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Effects of light and temperature on *Chara vulgaris* (Charophyceae) gyrogonite productivity and polymorphism – palaeoenvironmental implications

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Abstract: To test the influence of light and temperature on intraspecific gyrogonite variation, two common European charophyte species, *Chara vulgaris* and *Chara globularis*, were studied indoors under controlled environmental conditions. A total of 60 plants of each species were incubated under different combinations of four irradiances (L1 = 30 μmol PAR m^{-2} s^{-1}; L2 = 150 μmol PAR m^{-2} s^{-1}; L3 = 500 μmol PAR m^{-2} s^{-1} and L4 = 1000 μmol PAR m^{-2} s^{-1}) and three water temperatures (T1 = 15°C; T2 = 25°C and T3 = 35°C). These parameters simulate temporary ponds from the Mediterranean region in the summer and help in understanding charophyte reproduction in shallow temporary ponds of past geological periods. After 102 days, gyrogonite productivity was evaluated, and a morphometric analysis of the gyrogonites produced was performed. Our results indicated that both irradiance and water temperature play a key role in *C. vulgaris* intraspecific gyrogonite variation. Gyrogonites tended to increase their size as irradiance and temperature increased. However, in extreme conditions (L4 and T3), an opposite trend was observed, with gyrogonites becoming significantly smaller. Our results agree with the distribution patterns of fossil gyrogonite assemblages. Using the present data, we hypothesise that charophytes in historic temporary floodplain water bodies produced smaller gyrogonites in response to the stressful environments induced by high irradiances and temperatures.

Key Words: Charophytes, Fossil, Gyrogonite, Mediterranean, Polymorphism, Palaeoecology, Palaeoenvironment

INTRODUCTION

Charophytes are a group of submerged freshwater or brackish-water macrophytes that are among relatives of bryophytes and vascular plants. Fossil records of charophytes are mainly based on their calcified fructifications, gyrogonites and utricles, dating from the Silurian period to the present (Grambast 1974; Feist *et al*. 2005). Gyrogonites are important biostratigraphic markers used worldwide to date continental rock formations and reconstruct historic non-marine aquatic environments. However, gyrogonites attract little attention in studies on extant species, since the taxonomic classification of living charophytes is mainly based on vegetative characters (Corillion 1972). Delimitation of extant charophyte species is sometimes controversial due to the morphological variability of their vegetative and reproductive organs in response to environmental variables (Bonis *et al*. 1993; Blindow *et al*. 2003). Irradiance, salinity and their combination can determine the growth pattern and morphology of some species of *Chara* (Flor-Arnau *et al*. 2006). Furthermore, branch orientation during growth of *Chara* was modified by irradiance to protect plants from excessive light (Schneider *et al*. 2006). Asaeda *et al*. (2007) reported that morphological (shoot structure) and reproductive acclimations of charophytes change according to water depth. Water depth highly influences the irradiance and also regulates the effects of water turbulence. Ellawala *et al*. (2011) showed in indoor experiments that high water turbulence clearly reduced shoot elongation and chlorophyll concentration in *Chara fibrosa* C.Agardh ex Bruzelius.

In such cases, the oospore wall, i.e. the organic pellicle coating the fructification inside the gyrogonite, provides additional taxonomic characters, such as the oospore general morphology and its external ornamentation, thus enabling differentiation between genera and even species (Soulié-Märsche 1989; John *et al*. 1990). Indeed, the oospore ornamentation pattern and size have helped distinguish particular species of *Chara* (Ray *et al*. 2001; Holzhausen *et al*. 2015).

The charophyte fossil record is mainly based on the calcified gyrogonites, the spiral cells surrounding the oospore that result from post-fertilisation development (Soulié-Märsche 1989; Soulié-Märsche & García 2015 and references therein). These calcified propagules are long-lasting organs that are biologically viable after long periods of burial (Rodrigo *et al*. 2010). To date, gyrogonites have been little studied in extant species. However, the first taxonomic analyses did discuss polymorphic gyrogonite features and hypothesised the environmental conditions affecting gyrogonite formation (Soulié-Märsche 1971; Soulié-Märsche *et al*. 1991). Moreover, several studies have been dedicated to establishing taxonomic relationships between gyrogonites

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and the plants producing them (Soulié-Märsche 1989). In this regard, Soulié-Märsche & García (2015) recently highlighted the necessity of clarifying the taxonomic relationship between gyrogonites and the charophytes producing them. This would help reconstruct quaternary palaeoenvironments using current environments with similar characters. These authors also pointed out the need for culture experiments to understand how physico-chemical parameters affect the production of oospores/gyrogonites and their morphology, which could be used to infer palaeoecological constraints on charophytes.

