# Bacterivory of the hydrothermal-vent-specific copepod *Stygiopontius senokuchiae* (Dirivultidae, Siphonostomatoida) from copepodite through adult stages

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## Abstract

*Stygiopontius senokuchiae* is a hydrothermal-vent-specific copepod species (Dirivultidae, Siphonostomatoida) and abundantly distributes near vent orifices. This species is thought to have an early juvenile planktic stage, and previous studies using bulk tissue stable-isotope and radioisotope data suggest that adults ingest chemoautotrophic microbes growing on vent chimneys. However, ontogenetic changes in their diets have not been investigated. We analysed gut contents of copepodite I to adult stages of *S. senokuchiae* collected from a hydrothermal-vent chimney at the Izu-Ogasawara Arc to check for the presence of bacterial cells in oral tubes and guts. We compared these results with an unidentified co-occurring calanoid and a species of *Ectinosoma* (Harpacticoida) and to other Siphonostomatoid copepods, namely *Hatschekia labracis* (Hatschekiidae), which was attached to a scarbreast tuskfin (*Choerodon azurio*, Labridae), and *Asterocheres* sp. 1 (Asterocheridae), which was attached to a spirastrellid sponge. Carbon isotope ratios of *S. senokuchiae* at different stages and *Ectinosoma* were measured to complement the nutritional insights obtained from gut-content analysis. Our results clearly showed that most *S. senokuchiae* individuals possessed bacteria in the oral tube or gut regardless of growth stage, whereas the coexisting *Ectinosoma* and calanoid did not. The carbon isotopic compositions confirmed that *S. senokuchiae* gains its nutrition from chemoautotrophic bacteria that use the rTCA carbon-fixation pathway. Comparisons with other Siphonostomatoida copepods suggest that Dirivultidae are specifically adapted to feed on bacteria at hydrothermal-vent chimneys, allowing their high dominance and evolutionary success in these habitats.

## Introduction

Deep-sea hydrothermal-vent ecosystems utilize organic matter (OM) produced by chemoautotrophic microbes. Because prey–predator relationships provide fundamental knowledge about the ecology of organisms and relevant biogeochemistry, many studies have reported on the feeding ecology of megabenthos and chemoautotrophic microbes (e.g. Cavanaugh *et al.*, 1981; Felbeck, 1985; Belkin *et al.*, 1986; Duperron *et al.*, 2006; Watsuji *et al.*, 2014). Meiofauna – organisms that pass through a 1-mm mesh sieve but are retained on a 63- or 32-μm mesh sieve (Giere, 2009) – have received less attention at hydrothermal-vent habitats (Vanreusel *et al.*, 2010), although meiofauna comprises a large biomass and plays essential roles in deep-sea ecosystems in general (e.g. Rex *et al.*, 2006; Gooday *et al.*, 2008). Among the deep-sea meiofauna, nematodes typically dominate the community, followed by harpacticoid copepods (e.g. Shimanaga *et al.*, 2007). Vanreusel *et al.* (2010) reviewed biogeographic data on nematodes associated with chemosynthetic environments in the deep sea and showed high similarity between vents and adjacent typical, non-vent sediment communities. Similar conclusions were reported from studies of vent meiofaunal communities at the East Pacific Rise (Gollner *et al.*, 2010b) and nematode communities at the hydrothermal-vent field and adjacent non-vent seafloor at the Izu-Ogasawara Arc, western North Pacific (Setoguchi *et al.*, 2014). These faunal similarities suggest low endemism in deep-sea vent meiofaunal species (cf. Gollner *et al.*, 2010b) and that current vent meiofauna invaded from adjacent sediments, a pattern presumably related to the absence of a planktonic life stage (cf. Vanreusel *et al.*, 1997). The low endemism of nematodes at hydrothermal vents suggests that most taxa do not rely solely on chemoautotrophic microbial production at vent chimneys.

The family Dirivultidae (Copepoda: Siphonostomatoida), an exceptionally abundant meiofaunal taxon at deep-sea hydrothermal vents, are free-living on hard substrates and often

co-occur with aggregations of various sessile vent megafauna (Gollner *et al.*, 2010a). Their distribution patterns, their mouthpart morphologies, and observations of partly digested bacteria in their foregut, suggest tolerance of a wide range of hydrothermal fluid flux regimes and strong nutritional relationships with chemoautotrophic microbes inhabiting hydrothermal-vent chimneys (Dinet *et al.*, 1988; Heptner & Ivanenko, 2002; Gollner *et al.*, 2010a). Among Dirivultidae, *Stygiopontius* is the most diverse genus in hydrothermal-vent ecosystems (currently 28 accepted species; Walter & Boxshall, 2021), implying a nutritional advantage in these ecosystems. Measurements of stable carbon isotopic composition ($\delta^{13}C$) and natural-abundance radiocarbon concentration confirmed that *Stygiopontius senokuchiae* described from the Izu-Ogasawara Arc hydrothermal-vent system (Uyeno *et al.*, 2018) depends largely on chemoautotrophic bacteria on the chimney walls (Nomaki *et al.*, 2019).

*Stygiopontius* copepods have a lecithotrophic mode of nutrition and are expected to have a planktic nauplius larval stage (Ivanenko *et al.*, 2007). However, details about their ecologies, particularly the development and life cycles of dirivultid copepods, are still limited (Ferrari & Dahms, 2007; Ivanenko *et al.*, 2007). The high genetic connectivity between dirivultid copepods at different hydrothermal-vent fields (Gollner *et al.*, 2016; Watanabe *et al.*, 2021) suggests that they have high dispersal ability across several tens to thousands of kilometres. Indeed, abundant copepodites of dirivultids have been found in the water column above a vent field, enabling them to disperse to or colonize other vent fields (Gollner *et al.*, 2015b). The coexistence of adult *S. senokuchiae* along with juveniles of different copepodite stages on the same hydrothermal-vent chimney walls at our study sites (Senokuchi *et al.*, 2018) suggests that both adults and juveniles feed on organic matter on hydrothermal-vent chimneys.

The $\delta^{13}C$ values of juvenile dirivultids were sometimes lower than those of adults (Nomaki *et al.*, 2019). The adult $\delta^{13}C$ values were almost identical to those of co-occurring *Paralvinella* spp., which rely for their nutrition on chemoautotrophic microbes that use the rTCA carbon fixation pathway. This implies that juvenile dirivultids get part of their nutrition other than from chemoautotrophic bacteria, whereas adult dirivultids may rely solely on such bacteria for their nutrition. These presumed changes in dirivultid feeding habits are expected to play important roles in their adaptation to hydrothermal-vent areas. However, the timing of their colonization on hydrothermal-vent chimneys and their dependence on chemoautotrophic bacteria during their development have not been investigated in detail.

