Effects of temperature on food isotopic integrity and trophic fractionation in *Chironomus riparius* in laboratory experiments

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Abstract Our experimental study was designed to assess the effects of temperature on food isotopic integrity and trophic fractionations (of carbon and nitrogen stable isotopes: $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) in chironomid larvae. A laboratory experiment was run using *Chironomus riparius* larvae at 15, 18 and 23°C, and under three pulverized food resources. There were significant changes in the stable isotopic composition of the uneaten food, revealing the difficulty of preventing food microbial degradation in rearing experiments. C/N ratios of chironomid larvae were also affected by temperature, regardless of the food resource, suggesting changes in lipid contents and associated effects on the larval $\delta^{13}\text{C}$ values. Therefore, differences in calculation methods (with vs. without lipid normalizations, fresh vs. old food isotopic baselines) induced large variability in $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values, but our trophic fractionation estimates were similar to those previously reported in literature.

Therefore, we conclude that temperature is not a major driver of the variability in trophic fractionations for chironomid larvae, and stable isotope composition of aquatic consumers can be used in food webs studies under changing temperature conditions. Variability in trophic fractionation estimates should, however, be considered to avoid misinterpretations of food web structure.

Keywords Carbon stable isotope · Nitrogen stable isotope · Trophic fractionation · Chironomidae · Aquatic food webs

Introduction

Stable isotope composition of aquatic consumers is a common tool to evaluate the trophic structure and resource dynamics in aquatic food webs (Peterson & Fry, 1987; Vander Zanden et al., 2016). This approach is based on two main assumptions: (1) that food resources at the base of aquatic food webs can differ significantly in their stable isotopic compositions (Rounick & Winterbourn, 1986; Finlay, 2001) and (2) that the trophic fractionation, defined as the difference in stable isotopic compositions between food and consumer, is well-known (DeNiro & Epstein, 1978). However, large variability in trophic fractionations has been reported for aquatic consumers (Post,

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2002; McCutchan et al., 2003), confounding the tracking of energy flows through food webs. While a range of biotic and abiotic factors could explain the large variability reported in trophic fractionation values, their influences are still poorly understood, and laboratory studies are needed to specifically address this (Martinez del Rio et al., 2009).

Biotic and abiotic factors, such as the balance between animal assimilation and respiration (Schindler, 1968), body conditions (i.e. lipid contents; Masclaux et al., 2012), and the quality of food (Goedkoop et al., 2006; Masclaux & Richoux, 2017) could affect trophic fractionation and contribute to the variability in trophic fractionations of carbon and nitrogen stable isotopes in consumers ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively, Gannes et al., 2009). The role of temperature on trophic fractionation, however, has retained very little attention (but see Power et al., 2003). Indeed, high fluctuations in environmental temperature could potentially induce substantial error in isotope-based approaches. Only a few experimental studies have quantified trophic fractionations for aquatic organisms across a wide range of temperatures (Power et al., 2003; Barnes et al., 2007; Schilder et al., 2015; Masclaux & Richoux, 2017). While these studies reported large uncertainties in trophic fractionation values with $\Delta^{13}\text{C}$ values ranging from -1 to $+3\text{‰}$ and $\Delta^{15}\text{N}$ values ranging from -0.2 to $+5\text{‰}$, they also reported contradictory and unclear relationships with temperature. While Power et al. (2003) and Masclaux & Richoux (2017) reported increase in $\Delta^{13}\text{C}$ values with increasing temperature, Schilder et al. (2015) found the opposite relationships, and they found similar contradicting observations for $\Delta^{15}\text{N}$. A range of confounding factors could explain this lack of consistency among studies (Masclaux & Richoux, 2017). For example, lipids exhibit lower $\delta^{13}\text{C}$ values than other constituents (protein) in animal tissues (DeNiro & Epstein, 1977), and a lipid normalization of larval $\delta^{13}\text{C}$ values could be required to get more reliable data interpretations (McConnaughey & McRoy, 1979; Smyntek et al., 2007). Finally, most of these studies have been conducted using a single zooplankton species (i.e. *Daphnia* sp., Cladocera), and little is known for benthic macroinvertebrate consumers. Hence, we still have a limited understanding of how temperature affects trophic fractionation of carbon and nitrogen stable isotopes in aquatic consumers.