Significant intraspecific gyrogonite variation has been documented within and between populations of charophytes and has been observed both in fossils and extant species. In palaeontological studies, this polymorphism is considered to be palaeoenvironmentally controlled. However, no studies have been carried out to elucidate the extent to which gyrogonite morphology is affected by environmental conditions of irradiance and water temperature, which are considered of prime importance for gyrogonite formation and calcification. The physico-chemical parameters controlling oospore/gyrogonite production and morphology are poorly understood in charophytes but are generally thought to be species-specific (Soulié-Märsche 1989). A broad range of intraspecific gyrogonite polymorphism has been described in extant species of the genera *Chara*, *Lamprothamnium*, *Nitellopsis* and *Lychnothamnus* (Pedrola & Acuña 1986; Soulié-Märsche 1989), as well as in the oospores of *Chara*, *Nitella* and *Lamprothamnium* (Ray *et al.* 2001; Holzhausen *et al.* 2015). Despite making significant taxonomic advances regarding charophyte gyrogonites, these studies have shed little light on how environmental factors affect intraspecific gyrogonite variation. Casanova (1997) suggested that the morphology of *Chara* oospores was controlled by genetic and environmental factors, such as water temperature, water chemistry and the allocation of resources to oogonia during their development. Moreover, Pukacz *et al.* (2012) concluded that weather oscillations during vegetative growth directly affected oospore morphology in *Chara baueri* A.Braun.

They demonstrated that oospores of this species from a Polish lake without water level fluctuations displayed larger sizes and a wider size range than those from a very shallow temporary lake from flooded areas in neighbouring Germany.

To date, no experiments have been performed to test gyrogonite productivity and intraspecific/interspecific variation in relation to environmental factors. However, a number of studies on fossil gyrogonites have formulated a hypothesis about the effects of the palaeoenvironment on the distribution of gyrogonite populations and intraspecific gyrogonite variation (Villalba-Breva & Martín-Closas 2013; Sanjuan & Martín-Closas 2015; Vicente *et al.* 2016). According to these reports, fossil gyrogonites from shallow temporary ponds of floodplain environments in warm climates with marked seasonality show smaller sizes than those from coeval permanent lacustrine environments.

The present study used laboratory-controlled conditions to evaluate the effects of irradiance and water temperature on the production, size and morphology of gyrogonites generated by the cosmopolitan charophyte species *Chara vulgaris* var. *longibracteata* Groves & Bullok-Webster and *Chara globularis* Thuiller. Water temperature and irradiance values were established to simulate the environmental conditions of shallow temporary Mediterranean ponds in the summer, similar to the warm shallow ponds of past geological periods. Thus, another aim of this study was to enhance the usefulness of gyrogonites in future palaeoecological analyses, particularly in interpreting fossil charophyte assemblages from temporary ponds such as those from floodplain environments.

MATERIAL AND METHODS

Chara vulgaris var. *longibracteata* and *Chara globularis* were collected from an artificial pond located in Laberint d'Horta city garden (Barcelona, Catalonia, Spain, UTM 41°26′26″N, 2°8′40″E) on 20 May 2014. Sediment and water for culture were collected from an irrigation tank supplied with phreatic water, belonging to the Can Pujades farm (Sant Llorenç d'Hortons, Catalonia, Spain, UTM 41°28′3″N, 1°48′14″E). This locality was selected for the properties of the water, especially its high alkalinity and low nutrient content ($NH_3 =$ 0.1 mg l^{-1}, $NO_2 = 0.02$ mg l^{-1}) with pH of 7.3. In addition, the general hardness (GH = 16.5) and carbonate hardness (KH = 15) simulated environmental conditions that promote the development of reproductive organs (oogonia and antheridia) and ensure their fertilisation and calcification (gyrogonite formation).

Growth experiments for gyrogonite production were carried out in a culture chamber. The controlled variables in the cultures were irradiation (L) and water temperature (T). Four light categories (L1 = 30 μmol photosynthetically active radiation (PAR) m^{-2} s^{-1}; L2 = 150 μmol PAR m^{-2} s^{-1}; L3 = 500 μmol PAR m^{-2} s^{-1} and L4 = 1000 μmol PAR m^{-2} s^{-1}) and three water temperatures (T1 = 15°C; T2 = 25°C and T3 = 35°C) were chosen for gyrogonite production. Light levels (L1, L2 and L3) were controlled by covering the lamps of the culture chamber with plastic meshes, using a photoperiod of 12:12 h. The culture chamber was adjusted to the minimum temperature (T1), while the warmer treatments (T2 and T3) were obtained using a small heater (220 volts and 35 watts) within the tanks. An independent thermostat regulated each heater. The combination of the different variables produced a total of 12 experimental conditions per species. Each experiment was carried out in a glass culture vessel (a closed tank) of approximately 4 dm^3. Plants of *Chara vulgaris* and *Chara globularis* were cut into 3 cm segments, leaving one or two nodes and the end of the last internodal cell free. Five 250-ml cylindrical transparent plastic cups were introduced within each tank. These plastic containers were first drilled identically to allow water circulation. One specimen was placed in each drilled cup by planting the thallus into the sediment at a depth of about 1.5 cm. A cotton layer was added to cover the sediment and prevent the development of new undesired plants. Thus, 60 apical sections of each species (total of 120) were planted, with five replicates in each of the 24 microcosms. To avoid water stratification, a diffuser system was connected to an air pump. The culture experiments ran for 102 days (from 23 May 2014 to 02 September 2014). On the last day, all the

Table 1. Values of the studied morphometric parameters for each experimental condition. Abbreviations: ISI, isolopolarity index (H/W × 100); H, gyrogonite height; W, gyrogonite width; *n*, number of samples; n.c., number of convolutions visible in the lateral view; SD, standard deviation.

