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ORIGINAL PAPER

Fossil chironomid $\delta^{13}C$ as a proxy for past methanogenic contribution to benthic food webs in lakes?

Maarten van Hardenbroek · Oliver Heiri · Jonathan Grey · Paul L. E. Bodelier · Frederike Verbruggen · André F. Lotter

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Abstract We used a series of experiments to determine whether stable carbon isotope analysis of modern and fossil larval head capsules of chironomids allowed identification of their dietary carbon source. Our main focus was to assess whether carbon from naturally ^{13}C-depleted methane-oxidizing bacteria (MOB) can be traced in chironomid cuticles using stable carbon isotope analysis. We first showed that a minimum sample weight of $\sim$20 μg was required for our equipment to determine head capsule $\delta^{13}C$ with a standard deviation of 0.5‰. Such a small minimum sample weight allows taxon-specific $\delta^{13}C$ analyses at a precision sufficient to differentiate whether head capsules consist mainly of carbon derived from MOB or from other food sources commonly encountered in lake ecosystems. We then tested the effect of different chemical pre-treatments that are commonly used for sediment processing on $\delta^{13}C$ measurements on head capsules. Processing with 10% KOH (2 h), 10% HCl (2 h), or 40% HF (18 h) showed no detectable effect on $\delta^{13}C$, whereas a combination of boiling, accelerated solvent extraction and heavy chemical oxidation resulted in a small (0.2‰) but statistically significant decrease in $\delta^{13}C$ values. Using culturing experiments with MOB grown on ^{13}C-labelled methane, we demonstrated that methanogenic carbon is transferred not only into the larval tissue, but also into chironomid head capsules. Taxon-specific $\delta^{13}C$ of fossil chironomid head capsules from different lake sediments was analyzed. $\delta^{13}C$ of head capsules generally ranged from −28 to −25.8‰, but in some instances we observed $\delta^{13}C$ values as low as −36.9 to −31.5‰, suggesting that carbon from MOB is traceable in fossil and subfossil chironomid remains. We demonstrate that stable carbon isotope analyses of fossil chironomid head capsules can give insights into dietary links and carbon cycling in benthic food webs in the past and that the method has the potential to reconstruct the importance of MOB in the palaeo-diet of chironomid larvae and, indirectly, to infer past changes in methane flux at the sediment water interface in lakes.

Keywords Chironomids · Methane-oxidizing bacteria · Stable carbon isotopes · Palaeolimnology · Lake sediments · Carbon cycle · Diet

M. van Hardenbroek (✉) · O. Heiri · F. Verbruggen · A. F. Lotter
Institute of Environmental Biology, Palaeoecology, Laboratory of Palaeobotany and Palynology, Utrecht University, Budapestlaan 4, 3584 CD Utrecht, The Netherlands
e-mail: m.r.vanhardenbroek@uu.nl

J. Grey
School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

P. L. E. Bodelier
Department of Microbial Wetland Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Centre for Limnology, Rijksstraatweg 6, 3631 AC Nieuwersluis, The Netherlands

Introduction

Non-biting midges (Insecta: Diptera: Chironomidae) are sensitive indicators for a variety of environmental parameters. The chitinous remains of chironomid larvae preserve well and remain identifiable in lake sediments. Consequently, fossil chironomid assemblages can be used to reconstruct the past chironomid fauna of lakes and infer past changes in physical and chemical variables from lake sediments. Examples of chironomid-based environmental inferences include the reconstruction of air or water temperature (Walker and Cwynar 2006; Brooks 2006; Heiri et al. 2007), total phosphorus (Brooks et al. 2001; Langdon et al. 2006), chlorophyll *a* (Brodersen and Lindegaard 1999), oxygen availability (Quinlan et al. 1998), and lake depth (Korhola et al. 2000). Recently, the potential of fossil chironomids for isotope studies has been demonstrated with regard to ^{14}C dating (Jones et al. 1993; Fallu et al. 2004) and to $\delta^{18}O$ as a palaeotemperature proxy (Wooller et al. 2004, 2008). Thus far, however, the composition of stable carbon isotopes in fossil chironomids has received less attention for reconstructing past environmental change. In a recent palaeolimnological study, Wooller et al. (2008) measured the carbon isotope ratio ($\delta^{13}C$) as well as $\delta^{15}N$ and $\delta^{18}O$ in chironomid fossils but for the most part they used chironomid $\delta^{13}C$ to constrain the interpretation of other stable isotopes measured on the same material, rather than as an independent geochemical proxy.