Chironomidae (Arthropoda; Diptera; Nematocera) are non-biting midges with a merolimnic life history composed of an aquatic larval development, a short pupal stage, and a terrestrial adult stage (Armitage et al., 1995). Larvae of many chironomid species can live in superficial lake sediments where they often are a predominant group in the benthic food webs (Armitage et al., 1995; Northington et al., 2010). Especially during the pupal stage, chironomids substantially contribute to the energy transfer from benthic to pelagic habitats (Vander Zanden et al., 2006; Wagner et al., 2012) as they are readily consumed by fish, making Chironomidae a keystone group in lake food webs. After each larval moulting, the sclerotized head capsules (HC) are archived in sediments (Walker, 2001), and their chemical compositions are preserved during the fossilization process in sediments (Verbruggen et al., 2009), allowing for the analysis of their stable isotopic composition (Heiri et al., 2012). Stable isotope analyses (mainly those of carbon, nitrogen and hydrogen) of chironomid HC are increasingly used in paleolimnological studies to reconstruct the effects of past climate change on energy flows in lake food webs (Belle et al., 2017, 2018), but the reliability of these inferences could be strengthened by a better understanding temperature effects on trophic fractionation in chironomid larvae.

Laboratory experiments allow for studies on effects of specific variables (Gannes et al., 2009) and can help to clarify causal relationships between temperature and trophic fractionations. However, temperature is also known to affect both growth and development of chironomid larvae (Pinder, 1986), implying that larvae incorporate new elements into biomass at different rates, and similar observations have been reported with food quality (Frossard et al., 2013; Belle et al., 2015). Therefore, experimental treatments with different temperatures should be terminated after different time intervals when larvae have reached the same developmental stage (see also Frossard et al., 2013; Belle et al., 2015). Furthermore, Goedkoop et al. (2006) demonstrated that food isotopic integrity is affected by microbial degradation, thus impacting on trophic fractionation in consumers. This implies that the reliability of trophic fractionation estimates is dependent on the capability of keeping constant stable isotopic compositions of food resources during experimental treatments of different duration, and

under different food resources. As a slower larval development is expected at low temperature than at high temperature, food isotopic drifts in experimental units could deeply impact on trophic fractionation estimates of consumers. Therefore, the assessment of food isotopic integrity is required to provide more accurate evaluation of temperature effects on trophic fractionation in chironomid larvae.

In this study, we investigated the effects of temperature on food isotopic integrity and trophic fractionations of different food resources in experiments with *Chironomus riparius* Meigen, 1804, a key benthic macroinvertebrate species. We aimed to evaluate how (1) carbon and nitrogen elemental and stable isotopic composition of food resources are affected by extended microbial degradation of food in experiment units and (2) to what extent temperature has effects on trophic fractionations of carbon and nitrogen stable isotopes across the food–consumer interface.

Material and methods

Laboratory culture of *C. riparius*

A population of *C. riparius* Meigen, 1804, was cultured in a 50-L aquarium system filled with 4 cm of sea sand and tap water in our lab. Larvae were grown at room temperature (20°C), under a natural photoperiod and fed ad libitum with pulverized TetraMin® (Tetra, Melle, Germany). Dissolved oxygen concentrations were maintained high using an aquarium air pump. For our experiment five newly spawned egg masses (< 1 day after spawning) were collected and placed in a 100-mL beaker until hatching.