		H (μm)		W (μm)		n.c.		ISI	
	n	min–max (mean)	SD	min–max (mean)	SD	min–max (mean)	SD	min–max (mean)	SD
L1T1	24	458–535 (496)	19.70	287–367 (332)	20.05	10–14 (12)	1.25	1.29–1.72 (1.50)	0.13
L1T2	92	470–609 (544)	29.41	323–440 (368)	24.62	10–15 (13)	1.14	1.18–1.88 (1.48)	0.11
L1T3	58	501–725 (369)	47.05	291–519 (396)	39.06	10–15 (13)	1.00	1.36–2.13 (1.62)	0.12
L2T1	41	447–616 (553)	37.09	273–409 (358)	30.54	10–14 (13)	0.90	1.32–1.98 (1.53)	0.15
L2T2	95	430–728 (591)	63.50	291–495 (378)	35.12	6–14 (12)	1.11	1.30–1.86 (1.56)	0.13
L2T3	26	497–656 (586)	40.03	311–402 (586)	21.34	11–15 (13)	1.09	1.33–1.79 (1.59)	0.12
L3T1	12	458–526 (484)	20.68	280–391 (337)	34.37	11–14 (13)	0.98	1.30–1.69 (1.47)	0.13
L3T2	77	451–660 (542)	39.72	302–456 (381)	28.21	10–14 (12)	1.00	1.18–1.71 (1.43)	0.12
L3T3	3	478–562 (550)	45.47	271–341 (305)	35.01	13–14 (14)	0.58	1.61–1.84 (1.76)	0.12
L4T1	68	446–681 (579)	52.70	308–447 (370)	32.03	11–14 (12)	0.92	1.27–2.09 (1.54)	0.14
L4T2	40	450–690 (594)	54.98	313–449 (372)	24.66	11–15 (13)	1.10	1.26–1.88 (1.58)	0.16
L4T3	47	474–627 (521)	34.98	338–402 (338)	24.60	12–15 (13)	0.83	1.38–2.01 (1.57)	0.14

plants were removed and dried. A few weeks after harvesting, gyrogonites were carefully picked from the dried plants using a stereomicroscope at 40× magnification.

Almost 600 gyrogonites of *Chara vulgaris* var. *longibracteata* were produced and measured ($n = 583$). The following morphometric parameters were measured: gyrogonite height and width (μm), number of spiral turns observed in the lateral view, and the isopolarity index (ISI) (gyrogonite height/gyrogonite width × 100). Heavily encrusted calcified integuments around the gyrogonites (called tunicae in palaeontological literature) were removed before undertaking measurements. Gyrogonites were measured using the software Motic Images Plus 2.0 ML in a Motic BA310 stereomicroscope (Xiamen, China). Selected gyrogonites from each experimental microcosm were photographed using a Quanta 200 scanning electronic microscope located in the Centres Científics i Tècnics (Universitat de Barcelona).

Statistical analysis was performed with SPSS version 21 software. Several types of analyses of variance were applied. Single factor ANOVA was used for all the measured variables (gyrogonite height, gyrogonite width, number of spiral turns in the lateral view and ISI). Owing to the very low number of gyrogonites produced in the experimental group L3T3 ($n = 3$), this group was not used in the analysis. Levene's test for homogeneity of variances was performed in order to detect normality absences and heteroscedasticity. The results show *P* values below 0.05 indicating non homogeneous variances. For that reason, a Tukey analysis has not been included in the results, since they are not considered appropriate with normality absences or heteroscedasticity of residuals. However, to avoid this heteroscedasticity, two post-hoc tests were undertaken: Tamhane's and Ryan-Einot-Gabriel-Welsch tests (Day & Quinn 1989).

