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The influence of diet and water on the stable oxygen and hydrogen isotope composition of Chironomidae (Diptera) with paleoecological implications

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Abstract Stable oxygen and hydrogen isotope analyses of fossil aquatic organisms, such as the chitinous head capsules of chironomid larvae (Chironomidae: Diptera), are promising proxies for inferring paleoecological conditions. In order for analyses of stable oxygen ($\delta^{18}O$) and hydrogen isotope ratios ($\delta^{2}H$) of fossil chironomid head capsules to be used effectively in paleoecological research, it is necessary to understand the factors controlling their stable oxygen and hydrogen composition. We cultured chironomid larvae in two isotopically distinct waters under controlled, replicated laboratory conditions. Chironomid larvae were fed on identical diets, to examine the degree to which water and diet influence the $\delta^{18}O$ and $\delta^{2}H$ of these organisms. We used a two-end member mixing model to determine the proportional contributions of oxygen and hydrogen from water to the oxygen and hydrogen of chironomid larvae. Our experiment demonstrated that 69.0 ± 0.4% of oxygen and 30.8 ± 2.6% of hydrogen in chironomid larvae are derived from habitat water. Our results show that oxygen isotopes from chironomid remains can better constrain past habitat water isotopic changes compared to hydrogen, due to 69% of the chironomid oxygen being influenced by habitat water. Our data add to a small but growing suite of comparative data on the sources of oxygen and hydrogen in animal tissues, and provide the first such analyses from aquatic insects.

Keywords Chironomids · Organic · Water · Diet · Thermochemical reactor/elemental analyzer–isotope ratio mass spectrometer

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Introduction

Stable oxygen and hydrogen isotope data derived from analyses of fossilized animal remains have been used as proxies of past environmental change (Cormie et al. 1994a, b; Ehleringer et al. 2008; Grocke et al. 2006; Hobson 1999; Levin et al. 2006; Miller et al. 1988, 1993; Wooller et al. 2004). Two assumptions inherent to all these studies are that organisms record the isotopic composition of source water in organic compounds, and that the source water is

primarily derived from precipitation (Crowley 2000; Ehleringer et al. 2008; Hobson 1999; O'Brien and Wooller 2007). The second assumption has been well validated: storm-track trajectories, moisture origins, seasonality, and climate conditions can result in substantial temporal gradients in the ^{18}O and ^{2}H composition of precipitation (Bowen and Revenaugh 2003; Bowen et al. 2005b; Bowen and Wilkinson 2002; Gat 1996; Gat and Alrey 2006). These variations have been examined using teeth, bones and the chitinous exo-skeletons of animal remains (Ambler et al. 1999; Cormie et al. 1994a, b; Fogel et al. 1997; Levin et al. 2006; Macko et al. 1999). However, the first assumption, that these materials faithfully record the isotope composition of precipitation, has not been examined for most animal remains in the fossil record.

Stable isotope analyses of subfossil chironomid head capsules preserved in lake sediments are a recent addition to the suite of tools used to reconstruct the stable isotopic composition of past lake water (Wang et al. 2008; Wooller et al. 2004, 2008), a proxy for assessing past precipitation patterns and hydrological history. Chironomid head capsules, which are composed primarily of chitin, are well preserved in lake sediments (Walker 2001). The stable isotope composition of chitin (carbon, nitrogen, oxygen and hydrogen) is not significantly altered by biological and thermal degradation (Schimmelmann and DeNiro 1986a, b). The family Chironomidae is one the most abundant and widespread of aquatic insects, and chironomids are even able to thrive in extreme environments such as Antarctica and the bottom of Lake Baikal (Gullan and Cranston 2000; Oliver 1971). Changes in chironomid faunal assemblages over time have been used to infer past environmental changes (i.e., temperature, salinity and oxygen availability) based on modern calibration data sets and transfer functions (Brodersen et al. 2004; Brooks 2000; Heinrichs et al. 1999; Langdon et al. 2006). The stable oxygen and hydrogen isotope analyses of chironomid chitin can provide supplemental information to reconstruct past environmental conditions using the same organisms (Wooller et al. 2004, 2008).

For data on ratios of stable oxygen ($\delta^{18}O$) and hydrogen isotopes ($\delta^{2}H$) from analyses of fossil chironomid chitin to be used effectively in paleoecological research, it is necessary to understand the factors controlling the stable oxygen and hydrogen composition of insect chitin (Miller et al. 1988; Wang et al. 2008). However, such fundamental knowledge is lacking. The first step to improving our understanding of how oxygen and hydrogen are derived in chitin is to determine what happens in bulk tissues. Although the $\delta^{18}O$ of habitat water seems to have a strong influence on the $\delta^{18}O$ of chironomids (Wooller et al. 2004), diet may also play a role in influencing ^{18}O composition (Grocke et al. 2006; Schimmelmann and DeNiro 1986a, c). The degree to which water and diet influence the $\delta^{18}O$ and $\delta^{2}H$ of aquatic organisms has not yet been quantified experimentally.