Experimental setup

Incubations ($n = 4$) were made in climate chambers at 15, 18 and 23°C with a 16 h/8 h light/dark cycle (light intensity $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$). Experimental units consisted of large crystalizing bowls (14 cm in diameter) filled with a 1.5 cm of sand (grain size 200–500 μm) and 600 mL of tap water (8dH; renewed by a third every week). Aeration was carried out using Pasteur pipettes and aquarium air pumps (approximately 4 bubbles s^{-1}). Prior to the addition of larvae,

units were allowed to acclimate to the experimental conditions for 2 days. Then, 15 newly hatched, first instar larvae (< 2 days old) were introduced to each of the experimental units. Three pulverized food resources were used to test the effects of food quality on trophic fractionations in *C. riparius* Meigen, 1804: TetraMin (Tetra, Melle, Germany), Alder leaves (*Alnus glutinosa* (L.) Gaertner) and *Chlorella* sp. (commercial food brand, SuperFruit). Larvae were fed three times per week at a rate of 0.4 mg C per larvae per day (Doi et al., 2007).

Four aliquots of the three food resources were collected for stable isotopes analyses before the start of the experiment, and stored in a freezer (− 20°C). These samples were used to provide $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of the fresh food resources. Each individual experiment (i.e. one replicate for one food at one temperature) was run until the first pupae was about to appear (i.e. recognized by a very large and swollen fourth instar larvae), indicating complete larval development for the experimental cohort (Frossard et al., 2013). Ending all experiments at the same time would not have been a relevant strategy as it would lead to the comparison of larvae at different stages in their development. All surviving larvae in each of the experimental units were then transferred to a beaker filled with aerated tap water to allow for 24 h-gut clearance (Feuchtmayr & Grey, 2003). Meanwhile, uneaten food deposits on the sand were collected by carefully resuspending food particles and sampling these from the overlying water by filtration (GF/F, Whatman™). All samples were then dried at 60°C to constant weight. Larvae and uneaten food scraped from the dried filters were ground and stored in a freezer (− 20°C).

Then, 0.5 mg of larvae and 1 mg of food samples (both fresh and uneaten food samples) were transferred to tin capsules and weighed. Carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopic compositions, and carbon and nitrogen contents were analysed using an Isotope Ratio Mass Spectrometer interfaced with an Elemental Analyser (EA-IRMS) at SLU Stable Isotope Laboratory (Umeå, Sweden). Results were expressed as the deviation (δ) from reference standards; Vienna Pee Dee Belemnite or atmospheric nitrogen as standards: $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ (‰) = $([R_{\text{sample}}/R_{\text{standard}}] - 1) \times 1000$; where $R = {}^{13}\text{C}/{}^{12}\text{C}$ or ${}^{15}\text{N}/{}^{14}\text{N}$. Sample measurement replications from internal standards (wheat and maize flours) produced analytical

errors (1σ) of $\pm 0.15\text{‰}$ for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values ($n = 24$).

Data analysis

Lipid-normalized $\delta^{13}\text{C}$ values of larvae ($\delta^{13}\text{C}_{\text{norm}}$) were calculated based on C/N ratios following the recommendations by Post et al. (2007; $\delta^{13}\text{C}_{\text{norm}} = \delta^{13}\text{C} - 3.32 + 0.99 * \text{C/N}$). Trophic fractionations of C and N stable isotopes ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively) were calculated as the difference between stable isotopic compositions of food resources and larvae. Calculations were done using both fresh (i.e. initial food samples) and altered (i.e. collected uneaten food at the end of the experiment) food isotopic baselines, and using larval $\delta^{13}\text{C}$ values with and without lipid normalization. A two-way ANOVA was used to test for full effects and the effects of the interaction between temperature and food resources on $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ values (with $\alpha = 0.05$). All statistical tests and graphical displays were performed using R 3.5.2 statistical software (R Core Team, 2018).