RESULTS

Gyrogonite productivity

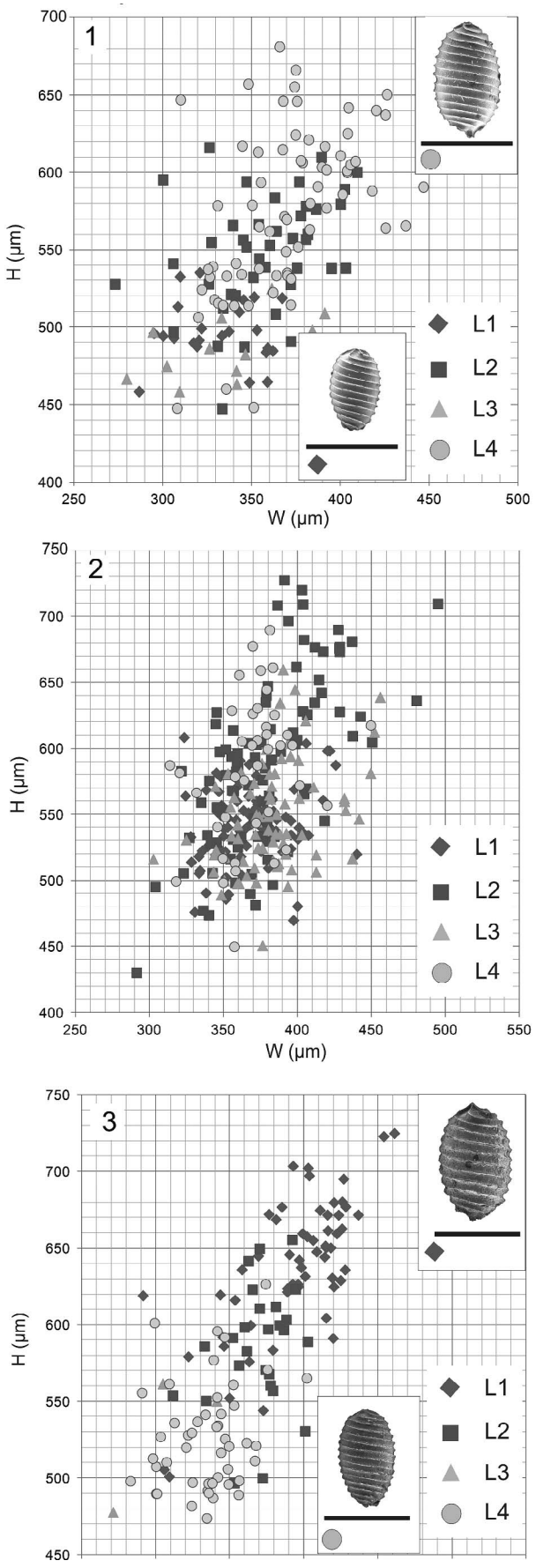
After 102 days, *Chara globularis* yielded negligible numbers of gyrogonites compared with the 583 produced by *Chara vulgaris*. In fact, *C. globularis* only produced gyrogonites (24 in total) in the microcosm L3T2. By contrast, *C. vulgaris* formed gyrogonites in all experimental conditions. Hence, morphometric analyses were performed only on gyrogonites produced by *C. vulgaris*. The best conditions for *C. vulgaris* gyrogonite production were L1T2, L2T2, L3T2 and L4T1, where plants generated over 65 propagules per microcosm. The highest gyrogonite productivity occurred at T2 for each light condition tested except in L4, which had the most gyrogonites at T1. In general, gyrogonite production at T2 was double that produced at T1 and T3.

Gyrogonite variation in relation to light irradiance and water temperature

IRRADIANCE: Changes in irradiance directly affected gyrogonite morphology. Dispersion graphs summarise the morphometric data, presented as maximum, minimum and mean values for gyrogonite height and width (μm), ISI and the number of spiral turns visible in the lateral view (c.n.) (Table 1). Plants exposed to lower temperatures (T1) produced larger gyrogonites as irradiance increased (Fig. 1). The gyrogonites produced at T1 under L4, were, on average, about 82 μm greater in height and 40 μm wider than those at L1. Charophytes subjected to medium temperatures (T2) bore gyrogonites that were not significantly affected by light conditions (Fig. 2). Finally, plants exposed to the warmest experimental temperature (T3) produced smaller gyrogonites as irradiance increased. At T3, gyrogonites at L4 were smaller (about 117 μm high and 57 μm wide) than those at L1 (Fig. 3).

Generally, changes in gyrogonite size resulting from different irradiances were mainly observed in gyrogonite height, with gyrogonite width varying less. Hence, ISI values were highly variable for the conditions tested, within a prolate shape, i.e. from 150 to 170. The number of spiral turns visible in the lateral view ranged from 10 to 14 in all the irradiance conditions; however, the mean value remained at 12–13, except for T3L3, where it was 14.

TEMPERATURE: Variation in gyrogonite morphology induced by temperature changes at a given light intensity was analysed. Plants cultured under a low irradiance (L1) produced larger gyrogonites with increasing water temper-



Figs 1–3. Dispersion graphs of gyrogonite height (H)/gyrogonite width (W) of *Chara vulgaris* at three different fixed temperatures (T1 = 15°C; T2 = 25°C and T3 = 35°C) under four different irradiances (L1 = 30 µmol photosynthetically active radiation (PAR) m^{-2} s^{-1}; L2 = 150 µmol PAR m^{-2} s^{-1}; L3 = 500 µmol PAR m^{-2} s^{-1} and L4 = 1000 µmol PAR m^{-2} s^{-1}). Abbreviations: H, gyrogonite height; W, gyrogonite width. Scale bar, 0.5 mm.

Fig. 1. Dispersion graphic with fixed T1.

Fig. 2. Dispersion graphic with fixed T2.

Fig. 3. Dispersion graphic with fixed T3.