In this study, we used transmission electron microscopy (TEM) to observe gut contents of *Stygiopontius senokuchiae* collected from a Myojin Knoll caldera hydrothermal-vent chimney. Different developmental stages of *S. senokuchiae*, from the first copepodite juvenile (CI) to adult, were observed in detail. The focus was mainly on the presence or absence of bacteria near mouth parts, in the oral tubes, and in the guts. For comparison, we also observed the gut contents of the co-occurring copepods *Ectinosoma* sp. 1 (Copepoda: Harpacticoida: Ectinosomatidae) and an unidentified calanoid copepod from the same hydrothermal-vent chimney. To highlight the uniqueness of bacteria ingestion by *Stygiopontius* among the order Siphonostomatoida, we further observed the gut contents of two other copepod species belonging to Siphonostomatoida: the parasitic copepod *Hatschekia labracis* (Copepoda: Siphonostomatoida: Hatschekiidae), which was attached to a scarbreast tuskfin (*Choerodon azurio*), and *Asterocheres* sp. 1 (Copepoda: Siphonostomatoida: Asterocheridae), which was attached to a spirastrellid sponge, both collected in Kagoshima Bay, Japan.

Furthermore, we measured stable carbon and nitrogen isotopic compositions of the fourth and fifth juvenile copepodite (CIV, CV)

and adult stages of *S. senokuchiae* and adult stages of *Ectinosoma* sp. 1 to identify any changes in nutritional ecology. We speculated that among Siphonostomatoida, which consists mainly of parasitic copepods, *Stygiopontius* is specialized for bacterivory. We further hypothesized that the dominance of *Stygiopontius* copepods at hydrothermal-vent chimneys (Uejima *et al.*, 2017; Senokuchi *et al.*, 2018) can be explained by this specialized feeding, which is not observed in co-occurring *Ectinosoma* sp. 1 or Calanoida.

## Materials and methods

### Sampling

We sampled the detritus on a hydrothermal-vent chimney at Myojin Knoll caldera, located in the Izu-Ogasawara Arc of the western North Pacific (Honsho *et al.*, 2016; Bernhard *et al.*, 2023), during cruise KT-18-3 of the RV 'Shinsei-maru' using the remotely operated vehicle (ROV) 'Hyper-Dolphin 4500' (Figure 1A). Hydrothermal-vent meiofaunal communities in the Izu-Ogasawara Arc area were previously described by Uejima *et al.* (2017) and Senokuchi *et al.* (2018).

The detritus on a *Paralvinella* polychaete colony on a hydrothermal-vent chimney was sampled with a suction sampler attached to the ROV with exchangeable sample containers equipped with a 30-μm mesh. On board, suction samples were split in half using a plankton splitter (RIGO Co. Ltd, Japan). One half of the sample was immediately fixed and preserved in 99.5% EtOH for study of molecular phylogeny. After removing excess seawater, the other half was preserved with 2.5% glutaraldehyde in filtered seawater at 4°C for TEM observations. In the laboratory on land, the samples fixed with glutaraldehyde were sieved with seawater, and the different developmental stages of *S. senokuchiae* and *Ectinosoma* sp. 1, and a single specimen of Calanoida, were isolated under a binocular microscope with the assistance of a differential interference microscope (Table 1, Figure 1B–F). The isolated specimens were put into 2.5% glutaraldehyde and kept at 4°C prior to further processing for TEM.

A parasitic copepod *Hatschekia labracis* associated with the scarbreast tuskfin (*Choerodon azurio*), and *Asterocheres* sp. 1, which might be a commensal species attached to a spirastrellid sponge, were collected by divers using scuba gear in Kagoshima Bay, Japan (Figure 1G–I). Copepods were removed from the host animals and sorted under a dissection microscope (Table 1). Subsequently, the copepods were fixed with 2.5% glutaraldehyde and kept overnight at room temperature. They were then kept chilled at 4°C until processing.

Hydrothermal-vent copepods were also sampled for measurement of stable carbon isotopic composition ($\delta^{13}C$). During cruise NT13-09 of the RV 'Natsushima', suction samples were collected from the surface of a *Paralvinella* colony at Myojin-sho caldera for the isolation of *S. senokuchiae* (Table 2). During cruise NT14-06 of the RV 'Natsushima', suction samples were collected from the surface of a bacterial mat at Bayonnaise caldera for the isolation of *Ectinosoma* sp. 1 (Table 2). The suction samples were preserved in 99.5% EtOH after removing excess seawater. On land, copepods were isolated from the samples under a binocular microscope and kept in 99.5% EtOH prior to sample processing for $\delta^{13}C$ analyses. Previous studies have reported that ethanol preservation sometimes causes a shift in $\delta^{13}C$ values, typically within a few ‰ (e.g. Enge *et al.*, 2018). However, these isotopic shifts are relatively small compared with the $\delta^{13}C$ variation in hydrothermal-vent ecosystems (typically around 30‰) (Bell *et al.*, 2016).

