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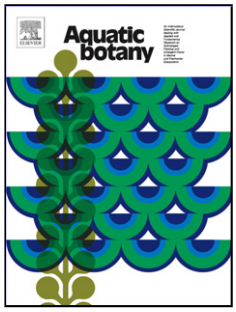
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TIME AND HEAT FOR SEXUAL REPRODUCTION: COMPARING THE PHENOLOGY OF *CHARA HISPIDA* OF TWO POPULATIONS AT DIFFERENT LATITUDES

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HIGHLIGHTS

1. Comparison of *Chara hispida* sexual reproductive phenology in Spanish and Swiss ponds

2. All phenological events happened 40 days earlier in the Spanish pond

3. *C. hispida* phenology agrees with the Hopkins' Bioclimatic Law prediction

4. Accumulated heat, a better predictor than daily mean temperature for *Chara* phenology

5. In the foreseeable warming scenario, Swiss *C. hispida* would lengthen its reproductive period

ABSTRACT

Geographical location affects the main ecological factors driving the timing of plants' life events. In addition, studying phenology is the simplest procedure to track current global warming and its effects on the success and survival of different populations of the same species. Little is known about the effect of water temperature and its corresponding accumulated heat on charophytes' phenology. We compared differences in water temperature and sexual reproductive phenology of *Chara hispida* in two ponds of two countries located at different latitudes (Spain and Switzerland) over the same year. We estimated the accumulated heat required to develop from one phenophase to another (unripe/ripe gametangia and oospores). Curve fitting techniques on water temperature showed an advance of 26 days in the Spanish spring

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onset. All phenological events happened for the first time around 40 days earlier in the Spanish pond, agreeing with the Hopkins' Bioclimatic Law prediction. *C. hispida* sexually reproduced in a daily mean temperature (DMT) range of 10-25°C and needed 600 growing degree-days (GDD) to ripen gametangia in the Spanish pond. The Swiss population required a higher DMT (15°C) to begin to reproduce, and ~700 GDD to initiate gametangia ripening. Temperature (as well as radiation) is one of the most important drivers of reproductive phenology, and accumulated heat is a better predictor than DMT for charophyte phenology. In the foreseeable warming scenario, we assume that *C. hispida* sexual events would advance by more than one month in Switzerland and expand at the end of the season, considerably lengthening its reproductive period.

Keywords: charophytes, temperature, Growing Degree Days, geographical factors, Hopkins' Bioclimatic Law.

1. Introduction

Phenology is the study of the seasonal timing of biological events. Organisms develop from one phenophase to another in their life cycle depending on their biology and "internal clock" (Resco et al., 2009). However, authors have also described how the initiation of phenological events is driven predominantly by climatic shifts associated with the change of seasons, rather than by intrinsic controls (Badeck et al., 2004). Unpredictability can affect the success of individuals and populations if it affects the precise timing of events -especially those regarding reproduction, such as flowering or fruiting in plants. Thus, organisms have to respond to environmental factors that are variable from year to year (Ausín et al., 2005). Factors related to light availability (such as photoperiod or irradiance), water availability (such as precipitation or hydroperiod) and other climatic factors (such as temperature) act as cues for phenology, as they inform about short-term environmental trends and foreseeable future conditions (Pau et al., 2011). Hence, phenology is considered to be the simplest process which allows us to track the current changing climate and its effects on the ecology of both animal and plant species (Walther et al., 2002).

All the environmental factors that affect phenology vary with the geographical factors in which the organisms are living (Pau et al., 2011), determining the distribution of species (Chuine, 2010). According to Hopkins' Bioclimatic Law, a variation in geographical position – as defined by latitude, longitude and altitude – implies a constant variation in climatic conditions and phenology across temperate

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areas (Hopkins, 1918). So, with other conditions being equal, there is a progression of spring phenological events from south to north, from west to east and from lower to higher elevation. Hopkins research was focused on the phenology of insects, forest trees, wild shrubs, farm crops and cultivated plants from gardens, all of them being from terrestrial ecosystems. Research based on remote sensing techniques and vegetation indexes (Schwartz, 1998; Pettorelli et al., 2005) delved deeper into the description of the spring phenology advance in terrestrial plants, the so called "green up", through latitudinal gradients. For aquatic ecosystems, recent examples of the application of these methods exist (Hestir et al., 2015; Luo et al., 2016), but no information is available concerning the effect of latitude on the reproductive phenology of submerged aquatic plant species or the fulfilment of Hopkins' Law.

