










## Effects of formaldehyde preservation time on the length-weight relationship of the ubiquitous neotropical cladoceran *Ceriodaphnia silvestrii*

Efeito do tempo de preservação por formaldeído na relação peso-comprimento do ubíquo cladóceros neotropical *Ceriodaphnia silvestrii*

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**Abstract: Aim:** In this study, the effect of preservation time on total body length and dry weight of adult specimens of the neotropical cladoceran *Ceriodaphnia silvestrii* preserved with 4% formalin solution were examined. **Methods:** The relationship between these variables was examined under increasing gradual time effects (i.e. 7, 30, and 60 days) after preservation using linear models and analysis of variance. **Results:** Total body length did not statistically differ between fresh and preserved cladocerans at any preservation time, whereas dry weight was drastically reduced with increasing preservation time, with 15, 47 and 57% weight losses. Length-weight relationships were significantly and positively related in all treatments, though higher values of slope were found for fresh and 7 days samples. **Conclusions:** We highlight that, for *Ceriodaphnia silvestrii*, the use of the formalin solution as a preservation fixative is not adequate when the major interest is biomass estimation. Also, we recommend that dry weight estimations from preserved samples should be done as soon as possible. Finally, considering the preservation losses and intra-specific composition of organisms, the application of correction factors is advised since preserved samples are important in the evaluation of long-term changes of biological communities.

**Keywords:** body length; Daphnidae; dry weight; fresh weight; secondary productivity.

**Resumo: Objetivo:** Neste estudo, examinamos o efeito do tempo de preservação no comprimento total do corpo e no peso seco de espécimes adultos do cladóceros neotropical *Ceriodaphnia silvestrii* fixados com solução de formalina a 4%. **Métodos:** A relação entre essas variáveis foi examinada sob efeito crescente do tempo (7, 30 e 60 dias) após a preservação usando modelos lineares e análise de variância. **Resultados:** O comprimento corporal total não diferiu estatisticamente entre os cladóceros não-preservados e preservados, enquanto o peso seco foi reduzido com o aumento do tempo de



preservação, resultando 15, 47 e 57% de perda de peso, respectivamente. As relações peso-comprimento foram significativamente positivas em todos os tratamentos, embora valores mais elevados tenham sido encontrados em amostras frescas e de 7 dias. **Conclusões:** Destacamos que, para *Ceriodaphnia silvestrii*, o uso da solução de formalina como método de preservação não é adequado quando o principal interesse é a estimativa de biomassa. Recomendamos que as estimativas de peso seco de amostras preservadas sejam feitas o mais rápido possível. Por fim, considerando as perdas atribuídas à preservação e às variações intraespecíficas da biomassa dos organismos, recomenda-se a aplicação de fatores de correção uma vez que amostras preservadas são importantes na avaliação de mudanças de longo prazo nas comunidades biológicas.

**Palavras-chave:** tamanho corporal; Daphnidae; peso seco; peso fresco; produção secundária.

## 1. Introduction

Biological and ecological studies often require biometrical measurements, biomass calculations or estimates as tools to evaluate community structure. Size *per se* influences functional and ecological traits of organisms and their role in ecosystem processes (Brown et al., 2004; Woodward et al., 2005). As a result, studying the influence of body size on trophic efficiency has become a prominent focus in ecology and applied biology, emphasizing the need for accurate and efficient individual and population measurements (LaBarbera, 1989; Wetzel et al., 2005; Costa-Paiva et al., 2007; Souza & Barros, 2017). Most often, measurements are based on dry weight (e.g. Platt et al., 1969; Dumont et al., 1975; Bottrell et al., 1976; Culver et al., 1985), which ideally should be taken on fresh, unpreserved material. However, an immediate sorting of sampled material, as well as any subsequent processing, are almost impossible under field conditions and samples need to be preserved immediately after collection. Thus, qualitative and quantitative measurements are mostly determined from preserved organisms.