A handful of previous studies have demonstrated that the proportional contribution of hydrogen derived from water and diet to the composition of organisms is fairly consistent (Ehleringer et al. 2008; Hobson et al. 1999; Kreuzer-Martin et al. 2003; O'Brien and Wooller 2007). In contrast, the proportional contribution of oxygen derived from water and diet to the composition of organisms varies greatly among different organisms (Ehleringer et al. 2008; Hobson et al. 1999; Kreuzer-Martin et al. 2003; O'Brien and Wooller 2007). For example, the contribution of drinking or habitat water to hydrogen in human hair, quail feathers, and microbial biomass was found to vary between 26% and 36%. In contrast, the contribution of oxygen from drinking water to organic compounds in these organisms varied from 27% in humans to 70% in microbes (Ehleringer et al. 2008; Hobson et al. 1999; Kreuzer-Martin et al. 2003; O'Brien and Wooller 2007; Sharp et al. 2003). It is possible that aquatic organisms like chironomids may be more strongly influenced by the isotopic signatures of their habitat water. However, the influences of water and diet on the stable oxygen and hydrogen isotope composition of aquatic organisms are largely unexplored and controlled experiments are necessary to understand the factors influencing the stable isotopic composition of organisms. Chironomids provide an excellent model for examining the influence of environmental parameters on their isotopic composition because they can be cultured under controlled laboratory conditions (Walker 1995).

Here we present the results of a controlled, replicated growth experiment designed to quantify how the $\delta^{18}O$ and $\delta^{2}H$ of water and diet influence the $\delta^{18}O$ and $\delta^{2}H$ of chironomid larvae. We grew chironomid larvae on an isotopically homogeneous diet, in two waters of contrasting $\delta^{18}O$ and $\delta^{2}H$ signatures. We then calculated the proportional contribution of oxygen and hydrogen from water and diet to larval tissue.

Materials and methods

Study species and culture setup

Chironomus dilutus were supplied as egg masses by the Environmental Protection Agency (EPA Mid-continent Ecology Division, Duluth, Minn.). This species has been used for toxicity studies by the U.S. Fish and Wildlife Service, National Fisheries Contaminant Research Center, and the EPA, and its rearing requirements are well known. The life cycle of *C. dilutus* is between 4 and 6 weeks at 20–23°C under ad libitum feeding conditions. Our experiment was conducted in an environmental chamber (Smith, San

Diego, Calif.) at the Water & Environmental Research Center (WERC) at the University of Alaska Fairbanks (UAF). Our culture operation was based on the EPA (Mid-continent Ecology Division) standard operating procedures (EPA 1997).

C. dilutus were cultured in clear plastic aquaria (28 × 16 × 20 cm), each containing 5 l of water. Aquaria were maintained at 23°C with a photoperiod of ~16-h light:8-h dark. A ~2-cm layer of fine silica sand (<0.2-mm grain size), pre-combusted for 24 h at 200°C, was provided as a substrate. The aquaria stood in a water bath (23°C) leveled with gravel to help buffer any fluctuations in environmental temperature. Each aquarium was aerated using an air-stone to maintain high levels of dissolved oxygen (DO) throughout the entire experiment (DO levels were >90% in the aquaria measured on a calibrated YSI 556 DO meter). One liter of water was replaced each day via a gravity feed system from a 1-l Nalgene bottle, and drained through a drainage hole 11 cm from the bottom of the aquarium. This minimized the concentration of ammonium and the decomposition of excessive food in the aquaria. The pH, ammonium levels, and ionic content of the water in each aquarium were monitored daily throughout the experiment to make sure they remained constant.

Experimental design

The experiment ran for ~8 weeks, determined by the larval development time. Two and one half egg masses were placed into each aquarium, and chironomid larvae were allowed to develop from eggs to fourth instar larvae (determined by body length measurements following EPA guidelines) (EPA 1997). Once the larvae had reached their fourth instar, they were harvested using a pair of fine forceps and stored in a freezer until their analysis. Larvae from all of the aquaria were terminated at the same time. The numbers of chironomid larvae harvested from each aquarium were not the same, which may have resulted from variability in hatching success rate for each aquarium due to inadequate egg mass fertilizations (Maier et al. 1990).