### TEM observations

Glutaraldehyde-fixed samples were post-fixed and embedded in resin following previously published protocols for TEM sample
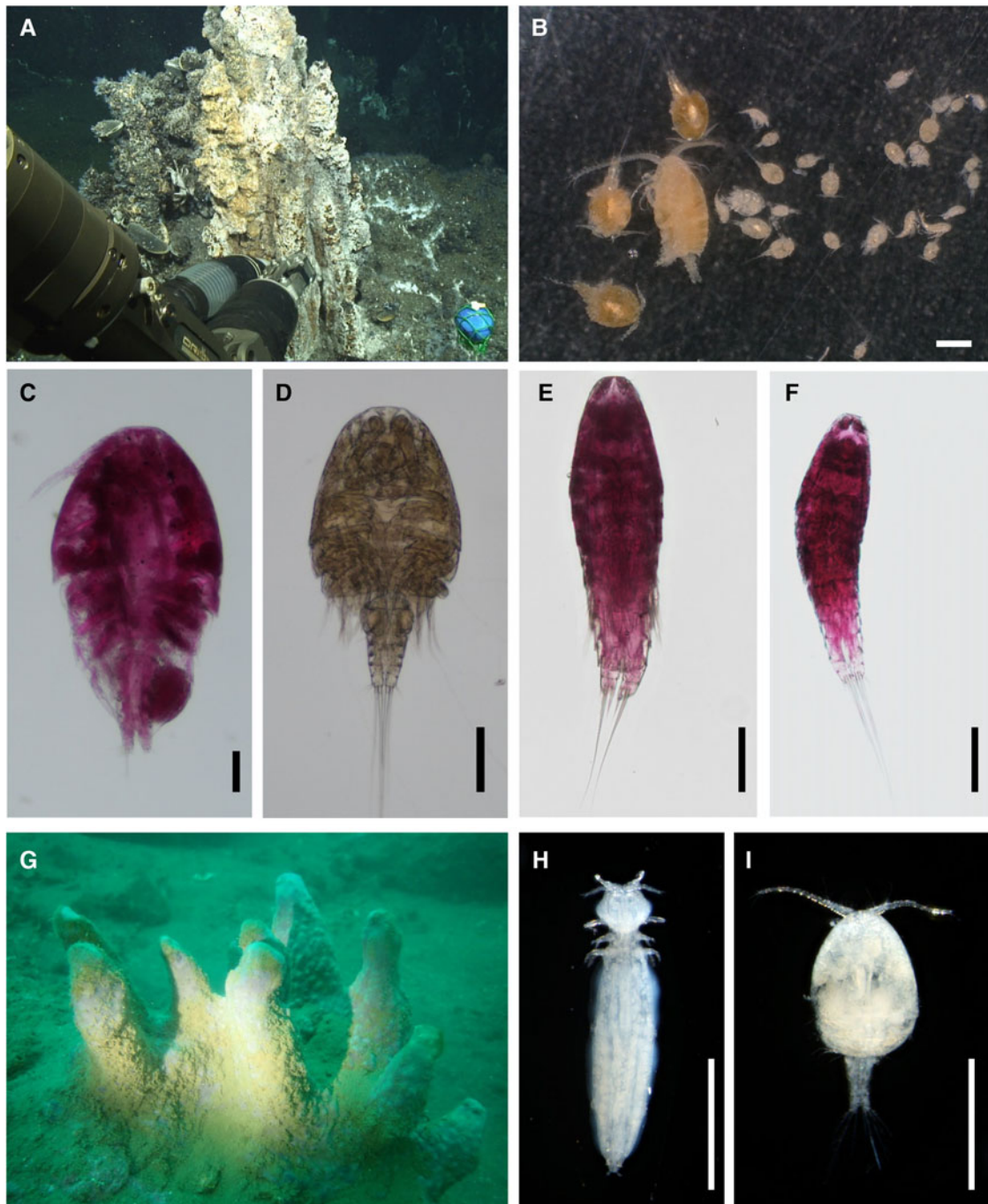
**Fig. 1.** Images of sampling sites and copepod species examined in this study. (A) Hydrothermal-vent chimney at Myojin Knoll caldera. (B) Copepod specimens isolated from glutaraldehyde-fixed detritus samples from the hydrothermal-vent chimney. (C) *Stygiopontius senokuchiae* (adult female) stained with rose-Bengal. (D) *Stygiopontius senokuchiae* (adult male). (E) *Ectinosoma* sp. 1 (adult female) stained with rose-Bengal. (F) *Ectinosoma* sp. 1 (adult male) stained with rose-Bengal. (G) Spirastrellid sponge off Sakurajima, Kagoshima Bay, Japan. (H) *Asterocheris* sp. 1. (adult female) collected from the spirastrellid sponge. (I) *Hatschekia labracis* (adult female) attached to a gill filament of the scarbreast tuskfin *Choerodon azurio* in Kagoshima Bay. Scale bars: B, 500 μm; C, D, E, F, 100 μm; H, I, 500 μm.

preparation (Nomaki *et al.*, 2015, 2018). In brief, the specimens were post-fixed with 2% osmium tetroxide in filtered artificial sea-water for 2 h at 4°C, dehydrated in a graded ethanol series, and embedded in epoxy resin (Quetol 651; Nisshin EM, Tokyo, Japan). Embedded specimens were sectioned into several semi-thin sections (500 nm thick) using an ultramicrotome (Ultracut S; Leica, Germany). Sections were observed with an optical micro-scope (BX51; Olympus, Japan) to assess the condition and appearance of oral tubes or gut contents. Selected specimens were further sectioned into ultra-thin sections (60 nm thick) (Table 1). These sections were stained with 2% aqueous uranyl acetate and lead staining solution (0.3% lead nitrate and 0.3%

lead acetate; Sigma-Aldrich, USA) and were observed by TEM (Tecnai G2 20; FEI, Hillsboro, OR, USA) at 200 kV.

### Analysis of stable carbon isotopic composition

EtOH-fixed copepod samples were rinsed twice in the ultrapure water and then put directly into pre-cleaned tin capsules and dried at 60°C to remove water and to determine the dry weight. Typically 5–15 individuals were pooled into one sample to pro-vide enough sample mass for analysis, with the exception of one adult female *S. senokuchiae* that had a large body size (Table 2). They were then decalcified with 0.1 M HCl and

**Table 1.** Sampling details for copepod specimens observed by transmission electron microscopy (TEM) and notes on bacteria appearance in TEM images

| Cruise ID | Dive ID | Sampling date | Sampling ID | Location | Latitude | Longitude | Water depth (m) | Species | Stage | Sex | Sample ID | TEM-confirmed bacterial ingestion |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KS-18-3 | HPD2056 | 4 Apr 2018 | C06 | Myojin Knoll caldera hydrothermal-vent chimney | 32°06.22′N | 139°52.15′E | 1220 | *Stygiopontius senokuchiae* | CI | – | C1_1 | Many spherical and some elongated bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CI | – | C1_3 | Absent |
| | | | | | | | | *Stygiopontius senokuchiae* | CII | – | C2_2 | Abundant spherical and elongated bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CIII | – | C3_1 | Abundant spherical and elongated bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CIII | – | C3_2 | Some degraded spherical bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CIII | – | C3_4 | Many degraded spherical bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CIV | – | C4_1 | Some degraded spherical bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_B1 | Abundant spherical and elongated bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_B2 | Absent |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_B4 | Abundant spherical and elongated bacteria in the gut |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_2 | Abundant spherical and elongated bacteria in the gut and oral tube |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_3 | Abundant spherical and elongated bacteria in the gut, oral tube, and around the setae at the mouth |
| | | | | | | | | *Stygiopontius senokuchiae* | CV | – | C5_11 | Degraded bacteria in the oral tube |
| | | | | | | | | *Stygiopontius senokuchiae* | Adult | Male | A_3 | Many spherical and elongated bacteria in the gut |
| KS-18-3 | HPD2056 | 4 Apr 2018 | C06 | | 32°06.22′N | 139°52.15′E | 1220 | *Ectinosoma* sp.1 | Adult | – | Kai_1_1 | Absent |
| | | | | | | | | *Ectinosoma* sp.1 | Adult | Female | Kai_1_2 | Absent |
| | | | | | | | | *Ectinosoma* sp.1 | Juvenile | – | Kai_1_5 | Absent |
| KS-18-3 | HPD2056 | 4 Apr 2018 | C06 | | 32°06.22′N | 139°52.15′E | 1220 | Calanoida sp. | Adult | | Calanoida_1 | Absent |
| – | – | | – | | 31°33.15′N | 130°37.36′E | 10 | *Hatschekia labracis* | Adult | Female | H1 | Absent |