Generally, buffered formalin solution and ethanol are considered to be suitable preservatives and have been commonly used as fixative for zooplankton samples and other invertebrate groups (Souza & Barros, 2017). Nearly one hundred years ago, several studies already described deleterious effects of those preservatives on dry weight of preserved animals (e.g.: Geng, 1925; Omori, 1978; Giguère et al., 1989). Impacts of preservatives on biometrical characteristics of macroinvertebrate fauna is also largely known (Mackay & Kalf, 1969; Donald & Paterson, 1977; Wiederholm & Eriksson, 1977; Maslin & Pattee, 1981), as well as for being also much less appropriate fixative (e.g. in genetic analysis) than absolute ethanol (Timm & Martin, 2015). However, studies evaluating the effects of preservation of freshwater zooplankton samples are scarce and most of the published

literature comes from temperate regions (e.g. Giguère et al., 1989; Omori, 1978; Pakhomov, 2003). Therefore, considering that methodological studies evaluating the reliability of primary data (e.g. density, composition and biomass) are critical for efficient environmental characterization such as trophic conditions (Sendacz et al., 2006; Klippel et al., 2020), and biomonitoring studies for ecological impact assessment (Leppänen, 2018). Thus, it is a critical step in environmental studies, and its broad objectives, to preserve samples' characteristics as much as possible for more reliable ecological interpretations (Huffman et al., 2020).

Length-weight regressions are one of the most commonly used methods for estimating zooplankton dry weight and biomass (Dumont et al., 1975; Bottrell et al., 1976; McCauley, 1984; Culver et al., 1985). Such analysis allows the assessment of a rather difficult measurement, the dry weight, by using a much easier biological parameter to obtain – the body length – plus applying linear regression equations. These relationships between zooplankton body length and weight are well documented in the literature, being often used in zooplankton studies for dry weight estimations (Blettler & Bonecker, 2006; Brito et al., 2013). As proposed by Edmondson & Winberg (1971) and Edmondson (1974), species biomass can be calculated from the average lengths and organisms' densities through formulae relating the dry weights of individuals to their lengths for the main cladocerans species, using the formula  $W = aL^b$ , where:  $W$  = weight,  $a$  = intercept,  $L$  = length, and  $b$  = slope. However, not all species have their respective specific formula, especially for the Neotropical species. Thus, while lacking specific equations, studies often apply the constants 'a' and 'b' obtained for different species of the same genus (Pace & Orcutt Junior, 1981; Santos et al., 2010) adding one more relevant confounding factor. Additionally, only few studies have been carried out to estimate dry weight losses due to chemical fixative agents on freshwater zooplankton samples (Omori, 1978). Such gap of

knowledge regarding preserved samples dry weight variation can drive scientists to misleading length-weight models as a consequence of not having accurate data. Therefore, it is highly important to assess and quantify such losses among zooplankton representatives in order to provide more reliable models that can also account for losses over the course of preservation time.

Concerning the zooplankton fauna, many are the issues for the direct weighing of individuals and assessing dimensional characters: i) the taxonomic diverse and small-sized taxa, ii) their differential and fragile body structures with variable effects on osmotic balance (Durbin & Durbin, 1978; Miliou & Moraitou-Apostolopoulou, 1991) that can be altered by chemical substances such as formaldehyde (Williams & Robins, 1982), iii) biomass variations related to habitat type, levels of nutrient enrichment and biotic factors such as predation pressure (Tessier et al., 1983; Culver et al., 1985), and iv) continuous reproduction and overlapping cohorts, which is the case of most neotropical species. To overcome these issues, specific methods are used to infer zooplankton production (Winberg, 1971; McCauley, 1984; Harris et al., 2000), including the widely used geometric-based method and the length-weight regressions (e.g., Edmondson & Winberg, 1971; McCauley, 1984; Castilho-Noll & Arcifa, 2007; Ghidini & Santos-Silva, 2009). For microcrustaceans (cladocerans and copepods), weight estimates from length-weight regressions have been the most frequently used technique. Cladocerans are important components of zooplanktonic communities of freshwater ecosystems, and account for almost 45-91% of the sizable fraction of secondary production (Pederson et al., 1976). In lentic aquatic systems, they occupy a central position within food chains and are important components of zooplankton in temporary and perennial lakes and ponds (Forró et al., 2008; Karuthapandi & Rao, 2016). Furthermore, due to their short life cycle, predominantly parthenogenetic reproduction rates and intermediate position in lake's food web (Jeppesen et al., 2011), cladoceran species are commonly used as indicators of environmental changes (Kurek et al., 2010; Brasil et al., 2019).