Two water treatments with different stable isotopic compositions [natural abundance (W_1) and isotopically labeled (W_2)] were used in this experiment, with three aquaria for each treatment. Water was collected from the Fox spring outside of Fairbanks, Alaska in March 2007 in two 200-l drums, and kept at 4°C throughout the experiment. W_1 consisted of natural abundance Fox spring water in one of the drums and was used for aquaria 1, 2, and 3. W_2 consisted of Fox spring water in the second drum that was isotopically labeled using water enriched in ^{18}O (>97 atom percent) (Iso-Solution, Ottawa, CA) and deuterium oxide ($^2H > 99.9$ atom %) (Cambridge Isotope Laboratories, Andover, Mass.). The labeled water was used for aquaria 4, 5, and 6. Ten milliliters of ^{18}O enriched water and 6.5 ml enriched deuterium oxide were added to W_2.

The diet for each treatment was constant throughout the experiment and consisted of fine powdered *Spirulina* algae (Aquatic Eco-Systems). Ten grams of dry *Spirulina* algae was mixed with 500 ml of water from each treatment and food mixes were stored in the refrigerator for use during the experiment. A final concentration of 0.02 mg/ml was achieved for each aquarium each day. Feeding stopped a week before harvesting to minimize larval gut contents.

Isotope sampling and analysis

Stable isotope ratios are expressed in δ notation in per mil (‰): $\delta\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000‰$, where R is the ratio of heavy to light isotopes (e.g., $^2H/^1H$, $^{18}O/^{16}O$). The isotope compositions of our results are expressed relative to international standards: Vienna Pee Dee belemnite for carbon, atmospheric nitrogen (air) for nitrogen and Vienna standard mean ocean water (VSMOW) for oxygen and hydrogen.

We sampled water from each aquarium at 2-day intervals throughout the experiment to check that the isotopic composition of the growth water remained constant. The aquaria did not have lids, and some evaporation did occur during the experiment. Water samples were collected into a 2-ml glass vial by pipette and were then crimped shut with no headspace in preparation for stable isotopic (oxygen and hydrogen) analysis. The vials were loaded into an autosampler (CTC Analytics A200SE liquid autosampler) and 0.2 µl of each sample was injected into an on-line pyrolysis, thermochemical reactor elemental analyzer (TCEA) (Finnigan ThermoQuest) coupled to a continuous flow (Conflo III) isotope ratio mass spectrometer (IRMS) (Finnigan MAT Delta V) at the Alaska Stable Isotope Facility (ASIF) at the UAF. $\delta^{18}O$ and δ^2H of gases (carbon monoxide and H_2) from each sample were measured relative to calibrated reference gases (carbon monoxide and H_2). The quality control scheme involved analyzing laboratory working standards after every seventh sample. Laboratory working standards were internally calibrated Duckering Building Millipore water (DMW), National Institute of Standards and Technology (NIST ref. 8535 VSMOW), Greenland ice sheet precipitation (GISP 8536) and standard light Antarctic precipitation (SLAP 8537) and measured versus expected had an R^2 of >0.99. Multiple ($n = 15$) $\delta^{18}O$ and δ^2H analyses of DMW conducted during the sample sequence yielded $1\sigma = 0.4‰$ and 1.7‰, respectively. Each sample and standard was analyzed in triplicate. Triplicate $\delta^{18}O$ and δ^2H analyses of separate DMW and water samples yielded 1σ of ≤0.3‰ and 1.6‰, respectively.

The $\delta^{18}O$ and $\delta^{2}H$ of larvae and diet were also measured at the ASIF. Between 0.1 and 0.3 mg of individual freeze-dried whole chironomid larvae and diet samples was weighed into tin capsules. Acknowledging the possibility of exchangeable hydrogen in organic samples (Kreuzer-Martin et al. 2003, 2006; Miller et al. 1988; Schimmelmann and DeNiro 1986a, c), we adopted a method consistent with that outlined by Wassennar and Hobson (2002), where samples were air equilibrated with ambient laboratory air moisture in the ASIF at room temperature for >96 h to minimize the influence of exchangeable oxygen and hydrogen. After equilibration, samples were freeze dried for >6 days, as recommended for organic samples such as keratin (Bowen et al. 2005a). We treated all of our samples in the same manner, allowing relative comparisons between samples. After comparative equilibration and freeze drying, all samples were loaded into a zero-blank autosampler that was purged with research grade helium. Measurements of the $\delta^{18}O$ and $\delta^{2}H$ of these organic samples were made using the same instrumentation as the water samples. $\delta^{18}O$ and the $\delta^{2}H$ of sample gases were then calibrated relative to internally calibrated organic standards, bowhead whale baleen keratin (BWBII) (Wassenaar and Hobson 2002) and international, calibrated standards [Australian National University sucrose (ANU sucrose), National Bureau of Standards 22 and 30 (NBS-22, NBS-30) and polyethylene foil (PEF-1)] (measured versus expected R^2 of >0.99). The $\delta^{18}O$ and $\delta^{2}H$ analyses of laboratory working organic standards, benzoic acid (lot no. 947459; Fisher Scientific), were also conducted throughout the run ($n = 10$) and yielded 1σ of 0.4‰ and 1.7‰, respectively. Blank tin capsules were also analyzed among the samples.