| Date | Location | | N | Coordinates | Species | Stage | Sex | Specimen | Observation |
|---|---|---|---|---|---|---|---|---|---|
| 15 Sep 2020 | Off Nenzaki, Sakurajima, attached to Choerodon azurio | – | | | Hatschekia labracis | Adult | Female | H2 | Abundant intact bacteria in the guts |
| | | – | | | Hatschekia labracis | Adult | Female | H3 | Absent |
| | | – | | | Hatschekia labracis | Adult | Female | Kai_2_1 | Abundant intact bacteria in the guts |
| | | | | | Hatschekia labracis | Adult | Female | Kai_2_2 | Absent |
| | | | | | Hatschekia labracis | Adult | Female | Kai_2_3 | A single bacterial cell in the oral tube |
| 9 Sep 2020 | Off Okogashima Island, attached to spirastrellid sponge | – | 20 | 31°32.61'N 130°37.01'E | Asterocheres sp. 1 | Adult | Female | Kai_3_1 | A few bacteria in the oral tube |
| | | | | | Asterocheres sp. 1 | Adult | Female | Kai_3_4 | Absent |
| | | | | | Asterocheres sp. 1 | Adult | Female | Kai_3_5 | Absent |
| | | | | | Asterocheres sp. 1 | Adult | Male | Kai_4_1 | A few bacteria in the oral tube |
| | | | | | Asterocheres sp. 1 | Adult | Male | Kai_4_3 | Absent (insufficient observation due to sectioning) |
| | | | | | Asterocheres sp. 1 | Adult | Male | Kai_4_5 | Absent |

completely dried again, and then the tin capsules were sealed using pre-cleaned forceps.

The stable carbon isotopic compositions were determined using an isotope ratio mass spectrometer (Delta plus XP; Thermo Finnigan, Cambridge, MA, USA) connected to an elemental analyzer (FlashEA1112; Thermo Finnigan) through a continuous-flow interface (ConFloIII; Thermo Finnigan) following the procedure and using standard materials reported previously (Ogawa et al., 2010; Tayasu et al., 2011; Isaji et al., 2020). We used six inter-laboratory verified organic standards (L-tyrosine, L-alanine, L-proline, L-glutamic acid, L-valine and nickel octaethylporphyrin; $\delta^{13}C$ values of $-34.17‰$ to $+0.18‰$) as working standards to calibrate isotope ratios. The L-tyrosine listed above was used as a standard for the quantification of TOC contents. Errors for repeated analyses (from 5–20) of these standards were $\pm0.12‰$ (SD, $1\sigma$).

## Results

### TEM observations of mouthparts, oral tubes and gut contents

Among 14 individuals of *Stygiopontius senokuchiae* that were observed with TEM, 12 specimens showed degraded to almost intact bacteria in their oral tube or gut, or even around mouthparts in some specimens (Table 1, Figure 2). There were no obvious trends in bacterial appearance across different developmental stages of *S. senokuchiae*. A seta in the oral tube seems to prevent a backflow of bacteria toward the mouth (Figure 2D, E). Bacteria with spherical or elongated shapes sometimes completely filled the oral tube (Figure 2F–I). In the gut, bacteria were observed in many different states of degradation (Figure 2J–L). The abundance of bacteria in the gut varied between specimens (Table 1).

There were no visible bacteria in the co-occurring calanoid (N = 1) or *Ectinosoma* sp. 1 (N = 3), regardless of the location observed (i.e. mouthparts, oral tubes and guts) (Figure 3). The calanoid had longer villi than *S. senokuchiae* with detritus of unidentifiable origin in their gut (Figure 3B), and their guts were filled with amorphous degraded contents (Figure 3D). The oesophagus and gut of *Ectinosoma* sp. 1 were almost empty (Figure 3F–H).

Among six *Hatschekia labracis* specimens observed, two had abundant bacteria in their guts (Figure 4B, C), and one (specimen Kai_2_3) had a single bacterial cell in its oral tube (image not shown). In general, their gut contents consisted of amorphous, degraded organic matter (Figure 4D).

There were no bacterial cells or remnants in the gut contents of six *Asterocheres* sp. 1 individuals, however two specimens had a few bacterial cells in their oral tube (Figure 4F, G). The content of their gut or oesophagus was mainly amorphous degraded organic matter of unknown origin (Figure 4H).

### Stable carbon isotopic composition

The $\delta^{13}C$ values of *S. senokuchiae* ranged between $-14.2$ and $-9.9‰$ (Table 2), which is a range similar to previously reported values for dirivultid copepods at Myojin-sho caldera (Nomaki et al., 2019). There were no significant differences across developmental stages, or between sexes of adults of this species (Kruskal–Wallis test, $P > 0.05$, Figure 5). Multiple comparisons did not detect any significant difference between any pair of copepodite stages of the species, either (Tukey tests, $Ps > 0.05$). *Ectinosoma* sp. 1 had consistently lower $\delta^{13}C$ values ($-19.9$ to $-17.5‰$) than *S. senokuchiae*. There were no significant differences in $\delta^{13}C$ values between adult females and males (Wilcoxon rank sum exact test, $P > 0.05$, Figure 5).