In this study, we evaluated the effect of 4% formalin solution buffered with sodium tetraborate (borax) on body length and dry weight of the widely spread neotropical cladoceran *Ceriodaphnia silvestrii* Daday 1902 (Daday, 1902) sampled in a tropical reservoir. We compared the body length

and dry weight of fresh-unpreserved and preserved specimens from fixation up to 7, 30 and 60 days to test the following hypotheses: i) the negative effect of preservation time with formalin is stronger on dry weight than on body length, and ii) slope differences and low correlations among treatments are expected for length-weight regressions of fixed specimens, being related to preservation time.

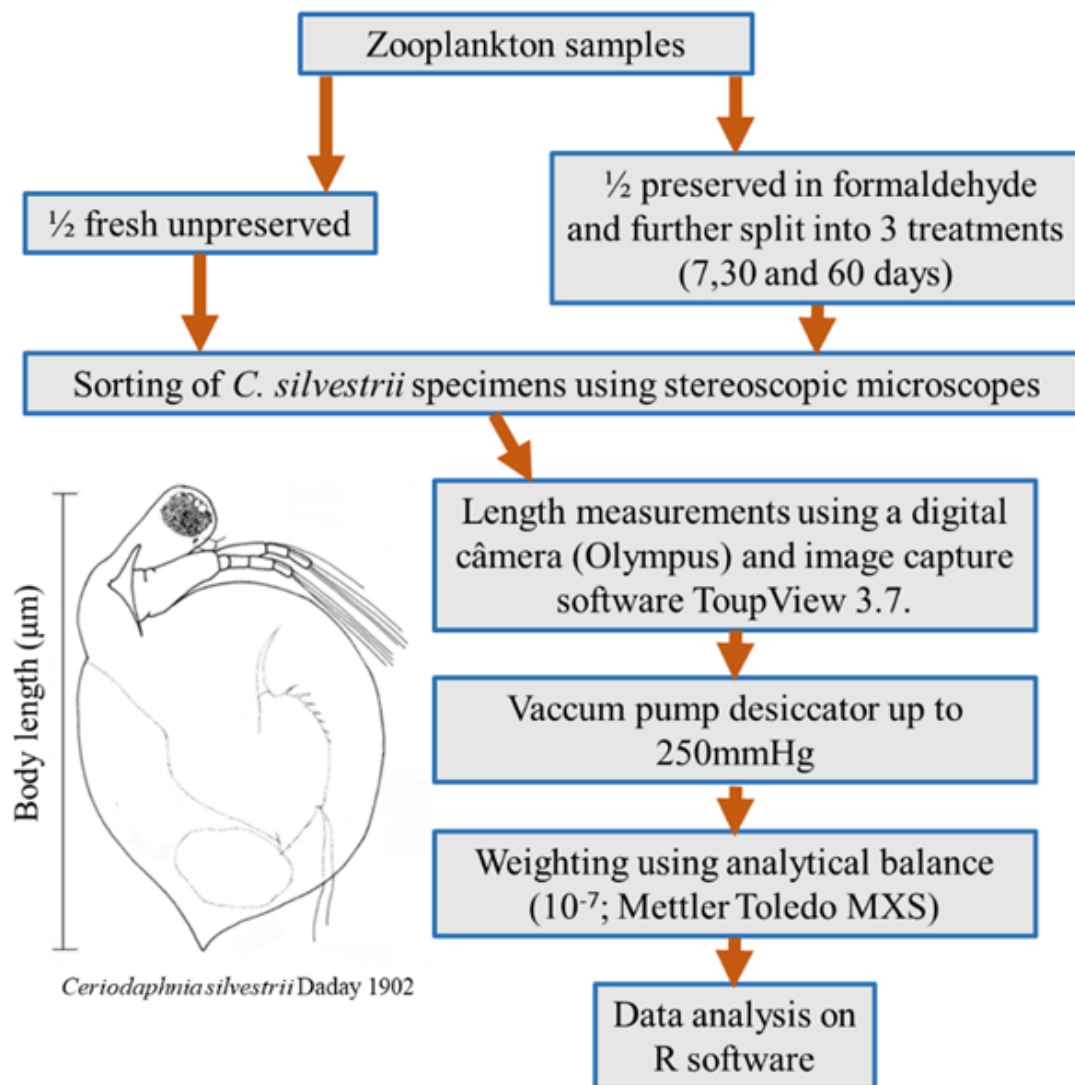
## 2. Material and Methods

### 2.1. Assay organism: *Ceriodaphnia silvestrii* Daday 1902 (Daphnidae)

Locally occurring *Ceriodaphnia silvestrii* was collected in Ribeirão das Lajes hydroelectric Reservoir (RLA; 22°43'S, 22°46'S; 44°30'W, 44°60'W), an oligotrophic system located in Southeastern Brazil, in Rio de Janeiro state. The reservoir is characterized by low chlorophyll-*a* and high water temperature, normally above 25° C (Guarino et al., 2005; Soares et al., 2008; Klippel et al., 2020). Due to these characteristics, the zooplankton community in RLA is composed by small-sized (e.g. *Bosmina hagmani* <0.5mm) and medium-sized cladocerans (e.g. *C. silvestrii* 0.5-1.0mm) (Macêdo et al., 2019). *C. silvestrii* was selected for three major reasons: i) medium-sized zooplankton species are generally more representative in terms of biomass than smaller ones (e.g. Hanazato, 1998), ii) being *Ceriodaphnia* a widely spread genus in Brazil (Elmoor-Loureiro, 2000), and iii) *C. silvestrii* is among the most abundant species in RLA (Macêdo et al., 2019).