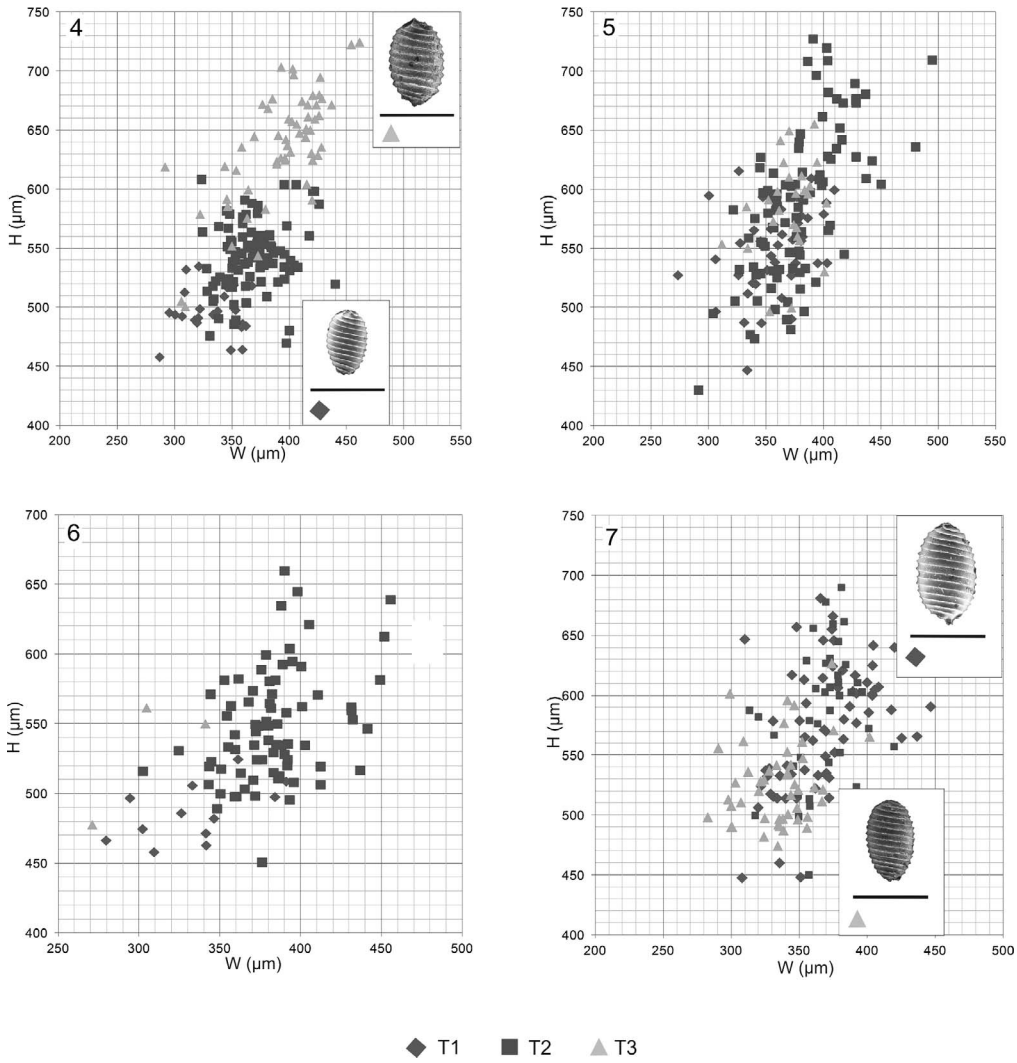
ature (Fig. 4). Thus, gyrogonites of plants exposed to the highest water temperature studied, T3, were, on average, about 142 µm greater in height and 63 µm wider than those grown at the coldest water temperature tested, T1 (15°C). Charophytes cultivated under L2 yielded gyrogonites that displayed great size variation. Thus, under L2, gyrogonites produced at T2 (very abundant, $n = 95$) and T3 ($n = 26$) were slightly larger (average of 32 µm high and 12 µm wide) and showed a notably wider range in heights than those at T1 ($n = 41$) (Fig. 5).

Plants at L3 had 77 gyrogonites at T2 but only 12 and three fructifications at T1 and T3, respectively. Despite gyrogonite productivity being lower under L3 than at L1 ($n_{L1T1} = 24$, $n_{L1T2} = 92$ and $n_{L1T3} = 58$) and L2 ($n_{L2T1} = 41$, $n_{L2T2} = 95$ and $n_{L2T3} = 26$), the yield at different temperatures followed the same pattern, with T2 giving the largest number of gyrogonites. The low number of gyrogonites produced at L3T3 was not sufficient for morphometric analysis. However, plants at T2 had gyrogonites averaging about 37 µm longer and 20 µm wider than those at T1 (Fig. 6).

The yield pattern observed at L1, L2 and L3 was not seen at L4 ($n_{L4T1} = 68$, $n_{L4T2} = 40$ and $n_{L4T3} = 47$), since L4 plants at T2 produced the lowest number of propagules. Moreover, in contrast to the lower irradiances tested, smaller gyrogonites were noted at T3 (about 57 µm in height and 30 µm in width on average) compared with those produced at T1 (Fig. 7).

Plants tended to produce gyrogonites with a higher number of convolutions, from 12 to 13, with increasing temperatures. Similar to that observed with irradiance, changes in gyrogonite size by altering water temperature were mainly in gyrogonite length instead of width, thus resulting in high variation within the prolate shape of gyrogonites.

In summary, changes in light and water temperature affected gyrogonite size. Between 15°C and 25°C, gyrogonites became larger or did not change with increasing irradiance. However, this pattern reversed at higher temperatures (35°C), with gyrogonites becoming smaller with increasing irradiance. A similar morphological pattern was observed at the highest irradiance studied. Plants cultivated between 30 and 500 µmol PAR m^{-2} s^{-1} had larger gyrogonites with increasing water temperatures; however, at 1000 µmol PAR m^{-2} s^{-1}, gyrogonites were smaller with rising temperatures. These results suggest that there is a light and temperature threshold beyond which gyrogonites become very small. However, further factorial experiments using a response surface methodology are required to confirm this.