Results

Larval development and experimental design

The degree-days required to complete larval development in treatments with TetraMin increased from 322°D at 23°C to 377°D at 15°C (Fig. 1). At a similar temperature, the number of degree-days for complete larval development was higher in treatments with

TetraMin than in those with *Chlorella* sp. (Fig. 1). As larval development differed among temperatures and food resources (Fig. 1), the experimental duration varied from 14 days for treatments with TetraMin at 23°C to 29 days for those with TetraMin at 15°C. Due to an unexpected high larval mortality in the Alder treatments at all temperatures, and in the *Chlorella* sp. treatment at 23°C, these treatments were omitted from further processing.

Elemental and isotopic compositions of food

As expected, food resources of aquatic origin (i.e. TetraMin, and *Chlorella* sp.) were more enriched in ^{15}N , ^{13}C and total N than Alder leaves (Fig. 2), with initial $\delta^{13}\text{C}$ values ranging from $-29.8 \pm 0.1\text{‰}$ for Alder leaves to $-20 \pm 0.1\text{‰}$ for *Chlorella* sp., and $\delta^{15}\text{N}$ values from $-1.6 \pm 0.1\text{‰}$ for Alder leaves to $6.9 \pm 0.1\text{‰}$ for TetraMin (Fig. 2A, B). Also C/N ratios for the food resources differed, with values ranging from 5.3 ± 0.1 for *Chlorella* sp. to 17.7 ± 0.2 for Alder leaves (Fig. 2C). At the end of the experiments, food isotopic and elemental compositions ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and C/N ratios) significantly differed from initial conditions (Fig. 2, Table 1 and Fig. S1). For example, comparisons between initial conditions and those at the end of the 15°C experiment revealed a significant decrease in Alder leaves' C/N ratios (from 17.7 ± 0.2 to 11.5 ± 0.2), an increase in $\delta^{15}\text{N}$ of TetraMin (from 6.9 ± 0.1 to $10.5 \pm 0.3\text{‰}$), and a decrease in $\delta^{13}\text{C}$ values of *Chlorella* sp. (from -20 ± 0.1 to $-22.3 \pm 0.3\text{‰}$; Fig. 2 and Table 1). These results show the sum effects of the conditioning on food particles.

Stable isotopic compositions and C/N ratios of chironomid larvae

The $\delta^{15}\text{N}$ values of larvae fed TetraMin ranged from 10.5 ± 0.3 to 11.0 ± 0.3 (Table 2), and were negatively correlated with temperature (Table 2). The $\delta^{13}\text{C}$ values of these larvae ranged from -22.2 to -21.5‰ , whereas C/N ratios increased by 0.9 across the temperature gradient (i.e. from 4.5 at 15°C to 5.4 at 23°C; Table 2). As an increase in C/N ratios suggests an increase in larval lipids contents, larval $\delta^{13}\text{C}$ values were lipid-normalized using C/N ratios. Larval $\delta^{13}\text{C}_{\text{norm}}$ values were very similar across the temperature gradient and ranged from -20.6 to -20.1‰

Fig. 1 Effects of temperature and food resources on chironomid larval development (number of degree-day)

Fig. 2 **A** Carbon stable isotopic compositions ($\delta^{13}\text{C}$), **B** nitrogen stable isotopic compositions ($\delta^{15}\text{N}$), and **C** C/N ratios of food resources (Alder leaves, *Chlorella* sp., and TetraMin) at initial conditions, and at the end of the experiments

Table 1 Statistical comparison of temperature effects on $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and CN ratios among the food resources

	A	T	C
$\delta^{13}\text{C}$			
ANOVA (<i>p</i> value)	0.1	< 0.001	< 0.001
Tukey test (significant differences)		Fresh < 15 Fresh < 18 Fresh < 23	23 > 15 Fresh > 15 Fresh > 18 Fresh > 23
$\delta^{15}\text{N}$			
ANOVA (<i>p</i> value)	< 0.001	< 0.001	< 0.001
Tukey test (significant differences)	18 < 15 Fresh < 15 Fresh < 18 Fresh < 23	18 > 23 Fresh < 18 Fresh < 23	Fresh < 15 Fresh < 18 Fresh < 23
CN			
ANOVA (<i>p</i> value)	< 0.001	< 0.001	< 0.001
Tukey test (significant differences)	Fresh > 15 Fresh > 18 Fresh > 23	18 < 15 Fresh > 15 Fresh > 18 Fresh > 23	Fresh < 15 Fresh < 18 Fresh < 23

A Alder leaves, T TetraMin,
C *Chlorella* sp.

