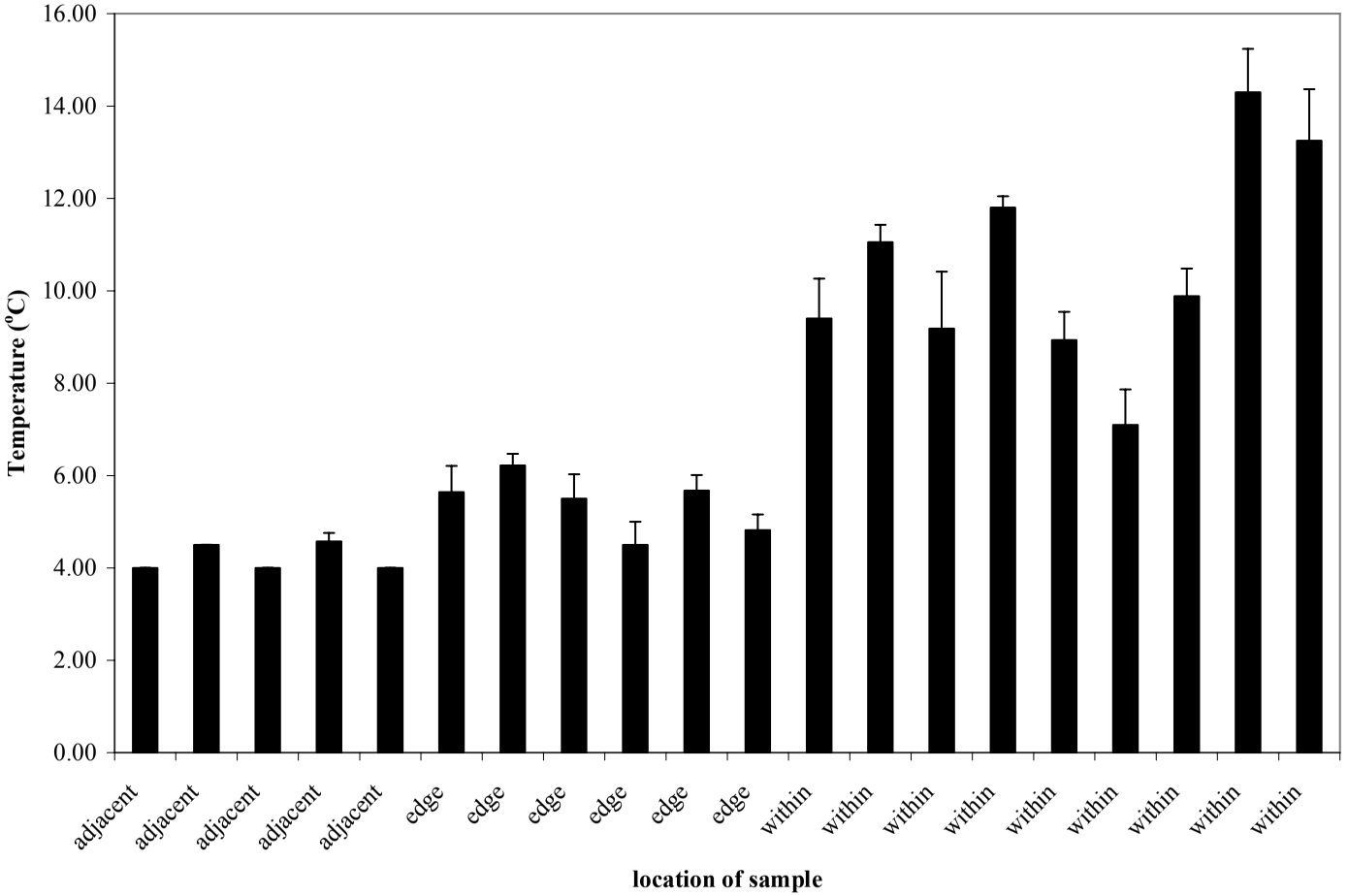


Figure 4.4 Mean temperatures measure outside of, on the edge of, and within fish holes. Temperatures are statistically higher within the fish holes than on the bare basalt outside of them.