To assess whether chironomid larvae had any additional sources of available food, we analyzed the $\delta^{13}C$ and $\delta^{15}N$ of food (*Spirulina*) and chironomid larva in an initial experiment in 2006. These larvae were freeze dried before weighing and analyzed for $\delta^{13}C$ and $\delta^{15}N$. Microorganisms were observed growing on the inner wall of the aquarium and these were also collected and analyzed for $\delta^{13}C$ and $\delta^{15}N$. The $\delta^{13}C$ and $\delta^{15}N$ values of the samples were determined using a Costech ECS4010 EA attached via a Conflo III to a continuous flow IRMS (Thermo Finnigan Delta^{plus} XL) at the ASIF. Multiple ($n = 7$) analyses of our laboratory working standard (peptone, lot no. 76F-0300; Sigma) yielded 1σ of 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$. The second and fourth larval instars were also measured for $\delta^{18}O$ and $\delta^{2}H$ using the procedure described above to examine whether the isotopic composition varied between larval stages. We chose second and fourth instars to conduct our stable isotopic analyses to examine whether different instars had different isotopic values. These two instars were largely chosen arbitrarily, although first instars were not considered since they were very small and did not possess sufficient sample mass to conduct individual larvae isotopic measurements.

Data analysis

The oxygen isotope composition of chironomid larvae is determined by the isotopic composition of its oxygen sources, weighted by their proportional contribution (Schwarcz 1991) and potentially offset by fractionation (Boutton et al. 1983):

$$\delta^{18}O_C = (\delta^{18}O_D + \varepsilon_D)(1 - p) + (\delta^{18}O_W + \varepsilon_W)p \quad (1)$$

where C = chironomid, D = diet, W = water, and p = the proportional contribution of water to chironomid tissues. The terms ε_D and ε_W are fractionation effects associated with diet and water uptake, respectively. Because $\delta^{18}O_D$ and ε_D are the same for both treatments, we can solve for p by using the two experimental water types as follows:

$$p = \frac{(\delta^{18}O_{C_{(W1)}} - \delta^{18}O_{C_{(W2)}})}{(\delta^{18}O_{W1} - \delta^{18}O_{W2})} \quad (2)$$

where W_1 is the natural abundance water and W_2 is the labeled water. The same equations can also be used to calculate the proportional contributions of hydrogen from diet and water to that of chironomids by substituting H into the equations where O is used. The possible contribution of atmospheric oxygen to the oxygen isotope composition of the chironomid larvae in both treatments were assumed constant for all aquaria. This contribution, if present, would be confounded with the proportional contribution of dietary O ($1 - p$).

Statistical analyses

All statistical analyses were performed using JMP IN 5.2.1 (JMP; SAS Institute, Cary, N.C.). Data from the two treatments were analyzed separately. Values of $\delta^{18}O_C$ ($\delta^{2}H_C$) and $\delta^{18}O_W$ ($\delta^{2}H_W$) were analyzed using ANOVA to compare among tanks within two treatments. We assessed the differences among chironomid larvae and water from different aquaria with Tukey honest significant difference (HSD) contrasts. Because calculations of p required $\delta^{18}O_C$ ($\delta^{2}H_C$) to be compared with $\delta^{18}O_W$ ($\delta^{2}H_W$), we used the mean of the $\delta^{18}O_C$ ($\delta^{2}H_C$) compared with the $\delta^{18}O_W$ ($\delta^{2}H_W$) (Eq. 2) of each tank. All these measurements are reported as the mean ± 1 SD. Brown and Forsythe's tests were performed to check the constant variances of $\delta^{18}O_C$ and $\delta^{2}H_C$ within each treatment. Isotope differences in two larval stages (2nd and 4th instar larvae) were assessed using a Student t-test. Unless otherwise stated, statistical significance is assessed at $P < 0.05$.

Results

Stable isotopic composition of water and diet

Labeled water treatments were heavily enriched relative to the natural abundance treatments throughout the entire experiment. There were no significant differences in $\delta^{18}O_W$ and $\delta^{2}H_W$ among the three natural abundance aquaria ($P = 0.211$ for $\delta^{18}O$ and $P = 0.235$ for $\delta^{2}H$) (Table 1). However, $\delta^{18}O_W$ and $\delta^{2}H_W$ were not identical among isotopically labeled aquaria, which may have been due to slight differences in evaporation levels from each aquarium (ANOVA, $P = 0.007$ for both $\delta^{18}O$ and $\delta^{2}H$). The $\delta^{18}O$ and $\delta^{2}H$ of the diet was 28.0 ± 0.9‰ and −152.3 ± 2.6‰, respectively.

