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ORIGINAL PAPER

Effects of pre-treatment procedures, mounting media, and slide storage on morphometric measurements of subfossil chironomid head capsules: recommendations for reconstructing phenotypic plasticity

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Abstract Conducting morphometric measurements on subfossil chironomid head capsules will be key to uncovering the long-term mechanisms of body size variations of aquatic invertebrates. However, the wider use of subfossil chironomids in phenotypic plasticity reconstructions has been limited by three main methodological gaps regarding the influence of laboratory procedures on the size of subfossil chironomids. This study aimed to investigate the effects of the most used chemical pre-treatments and mounting media on chironomid head capsule lengths and to quantify the influence of storage time on chironomid head capsule measurements. Results showed no effect of mounting media and chemical pre-treatments on chironomid head capsule lengths and that measurements did not significantly change with increasing slide storage time, after 1 and 5 months. These results therefore indicate that it is possible to re-examine existing slide collections regardless of laboratory procedures, mounting media, and storage time. Furthermore, since measurements taken from HC mounted in water did not differ from other procedures, HC may be re-used after morphometric measurements for stable isotope analyses. Combining these two promising approaches will enable us to disentangle the influence of food source and temperature variations on chironomid body size. Filling these three methodological gaps will allow us to make reliable inferences about long-term changes in phenotypic plasticity using subfossil chironomids, thus contributing to better predictions of future climate-related changes in body size of aquatic invertebrates.

Keywords Chironomidae · Paleolimnology

Introduction

Phenotypic plasticity may allow ectothermic species to persist under changing thermal conditions, unless thermal tolerance limits are reached. Declines in body size have been widely observed in aquatic invertebrates along gradients of increasing temperature (Bonacina et al. 2023), but long-term trends in aquatic invertebrate body size in nature remain largely unknown. The study of lake sediments is a promising approach to extend the temporal scale over which body size variation is usually studied allowing us to unravel, among others, the influence of different climate characteristics on phenotypic plasticity. In particular, the head capsules (HC) of Chironomidae (Arthropoda; Diptera; Nematocera), one of the most diverse and abundant insect families in lakes, are morphologically well preserved in lake sediments

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and can serve as surrogates for broader biodiversity in aquatic ecosystems (Walker 2001). Like other aquatic invertebrates, temperature affects chironomid larval size (Frouz et al. 2002; Baek et al. 2012), conducting morphometric measurements on subfossil chironomid HC will be a promising approach to uncovering the long-term mechanisms of phenotypic plasticity in aquatic invertebrates. This will allow us to make better predictions of future climate-related changes in body size of aquatic invertebrates.

However, the wider use of subfossil chironomids in phenotypic plasticity reconstructions is limited by three major methodological gaps. First, the preparation of subfossil chironomid HC often involves using chemical solutions to deflocculate particles and remove carbonates from sediment samples (Walker 2001). Numerous laboratory procedures have been used with variations in the type and concentration of chemicals used, the time spent in these chemicals, and the temperatures (Luoto 2009; Heiri et al. 2011; Belle et al. 2022; de Mendoza et al. 2024). In contrast to the large existing literature focusing on the effects of preservatives on invertebrate body size (Nolte 1990; von Schiller and Solimini 2005), the effect of sediment pre-treatments on the morphometric characteristics of subfossil chironomid HC is still largely unknown. Second, chironomid HC are mounted on microscope slides using various mounting media for long-term preservation, including Euparal® (Tátosová et al. 2006) and Aquatex® (Belle et al. 2024), and other media have also been used (Pro-Texx® in Massaferro et al. 2009; Permount® or Entellan in Porinchu et al. 2009; Berlese mounting medium in Hamerlik et al. 2022). Mounting media may cause immediate shrinkage or expansion of the chironomid remains due to, for example, changes in water content, but their effect on the morphometric characteristics of chironomid HC is not yet quantified. Third, microscope slide preparations may shrink over time due to the aging of the mounting medium, as evidenced by cracks and bubbles frequently observed in archived slides. Neuhaus et al. (2017) reported that the deterioration of microscope slides can be significantly influenced by the type of mounting medium used, noting that permanent mounting media tend to provide greater long-term stability compared to water-based alternatives, highlighting the importance of medium selection in collection management. The influence of mounting procedures on HC size measurements remains, however, unstudied. If the length of mounted chironomid HC does not further change with increasing preservation time, this will allow us to re-examine existing subfossil chironomid slide collections. Addressing these three methodological gaps will enable robust inferences about long-term changes in phenotypic plasticity in subfossil chironomids, regardless of differences in laboratory methods, mounting procedures, or slide storage.