Many species of chironomid larvae are benthic, and their habitats include the interface between sediments and the water column, where the larvae either live on the sediment surface or in the uppermost few cm of the sediments. Therefore, larvae have access to freshly deposited algal material as well as to organic matter and associated microorganisms in and on the sediments. The carbon isotopic signature of insects is determined by their diet (DeNiro and Epstein 1978). It can therefore be expected that $\delta^{13}C$ of chironomid tissue reflects the isotopic values of the food ingested by the larvae, and that $\delta^{13}C$ will provide insight into the carbon cycling within lake food webs. Algal biomass $\delta^{13}C$ commonly ranges between −30 and −25‰ (Boutton 1991; Meyers and Lallier-Vergès 1999). A stable isotope study of lake ecosystems worldwide (Jones et al. 2008) revealed that many chironomid larvae dwelling in or on sediments have carbon isotope values that are substantially ^{13}C-depleted ($\delta^{13}C$ as low as −64‰) relative to the algae or bulk sediment on which they feed. Such extremely low $\delta^{13}C$ values are similarly found in biogenic methane produced in lake sediments by methanogenic archaea (Whiticar 1999) as well as in methane-oxidizing bacteria (MOB) that utilize methane as their carbon source (Templeton et al. 2006). Molecular studies of two contrasting German lakes (Eller et al. 2005) and laboratory experiments with ^{13}C-labelled methane (Deines et al. 2007) recently provided more direct evidence that low $\delta^{13}C$ values of chironomid larvae are derived from MOB.

The relation between chironomid $\delta^{13}C$, MOB and methane production has only recently been discovered and bears great potential for applications in palaeoenvironmental studies. Since the carbon isotopic signature of MOB is distinct and is incorporated into chironomid biomass, it can be expected that fossil chironomid cuticles also preserve this signal. As a consequence, analysis of $\delta^{13}C$ in subfossil and fossil chironomid remains might provide a means of evaluating the importance of MOB in the past diet of these larvae. The abundance of MOB is dependent on methane originating from the sediment (Templeton et al. 2006; Deines et al. 2007). Therefore, $\delta^{13}C$ in fossil chironomids could potentially provide information on past changes in methane production from lake sediments without the need for long-term monitoring.

However, a number of uncertainties and challenges remain before $\delta^{13}C$ in fossil chironomids can be used to reconstruct the carbon isotopic signature of their ingested food and the importance of MOB in their diet. First, it is unclear how much material is necessary to measure fossil chironomid $\delta^{13}C$ using the available analytical methods. Ideally the method should be applicable to small sample weights so that taxon-specific measurements are possible. This would allow $\delta^{13}C$ of deposit feeders to be compared with values measured on chironomids with a different feeding behaviour (e.g. grazers or predators). Second, it has not yet been explored whether the chemical pre-treatment and processing methods commonly used for preparing sediment for fossil chironomid analysis affect $\delta^{13}C$. Third, the extent to which the carbon isotopic signature of MOB is expressed in the larval exoskeletons and consequently in the fossil record is still unclear. Insect cuticle formation is

complex with the new cuticle formed both from material re-digested from the old exoskeleton and from material assimilated by the larvae prior to moulting. Although it has been shown that soft tissue in chironomid larvae can reach $\delta^{13}C$ values close to those of MOB (Deines and Grey 2006), it has not yet been determined whether the chironomid exoskeleton and fossil chironomid remains demonstrate similar values.

Here, we assess the minimum sample weight required for $\delta^{13}C$ analysis of chironomid head capsules using standard-sized combustion columns and test if different sediment processing and pre-treatment steps affect $\delta^{13}C$ values of head capsules. We use culturing experiments with living chironomids to determine whether the carbon isotopic signature of methane can be traced into chironomid exoskeletons. Finally, we report stable carbon isotope values from subfossil and fossil invertebrate remains to determine whether chironomid head capsules might contain methane-derived carbon, and we indicate next steps necessary for further developing fossil chironomid $\delta^{13}C$ as a means for reconstructing the past importance of methanogenic carbon in the diet of chironomid larvae.

Methods

Sample size and chemical sample pre-treatment

In order to develop the methodology for measuring $\delta^{13}C$ on head capsules of larval chironomids, several analytical tests were carried out on cuticles originating from modern chironomid larvae. For this purpose we obtained cultured larvae of *Chironomus riparius* from a local aquarium food supplier (Marsilea Lelystad, The Netherlands). Except where indicated otherwise, the larvae were chemically fossilized before further chemical treatment by removing soft tissue by heating in 10% KOH for 1 h at 70°C. In all of these tests the head capsules were manually separated from the cuticle of the larval body after rinsing twice in demineralized water and isolated for further treatment.