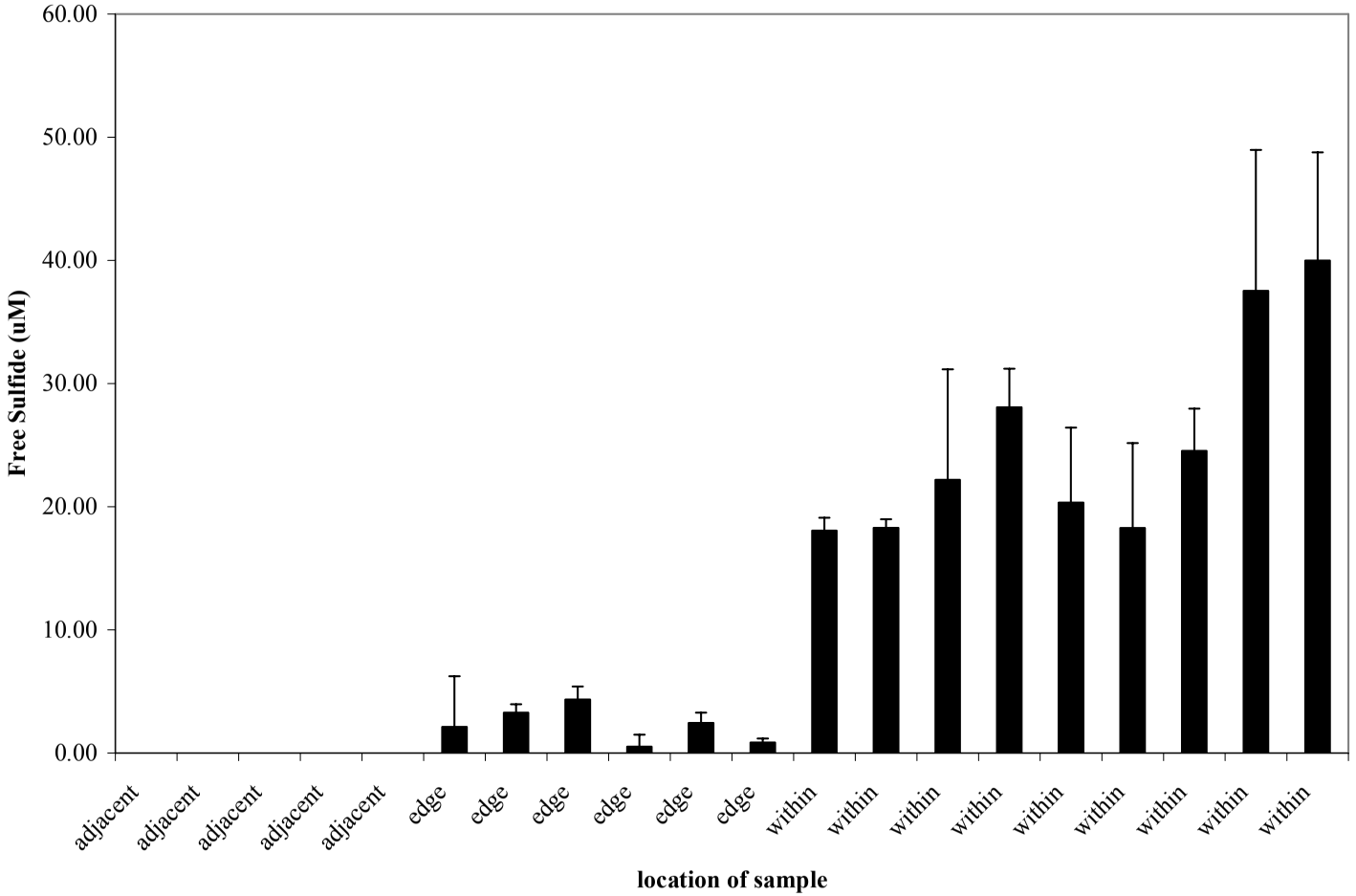


Figure 4.5 Free sulfide concentrations within fish habitat. Sulfide was non-detectable when measured adjacent to, but not within the fish hole. Sulfide concentrations are significantly higher within the fish hole than either on the edge of or outside.

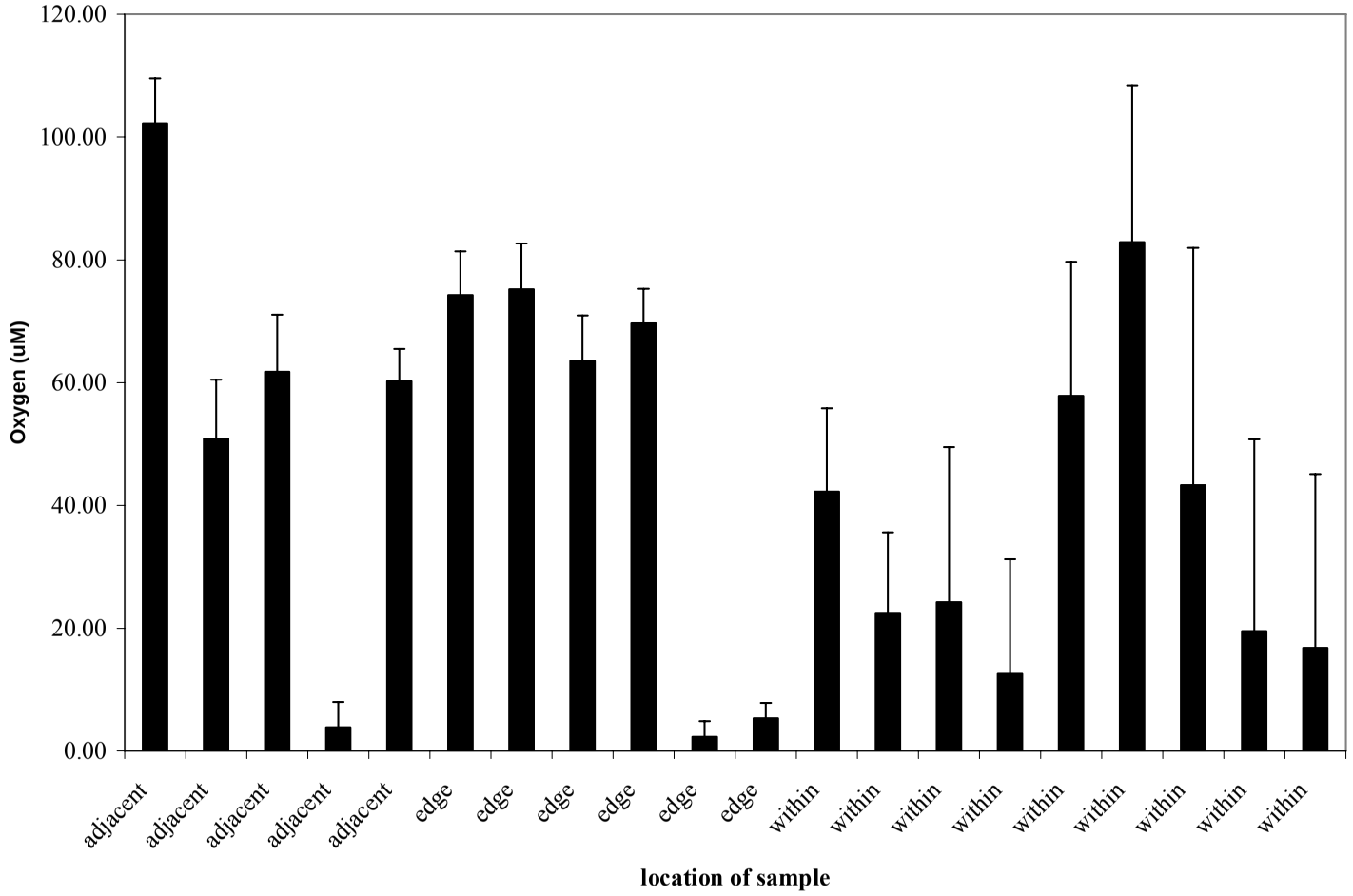


Figure 4.6 Mean oxygen concentrations within, on the edge of and adjacent to sampled fish holes. The chemistry is variable within the fish holes, yet in general they exhibit qualitatively lower oxygen concentrations within than without.