Figs 4–7. Dispersion graphs of gyrogonite height (H)/gyrogonite width (W) of *Chara vulgaris* under four fixed irradiances (L1 = 30 µmol PAR m^{-2} s^{-1}; L2 = 150 µmol PAR m^{-2} s^{-1}; L3 = 500 µmol PAR m^{-2} s^{-1} and L4 = 1000 µmol PAR m^{-2} s^{-1}) at three different water temperatures (T1 = 15°C; T2 = 25°C and T3 = 35°C). Abbreviations: H, gyrogonite height; W, gyrogonite width. Scale bar, 0.5 mm.
Fig. 4. Dispersion graphic with fixed L1.
Fig. 5. Dispersion graphic with fixed L2.
Fig. 6. Dispersion graphic with fixed L3.
Fig. 7. Dispersion graphic with fixed L4.

Statistical analysis

We performed an ANOVA test, which demonstrated that irradiation (L), water temperature (T) and their combination significantly affected ($P = 0.001$) all the gyrogonite morphometric parameters analysed: gyrogonite height, gyrogonite width, convolution number in lateral view and Isopolarity Index (ISI)

According to the test for homogeneity of variances, gyrogonite height, gyrogonite width and convolution number in lateral view were significant ($P < 0.005$), showing different variances (heteroscedasticity). (Table 2 and tables of supplementary data).

Homogeneity group analysis was performed for 11 experimental conditions (L1T1, L1T2, L1T3, L2T1, L2T2, L2T3, L3T1, L3T2, L4T1, L4T2 and L4T3), revealing well-defined groups only for gyrogonite height and gyrogonite width. The gyrogonite height parameter had four groups, while the gyrogonite width parameter had five groups; however, many of these groups overlapped. Hence, the number of groups was reduced to three for gyrogonite height and three for gyrogonite width (Figs 8 and 9). This statistical analysis identified groups with similar mean values of gyrogonite height and width across all the experimental conditions tested.

The post-hoc Tanhame's test results agreed with the groups created by the homogeneity group analysis. Moreover, the correlation test performed at the end of the analysis indicated a positive significant correlation between temperature and gyrogonite height, gyrogonite width and ISI. By contrast, the correlation test only revealed one positive significant correlation for irradiance, which was with gyrogonite width. However, the significance values were quite low, hindering future predictive analyses.

Table 2. Summary of the one-way factor ANOVA.

	Sum. of Squares	df	Mean Square	F	Sig.
Light Levels					
Height					
Between Groups	90,698.125	3	30,232.708	9.622	0.001
Within Groups	1,816,054.353	578	3141.963		
Total	1,906,752.478	581			
Width					
Between Groups	20,528.551	3	6842.850	5.816	0.001
Within Groups	680,031.002	578	1176.524		
Total	700,559.553	581			
Spiral Turns					
Between Groups	6.343	3	2.114	1.798	0.146
Within Groups	679.678	578	1.176		
Total	686.021	581			
ISI					
Between Groups	0.965	3	0.322	17.217	0.001
Within Groups	10.794	578	0.019		
Total	11.759	581			
Temperature Levels					
Height					
Between Groups	106,360.040	2	53,180.020	17.103	0.001
Within Groups	1,800,392.438	579	3109.486		
Total	1,906,752.478	581			
Width					
Between Groups	33,081.249	2	16,540.625	14.348	0.001
Within Groups	667,478.303	579	1152.812		
Total	700,559.553	581			
Spiral Turns					
Between Groups	37.058	2	18.529	16.532	0.001
Within Groups	648.962	579	1.121		
Total	686.021	581			
ISI					
Between Groups	0.876	2	0.438	23.300	0.001
Within Groups	10.883	579	0.019		
Total	11.759	581			
Temperature–Light Levels					
Height					
Between Groups	729,753.121	11	66,341.193	32.128	0.001
Within Groups	1,176,999.357	570	2064.911		
Total	1,906,752.478	581			
Width					
Between Groups	182,504.026	11	16,591.275	18.255	0.001
Within Groups	518,055.526	570	908.869		
Total	700,559.553	581			
Spiral turns					
Between Groups	77.666	11	7.061	6.615	0.001
Within Groups	608.354	570	1.067		
Total	686.021	581			
ISI					
Between Groups	2.014	11	0.183	10.708	0.001
Within Groups	9.745	570	0.017		
Total	11.759	581			

Statistical analyses were consistent with our descriptive results, shedding new light on the ecological constraints affecting gyrogonite size in *Chara vulgaris*. The homogeneity group analysis indicated that the best experimental conditions for producing the largest *C. vulgaris* gyrogonites was L1T3, with a low irradiance of 30 μmol PAR m^{-2} s^{-1} and a very high water temperature of 35°C (Fig. 9). On the other hand, smaller gyrogonites were produced by plants exposed to three conditions: L1T1, with a low irradiance of 30 μmol PAR m^{-2} s^{-1} and a low water temperature of 15°C; L3T1, with a high irradiance of 500 μmol PAR m^{-2} s^{-1} and a low water temperature of 15°C and L4T3, with a very high irradiance of 1000 μmol PAR m^{-2} s^{-1} and a very high water temperature of 35°C. The correlation test performed at the end of the analysis suggested that temperature affected gyrogonite size more than irradiance. The L1T1 and L3T1 conditions produced an expected trend, since very low water temperatures restrict gyrogonite development. However, L4T3 generated an unexpected trend that was opposite to the general growth pattern.