Some of the observed exceptions to the general pattern of decreasing body size in response to warming (Hayden et al. 2017; Bonacina et al. 2023) might reflect the effects of additional environmental drivers that regulate the body size of aquatic invertebrates (Davidowitz et al. 2004). Among others, variations in the diet of aquatic ectotherms can affect their final body size with effect size comparable to that of temperature and, for example, a lipid-rich diet usually results in producing larger individuals (Vos et al. 2000). Therefore, understanding size variations in nature requires disentangling the effects of these additional environmental drivers in regulating ectotherm body size. Subfossil chironomid HC are also chemically well-preserved in sediments (Verbruggen et al. 2010), and stable isotope analysis, mainly of carbon ($\delta^{13}C$), of chironomid HC offers both quantitative and qualitative insights into resource partitioning and the contribution of carbon sources to consumer biomass (Belle et al. 2017). This approach has been successfully applied to reconstruct long-term changes in food sources incorporated into chironomid biomass over the last decades (Wooller et al. 2012; Frossard et al. 2014; Belle et al. 2018). Combining carbon stable isotope and morphometric measurements of subfossil chironomid HC should be a promising approach to disentangle the respective influence of food source and temperature variations on body size. However, HC suitable for both morphometric and stable isotope analyses typically share the same characteristics (i.e., unbroken and belonging to the 4th instar larvae) and such specimens are often scarce in lake sediments. This scarcity limits the feasibility of combining these two promising approaches. Alternatively, HC may be usable for both stable isotope analyses and morphometric analyses if measurements taken from HC temporarily mounted in water produced comparable measurements; however, this method requires further

testing to assess the potential effects of mounting procedures on HC size.

In this study, the effects of (*i*) the most applied chemical pre-treatments, (*ii*) mounting media on chironomid HC lengths were investigated and (*iii*) the influence of slide storage duration on HC measurements was quantified. HC measurements were hypothesized to remain unaffected by laboratory procedures and slide storage, thereby supporting the use of subfossil chironomid HC in phenotypic plasticity reconstructions.

Material and methods

Using five newly spawned egg masses (< 1 day after spawning), a population of *Chironomus riparius* Meigen was cultured in December 2024 in a 50 L aquarium system filled with 4 cm of sea sand and tap water, and high-oxygen condition was maintained using an aquarium air pump. Larvae were grown at room temperature (20 °C), under a natural photoperiod, and fed with PRO NOVO BEL® (JBL) reduced to a fine powder. When sufficient chironomids (> 300) emerged, the aquarium substrate was sieved through a 200 µm mesh sieve and > 300 HC were picked out for further analysis. Only HC belonging to the fourth larval instar was collected and further used for morphometric purposes.

To quantify the effects of chemical pre-treatments on chironomid HC lengths, HC were treated following the different procedures detailed in Table 1. Each treatment was reproduced three times with 10 HC in each replicate. Briefly, HC were washed using distilled H_2O at room temperature (control treatment); distilled H_2O heated at 70 °C for 1 h; NaOH solution at 10% for 15 min; NaOH solution at 10% heated at 70 °C for 30 min; or NaOH solution at 10% for 15 min followed by HCl solution at 3.7% for 5 min. Then, HC were rinsed using distilled water and mounted between microscope slides using Aquatex®. After one day of drying, measurements were performed using a microscope camera coupled to the Nikon Digital Sight 1000 software. The measurements consisted of taking the length between the top of the median tooth of the mentum and the base of the HC as shown in ESM1. Those measurements were performed on the axial plane of the HC, thus being not influenced by variations in slide preparation (e.g., not sensitive to the flattening between slides unlike measurements of HC widths).