**Table 2.** Details of hydrothermal-vent copepods used for carbon isotopic composition analysis

| Cruise ID | Dive ID | Sampling date | Sampling ID | Location | Latitude | Longitude | Water depth (m) | Species | Stage | Sex | Number of individuals | δ¹³C (‰ VPDB) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NT13-09 | 1518 | 24 Apr 2013 | C01 | Myojin-sho hydrothermal-vent chimney | 31°53.04′N | 139°58.22′E | 795 | *Stygiopontius senokuchiae* | Adult | Male | 5 | −9.9 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | Adult | Male | 5 | −10.3 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 2 | −12.7 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 2 | −11.2 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 2 | −13.3 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 2 | −12.1 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CV | – | 12 | −10.8 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CV | – | 11 | −11.7 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CV | – | 11 | −10.7 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CV | – | 12 | −12.1 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CV | – | 12 | −10.1 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CIV | – | 12 | −14.2 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CIV | – | 12 | −12.1 |
| NT13-09 | 1518 | 24 Apr 2013 | C01 | | | | | *Stygiopontius senokuchiae* | CIV | – | 11 | −11.5 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | Myojin-sho hydrothermal-vent chimney | 31°53.04′N | 139°58.22′E | 795 | *Stygiopontius senokuchiae* | Adult | Male | 6 | −8.5 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Male | 5 | −10.7 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Male | 5 | −10.5 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Male | 5 | −8.0 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 3 | −7.3 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 3 | −8.9 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 3 | −8.5 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 2 | −11.1 |
| NT13-09 | 1518 | 24 Apr 2013 | C04 | | | | | *Stygiopontius senokuchiae* | Adult | Female | 1 | −6.4 |
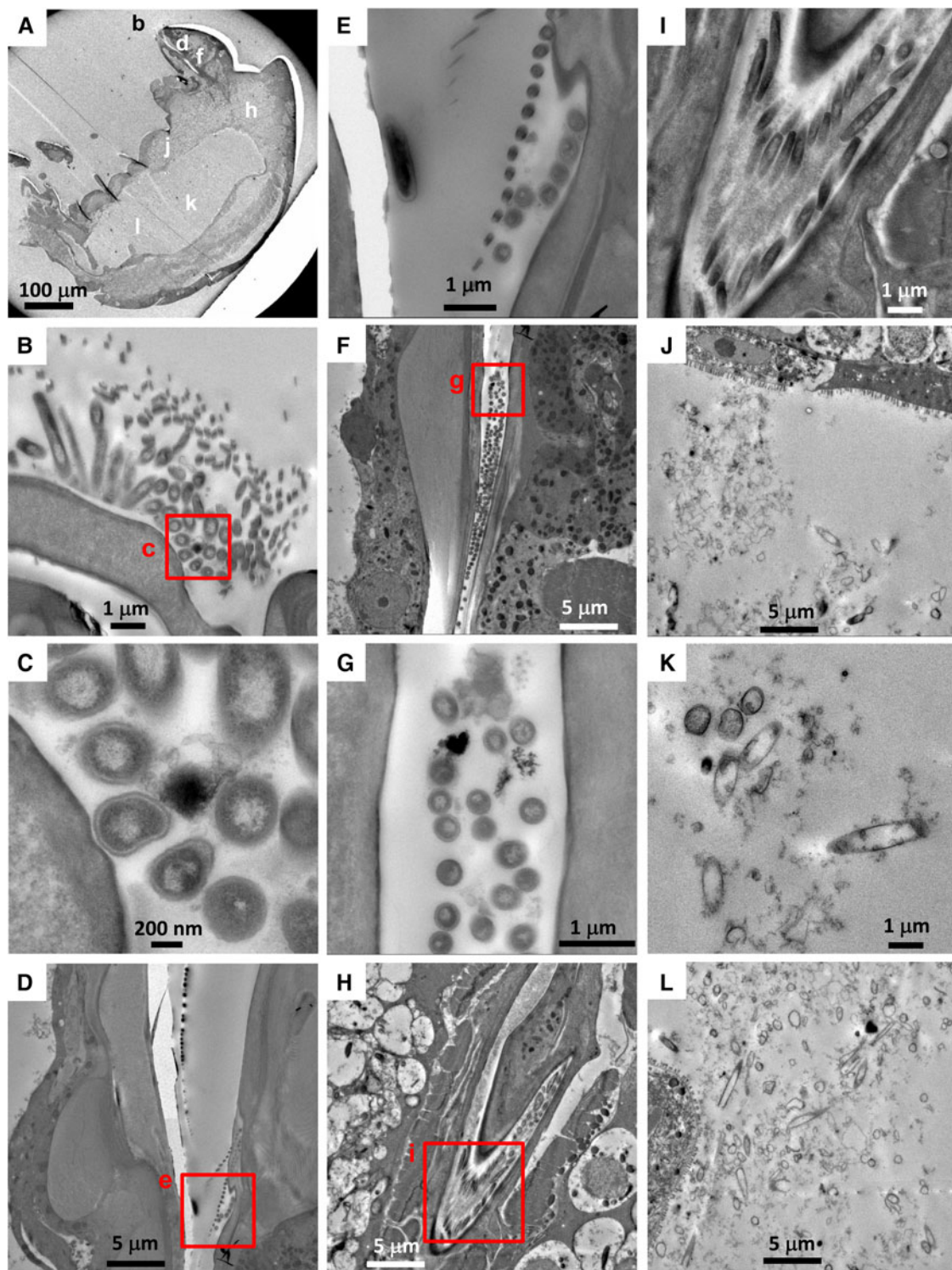
**Fig. 2.** Transmission electron microscopy (TEM) images of a stage CV male *Stygiopontius senokuchiae* (specimen C5_3 in Table 1) collected from Myojin Knoll caldera. (A) Low magnification view showing entire specimen and the regions shown at greater magnification in B–L. (B) Close-up view of mouthparts showing putative bacteria surrounded by setae. (C) Close-up view of rectangle (c) in image B showing putative bacteria. (D) Oral tube showing spherical bacteria inside. (E) Close-up view of rectangle (e) in image D showing bacteria trapped by setae. (F) Abundant spherical bacteria in the oral tube. (G) Close-up view of rectangle (g) in image F showing spherical bacteria. (H) Elongated bacteria filling the oesophagus. (I) Close-up view of rectangle (i) in image G showing abundant elongated bacteria. (J, K, L) Gut contents showing degraded spherical and elongated bacteria.