4.3.2 Image analysis

The time-lapse camera successfully imaged the central fish hole for approximately 2 days and 16 hours with a total of 1,024 images analyzed. During this time period there were on average significantly (t-test, $p<0.001$) more fish inside of the central hole (mean 3.7 fish/image) than outside of it (mean 2.7 fish/image) with a mean of 6.4 fish per image total (Figure 4.7). Other fauna observed included numerous bythograeid crabs (mean 4.1 per image) and the occasional octopod.

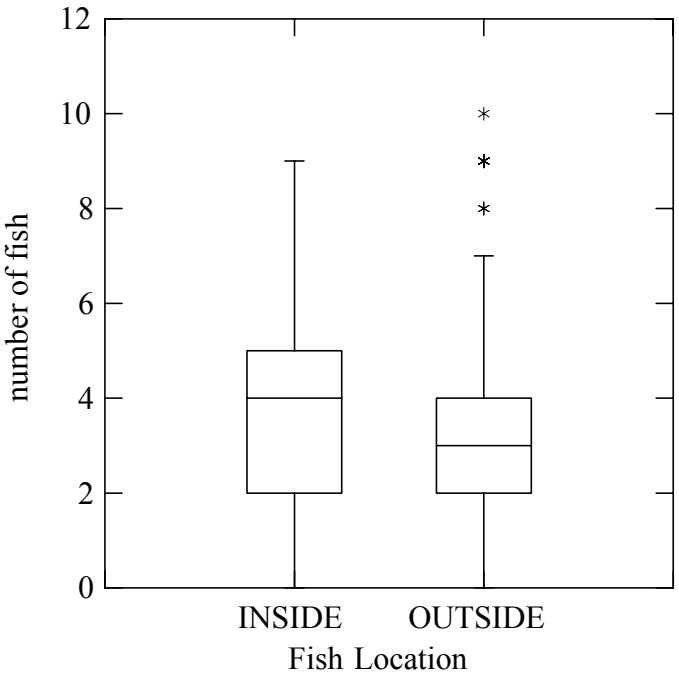


Figure 4.7 Median and range of *Thermichthys hollisi* individuals imaged inside of and outside of the central fish hole using the RatCam. "Inside" fish have all or part of their body below the level of the basalt crust at the edges of the fish hole. Asterisks are considered outliers.

4.3.3 Gene expression and relationship

Primers were designed to amplify fragments of genes encoding for metallothioneins, superoxide dismutase, hypoxia inducible factor, heat shock proteins, and glutathione s transferase (Table 4.1). 17 forward and 12 reverse primers were tested for metallothioneins with some expected to be specific to a particular metallothionein isoform and others more generic. Numerous bands up to ~900bp successfully amplified utilizing annealing temperatures ranging from 45-58°C with most amplification occurring near 50°C. Over 20 bands were subsequently sequenced with most exhibiting non-specific, short-sequence length matches to sequences in GenBank. Matches to vertebrate zinc fingers, HLA-B associated transcripts, and myosin light chain occurred more than once with 80% or less sequence similarity. Two sets of primers were tested for superoxide dismutase, one universal set and one specific to Cu, Zn SOD. Numerous bands less than 600bp in length were obtained and 12 bands sequenced. Again most matches were non-specific, but a fragment of Cu, Zn SOD was obtained. One set of primers were tested for heat shock protein 70. At an annealing temperature of 50°C there was no amplification of *T. hollisi*, but there were non-specific bands amplified for *T. cerberus*. Two primer sets were tested specific to hypoxia inducible factor, only one of which yielded products (multiple bands <500bp) when annealing temperatures ranging from 48-55°C were utilized. Two of these bands were successfully sequenced but again yielded non-specific matches. Two primer sets targeting glutathione S transferase (GSTpi and GSTtheta respectively) were tested with only GSTpi primers amplifying under similar conditions to those outlined above. Multiple bands less than 700bp length

were amplified, one of which when sequenced matched a guanine nucleotide binding protein with >85% sequence similarity. RNA quality was not specifically assessed, and thus it is difficult to evaluate whether the number of short sequences obtained was due to partial RNA degradation or not.

Of the primers tested, we were only able to successfully obtain fragments for the target gene for Cu, Zn-SOD. *T. hollisi* partial SOD gene sequence shares an 81% pairwise similarity (51% identical) in amino acid composition when compared with 15 other teleost, a shark, and an oyster Cu, Zn-SOD amino acid sequences (Figure 4.8). The nucleotide neighbor-joining tree generated from the SOD fragment indicates that the closest related SOD analyzed is that of *Oreochromis mossambicus* (Figure 4.9). The amino acid neighbor-joining tree (Figure 4.10) showed even less definition with no distinguishable relationship between *T. hollisi*, the other teleosts and the oyster outgroup.

Cu, Zn SOD gene expression was similar in all tissue types within an individual fish (Figure 4.11). There are no clear expression patterns among individuals (Figure 4.12). Although gill relative expression appears slightly elevated in comparison to that of the liver and muscle for two of the individuals, the difference is not significant, though small sample size diminishes the power of statistical tests. A larger sample size is necessary to assess the presence of significant expression patterns within the collected population.