DISCUSSION

Our results showed that irradiance and water temperature are interlinked parameters that play a key role in gyrogonite production and morphological variation in *Chara vulgaris*. Both light and temperature, as well as their combination,

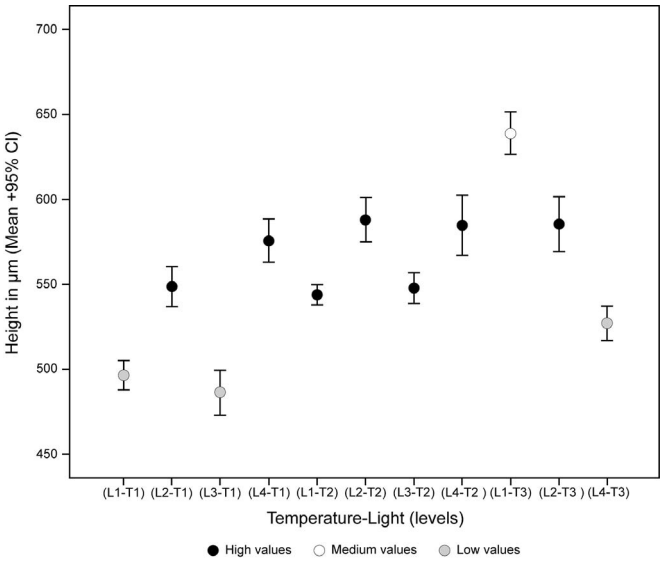


Fig. 8. Graph of the mean value and confidence interval (CI, 95%) of gyrogonite height for the studied combinations of irradiances and water temperatures.

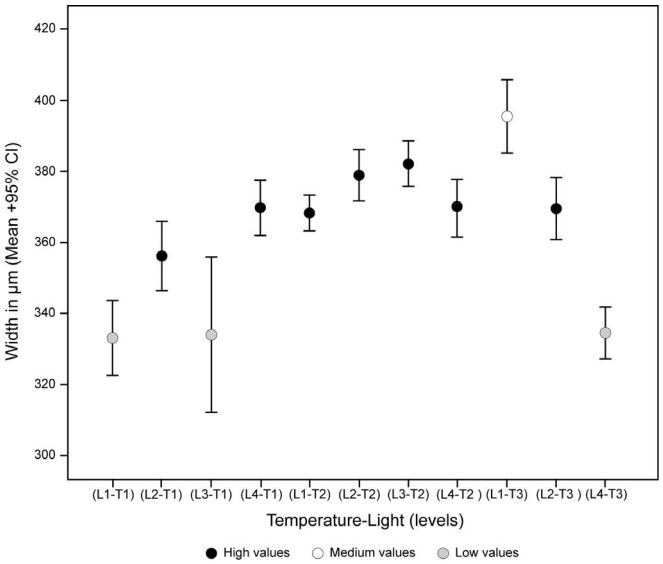


Fig. 9. Graph of the mean value and confidence interval (CI, 95%) of gyrogonite width for the studied combinations of irradiances and water temperatures.

significantly affected all the gyrogonite morphometric parameters measured.

Studies on fossil gyrogonites have highlighted the dominance of species with small gyrogonites in floodplain areas inferred to correspond to warm shallow temporary ponds (Sanjuan & Martín-Closas 2014, 2015; Vicente *et al*. 2015, 2016). Sanjuan & Martín-Closas (2015) documented this pattern in a morphometric study of gyrogonite populations of *Lychnothamnus vectensis* (Groves) Soulié-Märsche (1989) in three coeval Eocene basins with different sedimentary compositions: the Hampshire Basin (Isle of Wight, UK), Paris Basin (France) and Eastern Ebro Basin (Catalonia, Spain). They concluded that smaller gyrogonites occurred in thin-marl beds from temporary ponds in floodplain environments of the Ebro foreland Basin, while larger gyrogonites of the same species occurred in thick-marl beds associated with permanent lakes close to the shoreline in the Hampshire rift basin. The same patterns were observed in Upper Cretaceous basins. Vicente *et al*. (2015, 2016) reported that Late Cretaceous gyrogonites from Vallcebre and Coll de Nargó basins (Catalonia, Spain) displayed different sizes depending on the sedimentary environment in which they occurred. These authors concluded that permanent lake facies hosted diverse assemblages of species bearing larger fructifications, whilst floodplain pond deposits housed a few species bearing small gyrogonites, mainly belonging to the fossil genera *Microchara* and *Peckichara*. They also highlighted the presence of extremely small-sized gyrogonites from *Microchara* in very shallow temporary floodplain ponds, suggesting that these small gyrogonites may represent an adaptation to environmental stress. Similar gyrogonite distributional patterns were observed in Lower Cretaceous (Vicente & Martín-Closas 2013) and Eocene (Sanjuan & Martín-Closas 2012) floodplain deposits, showing that characean plants display a long history of adaptation to shallow temporary ponds by producing a high number of small gyrogonites that probably indicate short life cycles and opportunistic strategies (Vicente *et al*. 2016).