Stable isotopic composition of chironomid larvae

Similar to the water, the $\delta^{18}O$ and $\delta^{2}H$ of chironomid larvae from the aquaria were distinctly different between the natural abundance (W_1) and enriched water treatments (W_2) (Table 1; Fig. 1). However, the magnitude of the difference between the two treatments is much smaller compared to that of their growth water (Fig. 1). This is a clear indication that diet also influenced the $\delta^{18}O_C$ and $\delta^{2}H_C$ of the larvae. Variation in $\delta^{18}O_C$ and $\delta^{2}H_C$ among aquaria were also observed within the same treatment; however, it was much smaller than the variability between the two growth water treatments. Differences in the larvae grown in W_1 aquaria were significant for $\delta^{18}O$ but not for $\delta^{2}H$ ($P = 0.01$ and $P = 0.33$ for $\delta^{18}O$ and $\delta^{2}H$, respectively). The larvae grown in the W_2 did not show significant differences for their $\delta^{18}O$ and $\delta^{2}H$ ($P = 0.34$ for $\delta^{18}O$ and $P = 0.53$ for $\delta^{2}H$) and variances among aquaria were also constant ($P = 0.09$ and $P = 0.63$, for $\delta^{18}O$ and $\delta^{2}H$, respectively) (Table 1). The $\delta^{18}O$ and $\delta^{2}H$ analyses of second ($n = 3$) and fourth larval instars ($n = 3$) from an initial experiment showed that there was no significant difference between these life stages ($P = 0.781$ and $P = 0.744$ for $\delta^{18}O$ and $\delta^{2}H$, respectively).

Proportional contributions of oxygen and hydrogen from water versus diet to chironomid larvae

We used two approaches to calculate the proportional contributions of oxygen and hydrogen from water to the organic composition of chironomid larvae. In the first approach, by neglecting the slight differences among replicate aquaria within the same treatment, we calculated the mean $\delta^{18}O$ and $\delta^{2}H$ of all water samples measured for each treatment (W_1 and W_2) and the mean $\delta^{18}O$ and $\delta^{2}H$ of chironomids associated with each treatment (Table 1). By using these data to solve Eq. 2, we found that 72% of chironomid oxygen was derived from water whereas 32% of their hydrogen is derived from water. This result is represented graphically in Fig. 2, where the slopes of the relationship between water and chironomid isotope values represent p. However, because there were small but significant isotopic differences between the water in replicate aquaria 4 and 5 and also among the $\delta^{18}O$ for chironomid larvae in replicate aquaria 2 and 3 (Tukey HSD), we also solved Eq. 2 for each individual aquarium. In order to do this, we calculated the proportional contribution (p) of oxygen and hydrogen from water for every possible paired combination of natural abundance (three) and enriched aquaria (three) (a total of nine combinations). We then solved Eq. 2 and calculated the p for both oxygen and hydrogen from the nine combinations and the mean p from these nine combinations (Table 2). Using this approach we estimated that 69 ± 4% of oxygen in chironomid larvae was derived from the water they lived in, whereas 31 ± 3% of hydrogen in chironomid larvae was derived from water. In both approaches, we calculated the proportional contribution using the mean chironomid $\delta^{18}O$ and $\delta^{2}H$ for each aquarium. Because more than 100 chironomid heads are used for a single isotope value in paleoresearch (Wang et al. 2008), each aquarium is analogous to a single

Table 1 The stable oxygen ($\delta^{18}O$) and hydrogen isotope ratios ($\delta^{2}H$) (mean ± 1 SD) of water (W) and chironomid (C) larvae from two water treatments