Among environmental factors, light and temperature especially affect phenology by directly influencing biochemical processes and the development rates of organisms (Franklin, 2009). To study the seasonality in environmental factors, curve fitting techniques have been applied, as they allow one to represent the annual pattern on a temporal axis and to characterize some key dates within the year that can be related to biological events. The onset, the maximum or the end of the growing season are some examples of these key dates (Pettorelli et al., 2005) that can be calculated based on temperature dynamic. Other methods can be used to explore phenological events, such as the broadly used growing degree day model, also called the thermal time model (Trudgill et al., 2005). It is based on the accumulation of heat above a base temperature, called the low threshold of temperature. This model assumes that each organism requires a particular heat sum to develop to the next phenophase in its life cycle, so when that required thermal summation is reached, the phenological event is triggered. There are many examples of the application of this model for terrestrial organisms, especially in plants (Zavalloni et al., 2006; Sacks & Kucharik, 2011; Kramer et al., 2012). However, its use for submerged macrophytes is scarce (Spencer et al., 2000; Spencer & Ksander, 2001), particularly for green macroalgae such as charophytes (Rey-Boissezon, 2014; Calero et al., 2015).

Charophytes play key roles in aquatic ecosystems (Schneider et al., 2015). They form dense stands in permanent waterbodies by means of their high capacity for vegetative reproduction. In spite of this, the disappearance of several charophyte species, and the decline of rarer ones, have already been reported for Europe (see, for example, Simons & Nat, 1996; Auderset Joye et al., 2002; Korsch et al., 2008; Auderset Joye & Schwarzer, 2012; Baastrup-Spohr et al., 2013). Reducing the risks to

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which charophytes are exposed requires a better knowledge of their ecology. Under the current scenario of changing climate (CH2011, 2011; IPCC, 2014) it is crucial to unravel how charophytes, and their phenological processes, will respond to these perturbations. Even many charophyte species can reproduce vegetatively, higher unpredictability and frequency of droughts are making sexual reproduction more important for charophyte populations. Climate change might affect interactions between temperature and charophyte reproduction, affecting their geographical distributions and threatening the survival of certain species (Auderset Joye & Rey-Boissezon, 2015).

As stated by Marchin et al. (2015), predicting future responses of phenology to climate change in general, and warming in particular, requires a broad perspective and the use of a range of approaches. For instance, differences in temperature between two countries over the same year due to distinct geographical locations could be comparable, with caution, to the increment in temperature that the country located at the higher latitude would experience in the future under the warming scenario. For example, in Switzerland there is a foreseeable increase of up to 4.8°C in the seasonal mean temperature by the end of the century under a non-intervention scenario (CH2011, 2011). Looking at the seasonal mean air temperature between this country and one located at a lower latitude such as Spain, which is approximately 5.5°C warmer in spring (in the period 1981-2010; AEMET, 2012; MétéoSuisse, 2015), means that Spain would be a suitable candidate for the comparison of spring phenology. Therefore, in this study we compared the temperature dynamics and the reproductive phenology of the same species during 2014 in two ponds located in Spain and Switzerland. We chose the submerged macrophyte *Chara hispida*, a European broadly distributed charophyte which lives in both freshwater and brackish water, with a degree of threat rated as vulnerable in Switzerland (Auderset Joye & Schwarzer, 2012). The aim of the study was to answer the following questions: (i) does *C. hispida* follow Hopkins' Bioclimatic Law? (ii) does the length of the *C. hispida* reproductive season change with the geographical location? and (iii) does the heat sum needed to develop from one phenophase to the next one within a life cycle depend on the geographical position? Summarizing, we tried to understand how geographical factors, through an important climate factor such as temperature, affect the reproductive phenology of the same particular aquatic species.