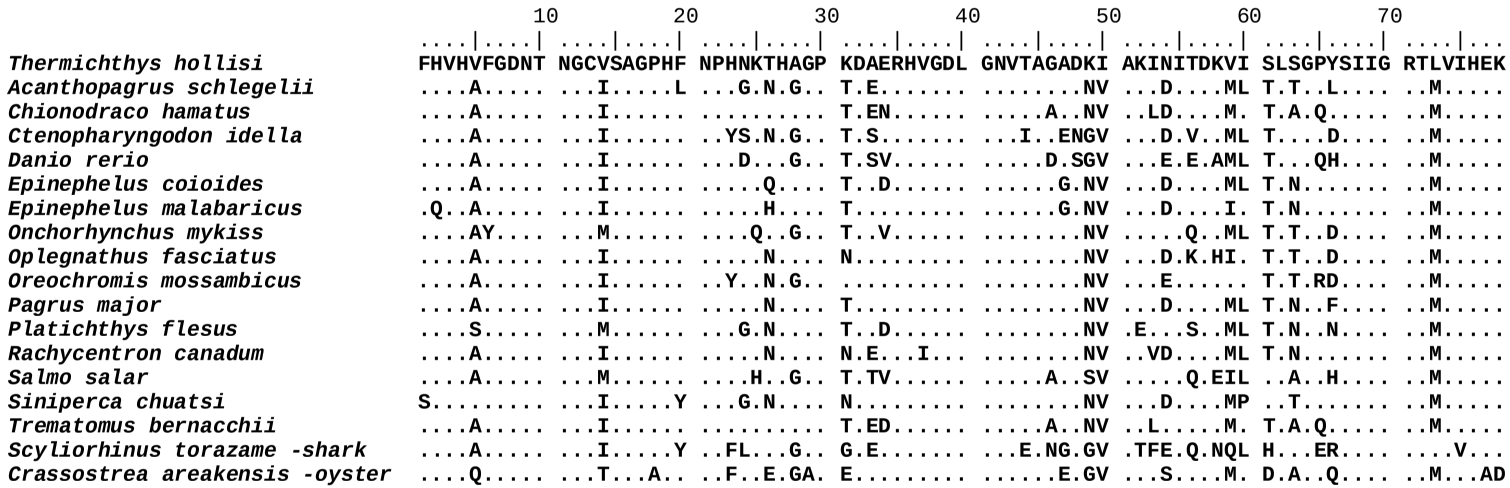


Figure 4.8 Protein alignment of *Thermichthys hollisi* in comparison with other fish (and one mollusk) Cu, Zn SOD sequences.

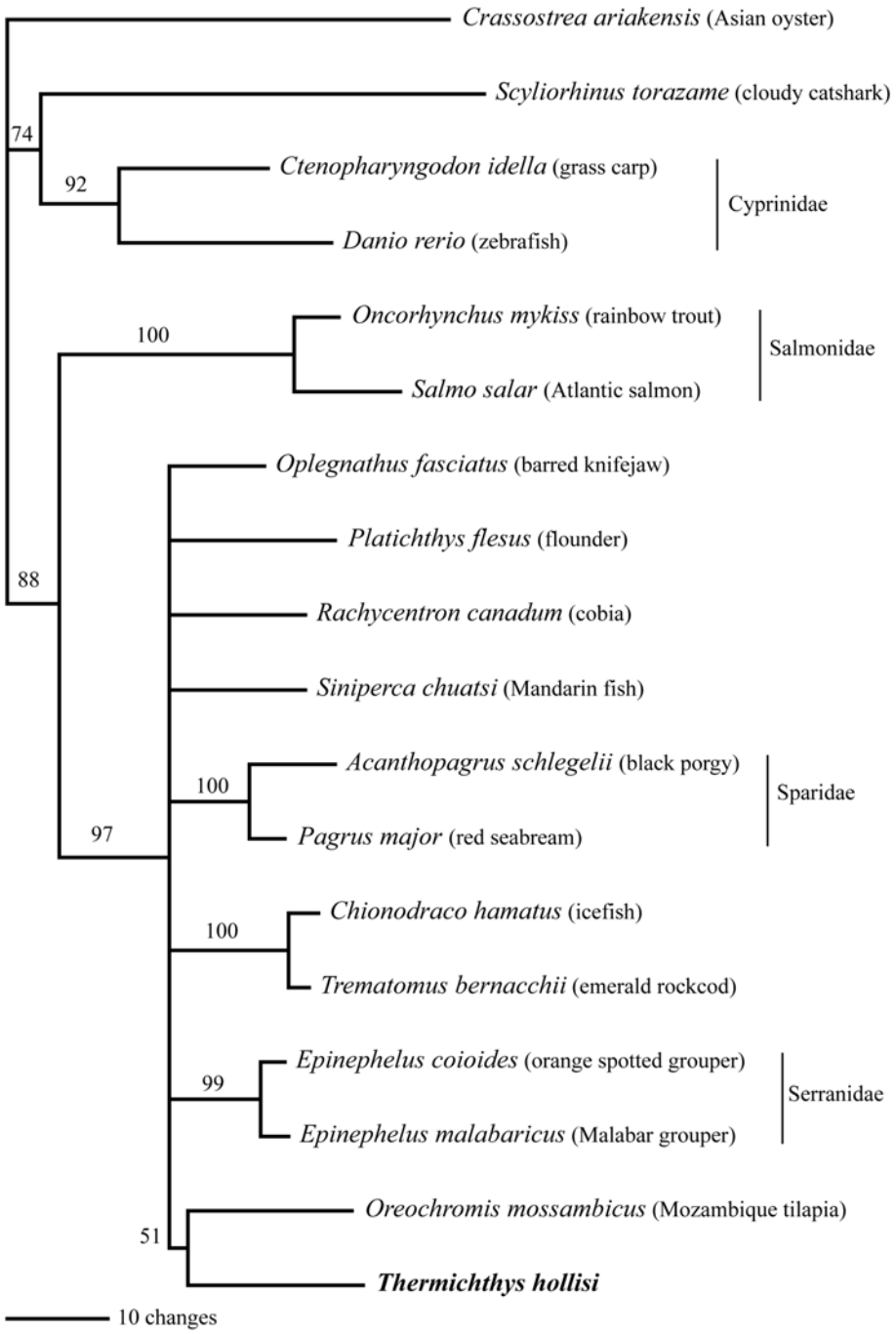


Figure 4.9 Neighbor-joining tree for fish Cu, Zn superoxide dismutase nucleotide alignment (oyster outgroup). Bootstrap values are indicated at the nodes and branches occupied by individuals from the same family are marked on the right.