## Discussion

### *Bacterivory of* S. senokuchiae

Among three copepod species examined at a hydrothermal-vent field, *Stygiopontius senokuchiae* almost consistently showed bacterial cells in its oral tube or gut contents (Table 1). In some cases, the oral tube was filled with spherical and elongated bacterial cells (Figure 2F, I). Guts were also filled with relatively intact to mostly degraded bacterial cells (Figure 2J–L). The existence of degraded bacteria supports the idea that *S. senokuchiae* digests ingested bacteria and gains nutrition from them. The presence of at least two morphotypes of bacterial cells suggests that *S. senokuchiae* does not selectively ingest specific bacterial species from the flourishing bacteria on hydrothermal-vent chimneys.
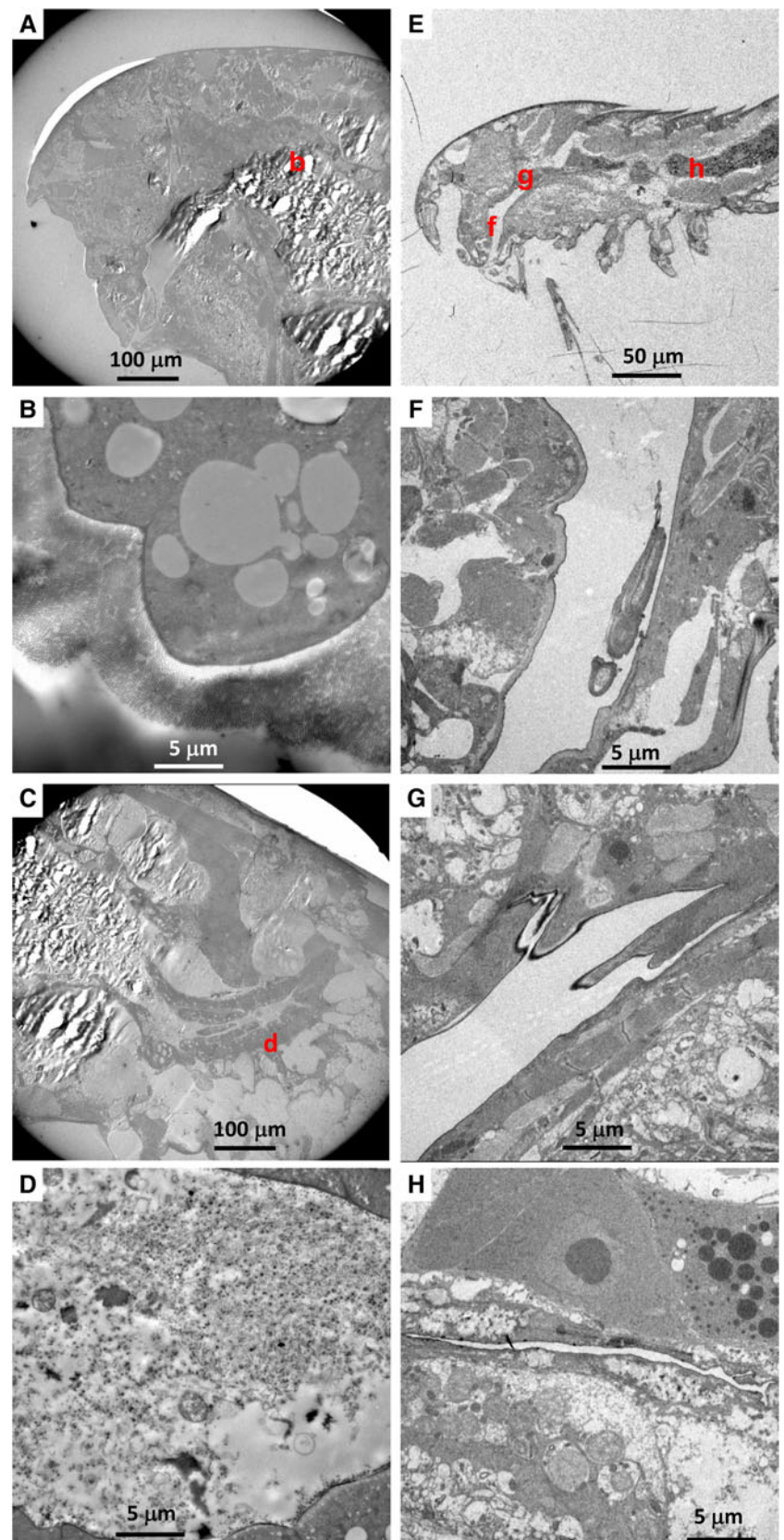
**Fig. 3.** (A–D) Transmission electron microscopy (TEM) images of a calanoid copepod collected from Myojin Knoll caldera. (A) Low magnification view of anterior, showing position of image B. (B) Intestinal wall filled by dense villi. (C) Low-magnification view of posterior, showing position of image D. (D) Amorphous degraded contents in the hindgut. (E–H) TEM images of an adult female *Ectinosoma* sp. 1 collected from Myojin Knoll caldera (specimen Kai_1_2 in Table 1). (E) Low-magnification view showing anterior and regions shown at higher magnification in F–H. (F, G) Empty oesophagus. (H) No obvious gut contents.

Limen *et al.* (2008) reported the selective ingestion of bacteria by *Stygiopontius quadrispinosus*, which has a small mouth opening. *Stygiopontius senokuchiae*, which has a similar mouth size, seems to ingest the bacterial mat in bulk without any obvious selection, probably by grazing (Heptner & Ivanenko, 2002).

There were no clear trends in gut contents or $\delta^{13}C$ values of *S. senokuchiae* with developmental stage, at least from CI to adult, in terms of gut contents (Table 1), or from CIV to adult, in terms of $\delta^{13}C$ values (Figure 5). After settling on a hydrothermal-vent chimney in the CI stage (Ivanenko *et al.*, 2007), *S. senokuchiae* may continuously utilize chemoautotrophic bacteria as a nutritional source. It should be noted that one sample of juvenile Dirivultidae, which consisted mainly of *Stygiopontius*, had $\delta^{13}C$ values as low as −21.2‰ (Nomaki
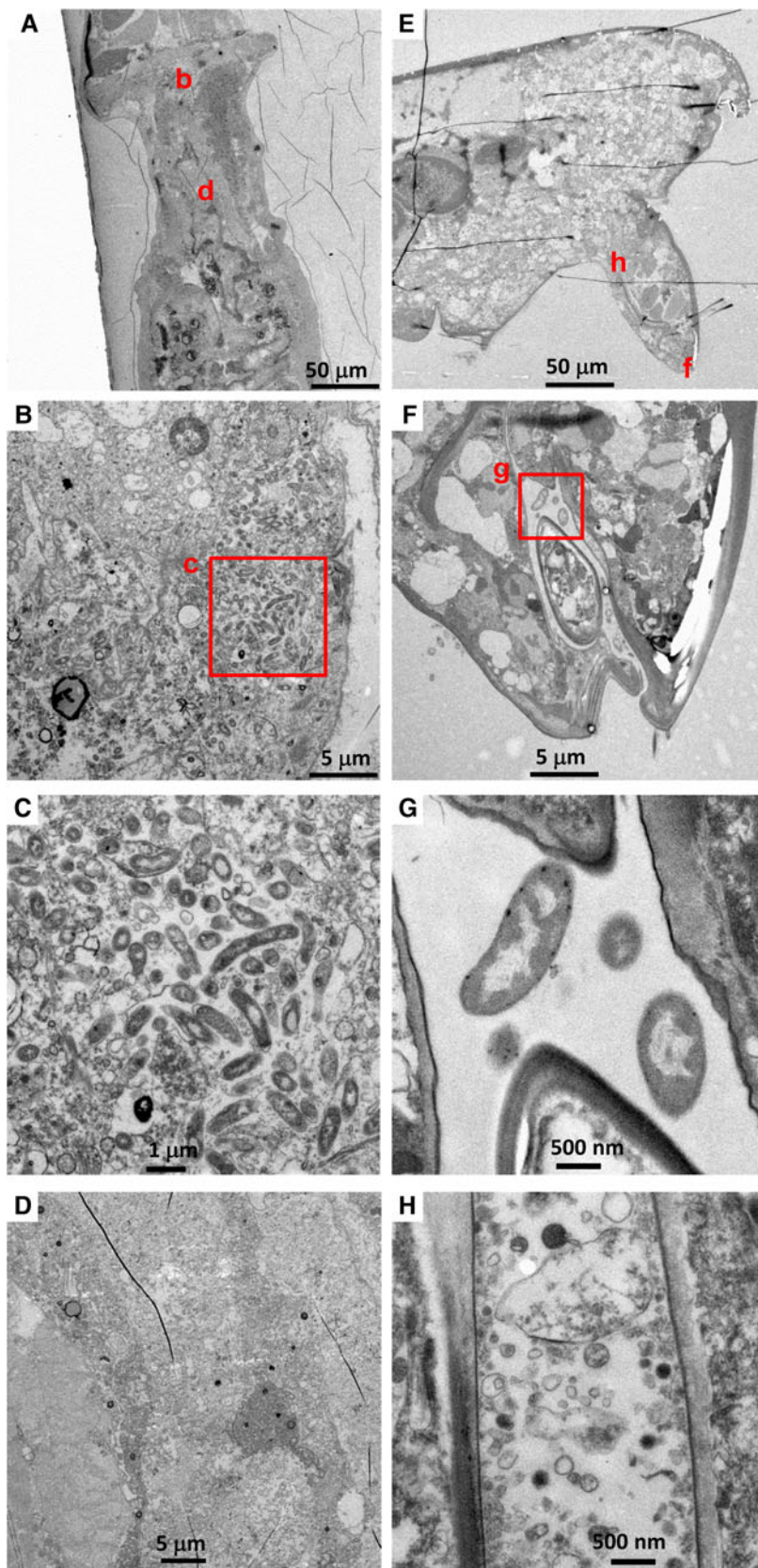
**Fig. 4.** (A–D) Transmission electron microscopy (TEM) images of an adult female *Hatschekia labracis* attached to a scarbreast tuskfin *Choerodon azurio* collected from Kagoshima Bay, Japan (specimen Kai_2_1 in Table 1). (A) Low-magnification view of anterior showing positions of images B and D. (B) Aggregation of bacteria found in the oesophagus. (C) Close-up view of rectangle (c) in image B showing elongated bacteria. (D) Amorphous, degraded contents in the guts. (E–H) TEM images of an adult male *Asterocheris* sp. 1 attached to a spirastrellid sponge collected from Kagoshima Bay (specimen Kai_4_1 in Table 1). (E) Low-magnification view of anterior, showing positions of images F and H. (F) Close-up view of mouthparts and oral tube. (G) Close-up view of rectangle (g) in image F showing bacteria in the oral tube. (H) Degraded contents in the oesophagus.