2. Methods

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2.1. Study sites

The two ponds under study were located in two countries within the European continent, differing in their latitude, longitude and altitude (Fig. 1). The *Llacuna Nova del Canyar* (LNC hereafter) is an oligohaline shallow pond created in 2007 within the *Albufera de València* Natural Park (Valencia, Spain) as part of the restoration of a sea-shore dune front. During 2014, scattered meadows of submerged macrophytes covered the sandy bottom, dominated by dense beds of *C. hispida* (Calero et al., 2015).

The *Étang Robert Hainard* (HAI hereafter) is a freshwater pond created in 1991 within the *Moulin de Vert* Nature Reserve (Geneva, Switzerland) in a former meander of the *Rhône* river that disappeared when the river was channelized in 1940. In 2014, charophytes dominated the macrophytes community and, although the pond was covered by ice during part of the winter, *C. hispida* grew perennially in permanent shallower and deeper waters.

2.2. In situ *temperature recording*

Several Onset HOBO® probes with data loggers (Onset Computer Corporation, Bourne, MA, USA) were set up within the ponds to hourly record the air and water temperatures. All underwater sensors were fixed to the bottom of the pond so that their depth relative to the surface changed with the water level. For this study, however, we defined the depth of each probe related to the water surface -by using the average water level in each case- to understand the phenology of the charophytes sampled at those same depths in other areas of the pond.

Two underwater temperature sensors were located within LNC at different areas with permanent water. One of the probes was located close to the shore of the pond, at a depth of 15 cm in an area 30 cm deep (LNC-15 cm hereafter), where charophytes were growing at the base of emergent vegetation. Another one was placed in open waters, at a depth of 30 cm in an area which is 120 cm deep (LNC-30 cm hereafter). By contrast, in HAI, four underwater sensors were placed at different depths in the same vertical line in an area 400 cm deep (HAI-50 cm, HAI-150 cm, HAI-250 cm, HAI-350 cm hereafter, respectively). See their location within the pond in Figure 1.

2.3. Calculations and statistics related to temperature data

To describe and compare both studied ponds, we calculated the daily mean temperature (DMT). DMT data from each location and depth were studied by curve

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fitting techniques to characterize the beginning of spring and autumn in order to relate them with biological events. Scaled data were fitted to two different bell-shaped functions for subsequent model selection (Johnson & Omland, 2004): the Fourier model of 2 terms (Los, 1998) and the Weibull model of 6 parameters (Rolinski et al., 2007). The goodness of fit for both models was estimated with the χ^2 parameter (significance calculated with Monte Carlo's methodology with 10,000 permutations), and the Akaike Information Criterion was applied to choose the better model in each case (Johnson & Omland, 2004). Spring onset was determined as the highest slope of increase in temperature in the fitted model, while the end of autumn was determined as the highest slope of decrease in temperature. The derivative function of the bell-shaped model was calculated so that the spring onset corresponded to the moment of minimum value; the end of autumn corresponded to the moment of maximum value; and the maximum temperature with the derivative function being equal to 0. Further information about this methodology, performed with MATLAB software (2015), is described in Benavent-Corai (2015).

Both air and water DMT data were analysed seasonally (approximating winter as 1 January-31 March, spring as 1 April-30 June, summer as 1 July-31 September, and autumn as 1 October-31 December). As temperatures are largely dependent on time and space, differences between each location and depth were analysed with the Tukey Mean-difference plot (also called the Bland–Altman plot in medical research), a simple but powerful graphical tool to compare paired observations of two directly comparable variables (Kozak & Wnuk, 2014). These statistics were performed with R software version 3.2.1 (R Core Team, 2015).