To quantify the effect of mounting media on chironomid HC lengths, HC were mounted using different media as summarized in Table 1. Each medium was reproduced three times, and 10 HC were used in each replicate. Briefly, HC were washed using NaOH solution at 10% for 15 min followed by HCl solution at 3.7% for 5 min, and mounted under microscope slides using water (control medium), Aquatex®, Naphrax®, and Euparal®. Using Naphrax® is not recommended for chironomid HC mounting. However, this procedure can potentially represent the most impactful procedure potentially damaging the remains as it involves heating the slide to evaporate the solvent and solidify the medium. The successive heating and cooling phases may therefore induce the expansion and/or the shrinkage of the mounted HC. After one day of drying at room temperature, measurements of HC length were performed as described above.

To quantify the effect of slide storage duration on chironomid size, HC lengths were re-measured after one and 5 months of storage in plastic slide boxes and in the dark at room temperature (Table 1). The same

Table 1 Summary of the different tested combinations of pre-treatments, mounting media, and storage durations

Pre-treatment	Time spent	Mounting medium	Storage time
H_2O	30 min	Aquatex	*t*0, *t*0 + 1 month, *t*0 + 5 months
H_2O 70 °C	60 min	Aquatex	*t*0, *t*0 + 1 month, *t*0 + 5 months
NaOH 10%	15 min	Aquatex	*t*0, *t*0 + 1 month, *t*0 + 5 months
NaOH 10% 70 °C	30 min	Aquatex	*t*0, *t*0 + 1 month, *t*0 + 5 months
NaOH 10% + HCl 3.7%	15 + 5 min	H_2O	*t*0
NaOH 10% + HCl 3.7%	15 + 5 min	Aquatex	*t*0, *t*0 + 1 month, *t*0 + 5 months
NaOH 10% + HCl 3.7%	15 + 5 min	Naphrax	*t*0, *t*0 + 1 month, *t*0 + 5 months
NaOH 10% + HCl 3.7%	15 + 5 min	Euparal	*t*0, *t*0 + 1 month, *t*0 + 5 months

path was followed on the microscope slide, starting from the top right and screening the slide by moving down and to the left in increments slightly less than one optical field, to ensure matching measurements from the same HC.

Data analysis

One-way ANOVAs were used to test for the significance (with $\alpha = 0.05$) of the individual effects of chemical pre-treatments, and mounting media. Differences between HC lengths measured one or five months after and measurements taken immediately after mounting were calculated, thus taking negative values if HC lengths decreased as storage progressed and positive values if HC lengths increased. One-way ANOVA was used to test for the significance (with $\alpha = 0.05$) of slide storage duration on HC lengths. Assumptions of normality and variance were tested, and as no violations were detected and data were, therefore, not transformed. All statistical analyses and plots were performed using the R 4.4.1 software (R Core Team 2024).

Results

For the first test, HC were washed using distilled H_2O at room temperature (control treatment); distilled H_2O heated at 70 °C for 1 h; NaOH solution at 10% for 15 min; NaOH solution at 10% heated at 70 °C for 30 min; or NaOH solution at 10% for 15 min followed by HCl solution at 3.7% for 5 min and HC mounted between microscope slides using Aquatex® (Table 1). HC treated with distilled water at room temperature and mounted with Aquatex® had lengths of 332.8 ± 16 µm and 359.4 ± 15.3 µm for males and females, respectively. All tested treatments gave comparable measurements (Fig. 1), and none of the pre-treatment showed significant differences with the control treatment (p-value > 0.05; Table 2).