*et al.*, 2019; Figure 5). This might be explained by the contribution from photosynthetic organic matter, or mixtures of chemoautotrophic bacteria that use CBB and rTCA carbon-fixation pathways with different degrees of isotope fractionation (House *et al.*, 2003). Although we do not have the exact data for the composition by developmental stage measured by Nomaki *et al.* (2019), it is possible that their samples contained smaller copepodite stages such as CI–CIII.

At venting chimneys, high *in situ* primary chemoautotrophic production is limited to areas of active hydrothermal flow, and those environmental stresses have a stronger negative effect on smaller meiofaunal species than on macrofauna (Gollner *et al.*,
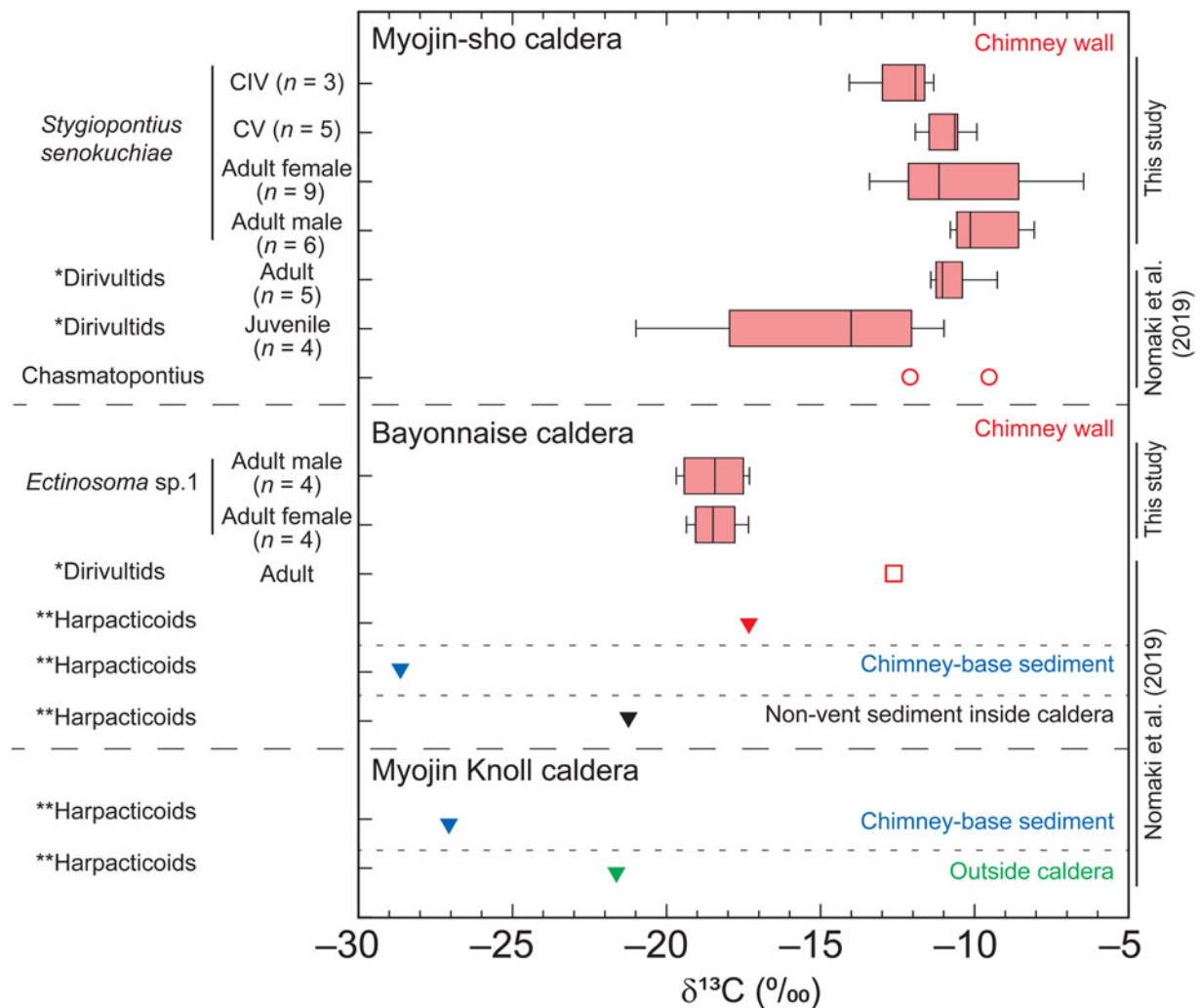
**Fig. 5.** Stable carbon isotope ratios ($\delta^{13}$C) of *Stygiopontius senokuchiae* collected from Myojin-sho, and of *Ectinosoma* sp. 1 collected from Bayonnaise Knoll. Box-and-whisker plot denotes minimum, maximum, lower and upper quartiles, and median of $\delta^{13}$C values of mixtures of 2–15 individuals for each category in cases with more than 3 replicates (Table 2). The $\delta^{13}$C values of copepods previously reported at these sites (Nomaki *et al.*, 2019) are plotted together for comparison. *Dirivultids, reported as 'Dirivultidae: mainly consist of *Stygiopontius* specimens'; **Harpacticoids, reported as 'Other copepods: other copepods than Dirivultidae, mainly (~90%) consist of harpacticoids' in Supplementary Table S3 in Nomaki *et al.* (2019). CIV, copepodite IV stage; CV, copepodite V stage.