(Table 2). The $\delta^{15}\text{N}$ values of larvae fed *Chlorella* sp. ranged from 12.0 to 12.5 at 15 and 18°C, respectively, while their $\delta^{13}\text{C}$ values ranged from -20.4 to -19.9‰ , and C/N ratios ranged from 4.5 at 15°C to 4.2 at 18°C (Table 2). After lipid normalization, larval $\delta^{13}\text{C}_{\text{norm}}$ values were higher and ranged from -19.6 to -18.8‰ (Table 2).

Trophic fractionations in *C. riparius*

Trophic fractionations of N stable isotope ($\Delta^{15}\text{N}$), calculated with both fresh and altered isotopic base-lines (Fig. 3, upper panel), were weakly affected by temperature only for the *Chlorella* sp. treatment (Table 3). Trophic fractionations of C stable isotope ($\Delta^{13}\text{C}$) were also firstly calculated without lipid normalization of chironomid $\delta^{13}\text{C}$ values. For fresh TetraMin, $\Delta^{13}\text{C}$ values ranged from 1 ± 0.2 to

Table 2 Carbon and nitrogen stable isotopic compositions ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), carbon and nitrogen contents, C/N ratios (mean $\pm$ 1 standard deviation), and carbon stable isotopic compositions after lipid normalization ($\delta^{13}\text{C}_{\text{norm}}$) of

Chironomus riparius Meigen, 1804, larvae fed under different food resources (*T* refers to TetraMin, whereas *C* refers to *Chlorella* sp.) and at three temperatures (15, 18 and 23°C)

Food	Temp. (°C)	<i>n</i>	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	Total C (%)	Total N (%)	C/N	$\delta^{13}\text{C}_{\text{norm}}$ (‰)
<i>T</i>	15	2	$-21.5 \pm 0.4\text{a}$	$11 \pm 0.3\text{a}$	45.7 ± 0.9	10.3 ± 0.9	$4.5 \pm 0.5\text{a}$	$-20.4 \pm 0.1\text{a}$
	18	4	$-22 \pm 0.4\text{ab}$	$10.9 \pm 0.3\text{a}$	46.7 ± 2.4	9.8 ± 0.2	$4.8 \pm 0.2\text{ab}$	$-20.6 \pm 0.4\text{a}$
	23	3	$-22.2 \pm 0.2\text{b}$	$10.5 \pm 0.3\text{a}$	48.3 ± 1	8.9 ± 0.2	$5.4 \pm 0.1\text{b}$	$-20.1 \pm 0.1\text{b}$
<i>C</i>	15	4	$-19.2 \pm 0.2\text{a}$	$12 \pm 0.3\text{a}$	46.8 ± 0.8	10.4 ± 0.5	$4.5 \pm 0.2\text{a}$	$-18.8 \pm 0.2\text{a}$
	18	4	$-20.4 \pm 0.5\text{a}$	$12.5 \pm 0.4\text{a}$	45.9 ± 0.4	11 ± 0.4	$4.2 \pm 0.2\text{a}$	$-19.6 \pm 0.6\text{b}$

Different letters indicate significant differences according to ANOVA

Fig. 3 Effects of temperature on trophic fractionations of carbon and nitrogen stable isotopes ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$, respectively) in *Chironomus riparius* Meigen, 1804, calculated with and without lipid normalizations and based on fresh and old food isotopic baseline