### 2.2. Sampling and preserving

Zooplankton samples were collected from the subsurface of the water column (total volume of 100L) in a pelagic zone using graduated bucket (20L), and then filtered using a plankton net (64µm mesh size). Thus, specimens were sampled once and from the same patch to minimize environmental influence on specimen's morphometry. Half of the samples were immediately preserved in formalin solution (final concentration 4%) buffered with sodium tetraborate (borax), and the other half was kept fresh and unpreserved (Figure 1). Formalin solution at 4% concentration was evaluated as it is one of the most commonly employed preservation techniques according to literature (Lincoln & Sheals, 1979) and has been known to cause changes in both length and weight in an array of organisms and tissues (Souza & Barros, 2017; Costa et al.,



**Figure 1.** Work flow chart showing the methodological steps of the experimental work.

2021), as well, for being predominantly used for *Ceriodaphnia* (Table 1).

### 2.3. Experimental design

A total of 840 specimens of *C. silvestrii* were split into four treatments: fresh (unpreserved) and preserved samples submitted to three different preservation times (7, 30, and 60 days). For each treatment, the specimens (N =210) were split into seven replicates containing 30 individuals. In the laboratory, intact organisms were carefully identified to the lowest level of taxonomic hierarchy aiming to avoid inter genus-variation, under an optical microscope (Olympus BX-50) at x400 magnification using specific literature (Elmoor-Loureiro, 1997). Eggs and embryos were carefully removed before drying and weighing processes. Specimens' body

length measurements were carried for each single individual from anterior margin of head (or crest) to posterior margin of valves (Figure 1) using an optical microscope (Zeiss Olympus BX-50) at 400 x magnification, a digital camera (Olympus), and image capture software ToupView 3.7.