For the second test, HC were treated using the same laboratory procedure (NaOH solution at 10% for 15 min followed by HCl solution at 3.7% for 5 min) but microscope slides were prepared by mounting HC with distilled water, Aquatex®, Naphrax®, and Euparal®. (Table 1). The HC mounted in water (control medium) had lengths of 327.6 ± 10.2 µm and 359.9 ± 15 µm for males and females, respectively

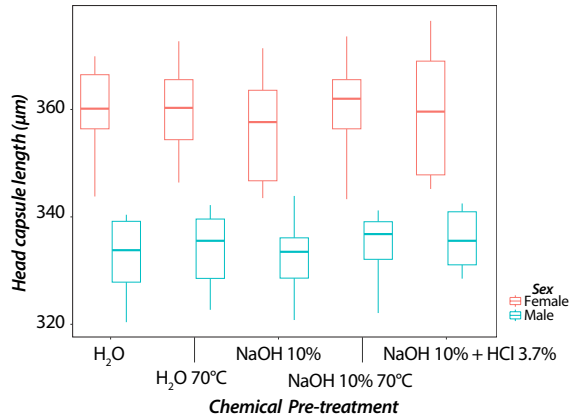

Fig. 1 Boxplots showing the effect of pre-treatments on head capsule lengths of *Chironomus riparius*, with male (in blue) and female (in red). The tested pre-treatments are summarized in Table 1, and head capsules were then mounted with Aquatex®. None of the differences were significant (p-value > 0.05)

(Fig. 2). None of the mounting media showed a significant difference with the control medium (p-value > 0.05; Table 2). As expected, the mounting procedure with Naphrax® induced severe damage to the HC and numerous HC were found broken under the microscope slides. However, HC measurements mounted with Naphrax® did not significantly differ from the other mounting media (Fig. 2).

After one and five months of storage, measurements were retaken from the same HC mounted in semi-permanent media (Table 1). Overall, the difference between HC measured one month and measurements taken immediately after mounting ranged from − 6.3 to 7.2 µm with an average of 0.2 µm (Fig. 3A), corresponding to a relative change ranging from − 1.9 to 2.2% (Fig. 3B). After five months of storage, these differences ranged from − 6.1 to 7.3 µm with an average of 0.8 µm (Fig. 3A), corresponding to a relative change ranging from − 1.7 to 2.3% (Fig. 3.B). None of the pre-treatments and mounting media combinations significantly changed with increasing storage time (p-value > 0.05).

Discussion

This study aimed to fill methodological gaps in the link between laboratory procedures and chironomid HC size to enable wider use of subfossil chironomids

Table 2 Results of one-way ANOVA testing the effects of chemical pre-treatments, and mounting media on head capsule lengths of *Chironomus riparius*

Pre-treatment					
		Estimate (µm)	Std. error	t value	*p*-value
Female					
$R^2 = 0.03$	H_2O (intercept)	359.4	2.3	158.2	< 0.001
p-value = 0.64	H_2O 70 °C	0.3	3.3	0.105	0.92
	NaOH 10%	− 3.3	3.1	− 1.06	0.29
	NaOH 10% 70 °C	1.1	3.1	0.349	0.72
	NaOH 10% + HCl 3.7%	0.1	3.3	0.043	0.96
Male					
$R^2 = 0.04$	H_2O (intercept)	332.8	1.7	192.4	< 0.001
p-value = 0.69	H_2O 70 °C	1.2	2.4	0.508	0.61
	NaOH 10%	− 0.4	2.6	− 0.15	0.88
	NaOH 10% 70 °C	1.9	2.8	0.723	0.47
	NaOH 10% + HCl 3.7%	3	2.5	1.188	0.24
Mounting media					
		Estimate (µm)	Std. Error	t value	*p*-value
Female					
$R^2 = 0.07$	H_2O (intercept)	358.9	3.3	108.8	< 0.001
p-value = 0.35	Aquatex	0.7	4.2	0.167	0.87
	Euparal	− 3.2	4.3	− 0.745	0.46
	Naphrax	− 6.4	4.6	− 1.367	0.18
Male					
$R^2 = 0.11$	H_2O (intercept)	332.6	1.4	230.8	< 0.001
p-value = 0.09	Aquatex	3.2	2.2	1.472	0.15
	Euparal	1.6	2.1	0.792	0.43
	Naphrax	− 2.4	2.1	− 1.12	0.27