$1.7 \pm 0.5\text{‰}$, while fresh *Chlorella* sp. ranged from -0.4 ± 0.5 to $0.1 \pm 0.2\text{‰}$ (Fig. 3, middle panel). The $\Delta^{13}\text{C}$ values for both these food resources was negatively affected by temperature (p value < 0.05 ; Table 3). For the altered food baseline (i.e. conditioned food), $\Delta^{13}\text{C}$ values ranged from 0.4 ± 0.3 to

$1.1 \pm 0.4\text{‰}$ for TetraMin, and from 1.9 ± 0.7 to $2.4 \pm 0.4\text{‰}$ for *Chlorella* sp. (Fig. 3, middle panel). A negative effect of temperature on $\Delta^{13}\text{C}$ values was only significant for the TetraMin treatment (p value < 0.05 ; Table 3). After lipid normalization, $\Delta^{13}\text{C}_{\text{norm}}$ values calculated with fresh food baseline

Table 3 Results of two-way ANOVA testing the combined effects of temperature and food resources on trophic fractionations of carbon and nitrogen stable isotopes ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) in *Chironomus riparius* Meigen, 1804

			Estimate	SE	<i>t</i> value	<i>p</i> value
Trophic fractionation values of carbon stable isotope were calculated with and without lipid normalizations ($\Delta^{13}\text{C}_{\text{norm}}$ and $\Delta^{13}\text{C}$, respectively), and based on fresh and altered food isotopic baseline	Fresh baseline					
	$\Delta^{15}\text{N}$ (‰)					
	$R^2 = 0.91$	Intercept	4.61	0.60	7.7	< 0.001
	p value < 0.001	Temperature: <i>Chlorella</i> sp.	0.09	0.04	2.3	0.03
		Temperature:TetraMin	− 0.04	0.03	− 1.2	0.25
	$\Delta^{13}\text{C}$ (‰)					
	$R^2 = 0.84$	Intercept	2.56	0.58	4.4	< 0.001
	p value < 0.001	Temperature: <i>Chlorella</i> sp.	− 0.16	0.04	− 4.6	< 0.001
		Temperature:TetraMin	− 0.07	0.03	− 2.3	0.04
	$\Delta^{13}\text{C}_{\text{norm}}$ (‰)					
	$R^2 = 0.84$	Intercept	2.69	0.81	3.3	< 0.001
	p value < 0.001	Temperature: <i>Chlorella</i> sp.	− 0.11	0.05	− 2.3	0.04
		Temperature:TetraMin	0.01	0.04	0.1	0.88
	Altered baseline					
	$\Delta^{15}\text{N}$ (‰)					
	$R^2 = 0.43$	Intercept	− 0.73	1.01	− 0.7	< 0.001
	p value < 0.001	Temperature: <i>Chlorella</i> sp.	0.14	0.06	2.3	0.03
		Temperature:TetraMin	0.08	0.05	1.5	0.16
	$\Delta^{13}\text{C}$ (‰)					
	$R^2 = 0.74$	Intercept	2.72	0.85	3.2	< 0.001
	p value < 0.001	Temperature: <i>Chlorella</i> sp.	− 0.03	0.05	− 0.7	0.52
		Temperature:TetraMin	− 0.11	0.04	− 2.4	0.02
	$\Delta^{13}\text{C}_{\text{norm}}$ (‰)					
	$R^2 = 0.30$	Intercept	2.84	1.13	2.5	0.02
	p value = 0.03	Temperature: <i>Chlorella</i> sp.	0.02	0.07	0.2	0.81
		Temperature:TetraMin	− 0.03	0.06	− 0.5	0.59