Treatment	Aquarium no.	$\delta^{18}O_W$ (‰)	$\delta^{2}H_W$ (‰)	$\delta^{18}O_C$ (‰)	$\delta^{2}H_C$ (‰)
Natural abundance water	1	−14.7 ± 1.1 ($n = 7$)	−121.5. ± 7.2 ($n = 7$)	9.8 ± 1.3 ($n = 10$)	−127.8 ± 4.4 ($n = 10$)
	2	−14.8 ± 1.2 ($n = 7$)	−122.1 ± 7.9 ($n = 7$)	10.6 ± 0.7 ($n = 4$)	−123.6 ± 7.3 ($n = 4$)
	3	−15.8 ± 1.2 ($n = 7$)	−128.1 ± 7.9 ($n = 7$)	8.5 ± 1.3 ($n = 9$)	−124.7 ± 5.6 ($n = 9$)
	Mean	−15.1 ± 1.2 ($n = 21$)	−123.9 ± 7.9 ($n = 21$)	9.4 ± 1.3 ($n = 23$)	−125.9 ± 5.6 ($n = 23$)
Isotopically labeled water	4	8.0 ± 1.3 ($n = 7$)	49.6 ± 13.7 ($n = 7$)	26.4 ± 2.9 ($n = 6$)	−72.6 ± 5.1 ($n = 6$)
	5	5.1 ± 1.5 ($n = 7$)	25.3 ± 15.6 ($n = 7$)	23.1 ± 1.3 ($n = 4$)	−76.6 ± 7.5 ($n = 4$)
	6	6.2 ± 1.8 ($n = 7$)	32.7 ± 9.6 ($n = 7$)	24.4 ± 0.2 ($n = 2$)	−76.0 ± 0 ($n = 2$)
	Mean	6.5 ± 0.7 ($n = 21$)	35.9 ± 16.0 ($n = 21$)	24.9 ± 2.6 ($n = 12$)	−74.5 ± 5.6 ($n = 12$)

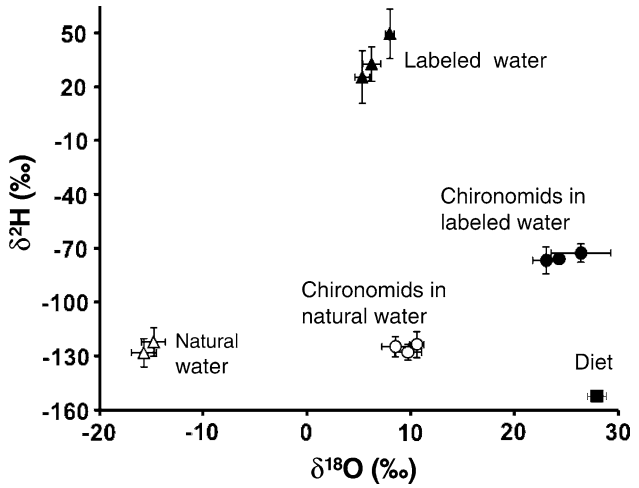

Fig. 1 The $\delta^{18}O$ and $\delta^{2}H$ of natural water (*white triangle*), isotopically labeled water (*black triangle*), chironomid larvae from both types of water (*white circle* and *black circle*, respectively), and the larval diet (*black square*) expressed as mean ± SD. The isotope compositions are expressed relative to the international standard, Vienna standard mean ocean water

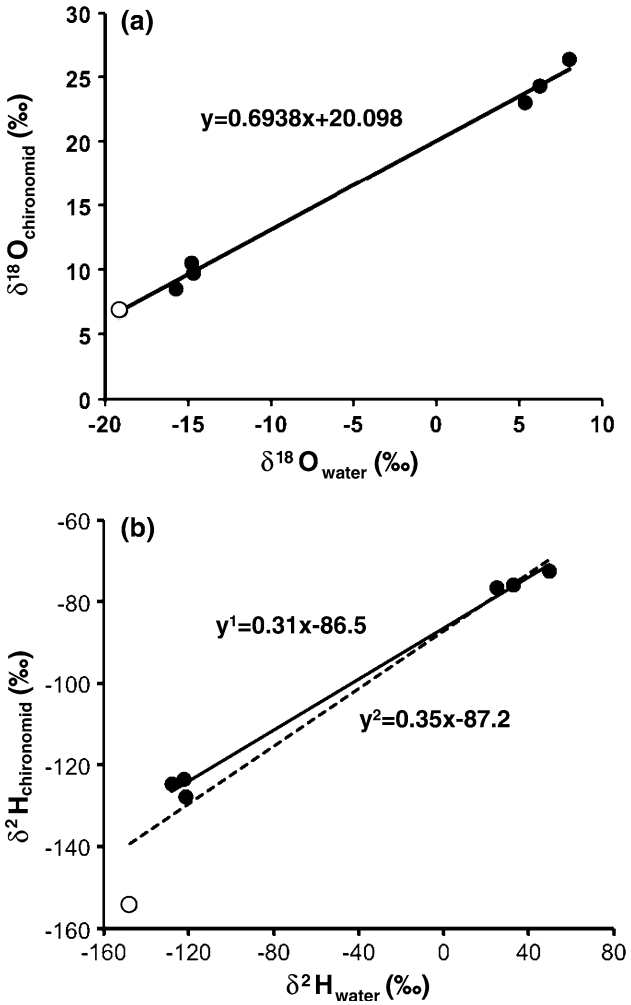

Fig. 2 **a** The $\delta^{18}O$ values of chironomid larvae versus their growth water $\delta^{18}O$ (*black circle*). **b** The $\delta^{2}H$ of chironomids versus $\delta^{2}H$ of their growth waters (*black circle*). The $\delta^{18}O$ and $\delta^{2}H$ of chironomids versus water from an independent culture trial (*white circle*) is superimposed on the line. The regression slope (*solid line*, y^1 equation) changes in **b** when a data point from an independent culture trial is added (*dotted regression line*, y^2 equation)

sample collected from sediment. These two approaches yielded very similar results: that only ~30% of oxygen in chironomid larvae originated from diet while ~70% of hydrogen in the larvae was derived from diet.