in phenotypic plasticity reconstructions. Results showed that the tested combinations of pre-treatments, and mounting media did not significantly affect HC measurements. Furthermore, the length of the chironomid HC did not show any further change with increasing storage time (up to 5 months), thus allowing us to re-examine existing slide collections regardless of the laboratory procedures, mounting media, and storage duration. However, there is no standard protocol for subfossil chironomids preparation and numerous laboratory procedures are frequently used in subfossil chironomid research. For example, Zheng et al. (2021) treated sediment samples using 10% KOH at 75 °C for 15 min and HC were mounted with Hydro-matrix®, while Lapellegerie et al. (2024) did not use any chemical pre-treatment and samples were simply sieved gently under water before mounting HC using Euparal®. Additionally, the present study focused only on three semi-permanent media (Aquatex®, Naphrax®, and Euparal®) which is a small subset of the mounting media frequently used for processing subfossil chironomids. For example, HC were mounted with Permount® or Entellan by Porinchu et al. (2009); with Hydro-matrix® by Zheng et al. (2021); with Berlese by Hamerlik et al. (2022); and dehydrated in 96% ethanol and mounted in Euparal® by de Mendoza et al. (2024). Whereas further rigorous tests are needed to rule out potential effects of these procedures, the untested media share similar chemical and optical properties with the tested ones (see also Neuhaus et al. 2017 for the most recent literature review on mounting media characteristics). As

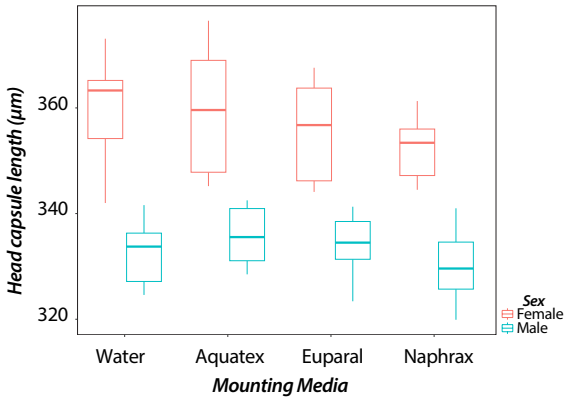

Fig. 2 Boxplots showing the effect of mounting media on head capsule lengths of *Chironomus riparius*, with male (in blue) and female (in red). Head capsules were previously washed using NaOH solution at 10% for 15 min followed by HCl solution at 3.7% for 5 min. The tested mounting media are summarized in Table 1. None of the differences were significant (p-value > 0.05)

a result, these slight variations in the type and concentration of chemicals used, the time spent in these chemicals, the temperatures, and the mounting media are not expected to influence the aging of the slides or chironomid HC sizes. It is, therefore, reasonable to assume that these different laboratory procedures should produce comparable results among studies. Based on these findings, no specific recommendations are needed to use subfossil chironomid HC in phenotypic plasticity reconstructions, and any laboratory procedures may allow making reliable inferences about long-term body size variations of subfossil chironomids.

Furthermore, measurements taken from HC mounted with water did not differ from the ones taken from other mounting media. This finding indicates that temporarily mounting HC with water is a possible strategy allowing us to reuse HC for other purposes. Specifically, stable isotope analysis of HC provides insights into long-term changes in food sources incorporated into chironomid biomass (Essert et al.