were between 2.8 ± 0.1 and $3.1 \pm 0.2\text{‰}$ for TetraMin and between 0.4 ± 0.6 and $1.3 \pm 0.3\text{‰}$ for *Chlorella* sp. (Fig. 3, lower panel) and were negatively correlated with temperature only for *Chlorella* sp. (p value < 0.05; Table 3). When $\Delta^{13}\text{C}_{\text{norm}}$ were calculated with altered food baseline, $\Delta^{13}\text{C}_{\text{norm}}$ values were generally higher and ranged from 2 ± 0.3 to $3.6 \pm 0.2\text{‰}$ (Fig. 3, lower panel), and none of the relationships with temperature were significant (p value > 0.05; Table 3). After combining the observed $\Delta^{15}\text{N}$ values with all existing data, no relationship was found between temperature and $\Delta^{15}\text{N}$ values (Fig. 4A). Furthermore, also a comparison of our $\Delta^{13}\text{C}$ values with all available data (i.e. calculated without lipid normalization and with fresh food baseline) showed no relationship with temperature (Fig. 4B).

Discussion

Our study is the first to assess combined effects of both temperature and food resources on trophic fractionations of carbon and nitrogen stable isotopes using *C. riparius* Meigen, 1804, as a model species. Despite some issues related to lipid contents and food isotopic integrity in experimental treatments of different duration, our controlled laboratory experiments provide trophic fractionation estimates that were similar to those previously reported in literature. We show that temperature is not a key driver per sé of the variability in stable isotope trophic fractionations for *Chironomus* and likely not for other aquatic consumers either.

Fig. 4 Relationship between temperature and trophic fractionations of carbon and nitrogen stable isotopes ($\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) in chironomid larvae. All values were calculated without lipid normalizations and based on fresh food isotopic baselines. Statistical relationships were not significant (p value > 0.05).

Food isotopic integrity in rearing experiments

As expected, food resources and temperature significantly affected the duration of larval development (Fig. 1), and experiments were therefore terminated after different time intervals to allow larvae to reach the same developmental stage (i.e. fourth larval instar just before pupation). In this context, the reliability of trophic fractionation estimates was mainly dependent on the capability of preventing microbial degradation in experiment units to keep constant stable isotopic compositions of food resources (Goedkoop et al., 2006). However, our study showed clear changes in the stable isotopic composition of uneaten food resources, food $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values changing up to 2.5 and 5‰, respectively.

The reported variability in the isotopic baseline of food confirmed the difficulty of keeping constant isotopic conditions in rearing experiments (Goedkoop et al., 2006; Wang et al., 2009; Belle et al., 2015). Microbial degradation of added food leads to a combination of changes in stable isotopic composition of food baselines and the production of altered resources available for larvae (Goedkoop et al., 2006). As chironomid larvae are selective feeders (Berg, 1995), they can select another resource than the added food in the experimental setups (Johnson et al., 1989; Åkerblom & Goedkoop, 2009). Microbial conditioning of food resources in aquatic ecosystems

often produces food (i.e. bacterial mats) with a higher digestibility and nutritional value than the degraded food itself (e.g. Berggren et al., 2010), thus confounding calculations of trophic fractionations. We hypothesized that trophic fractionation values calculated using uneaten $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ food (i.e. old food baseline) values might provide more realistic estimates of trophic fractionation. Some improvements of the experimental design could also increase the accuracy of trophic fractionation estimates for different food resources. Among others, the combined analysis of fatty acids and stable isotopes can help to identify the actual food resource assimilated into consumer biomass (Goedkoop et al., 2006; Masclaux & Richoux, 2017).