Replicates weighing was done in the laboratory using the following protocol: 30 individuals of each replicate were pooled into a petri dish, dried at 60°C for 24 hours, then cooled in a vacuum pump desiccator up to 250mmHg at room temperature before weighing in an analytical balance (10<sup>-7</sup>; Mettler Toledo MX5).

### 2.4. Data analysis

First, we calculated the mean length and weight for each different treatment as individuals

**Table 1.** Previous studies using fresh or formalin-preserved specimens of freshwater cladocerans. NM = not mentioned.

Reference	preserved or fresh	preservation method	preservation time
Matsumura-Tundisi et al., 1989	fresh	-	-
Mangas & Garcia, 1991	preserved	4% formalin	NM
Nandini et al., 2005	preserved	10% formalin	NM
Maia-Barbosa & Bozelli, 2005	preserved	4% formalin	~1year
Sendacz et al., 2006	preserved	4% formalin	NM
Corgosinho & Pinto-Coelho, 2006	preserved	4% formalin	NM
Bonecker et al., 2007	preserved	4% formalin	NM
Guevara et al., 2009	preserved	4% formalin	NM
Santos et al., 2010	preserved	4% formalin	NM
Bonecker et al., 2011	preserved	4% formalin	NM
Brito et al., 2013	preserved	4% formalin	>3 years
Silva et al., 2014	preserved	4% formalin	NM
Burgis, 1974	preserved	5% formalin	about 6 weeks
Dumont et al., 1975	preserved	4% formalin	few minutes to several years
Irvine & Waya, 1999	preserved	4% formalin	NM
Melão & Rocha, 2000	preserved	4% formalin	NM
Saint-Jean & Bonou, 1994	preserved	5% formalin	NM

were weighted in groups of 30 and measures were taken individually. To reduce data skewness, we transformed data using  $\ln(x)$ . Length-weight regressions were carried out using  $Y = a x^b$ , where  $Y = \ln W(\mu\text{g})$ ,  $x = \ln L(\text{mm})$ ,  $a$  = estimate of intercept,  $b$  = estimate of slope (Dumont et al., 1975; Culver et al., 1985). We performed ANOVA to evaluate the differences in length and weight among the different treatments. Further, we evaluated the effect of preservation on the length-weight relationships over time, using one-way analysis of covariance (ANCOVA). Briefly, the main goal was to include the covariate as a statistical control to explain variation on dry weight, reducing the error variation and increasing the statistical power on the underlying treatment. Subsequently, estimated marginal means (EMM) were calculated in order to provide pairwise comparison between slopes. Preliminarily, the following assumptions of ANCOVA were verified: i. independence between independent variable (treatment) and co-variable (length). In other words, body length was not significantly different between treatments (ANOVA,  $df=3$ ,  $F=2.73$ ,  $p=0.066$ ); ii. co-variable and dependent variables showed linear relationship; iii. homoscedasticity of regression parameters was verified by comparing the regression slopes (Levene's test  $p=0.656$ ); iv. residuals followed normal distribution (Shapiro-Wilk test,  $p=0.447$ ). All analyses were performed in R 4.0.1 (R Core Team, 2020) and plots were made using ggplot2 (Wickham, 2016).