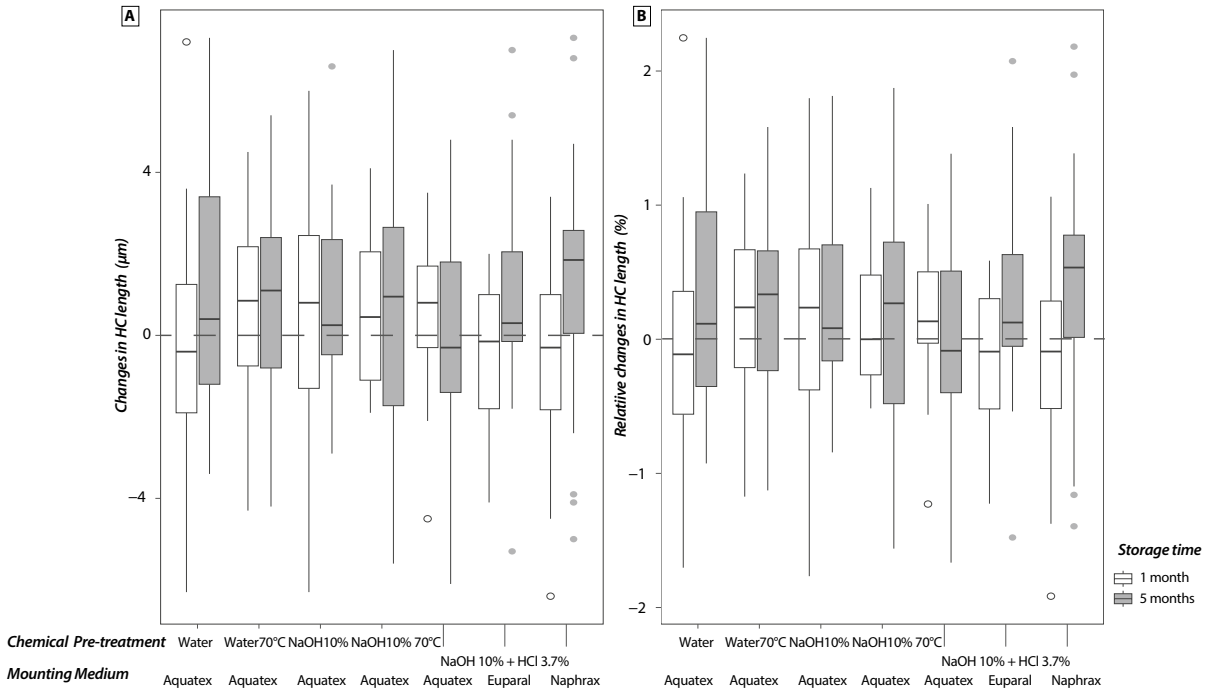

Fig. 3 Boxplots showing the differences in head capsule lengths of *Chironomus riparius* measured after one (in white) and five (in grey) months of storage at room temperature and immediately after mounting. Differences were negative values if head capsule lengths decreased as storage progressed and were expressed as actual differences (**A**; in µm) and relative changes (**B**; in %). The tested pre-treatments are summarized in Table 1. None of the differences were significant (p-value > 0.05)

2024) and could be a promising approach to disentangle the respective influence of food sources and temperature variations on body size. However, this type of analysis typically requires collecting a high number of HC (van Hardenbroek et al. 2010), and suitable HC are usually scarce in lake sediments. Therefore, using HC for both morphometric measurements of subfossil chironomids and then carbon stable isotope could be a promising approach to deal with the scarcity of HC in lake sediment and provide insights into potential additional drivers of phenotypic plasticity of subfossil chironomids. Understanding how body size of aquatic invertebrates was affected by climate change and other environmental changes in the past should be a crucial step towards realism that will allow us to make better predictions of future climate-related changes in the body size of aquatic invertebrates.

Author contributions SB conducted the study and wrote the paper.

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Data availability Data will be made available upon request.

Declarations

Conflict of interest The authors declare no competing interests.

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