Trophic fractionations of nitrogen and carbon stable isotopes

C/N ratios of chironomid larvae were affected by temperature, regardless of the food resource (Table 2). Even though chironomid larvae store most of their energy as glycogen (Hamburger et al., 1996), our results indicated an increase in lipid contents of chironomid larvae during the experiment (see Post et al., 2007). Such an increase in larval lipids contents might lead to a potential decrease in larval $\delta^{13}\text{C}$ values (see DeNiro & Epstein, 1977). For the TetraMin® treatment, we found correlations between larval $\delta^{13}\text{C}$

values, C/N ratios and temperature (Table 2), matching well with the theoretical effects of lipids contents on consumer $\delta^{13}\text{C}$ values. Therefore, a lipid normalization procedure of chironomid $\delta^{13}\text{C}$ values (McConnaughey & McRoy, 1979; Post et al., 2007; Smyntek et al., 2007) was required to account for potential changes in larval conditions regardless of incorporated food resource, and to get more accurate estimates of trophic fractionation. Although our data met the requirements of the lipid normalization procedure (Post et al., 2007; Smyntek et al., 2007), the applied lipid correction may not be accurate for chironomid larvae and may need further refinements (Kiljunen et al., 2006), e.g. by direct comparisons of chironomid $\delta^{13}\text{C}$ values with and without lipid extraction (see Logan et al., 2008).

Uncertainties associated with measurements of larval $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in our study were very low, indicating that the observed differences between treatments were accurate and reproducible. Trophic fractionations of nitrogen stable isotope ($\Delta^{15}\text{N}$) calculated using fresh (i.e. initial food samples) and altered (i.e. collected uneaten food at the end of the experiment) food was significantly lower with altered food $\delta^{15}\text{N}$ values. Our $\Delta^{15}\text{N}$ values ranged from 0.6 to 6.3‰ and were similar to those previously reported for *C. riparius* at 20°C (Goedkoop et al., 2006) and at 23°C (Wang et al., 2009), suggesting the absence of profound effects of temperature (Fig. 4A). Our findings of $\Delta^{15}\text{N}$ estimates also confirmed that any temperature effects on $\Delta^{15}\text{N}$ values for aquatic consumers are not straightforward. Indeed, findings from other aquatic organisms suggested a general trend of decreasing $\Delta^{15}\text{N}$ values with increasing temperature (Power et al., 2003; Barnes et al., 2007), whereas other studies reported an opposite trend (Schilder et al., 2015; Masclaux & Richoux, 2017). In our study, temperature effects on $\Delta^{13}\text{C}$ followed different patterns when calculated with fresh or altered isotopic baselines, and with or without larval lipid normalization. Indeed, temperature significantly affected the $\Delta^{13}\text{C}$ values when calculated with fresh isotopic baselines and without lipid normalization, but no effects were observed when lipids contents and microbial alteration of food resources were taken into account (Table 3). However, the observed $\Delta^{13}\text{C}$ values between − 0.6 and 1.6‰ were similar to those previously reported at 20°C (Goedkoop et al., 2006; Frossard et al., 2013) and at 23°C (Wang et al., 2009;

Fig. 4B). Hence, we conclude that there are no effects of temperature on $\Delta^{13}\text{C}$ values for chironomid larvae (p value > 0.05; Fig. 4B). This conclusion, however, could conflict with previous studies on *Daphnia* sp., Cladocera (Power et al., 2003; Schilder et al., 2015; Masclaux & Richoux, 2017) that reported inconsistent but significant effects of temperature on $\Delta^{13}\text{C}$ values.

Conclusion

We conclude that temperature likely is not a key driver of the variability in trophic fractionation values of both C and N stable isotope for aquatic consumers. This strengthens the use of stable isotope composition of aquatic consumers for the evaluation of the trophic structure and resource dynamics in aquatic food webs also under ongoing climate change and for long-term change such as in paleolimnological studies. However, the reported high variability in trophic fractionation estimates could lead to large uncertainties regarding resource partitioning and trophic relationships in food webs, and variability in trophic fractionation estimates should, however, be considered. Our results illustrate confounding effects of microbial degradation of food particles in rearing experiments, potentially resulting in erroneous estimates of trophic fractionation. Our observed effects of temperature on C/N ratios of chironomid larvae suggest potential changes in lipid contents and associated effects on larval $\delta^{13}\text{C}$ values.

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