Diet of chironomid larvae from the initial experiment

Spirulina ($n = 3$) (i.e., diet) yielded a mean $\delta^{13}C$ and $\delta^{15}N$ of -25.8 ± 0‰ and -1.4 ± 0.5‰, respectively, while the chironomid larvae ($n = 3$) yielded a mean $\delta^{13}C$ and $\delta^{15}N$ of -25.1 ± 0.2‰ and 2.0 ± 0.1‰, respectively. Thus, the larvae had mean $\delta^{13}C$ and $\delta^{15}N$ values that were 0.7‰ and 3.4‰ higher than the diet, consistent with the diet being their sole food source (Fig. 3). The microorganisms collected from the walls of the aquarium had a distinctly different $\delta^{13}C$ and $\delta^{15}N$ values of -34.4 ± 2.1‰ and -6.0 ± 1.5‰.

Discussion

The $\delta^{18}O$ and $\delta^{2}H$ of chironomid larvae grown from two water treatments were distinctly different from each other, despite having been fed the same diet. These differences are consistent with the $\delta^{18}O$ and $\delta^{2}H$ of their growth water. However, the magnitude of differences between chironomid larvae from the two treatments is smaller than that of two types of growth water. These results demonstrate that both water and diet affect the oxygen and hydrogen isotope composition of chironomid larvae. By providing a diet with constant isotopic composition and water with two different isotopic compositions, we were able to quantify the proportional contributions of oxygen and hydrogen from water to chironomid larvae. Our data show that ~70% of the oxygen in the total organic composition of the chironomid larvae is derived from the water of the larval habitat. In contrast, diet dominates the hydrogen isotope ratios of chironomid larvae (~70%), because only 30% of hydrogen in the chironomid larvae was derived from water. Interestingly, our findings for both oxygen and hydrogen are identical to the proportional contributions of oxygen and hydrogen from water and growth substrate supplied to microbial spores, where 70% of oxygen and 30% of hydrogen of microbial spores were found to derive from water, whereas the remainder was derived from the organic compounds supplied as substrate (Kreuzer-Martin et al. 2003).

Table 2 The proportional contribution (p) of oxygen and hydrogen derived from water to the organic composition of chironomid larvae

	Aquaria	p of oxygen from water (natural abundance water)			p of hydrogen from water (natural abundance water)		
		1	2	3	1	2	3
Isotopically labeled water	4	0.733	0.692	0.751	0.299	0.273	0.270
	5	0.663	0.618	0.688	0.349	0.318	0.313
	6	0.695	0.652	0.717	0.336	0.307	0.303
Mean of 9		0.690			0.308		
SD		0.041			0.026		

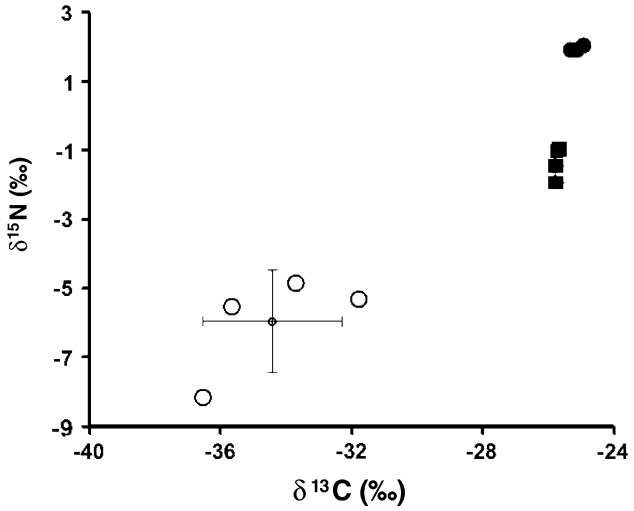

Fig. 3 The δ^{15}C and δ^{15}N of chironomid larvae (*black circle*, $n = 3$), their diet of *Spirulina* algae (*black square*, $n = 3$), and the microorganism (*white circle*, $n = 4$) growing on the aquarium walls, mean ± SD. The isotope compositions are expressed relative to the international standard, Vienna Pee Dee belemnite

Our finding that ~30% of chironomid total organic hydrogen derives from water is consistent with the handful of published studies of animals, both observational and experimental. Despite differences in tissues, the influence of water is remarkably consistent. Estimates of the drinking water contribution to hair hydrogen in humans range from 27% to 35% (Ehleringer et al. 2008; O'Brien and Wooller 2007; Sharp et al. 2003). Similarly, the contribution of water to hydrogen in bird feather keratin was between 26% and 32% (Hobson et al. 1999). These results confirm that the majority of the hydrogen in lipids, protein and keratin are derived from diet rather than water.