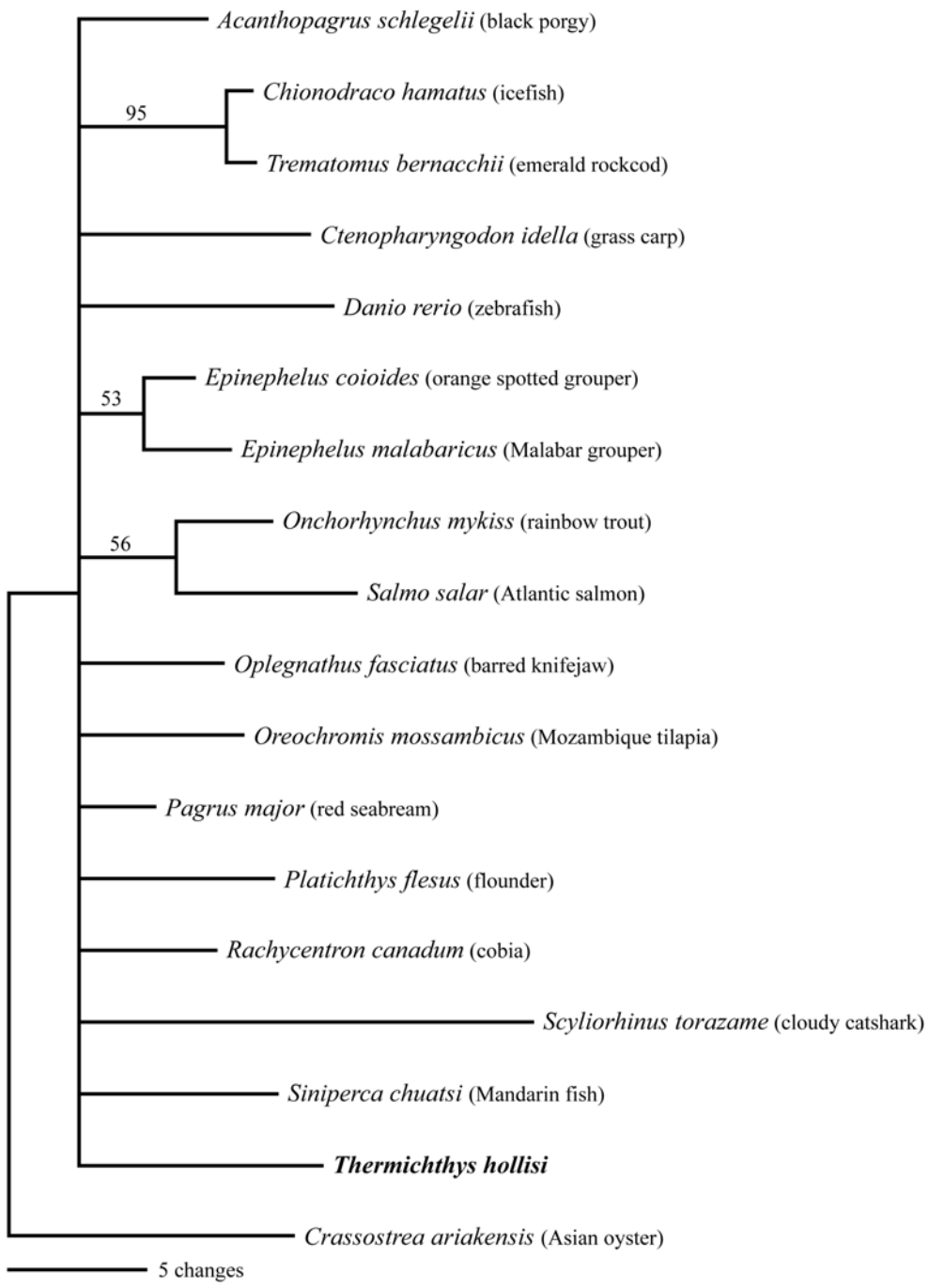
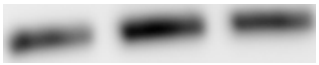


Figure 4.10 Neighbor-joining tree constructed from partial Cu, Zn-SOD amino acid fish sequences (oyster outgroup). Bootstrap values are indicated at the nodes.

liver gill muscle


18s


Cu, Zn SOD

Figure 4.11 Example of pcr gel image. The upper panel indicates 18s expression from Th5 and the lower panel indicates SOD expression. The levels of expression of both genes are similar over all tissue types.