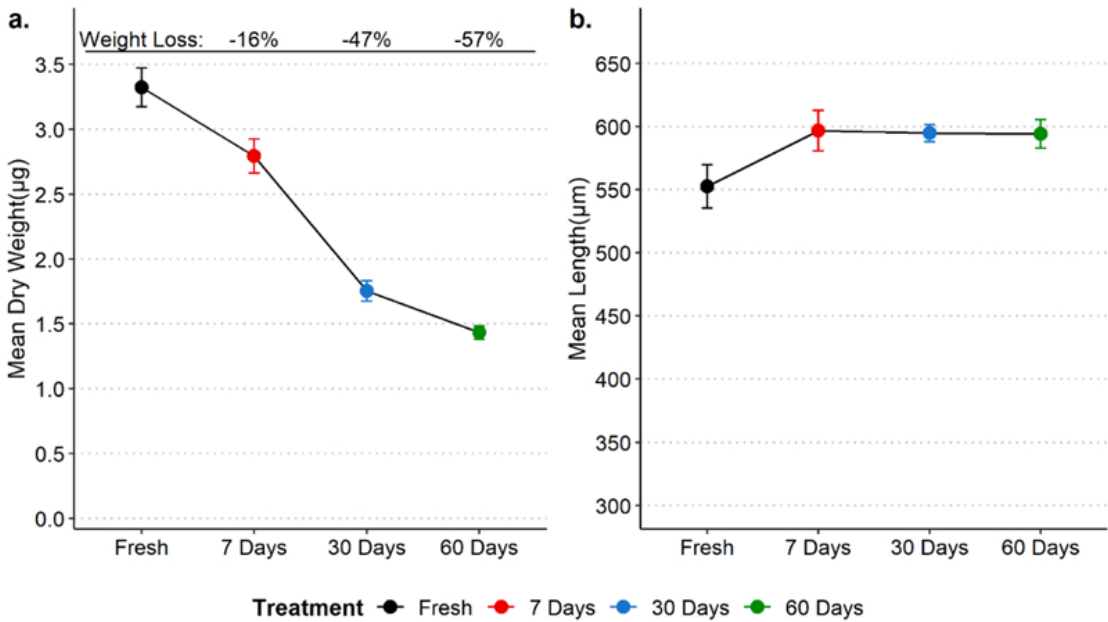
### 3. Results

Preservation negatively affected both length and dry weight measurements of *Ceriodaphnia silvestrii* (Figure 2). Organisms from fresh samples were smaller than those from preserved treatments (ANOVA  $df=3$ ,  $F=2.73$ ,  $p=0.066$ ), however, they accounted for higher values of dry weight (ANOVA  $gl=3$ ,  $F=64.772$ ,  $p<0.001$ ) (Figure 2). The increase in body length after the fresh period and its subsequent decrease in the following treatments was not statistically significant. Differently, dry weight significantly decreased as preservation time increased. Mean dry weight decreased by 15.96% after seven days. Much of the weight loss occurred after 30 days with a decrease of 47.28% (Figure 2a). Afterwards, the highest loss percentage along two months reached 56.92%, the maximum loss.

The linear models between fresh and preserved length-weight relationships showed significant p-values (Table 2). However, the  $R^2$  of fresh (0.66) and seven days (0.59) models were higher than those from 30 (0.36) and 60 (0.41) days.

The covariate, preservation time (treatment), was significantly related to the dry weight,  $F(3,23) = 161.1$ ,  $p<0.001$ . There was also a significant effect of the length of the specimens on the dry weight after controlling for the effect of the treatment  $F(1,23) = 23.2$ ,  $p<0.001$ .

Slopes were significantly different (Table 3), so treatment has a significant effect on the dependent variable which in this case can be interpreted as a significant difference in 'intercepts' between the



**Figure 2.** Mean and standard error of dry weight (a.) and body length (b.) by treatments.

**Table 2.** Regression coefficients for dry weight-length relationships in the different treatments (fixation time).

	Ln(y) = a + b ln(x)			R <sup>2</sup>	p-value
	a	b			
Fresh	-6.494	1.218		0.66	0.02(*)
7 days	-7.836	1.385		0.59	0.03(*)
30 days	-14.126	2.298		0.36	0.02(*)
60 days	-7.189	1.181		0.41	0.01(**)

**Table 3.** ANCOVA pairwise comparison of slopes by groups (treatments) using Tukey's post-hoc test.

Group 1	Group 2	df	F	p
Fresh	7 Days	23	5.66	p<0.001(*)
Fresh	30 Days	23	15.12	p<0.001(*)
Fresh	60 Days	23	19.24	p<0.001(*)
7 Days	30 Days	23	10.47	p<0.001(*)
7 Days	60 Days	23	14.93	p<0.001(*)
30 Days	60 Days	23	4.47	p<0.001(*)

\*Significant p-values.