First, we determined the minimum weight required for $\delta^{13}C$ analyses. Samples ranging from 7 to 100 µg were carefully weighed into tin capsules and analysed. Based on past experience with the Elemental Analyser-Isotope Ratio Mass Spectrometer (EA-IRMS) equipment we used, a minimum current of 1.0 V is required for reproducible $\delta^{13}C$ results. One of the aims of our chironomid $\delta^{13}C$ analyses was therefore to assess how much mass and how many individuals of chironomid head capsules were necessary to produce this voltage during measurement. In a second step, we tested whether different methods that are commonly used for chemical pre-treatment of sediment samples in palaeolimnological or organic geochemical studies affect $\delta^{13}C$ measurements of the head capsules. These pre-treatments included exposure at room temperature for 2 h in 10% KOH, for 2 h in 10% HCl and for 18 h in 40% HF solution. For comparison, we also assessed the $\delta^{13}C$ of head capsules of larvae allowed to decay in demineralized water for 3 months without any additional treatment or chemical fossilization. Finally, a combination of techniques was used to degrade chemically fossilized head capsules: first, head capsules were boiled in demineralized water for 2 h. Second, we used accelerated solvent extraction (ASE) to imitate the effect of elevated temperature and pressure with liquid solvents (Richter et al. 1996). Third, we used a solution of sodium chlorite and glacial acetic acid as described for the processing of cellulose by Leavitt and Danzer (1993) to assess the effect of heavy oxidation on head capsules. Head capsules were wrapped in Whatman GF/C filters for this combined treatment.

Samples were analyzed for $\delta^{13}C$ on a Fisons NA 1500 NCS Elemental Analyser coupled to a Thermo Electron Delta plus isotope ratio mass spectrometer. All resulting $\delta^{13}C$ values are expressed relative to Vienna Pee Dee Belemnite (VPDB) in units of per mille (‰). The reference material used was a secondary standard of known relation to the international standard of VPDB. Replicate sample measurements ($n = 20$) on the internal standard gave an analytical error of ± 0.26‰ (2σ). We used an unpaired *t*-test (95% confidence interval) in Prism 5 for Windows to test for significant differences in $\delta^{13}C$ values between the standard 10% KOH treatment and the other chemical treatments.

Culturing experiment

In addition to using *C. riparius* larvae obtained from commercial sources for our experiments, we also

cultured *C. riparius* larvae from egg masses in the laboratory. Egg masses were obtained from a local ecotoxicology laboratory (GrontmijlAquasense, Amsterdam, The Netherlands). Larvae were grown from eggs in tap water at 27°C on a 16:8 h light:dark rhythm and fed on fish food (Tetramin, manufactured by Tetra GmbH, Melle, Germany) with an isotopic composition of -22.3 ± 0.2‰ (VPDB). One sub-sample was grown until the larvae reached the final larval stage. Subsequently, the larvae were sieved from the sediments and placed into filtered tap water for 24 h to allow gut clearance (Feuchtmayr and Grey 2003). Excess faecal material was removed periodically during this step to prevent coprophagy.

A second sub-sample of larvae was transferred to a series of experimental glass tubes once they reached the second larval stage to determine whether the carbon isotopic signature of methane was taken up into soft larval tissue and cuticles. Each glass tube was prepared following Deines et al. (2007): sediment was collected from Ranworth Broad, UK (52°28′N, 1°42′E), and was sieved with a 0.5 mm mesh to remove macroinvertebrates and debris. Five 1.0 l screw-cap glass bottles each were filled with 0.6 l of the pre-sieved sediment and closed with butyl rubber stoppers. From the headspace, 180 ml air was removed and replaced by 200 ml of a mixture of $70\%_{vol}$ $^{12}CH_4$ and $30\%_{vol}$ $^{13}CH_4$ (99% pure $^{13}CH_4$; Isotec, Miamisburg, Ohio, supplied by Sigma–Aldridge, Zwijndrecht, The Netherlands). This mixing ratio was used to prevent an inhibition of methane oxidation by too high concentrations of $^{13}CH_4$. The resulting $\delta^{13}C$ value of the CH_4 was 12,200‰ (VPDB). Pre-incubation of the sediment was carried out at 20°C on a rotary shaker. After 5 days, considered to be sufficient for a MOB-enriched micro-flora to develop, the sediment from all five bottles was combined, mixed and used to fill three experimental tubes (diameter: 4.5 cm, height: 38 cm; Ochs, Bovenden, Germany) to half of their total volume. Tubes were filled up with 100 µm filtered rain water and 20 ml of the $^{12/13}CH_4$-mixture was injected in reservoirs under the tubes. This methane was allowed to diffuse through a glass-sintered separation into the tubes to provide MOB with a ^{13}C labelled CH_4 source during the experiment (Deines et al. 2007). The sediment was allowed to settle for 24 h before the experiment was started by placing 40 second-instar chironomid larvae into each tube. Oxygen supply to the water column and to sediments was ensured by aerating the water using an aquarium air stone placed 5 cm above the sediment surface. Larvae were allowed to feed on and grow in these sediments from second to final (fourth) larval stage for a period of 22 days at 20°C after which the fourth-instar larvae were sieved from the sediments, allowed to clear their guts and freeze dried. The larval heads were separated from the rest of the body with a surgical lancet, a sample of muscle tissue was obtained from each larva, and head capsules were cleaned in 10% KOH for 2 h to remove the remaining soft tissue. Muscle tissue, head capsules and sediment samples were analysed separately for stable carbon isotopes on a Euro Vector Elemental Analyser coupled to a Thermo Electron Delta V advantage IRMS. The reference material used was a secondary standard of known relation to the international standard of VPDB. Replicate sample measurements ($n = 8$) on the internal standard gave an analytical error of ± 0.15‰ (2σ).