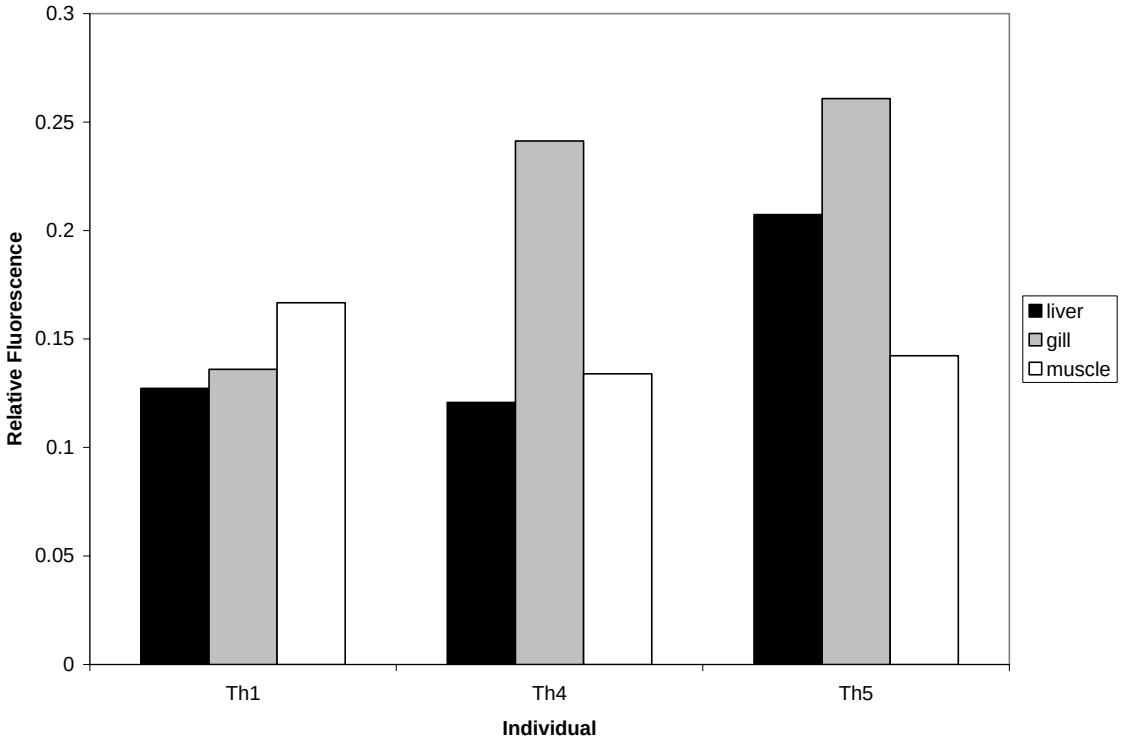


Figure 4.12 Relative SOD expression in liver gill and muscle tissue in three *Thermichthys hollisi* individuals,illustrating the lack of consistent differences among tissue types or between individuals.

4.4 Discussion

The vent fish *Thermichthys hollisi* has been observed to live in the periphery of Eastern Pacific vent fields and in enigmatic aggregations in collapse pits and cracks termed "fish holes", where they had previously been considered relatively unexposed to hydrothermal fluid influence. A study of enzyme Tm on a single *T. hollisi* individual found the lactate dehydrogenase of *T. hollisi* is less functionally tolerant of elevated temperatures than that of *Thermarces andersoni/cerberus*, the other species of vent-endemic fish on the East Pacific Rise, leading the investigators to hypothesize that *T. hollisi* is less well-adapted to live at vents (Dahlhoff et al., 1990). Despite this assumption, it has been shown through otolith chemistry (Buckman et al., in prep) that *T. hollisi* experiences a habitat with different chemical composition than would be considered typical for a deep-sea fish, and that the influence of hydrothermal fluids is evident throughout the majority of their lifespan.

These findings are supported by the current study. Electrochemical measurements indicated that fish holes are elevated in temperature and free sulfide concentration, and generally depleted in dissolved oxygen (at times near hypoxic levels) relative to water outside of the fish-hole, suggesting a greater influence of hydrothermal activity within the habitat than previously suspected. *T. hollisi* individuals are more likely to be found in association with fish holes than not. Time-lapse camera images indicated that mean numbers of fish were greater within the imaged fish hole than outside of it for the period of observation. These data may actually underestimate the number of fish associated with "fish holes" as imaged fish that were counted as outside of the fish

hole may have been associated with nearby fissures or collapses and not the central hole. The combined chemistry and image data indicated that *T. hollisi* are preferentially choosing habitats and thus experiencing environmental conditions associated with exposure to hydrothermal fluid (elevated temperatures, levels of metals and sulfides, and decreased oxygen). As such, they are likely to harbor behavioral and/or gene level adaptations to their habitat conditions.

The amplified fragment of SOD from *Thermichthys hollisi* shares amino acid sequence similarities with characterized SOD from other teleosts (Figure 4.6), in accordance with previous work suggesting that this gene is well conserved (Cho et al., 2006; Nam et al., 2006). A neighbor-joining tree derived from the 236bp nucleotide fragment and available GenBank sequences indicated that *T. hollisi* Cu, Zn-SOD is most similar to that of *Oreochromis mossambicus*, a freshwater tilapia. Though some individuals from the same families (serranids, salmonids, sparids) clustered well together, the relationship between *T. hollisi* SOD and that of other fish species is not well resolved (see bootstrap values). Surprisingly, the cyprinids' SOD was found to be most closely related to that of a shark, and not to the other teleosts as would have been expected. The amino acid neighbor-joining tree is even less well-resolved; however, groups consisting of serranids, salmonids, and Antarctic fish continue to be present. The grouping of the Antarctic fish *Trematonus bernacchii* and *Chionodraco hamatus* is interesting in that it suggests that SOD may be evolving to suit the environment or habitat characteristics rather than along taxonomic lines. The current study is the first example of SOD from a

deep-sea fish, and further investigation of the evolutionary relationships and conservation of teleost Cu, Zn-SOD with better taxonomic representation remains.

The results of the current exploration of Cu, Zn SOD gene expression within *Thermichthys hollisi* tissues were inconclusive, yet they highlighted a number of conditions to consider when conducting future gene expression studies of vent fauna. Despite its use in the current study (due to the lack of consistent and correct amplification of other genes), SOD may not be an ideal gene with which to examine environmental stress, particularly when considered independent of other stress response genes. SOD is an integral component of the regulation of cellular ROS levels, catalyzing the transformation of the super oxide anion to H_2O_2 and O_2, and through this process helping to decrease cell stress and damage (Fridovich, 1995). Yet SOD has been shown to actually decrease its activity upon initial exposure to heavy metals (whose presence facilitates the generation of ROS and oxidative stress) (Company et al., 2006; Lushchak et al., 2009). Organisms with chronic metal exposure in contrast may have elevated levels of SOD activity and transcription in comparison to unexposed individuals (Cho et al., 2006; Ruas et al., 2008). For oxidative stress response genes, a traditional assay of enzyme activity in combination with gene expression may prove to be more informative of levels of oxidative stress in vent fauna.

In addition, a different method of collection and preservation of vent fauna would greatly improve expression studies. Though we were able to successfully preserve, extract, reverse-transcribe and amplify RNA from specimens after their recovery from the seafloor, this method may confound future gene expression studies as it will be difficult

to distinguish expression patterns related to the stresses from recovery to the surface from those caused by habitat conditions, especially for genes with rapid response times. Additionally, extended periods of time between collection and processing may lead not only to altered expression patterns, but also to RNA degradation, which will limit the methods that can be successfully utilized to analyze expression patterns. A mechanism for preserving fauna *in situ* would eliminate these problems, and would allow for better correlation of individual expression profiles with measured environmental variables. Such mechanisms have been developed for use with small vent invertebrates (Shank et al., in prep), but their use with vent fish may be currently unrealistic.