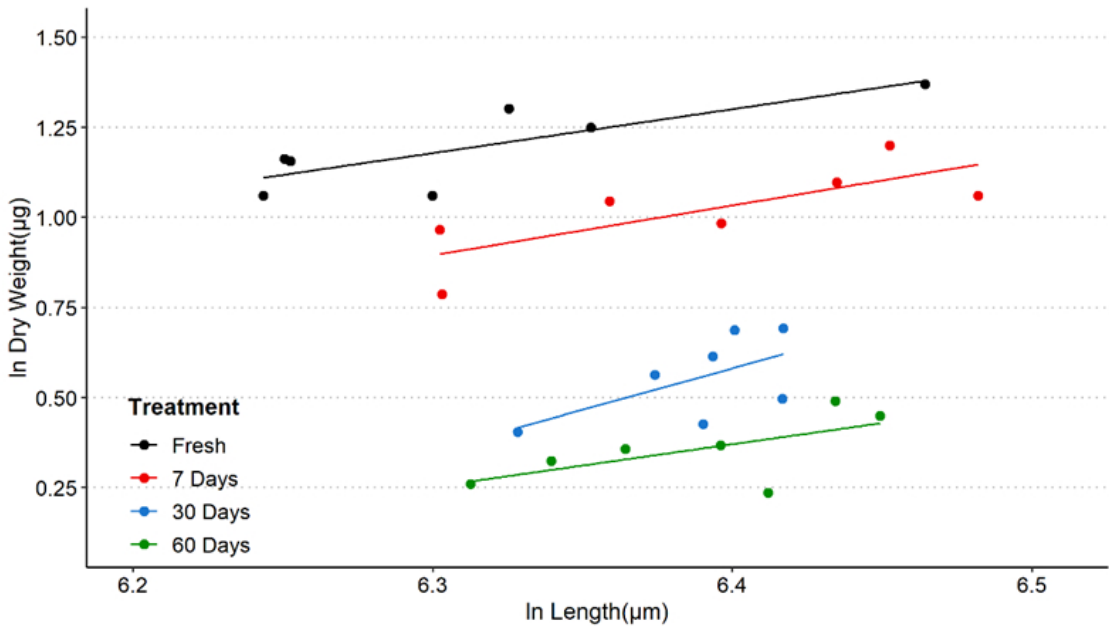
regression lines of treatments (fresh, 7, 30 and 60 days).

Despite the sustained relationship between length and dry weight in all treatments (Table 2), preserved samples showed lower predicted values for dry weight (Figure 3). Post hoc (Tukey) analysis underlined pairwise differences in length-weight regression among all treatments (Table 3).

#### 4. Discussion

Length-weight regressions have been frequently used to estimate the individual biomass of

cladocerans size classes (Edmondson & Winberg, 1971; Blettler & Bonecker, 2006). Similarly, length significantly predicts dry weight values, independently of the preservation time. More interesting is that when the effect of length is removed, the effect of preservation time becomes significant on dry weight of *Ceriodaphnia silvestrii*. Despite these findings, herein, we do not advocate for the elimination of chemical preservation in ecological studies, since it is considered the most suitable and convenient method in the field. Moreover, due to their small size, counting and



**Figure 3.** Scatterplot of dry weight (response variable) against body length (the covariate variable), on the experimental treatments.

measuring zooplankters are time-consuming and require undamaged specimens which can be easily solved by preservation.