$\delta^{13}C$ of fossil head capsules

Head capsules of chironomid larvae from three different sediments were analysed to obtain a first estimate of the range of $\delta^{13}C$ in fossil material. Sediments with high head capsule concentrations and similar taxa were selected to compare interspecific and intraspecific variability. Surface sediment was collected from a tundra pond in arctic Siberia, located near the River Elon, 25 km from the town of Chokurdakh, Yakutia. Sediment was also analysed from subalpine Hinterburgsee, Switzerland (Heiri et al. 2003), from a depth of 5–7 cm below the sediment water interface, representing an age of ca. 30 years. The oldest sediment originated from the Slotseng lake basin at an archaeological site in Denmark and has an age in the range of 14,800–12,800 calibrated ^{14}C years BP (Mortensen 2008). Details for the different sediments can be found in Table 1.

The sediments were deflocculated in 10% KOH for 2 h at room temperature, sieved with 200- and 100-µm sieves and rinsed with demineralized water. Head capsules were picked with a forceps and identified to the highest taxonomic level possible under a dissecting microscope at 40–100× magnification following Brooks et al. (2007). Samples were

Table 1 Main characteristics of the lake sediments analyzed for fossil chironomids in this study

Location	Tundra pond, Russia	Hinterburgsee, Switzerland	Slotseng, Denmark
Coordinates	70°49′46″N/147°29′13″E	46°43′5″N/8°4′2″E	55°19′48″N/9°16′17″E
Approximate age	Modern	Twentieth century	14.8–12.8 cal. ^{14}C ky BP
Altitude (m a.s.l.)	50	1,515	40
Dry weight of analyzed sample (g)	0.60	1.37	1.98
Number of head capsules per g dry weight	476	64	979

placed into Eppendorf tubes, treated with ultra clean 1% HCl for 2 h at room temperature to remove all carbonate particles and rinsed twice with ultrapure water (milliQ) using a centrifuge (5 min at 2,300 rpm) to concentrate the material. The head capsules were then transferred into pre-weighed ultraclean tin cups and dried on a hotplate at 50°C for 6 h after which the tin cups were re-weighed and crimped for stable isotope analysis. Samples were analyzed on a Fisons NA 1500 NCS Elemental Analyser coupled to a Thermo Electron Delta plus IRMS. The reference material used was a secondary standard of known relation to the international standard of VPDB. Replicate sample measurements ($n = 10$) on this internal standard gave an analytical error of ±0.06‰ (2σ).

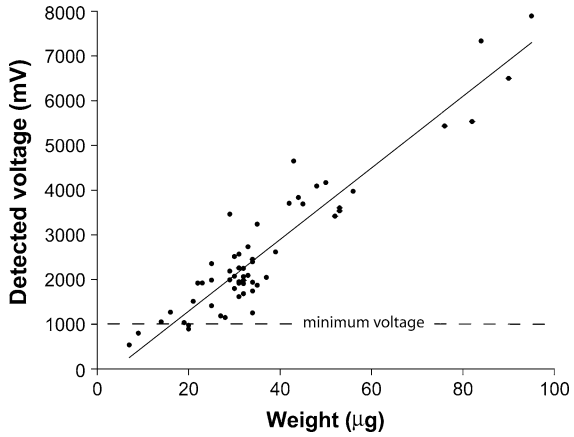

Fig. 1 Detected voltage measured during EA-IRMS for chironomid samples of different weight. The minimum voltage of 1.0 V is based on previous measurements with the analytical equipment used