2015a). Among meiofaunal copepods, dirivultids appear to be exceptionally successful in those extreme environments, probably using their larger body sizes (adult females are usually >1 mm long) and higher swimming speeds to avoid sudden environmental changes (Gollner *et al.*, 2015a). On the other hand, their youngest copepodites are much smaller (<0.3 mm in this study) and probably slower than the adults. Thus, it is plausible that the smallest *Stygiopontius* copepodites avoid areas with the most active hydrothermal eruptions and utilize detritus derived from photosynthetic organic matter deposited ubiquitously on chimneys, although they also ingest bacteria (Table 1). It should also be noted that gut contents analysis and biomass $\delta^{13}$C values reflect feeding habits at different time scales; gut contents show information about food items at a scale from hours to a few days, whereas $\delta^{13}$C values represent the average signature of food items over days to months, depending on turnover rates and the timing of moults.

Co-occurring Calanoida and *Ectinosoma* sp. 1 did not show any signs of bacterial ingestion (Table 1, Figure 3). Because calanoids are planktonic or epibenthic, the specimen found in our sample may have drifted from an adjacent ecosystem (Heptner & Ivanenko, 2002) and thus relied on nutritional sources other than bacteria. The empty gut contents of *Ectinosoma* sp. 1 might reflect regurgitations of gut contents during sample

recovery or the process of glutaraldehyde fixation, making interpretation difficult. However, their $\delta^{13}$C values (−19.9 to −17.5‰) do not indicate that their main nutritional source is chemoautotrophic bacteria fixing carbon through the rTCA pathway as observed for *Stygiopontius* (Figure 5; Nomaki *et al.*, 2019). Furthermore, Nakasugi *et al.* (2022) found that the distributional patterns of harpacticoids, including Ectinosomatidae, were not related to the availability of chemoautotrophic bacteria on hydrothermal-vent chimneys. The $\delta^{13}$C values of *Ectinosoma* in this study are attributed to the large contribution of photosynthetic organic matter, as observed in harpacticoids at surrounding non-vent areas, or to mixtures of chemoautotrophs (Nomaki *et al.*, 2019). Because the majority of harpacticoid species at vent sites also inhabit surrounding non-vent sites and are thought to have colonized vent sites from there (Gollner *et al.*, 2010b, 2020), patterns of $\delta^{13}$C values with developmental stage may shed light on their mechanisms for adapting to hydrothermal-vent chimneys.

TEM observations and carbon isotope ratio measurements of *S. senokuchiae* were carried out using samples from different hydrothermal vents – Myojin-sho and Myojin Knoll calderas – which are about 20 km apart. The $\delta^{13}$C values of Dirivultidae, mainly consisting of *S. senokuchiae*, were similar at Myojin-sho and the neighbouring seamount Bayonnaise Knoll (Figure 5;

Nomaki *et al.*, 2019). Furthermore, haplotype network analysis of this species revealed that populations at those knolls have almost the same genetics (Watanabe *et al.*, 2021). These observations suggest that all *S. senokuchiae* in this hydrothermal-vent area have comparable ecologies and similar stable carbon isotopic compositions.

### Bacterivory in the order Siphonostomatoida

We presume that both *Hatschekia labracis* and *Asterocheres* sp. 1 are parasites of *Choerodon azurio* and the spirastrellid sponge, respectively, because the amorphous contents in the oral tube and gut of both copepods suggest the digestion of blood plasma or other components of the host organisms. We observed putative bacterial cells in the oral tube or gut (Figure 4) of two or three specimens among the six examined for each species (Table 1). Although the origins of the observed bacteria are unknown, it is possible that these copepods accidentally suck bacterial cells from the host or from the surrounding seawater, and those cells could be part of their nutrition. However, bacteria were not observed in the gut of either *Mihbaicola sakamakii* (Hatschekiidae) or *Phrixocephalus cincinnatus* (Pennellidae), which are other members of the order Siphonostomatoida (Perkins, 1994; Hirose & Uyeno, 2014), implying that nutritional dependence on bacteria is not common within this order.

Heptner & Ivanenko (2002) mentioned that the 47 species belonging to the order Siphonostomatoida (i.e. 45 species in nine genera of the family Dirivultidae and one species each in the families Ecbathyriontidae and Asterocheridae) feed on bacteria. Furthermore, they speculated that the order Siphonostomatoida was originally parasitic, and the Dirivultidae evolved to graze bacteria on hydrothermal-vent chimneys. Our gut content observations and carbon isotopic composition analyses confirmed that *S. senokuchiae* is highly specialized to feed on chemoautotrophic bacteria at CI through adult stages, which is apparently different from co-occurring copepods and other siphonostomatoids. However, the morphological and nutritional adaptations of Dirivultidae, including *Stygiopontius*, are still largely unknown. Further investigations of the general trends in nutritional dependency of Siphonostomatoida should provide a clearer idea of how the Dirivultidae evolved and adapted to hydrothermal-vent fields as bacterivores.

### Conclusions

*Stygiopontius senokuchiae*, a hydrothermal-vent-specific dirivultid copepod, ingests bacterial cells on hydrothermal-vent chimneys from copepodite stage I to the adult stage. We found no clear differences in feeding habits between developmental stages or between males and females from gut content observations and stable carbon isotope measurements. Neither a coexisting calanoid species nor *Ectinosoma* sp. 1 showed any bacterial ingestion, implying that these copepods are temporarily distributed on the hydrothermal-vent chimneys or utilize food sources other than chemoautotrophic bacteria. Although two species of shallow-water siphonostomatoids sometimes possessed bacterial cells in their oral tube or gut, bacterivory seems to be specific for the family Dirivultidae among the order Siphonostomatoida. Further investigations are necessary to address the adaptation of Dirivultidae to the organic-rich, isolated, hydrothermal-vent habitat.

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