Of equal importance is the need to establish a comparative framework in order to assess organism response to variable conditions, and whether these responses are actually adaptive to the vent environment. Individuals of the same species should be collected from multiple sites, preferably with different habitat chemistry at each site, and gene expression and/or enzyme activity levels measured to assess between site differences. Comparisons can be made to expression levels and enzyme acitivities within and among other vent-endemic fish species in similar habitats to examine between species differences within the vent environment. It has been suggested that *Thermarces cerberus* may be better adapted to hydrothermal vent conditions than *Thermichthys hollisi*, and examining differences and similarities between gene nucleotide sequences as well as gene expression levels and enzyme activities is a first step towards examining this hypothesis. Enzyme sensitivity to changing conditions (temperature, pressure) can be measured within vent and non-ventfish tissues as in Dalhoff et al. (1990) to assess

functionality under conditions that the fish may experience in disparate habitats. Ideally, controlled experiments comparing transcription-level responses of vent-endemic fish to those of closely related non-vent species in response to specific environmental parameters (hypoxia or controlled additions of cadmium) could be conducted. Though the current expenses and difficulties involved in capturing and maintaining live deep-sea fish for laboratory experiments may be prohibitive, a good initial step in addressing adaptation of vent vertebrates would be to compare and contrast enzyme functionality between closely related species and from there move on to an examination of *in situ* expression profiles.

Summary and Future Work

This study provided the first characterization of *Thermichthys hollisi* abundance, habitat use and environmental parameters. It was found that *T. hollisi* are significantly and preferentially associated with habitats featuring elevated temperatures and sulfide levels, and decreased oxygen concentration in contrast to the ambient bottom water, which may influence their physiological adaptations. Cu, Zn SOD protein is similar to that of other organisms, and expression of this gene was observed in all tissue types. Further exploration and controlled experimentation of *Thermichthys hollisi* temperature tolerances and exposure to metals with coincident measurements of gene expression and enzyme activity will provide greater understanding of the physiological constraints and stresses experienced by vent endemic fish.

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Chapter Five

Summary of Findings

This thesis represents the first comprehensive ecological study of a hydrothermal vent-endemic fish. *Thermichthys hollisi*, a bythitid native to vents in the eastern Pacific, was the focus of this study; undertaken with the goals of elucidating the habitat preferences, trophic interactions, natural history, and potential adaptations to its environment. Gut content and stable isotope analyses provided the first direct evidence that *T. hollisi* feeds almost exclusively on other vent-endemic fauna, including brachyuran crabs, *Alvinocaris* shrimp, polychaetes, and the zoarcid fish *Thermarces cerberus*, placing *T. hollisi* within the upper if not the uppermost level of the trophic network within East Pacific Rise vent communities. As a top predator, *T. hollisi* may be influencing invertebrate community structure directly through predation on the invertebrates themselves and indirectly through removal of *T. cerberus* which is known to selectively prey upon limpest and amphipods.

Otolith isotope chemistry, *in situ* electrochemistry and time-lapse imagery all indicated that the preferred habitat of *T. hollisi* is comprised of collapse pits and fissures within the basalt, and that these areas are influenced by diffuse hydrothermal fluids despite being devoid of the vent invertebrate fauna typically associated with diffuse hydrothermal fluid flow. There are differences between the otolith chemistries of *T. hollisi* and *T. cerberus* which are reflective of their habitat preferences. *T. cerberus* appeared to experience greater exposure to the vent fluids and to spend the entirety of its

life within the vent system whereas *T. hollisi* otoliths reflected less direct exposure to vent fluids and showed significantly different elemental signatures within the core of the otolith. *T. hollisi* core otolith element chemistry was strikingly similar to that of non-vent fish measured, suggesting that *T. hollisi* individuals are spending some portion of their larval or juvenile stages away from vent ecosystems (Figure 5.1). This behavior has implications not only for larval transport and population connectivity, but may also represent a previously unconsidered pathway for carbon exportation away from vents in the form of migrating gravid females.

As *T. hollisi* is exposed to hydrothermal fluids, it can be expected to harbor adaptations to life within the vent environment that may be analogous to invertebrate adaptations to sulfide exposure, elevated temperatures and metal exposure. *In situ* electrochemistry indicated that fish holes, where the *T. hollisi* is typically found, have elevated sulfide levels and temperatures, and decreased oxygen levels in comparison to uninhabited basalt areas outside of the hole, supporting the hypothesis that these fish may be have biochemical adaptations to enable them to exist and thrive in the vent environment. Cu, Zn superoxide dismutase was amplified for the first time from a deep-sea fish. The protein structure exhibited a number of differences when compared with other shallow-water teleost sequences, but major binding residues were conserved. The evolutionary relationship of *T. hollisi* Cu, Zn SOD to other fish remainss unclear, and the addition of other vent and deep-sea fish species SOD sequences is necessary to interpret whether *T. hollisi* SOD protein structures are unique. Although SOD gene expression was evident in liver, gill, and muscle tissues from all individuals tested, there were no

differences in expression levels evident that could be related to environmental parameters.