Results and discussion

Sample size and chemical sample pre-treatment

A minimum sample size of ~20 µg of larval head capsules is needed to generate a signal of at least 1.0 V, the minimum voltage considered to produce reliable δ^{13}C analyses with the EA-IRMS equipment used for our experiment (Fig. 1). About 20 µg corresponds to a minimum of ~10 large head capsules such as the fourth instar head capsules of *C. riparius*. However, because our measurements relating the weight of chironomid head capsules with the measured current show a considerable scatter (Fig. 1), we recommend using a larger minimum sample size of at least 30 µg if enough material is available. Wang et al. (2008) observed a systematic error in δ^{18}O measurements for samples of fossil chironomids of low weight. We could not find evidence for a similar bias in δ^{13}C measured on chironomid samples with a low weight (Fig. 2), although our samples produced a higher voltage than the threshold of 0.5 V, below which Wang et al. (2008) observed their bias.

The percentage of carbon (%C) in the samples is 46.0 ± 2.1%C ($n = 5$) for head capsules that had decayed naturally, 36.5 ± 11.3%C ($n = 27$) for the KOH treatment, 52.5 ± 11.3%C ($n = 3$) for the HCl treatment, 43.3 ± 4.0%C ($n = 3$) for the HF treatment and 28.9 ± 3.7%C ($n = 8$) for the combined boiling, ASE and oxidation treatment. δ^{13}C measurements of head capsules produced a standard deviation of 0.23‰ ($n = 5$) for head capsules that had decayed naturally, 0.26‰ ($n = 27$) for the KOH treatment, 0.15‰ ($n = 3$) for the HCl treatment, 0.31‰ ($n = 3$) for the HF treatment and 0.09‰ ($n = 8$) for the combined boiling, ASE and oxidation treatment. All measurements regardless of chemical treatment fall within a range of ±0.5‰ from the average (Fig. 2) indicating that δ^{13}C in head capsules can be measured with high precision relative to the values of

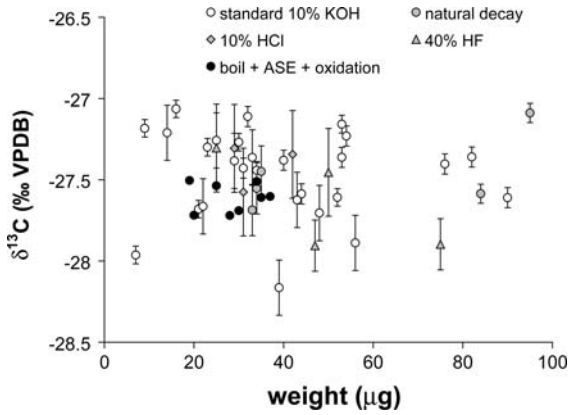

Fig. 2 $\delta^{13}C$ in head capsules of chironomid larvae from the same commercial source processed with different chemical treatments: standard 10% KOH (*open circles*); natural decay (*grey circles*); 10% HCl (*grey diamonds*); 40% HF (*grey triangles*); combination of boiling, accelerated solvent extraction (*ASE*) and oxidation (*closed circles*). *Error bars* indicate reproducibility of laboratory standards measured for the different EA-IRMS runs

−70 to −20‰ we expect to encounter in natural chironomid populations (Jones et al. 2008). To some extent, the observed variability in $\delta^{13}C$ might be a consequence of the natural variability between chironomid specimens used for our experiments. Although we have no information about the diet of the chironomids supplied from an external source, all chironomid larvae originate from the same batch. We therefore consider it unlikely that the variability of these supplied chironomids exceeds variability of larvae feeding on a similar diet in natural systems. Goedkoop et al. (2006) found a similar ±0.53‰ variation in $\delta^{13}C$ of *C. riparius* larvae that were grown on artificial sediment consisting of ground peat, sand, kaolin clay and $CaCO_3$. Of the chemical treatments tested in our experiments, only the combination of boiling, ASE and oxidation showed a small, but significant decrease in $\delta^{13}C$ of 0.2‰ (*t*-test, $P = 0.02$ at 95% confidence) relative to the standard method consisting of exposure of the head capsules to 10% KOH for 2 h. However, the effect on chironomid $\delta^{13}C$ values was small since head capsules treated in this manner had only on average 0.14‰ lower $\delta^{13}C$ than the mean $\delta^{13}C$ value of all the measurements (Fig. 2).