Our estimate that ~70% of total organic oxygen in chironomid larvae derived from water is about twice as high as has been reported in two previous human studies (Ehleringer et al. 2008; O'Brien and Wooller 2007). It is not clear why proportional contributions of oxygen from water in aquatic organisms [chironomids (this study) and microbes (Kreuzer-Martin et al. 2003)] should be different from humans, and it would be interesting to assess whether this pattern holds over a broader sample of aquatic and terrestrial organisms. We also note that neither this study nor previous studies have directly assessed the contribution to tissue oxygen from dissolved or bound O_2, which may make a small but measurable contribution to tissue oxygen. Because chironomid larvae vary in whether they possess hemoglobin, the magnitude of this contribution could vary taxonomically. Subsequent investigations in a broader sample of organisms will help to resolve these questions.

To assess the consistency of our results, we plotted the mean δ^{18}O and δ^{2}H values of chironomid larvae and growth water from both our preliminary culture trial and the main experiment (Fig. 2). The preliminary δ^{18}O data did not change the slope of the regression line from the main experiment (Fig. 2a). However, the preliminary δ^{2}H data shifted the slope from 0.31 to 0.35 (Fig. 2b) and while not exactly identical, the data are consistent with our estimated proportional contribution of water to chironomid total organic hydrogen (31 ± 3%).

It was important for our study that the chironomid larvae consumed only the experimental diet provided to them. The experimental diet to tissue fractionation for C and N found in this study ($\varepsilon\delta^{13}C = 0.7‰$ and $\varepsilon\delta^{15}N = 3.4‰$) (Fig. 3) is consistent with a classic trophic level fractionation ($\varepsilon\delta^{13}C = \sim 1‰$, $\varepsilon\delta^{15}N = \sim 3‰$) (Fry and Sherr 1984; Peterson and Fry 1987). In contrast, the δ^{13}C and δ^{15}N values of trace microorganisms found growing on walls of the aquarium were distinctively different from those of the diet, which shows that the microorganisms were not likely to have contributed significantly to the diet. Thus our calculations of the contributions of oxygen and hydrogen from diet and water were not affected by alternate diet sources.

Further studies are needed to establish the relationships between specific compounds (e.g., chitin) from chironomids. Previous studies have shown that hydrogen in different tissues (such as muscle, lipids and feathers) in birds can derive from different proportions of hydrogen from water (Hobson et al. 1999). The proportional contribution of oxygen and hydrogen to whole chironomid larvae, which include lipids and proteins, may differ from those of chitin, the predominant material preserved in the fossil record (Walker 1987). Furthermore, fractionation

factors (ε) associated with both water and diet in our experiment cannot be determined using Eq. 1 because there are three unknowns (p, ε_W, ε_D). A labeling study of nutritionally identical diets with different isotope ratios would make it possible to solve all the unknowns (Sessions and Hayes 2005) including the p, ε_W and ε_D.

Our findings support that the stable isotopic composition of chironomid subfossils can be used to explain changes in the oxygen and hydrogen isotope values of past environments in concert with other geological evidence (Wang et al. 2008; Wooller et al. 2008). The stable oxygen isotope composition of chironomid larvae provides a stronger marker of habitat water isotopic values compared with hydrogen isotopic data derived from chironomids since a greater proportion ($\sim$70%) of chironomid larvae oxygen is derived from water compared with hydrogen ($\sim$30%). Conversely, hydrogen isotope analyses of chironomid larvae better constrain the hydrogen isotopic composition of diet. In our experiment, the magnitude of changes in $\delta^{18}O_C$ and $\delta^{2}H_C$ is based on the mean value of chironomid larvae within each aquarium instead of individual larvae. The systematic variation in each aquarium resembles paleoecological research, where one measurement is based on more than 100 fossil chironomid head capsules. Our research is the first of its type on aquatic invertebrates to calculate the proportional contribution of oxygen and hydrogen from water versus diet. Our findings add to the growing suite of comparative data available on the proportional contribution of oxygen and hydrogen deriving from water and diet available to organisms.

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