In general, the range of variations in length was similar among treatments, however, fresh animals were smaller but also accounted for higher dry weight values (Figure 2, Table 2). Additionally, samples with more than 60 days of preservation showed more than 50% of dry weight loss. The weight losses through a short-term experiment (Figure 2) generates concern as it may represent a great loss of information for both water quality monitoring and ecological studies assessing the secondary production, which may mislead results associated with these organisms (Pederson et al., 1976; Arcifa, 1984). Moreover, cladocerans are important representatives of planktonic and benthic freshwater communities, being the species of *Daphnia* and *Ceriodaphnia* valuable indicators of water quality conditions and ecosystem functioning (ABNT, 2005; Pakrashi et al., 2013; Mansano et al., 2018) accounting for 45-91% of the sizable fraction of secondary production (Pederson et al., 1976).

The regressions between length and dry weight were different between treatments (Tables 2 and 3). This difference in allometric growth manifested itself as a different slope in all four regression lines. The interaction is significantly different meaning that the effect of the continuous covariate (length) on the response (dry weight) depends on the level

of the categorical factor (preservation time). Also, 30- and 60-days treatments showed higher residuals and therefore, smaller  $R^2$  values, whereas fresh and 7 days treatments indicated higher  $R^2$  values when compared to the aforementioned treatments. Such discrepancies indicate that the relationship between body length and dry weight weakens with longer preservation times.

Our assay organism is a filtering herbivore widely known to feed on micro algae in freshwaters. As expected, our populations showed lower values of length and consequently dry weight, comparatively to other systems with higher trophic state and thus, theoretically, with more resource availability. Further, cultivated organisms also showed higher dimensions as they have constant input of good quality food and much less biotic and abiotic stress compared to natural habitats. In this regard, the experimental findings allied to a literature review on biometrical measurements of *C. silvestrii* (see Table 1), suggest that estimates of biomass and production of aquatic invertebrates are often done without compensation for losses in dry weight and are certainly underestimated. Predicted weights from non-specific regressions also include an error factor since regression parameters (body length and dry weight) are sensible to habitat types, temperature, food type and availability (Michaloudi, 2005). Moreover, the sensibility of body length and dry weight may vary among

individuals of the same species (Dumont et al., 1975; Bottrell et al., 1976). Therefore, it is suggested that the body length of *C. silvestrii* and other tropical cladocerans from preserved samples can be used as a measure for the calculation of biomass from the length-weight relationship if weight loss is taken into consideration.

Dry weight measurements followed the expected pattern, decreasing along with preservation time. Accordingly, deleterious effects of preservation time were found on body length and dry weight relationship as *C. silvestrii* (Table 2 and 3) size has a significant and positive effect on the dry weight. In this sense, 4% formaldehyde (final concentration) in the first two months affected significantly mainly the dry weight of zooplankton organisms. As zooplankton weight and length are largely used to estimate production in aquatic ecosystems, our results indicate that interpretations on zooplankton secondary productivity based on those variables determined from samples preserved for extended periods of time may be greatly misleading.

Previously performed experimental studies evaluating sample preservation time alongside this study showed an underestimate of biomass quantification, but also contradictory results for body size alterations through time, showing the need of a standardization of preservation periods for the ecological analysis aiming reproducibility. Nonetheless, assessments of formalin effects on quantitative data for neotropical fauna are scarce, and despite require laborious laboratory work and taxonomic resolution effort our study advocate to future researches to restore this debate. We also suggest, different substances for evaluating the effects of preservation of different taxonomic groups of zooplankton and their development stages. Also, studies considering longer preservation periods are advised to match the average time lapse between preservation and biomass estimations. Accordingly, we also suggest studies in other regions since the local features of the watersheds regarding soil composition and inorganic components may be different between these regions (Stefanelli-Silva et al., 2019; Klippel et al., 2020) and may affect preservative solutions.

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