Schimmelmann and DeNiro (1986) treated chitin with solutions of 2N HCl at room temperature for 14 h and 1N NaOH at 100°C for 0.5 h and found a change of less than 0.2 ± 0.1‰ in $\delta^{13}C$ values. However, long-term (up to 600 h) deproteination/deacetilation in a 1N NaOH solution at 25°C caused a 1.5‰ increase in $\delta^{13}C$, although the authors highlight that these treatments were tested on powdered crab chitin that is not as sclerotized and robust as chitin in insect cuticles (Schimmelmann and DeNiro 1986). Furthermore, Schimmelmann et al. (1986) tested the effects of biodegradation on chitin in crustacean carapaces allowed to decay for 10 weeks in moist soil and marine sediment. They found a maximum increase in $\delta^{13}C$ values of 0.4 ± 0.2‰ in the decayed carapaces relative to untreated exoskeletons. However, this difference in $\delta^{13}C$ is smaller than the 0.5‰ standard deviation in $\delta^{13}C$ values that we found in analyses of chironomid head capsules.

Based on the close agreement between $\delta^{13}C$ of chironomid head capsules processed with different chemical treatments, we conclude that standard palaeoecological pre-treatment methods, such as exposure to 10% KOH for 2 h at room temperature to deflocculate sediments, or elimination of carbonate particles with 1% HCl for 2 h at room temperature, were not influencing $\delta^{13}C$ of head capsules to a significant extent. Our results and those of others described in the literature (e.g. Schimmelmann and DeNiro 1986) suggest that more intensive chemical pretreatment such as strong oxidation or extended deproteinization/deacetilization would be necessary to produce a noticeable bias in chironomid $\delta^{13}C$.

Culturing experiment

In order to assess the influence of a MOB-enriched diet on $\delta^{13}C$ of chironomid cuticles, we cultured chironomid larvae on sediments enriched with MOB grown on ^{13}C-labelled methane. The switch to a MOB-enriched diet had a strong effect on $\delta^{13}C$ of larvae and exoskeletons. On average, $\delta^{13}C$ values of tissue and head capsules of fourth instar larvae cultured on labelled MOB from the second larval instar onwards became 39 ± 10.9‰ and 53 ± 4.2‰ enriched, respectively, relative to $\delta^{13}C$ of larvae fed with the same standard diet during their entire development (Fig. 3). In an almost identical experimental setup, Deines et al. (2007) found a somewhat larger (52 ± 20‰) shift in $\delta^{13}C$ in larval tissue. However, there is a clear overlap of the standard deviations of the different measurements and this apparent difference is therefore not significant.

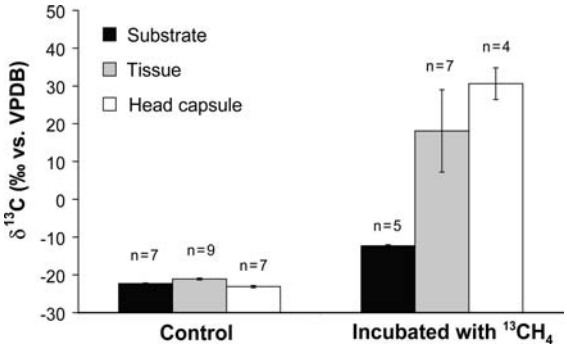

Fig. 3 Effect of labelled, methanogenic carbon in the diet of chironomid larvae on $\delta^{13}C$ of larval tissue and head capsules. The *left bar graph* shows replicate samples from a control group of fourth instar larvae that were cultured on a diet of commercially available fish food ($\delta^{13}C = -22.3 \pm 0.2‰$). The *bar graph* on the *right* indicates replicates from a group of fourth instar larvae that was first raised on a diet of fish food before being cultured from the second larval stage onwards in sediments incubated with ^{13}C-labelled methane ($\delta^{13}C$ of labelled $CH_4 = 12{,}200‰$)

Previous studies have indicated that bulk tissues of lacustrine chironomid larvae often can be found with markedly depleted ^{13}C values typical of methanogenic carbon (Deines and Grey 2006; Jones et al. 2008). In this study we have demonstrated experimentally that a shift in $\delta^{13}C$ in the diet of chironomids is transferred not only into the larval tissue but also into the sclerotized head capsules of the larvae. This implies that unless taphonomic processes affect the chemistry of chironomid cuticles, the $\delta^{13}C$ signal of formerly ingested food will be preserved in the head capsules when they fossilize in lake sediments. It has been shown that chitinous insect cuticles are very resistant to degradation and can be recovered from sediments up to 25 million years old (Stankiewicz et al. 1997). Remains of chironomid larvae have been reported from sediments as old as early Oxygen Isotope Stage-3 (Helmens et al. 2007) or the Eemian Interglacial (Brodersen and Bennike 2003; Ilyashuk et al. 2006) and are common in many younger lake sediment records (e.g. Walker and Cwynar 2006; Brooks 2006). Therefore, $\delta^{13}C$ of chironomid exoskeletons might provide insights into the past contribution of methanotrophic carbon to benthic food webs of lakes over a range of timescales. In our culturing experiment, neither larval tissue nor cuticles approached isotopic equilibrium with the methane on which we grew the MOB. Partially, this might be due to our experimental design in which larvae were first grown on a diet of fish food before being transferred to the MOB-enriched sediments. However, it is also likely that the *C. riparius* larvae ingested other food items available in our experimental containers, such as other organic sediment components and microorganisms growing on them. Furthermore, it is conceivable that fractionation processes disproportionately affect chironomid $\delta^{13}C$ if the larvae feed on substrates with unnaturally high $\delta^{13}C$ such as the 12,200‰ of the methane used to culture MOB in our experiments. An important next step would therefore be to explore the relationship between $\delta^{13}C$ of MOB and chironomids feeding on them if these micro-organisms form the diet of the larvae throughout their entire development.