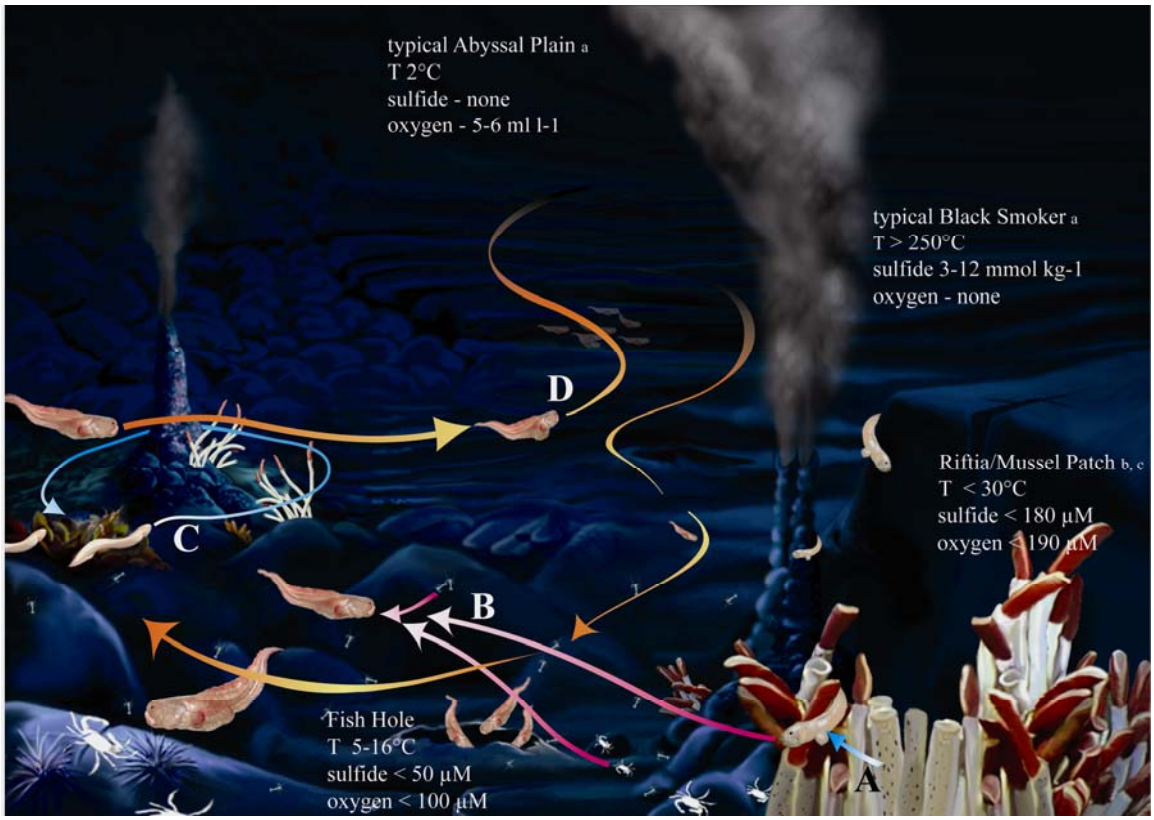

Figure 5.1 Composite illustration indicating generic habitat parameters experienced by vent-endemic fish on the East Pacific Rise as well as prey preference of zoarcids (A. limpets & small crustaceans (Micheli et al., 2002; Sancho et al., 2005)); prey preferences of bythitids (e.g. B. shrimp, crabs, zoarcids); and proposed life history strategies of zoarcids (C. whole life within venting environment) and bythitids (D. early portion of life away from vent fluid influence).

a. Van Dover, 2000 b. Luther et al., 2008 c. Nees et al., 2008

Future Directions

The studies undertaken as part of this thesis have advanced the current state of knowledge regarding East Pacific Rise hydrothermal vent-endemic fish ecological interactions with their habitat and their surrounding community. Yet, ecological knowledge of vent-endemic fish is far from complete, with numerous questions left to be clarified and answered. This work provides a building block for future studies regarding the ecology of vent-endemic vertebrates from all venting environments, not just the East Pacific Rise. Compound specific stable isotope studies are gaining popularity and may prove to be helpful in further elucidating trophic relationships within vent fields. Pond et al. (2008) studied fatty acid signatures within *Thermarces cerberus* to elucidate trophic interactions predicted from gut content studies. They discussed the hypothesized differences between invertebrate fatty acid acquisition at Atlantic versus Pacific vent sites, and comparative behavioral, feeding and isotopic studies of similar vent vertebrates between these disparate areas could be conducted to address unanswered questions regarding vent community fatty acid origins.

Otolith isotope analyses from the present study have illuminated interesting patterns within both species of vent fish examined. Strontium in particular bears further investigation as discussed in Chapter Three. The Sr:Ca values measured within the vent endemic fish otoliths, particularly within the zoarcids are higher than any previously measured Sr:Ca otolith values. The effects of temperature and environmental strontium concentrations on incorporation into the otolith bear further investigation, the results of

which may provide further possibilities for studying and understanding the relationships defining otolith chemistry and the utility of vent fish otoliths as environmental proxies. The current otolith study has also provided a basis for vent fish population genetic studies. The similar chemical patterns over the lifetime of the fish examined suggest that they experience similar environmental chemical parameters and thus are perhaps restricted in their dispersal or living in cohorts that maintain "local" lineage groups in association with specific vent fields or sets of neighboring vent fields. Population genetic studies would allow for further investigation of this hypothesis, and would add to the growing body of knowledge regarding vent fauna connectivity, dispersal, and recruitment.

Genetic studies need not be restricted to population genetics. Molecular phylogenetic approaches can also help elucidate evolutionary relationships between vent fauna around the world. The family Zoarcidae, members of which are seemingly ubiquitous in both the non-vent deep-sea and in venting environments may be a good candidate for exploring questions regarding radiation and evolution in the deep-sea. The current work proved that gene expression studies within venting environments are feasible with careful design and improved sampling procedures. As discussed in Chapter Four, in combination with studies of gene sequence similarity and evolution in related species, gene expression studies of vent fish may help to answer questions regarding how vent fauna are adapted to take advantage of their habitat when they do not appear to differ physically from other fish species. Population genomics (reviewed in Neilsen et al., 2009) may provide another tool for learning about adaptation within fish species.

The presence of fish within hydrothermal venting areas has been noted for as long as vents have been studied. Yet, with a few notable exceptions, the majority of publications regarding vent fish have been restricted to species descriptions or observational notes. Vent fish are integral members of the community, and as such deserve further examination in order to truly understand vent ecosystem function and dynamics. As this thesis has shown, there are ample opportunities for studying hydrothermal vent-endemic fish beyond basic species identification, and the fields of vent vertebrate behavior, ecology, and evolution remain wide open for future study.

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