Here, our results on extant *Chara* support the above hypotheses of environmental conditions affecting gyrogonite populations, demonstrating that water temperature and irradiance are two key environmental factors regulating gyrogonite production and morphology. Our observation of high temperatures and light intensities inhibiting the development of large gyrogonites suggests that small gyrogonite production is an adaptation of reproduction in *Chara vulgaris* to stressful environmental conditions. This agrees with the colonising pattern and worldwide geographical distribution of this species. Indeed, irradiance and water temperature can reach very high values during the summer in shallow ephemeral inland ponds in temperate regions, such as the Mediterranean, similar to the high values used in the present study, i.e. 1000 µmol PAR m^{-2} s^{-1} and 35°C. Our results are comparable to those from the morphometric analysis by Pukacz *et al*. (2012) on oospores of *Chara baueri* from Poland and Germany. They demonstrated that oospores from permanent lakes had a larger size range ($\sim$ 150 µm in height and $\sim$ 49 µm in width) than those from shallow temporary lakes.

Fig. 10 shows a hypothesised gyrogonite distribution in a lakeshore environment, based on data from the present study. We would expect *Chara vulgaris* meadows in the deepest parts of the lake (plants exposed to cold and low light, i.e. L1T1 conditions) to produce small gyrogonites, while meadows located in well-illuminated subaquatic lacustrine belts (plants exposed to moderate temperature and light, i.e. L2T2 and L3T2 conditions) would yield large propagules. By contrast, *Chara* meadows located in the shallowest lakeshore areas or temporary ponds (plants exposed to very high temperatures and light, i.e. L4T3 condition) would produce small gyrogonites (Fig. 10).

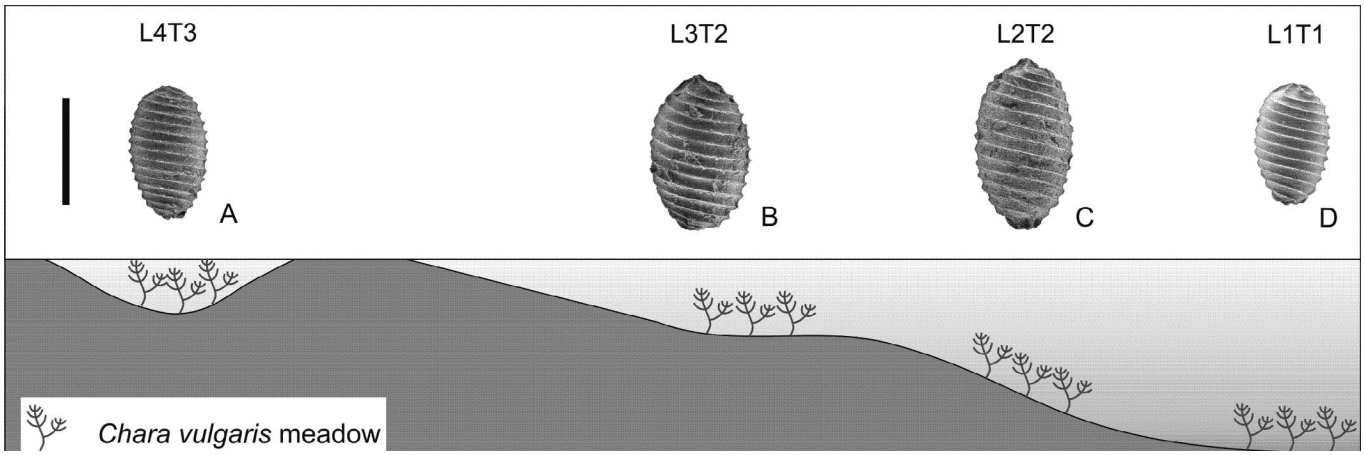


Fig. 10. Hypothetical distribution of *C. vulgaris* gyrogonites in an environmental reconstruction of a floodplain, based on results from the present study. The average morphology and size of the gyrogonites produced under experimental conditions are shown. Scale bar of photographs, 0.4 mm. (A) L4T3 (L4 = 1000 μmol PAR m^{-2} s^{-1}; T3 = 35 °C); (B) L3T2 (L3 = 500 μmol PAR m^{-2} s^{-1}; T2 = 25°C); (C) L2T2 (L2 = 150 μmol PAR m^{-2} s^{-1}; T2 = 25°C) and (D) L1T1 (L1 = 30 μmol PAR m^{-2} s^{-1}; T1 = 15°C).

Similar large ranges of gyrogonite size also have been observed for *Chara vulgaris* in their natural habitats. The mean values of gyrogonite height from five different ponds with different water depths and turbidity, and, thus, different light intensities, differed by up to 100 μm (Soulié-Märsche 1989). This highlights the need for experimental studies as a reference for interpreting gyrogonite variation in fossil charophytes.

The present study investigated only two environmental factors, irradiance and temperature. Other parameters could be explored in future studies, such as salinity and nutrients, since they also play a key role in the developmental and reproductive strategies of many charophyte species (Winter *et al*. 1996; Casanova *et al*. 2011). These types of studies are crucial for facilitating palaeoecological analyses, enabling fossil charophyte remains, more specifically gyrogonites, to be a valuable proxy for inferring palaeoenvironments, palaeotemperatures and fluctuations in lake water levels in historic wetlands.

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