$\delta^{13}C$ of fossil head capsules

We measured $\delta^{13}C$ values of (sub)fossil chironomid head capsules and other chitinous remains from three different lake sediments selected based on their high chironomid content (Table 1). Since our initial test indicated that $\delta^{13}C$ can be measured at weights as low as 20–30 µg, we prepared samples consisting of head capsules from a single species or morphotype where possible and samples consisting of individuals from the same tribe or subfamily from sediments in which fossil head capsules were sparser (Table 2). A sample of *Chironomus anthracinus*-type head capsules with a $\delta^{13}C$ value of −36.9‰ was found in sediments from a Siberian tundra pond. This is comparable to $\delta^{13}C$ values reported in living *Chironomus* larvae from Arctic lakes in Alaska, Sweden and Finland (Hershey et al. 2006; Jones et al. 2008). *Chironomus* species are the taxa most commonly reported to incorporate methane-derived carbon by feeding on MOB (Grey et al. 2004; Jones et al. 2008). The $\delta^{13}C$ value of −36.9‰ measured on *C. anthracinus*-type subfossils from Siberia, distinctly lower than the −30 to −18‰ typical of algal biomass reported in the literature (Boutton 1991; Meyers and Lallier-Vergès 1999), suggests a contribution from methane-derived carbon to this taxon's diet in the studied lake ecosystem.

In contrast, the −26.9‰ measured for a sample of head capsules of *C. anthracinus*-type from the Slotseng site does not indicate depleted values. The $\delta^{13}C$ value at this site is within the range typical for lacustrine algae, suggesting a food source without or

Table 2 $\delta^{13}C$ values of chironomid head capsules and cladocera ephippia from different lake sediments

Material	Location	Age	Number of head capsules in sample	Weight (μg)	$\delta^{13}C$ (‰ PDB)	%C
Chironomus anthracinus-type	Tundra pond	Modern	21	30	−36.9	46.7
Tanypodinae	Hinterburgsee	Twentieth century	44	30	−31.5	38.1
Tanytarsini	Hinterburgsee	Twentieth century	18	18	−30.4	22.3
Chironomus anthracinus-type	Slotseng	Late-glacial (14.8–12.8 cal ^{14}C kyr BP)	25	32	−26.9	39.8
Tanypodinae	Slotseng	Late-glacial	24	31	−25.8	41.8
Corynocera ambigua	Slotseng	Late-glacial	26	32	−27.0	35.5
Corynocera ambigua	Slotseng	Late-glacial	22	26	−27.1	43.2
Corynocera ambigua	Slotseng	Late-glacial	20	30	−27.2	38.3
Corynocera ambigua	Slotseng	Late-glacial	21	28	−27.1	37.5
Corynocera ambigua	Slotseng	Late-glacial	22	28	−27.2	42.0
Corynocera ambigua	Slotseng	Late-glacial	20	26	−27.1	45.2
Corynocera ambigua	Slotseng	Late-glacial	20	30	−27.0	32.4
Daphnia ephippia	Slotseng	Late-glacial	11	32	−28.0	43.1
Daphnia ephippia	Slotseng	Late-glacial	18	52	−26.5	38.1

Each row represents one sample

with a very low proportion of methanogenic carbon. Tanypodinae and Tanytarsini in Hinterburgsee also show lighter $\delta^{13}C$ values (−31.5 and −30.4‰, respectively) than comparable taxa in Slotseng (−25.8 and −27.1‰, respectively; value for Tanytarsini calculated as the average of $\delta^{13}C$ of *Corynocera ambigua*). The former values are low enough to suggest a contribution from a methane-derived carbon source in the diet of chironomid larvae. Tanypodinae are mostly predators and it is not likely that they assimilate MOB directly. However, they might feed on smaller chironomid larvae or oligochaete worms that inhabit the hypoxic sediments and might ingest MOB. Jones et al. (2008) also report values as low as −36.1‰ for the tanypodine genus *Procladius* in Blelham Tarn. At this site *Chironomus* is even further depleted in ^{13}C. Possibly methane-derived carbon is passed on to higher trophic levels in Blelhalm Tarn by predation on *Chironomus* larvae (Jones et al. 2008). Tanytarsini are generally considered to be collectors/gatherers (Ferrington et al. 2008) and are not known to prey on other invertebrates. The low $\delta^{13}C$ values of Tanytarsini head capsules isolated from the Hinterburgsee sediments suggest that methanogenic carbon might also be incorporated by these chironomids to some extent.

For the Slotseng sediments, it was possible to measure replicate samples of head capsules of *C. ambigua*, the dominant chironomid taxon. $\delta^{13}C$ values were very similar for the different replicates, with a mean value of -27.1 ± 0.1‰ ($n = 7$). Twenty to twenty-six head capsules were amalgamated per replicate sample (Table 2). This certainly averages variability between individuals (Grey et al. 2004). Nonetheless, these replicate measurements demonstrate that $\delta^{13}C$ can be analysed on fossil chironomid head capsules from an individual species with a high degree of reproducibility.

Potentially, other chitinous invertebrate remains in lake sediments can be analysed in a similar fashion as chironomid head capsules to study trophic links and carbon cycling within a lake ecosystem in the past. Amongst such remains are cladoceran ephippia, head capsules of non-chironomid midge larvae such as Ceratopogonidae, Simuliidae, or Thaumaleidae and mites. Indeed, methane-derived carbon has been detected in *Daphnia* biomass in modern lake ecosystems with $\delta^{13}C$ values reported as low as −50.3‰ (Jones et al. 1999; Kankaala et al. 2006; Taipale et al. 2007). Fossil ephippia of the cladoceran *Daphnia* from the Slotseng sediments show $\delta^{13}C$ values between −28.0 and −26.5‰ (Table 2). This range

of 1.5‰ is larger than the difference between Tanypodinae and *C. ambigua* in the same sediments. Such a relatively large variability might be explained by the lack of taxonomic control in our analyses, since the ephippia might originate from different species of *Daphnia*. Alternatively, these cladocerans might feed on algae in different parts of the water column, or the range of ephippial $\delta^{13}C$ might represent seasonal changes in the carbon isotopic signature of particulate organic matter in the water column (Bernasconi et al. 1997). Irrespective of the cause for the larger spread in *Daphnia* $\delta^{13}C$, the carbon isotopic composition of these cladoceran remains indicate that benthic and planktonic organisms in Slotseng fed on food items with a similar carbon isotopic composition, with no evidence that methanogenic carbon played an important role for macroinvertebrates in this lake ecosystem.

Conclusions

We showed that $\delta^{13}C$ is measurable on chironomid head capsules with a high precision (±0.5‰) relative to the 20–40‰ difference between methanogenic carbon and other carbon sources in lacustrine ecosystems. $\delta^{13}C$ analyses on chironomid head capsules are possible on samples with a comparatively low weight (20–30 μg), allowing taxon specific measurements of chironomid remains belonging to the same species, morphotype, subfamily or tribe. Our culturing experiments with labelled methane clearly show that $\delta^{13}C$ values of food are not only transferred to the larval tissue but also recorded in the chironomid cuticles. Moreover, $\delta^{13}C$ analyses on fossil chironomid head capsules showed depleted $\delta^{13}C$ values as low as −36.9‰ in one of our sediments, which confirms that methanogenic carbon is incorporated in chironomid exoskeletons. This demonstrates the potential to reconstruct the importance of MOB in the palaeo-diet of chironomid larvae and, indirectly, to provide information on past changes in methane flux from lake sediments.

Although the relation between chironomid larvae and their ingested food should be explored by more extensive rearing experiments, our results show that stable carbon isotopes in fossil chironomid head capsules can give insight in dietary links and carbon cycling in benthic food webs in lakes in the past. An important question that remains is whether it will be possible to quantify the relation between $\delta^{13}C$ in head capsules and the relative amount of MOB ingested by the larvae. Assuming at least a semi-quantitative relationship between the abundance of MOB and the methane production from sediments, this would ultimately allow an estimation of methane flux at the sediment-water interface based on $\delta^{13}C$ of fossil chironomids. To further develop chironomid $\delta^{13}C$ as an indicator for past benthic food-web changes, additional experiments and analyses are required to test the effect of preferential feeding, temperature dependent fractionation and species-specific fractionation on $\delta^{13}C$ values of head capsules in lakes with different environmental conditions.

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