The Growing Degree-Days (GDD) parameter was calculated with the Actual Temperature Method (no cutoff) from the HOBOware® Pro Growing Degree Days Assistant (Onset Computer Corporation, Bourne, MA, USA). This method performs a numerical integration, and uses the sampling interval of temperature data (1 hour in this case) to compute the area between the curve of temperature and the low threshold. There is not a well-defined low threshold of temperature for the development of charophytes (Rey-Boissezon, 2014), but *C. hispida* bears water temperatures down to at least 4°C, the minimum temperature mostly recorded in temperate ponds. At this value, water has the highest density/weight relation favouring the mixing of the water column. Besides this, we used 4°C as the low temperature threshold to allow for comparisons with previous studies (Rey-Boissezon, 2014). The 1 January 2014 was set as the starting date for calculation of GDD (biofix). However, as the population of *C. hispida* from LNC – 15 cm was already

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fertile in January, we examined temperature data from the last months of 2013 to find an appropriate starting date to describe the life cycle of the species. The 1 November, also used in previous studies on submerged plants (Spencer et al., 2000), was finally considered as the biofix for that particular site as the minimum temperature in 2013 happened within that month.

2.4. Sunlight data

Accumulated sunlight from the beginning to the end of the year (in hours of sun) was calculated in each latitude to provide additional information about the environmental conditions affecting both populations. Data were obtained from the European Climate Assessment & Dataset (Klein Tank et al., 2002) and MétéoSuisse (2015).

2.5. Measurement of other limnological variables

Some physical and chemical features of the water were analyzed monthly using portable field equipment: a WTW Meter (WTW GmbH, Weilheim, Germany) for conductivity and pH, and a Hach LDO1 Meter (Hach Company, Loveland, CO, USA) for dissolved oxygen. Water samples were taken to analyze total nitrogen (TN) and total phosphorus (TP) concentrations, as well as solute composition of water. The IIAMA laboratory (*Universitat Politècnica de València*) and *Laboratorios Tecnológicos de Levante* in Spain, and the SECOE laboratory (*Service de l'Ecologie de l'Eau - Etat de Genève*) in Switzerland performed these analyses using standard methods.

2.6. Specimen collection, observation and analysis

C. hispida specimens were collected from both ponds by hand, or using a hook from an inflatable boat. Species identification followed Krause (1997). Collection was less frequent in autumn and winter (once a month) and more frequent in spring and summer (approximately every 15 days) for a more accurate detection of reproductive events. After washing the fresh material to remove sediment and organic matter, charophytes were kept in a refrigerator or fixed in ethanol 70% for further observation. Between 15 and 30 specimens (less observation effort when charophytes were sterile) were generally observed by sampling date and depth with an Olympus® SZ61 binocular loupe (Olympus Corporation, Shinjuku, Tokyo, Japan) and a Leica® stereomicroscope M205C (Leica Camera AG, Wetzlar, Germany) with maximum magnifications of 67.5x and 160x, respectively. Only those specimens with an intact apex and at least 8 whorls were selected, so that we could detect the presence of

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sexual organs (called gametangia) in each whorl. The whole thallus, including the side-branches, were observed to monitor the emergence and maturation of antheridia (male gametangia), oogonia (female gametangia) and oospores (the product of fertilisation of gametes) of *C. hispida* from LNC and HAI.

Each phenological stage was identified based on the size and colour of the gametangia. Unripe antheridia in charophytes go from light orange to light red, while ripe antheridia present a dark red colour and defined shield cells (given that when maturation is complete, the shield cells fall apart). Oogonia in *C. hispida* range from light orange to dark green or brown, and were considered as ripe when swollen. After that stage, oogonia become abortive or are fertilised, forming the oosporangium. Inside it, oospores start ripening, until they get black and finally start a calcification process (Soulié-Märsche & García, 2015). For this comparative study, we did not consider the gyrogonite (calcified oospore) as a phenological stage, as the fructifications that remained attached to the shoot in the Spanish population did not show a highly calcified aspect.

The difference in number of days between the first date of detection of each phenological stage at both populations was calculated and compared with that stated by Hopkins (1938): "Other conditions being equal, the south north progression of spring phenological events in temperate portions of North America is delayed by 4 days for each degree of latitude northward, for each 5° of longitude eastward, and for each 400-foot increase in elevation". According to the geographical coordinates of both study sites, we expected a delay in the sexual reproduction of the Swiss population of 44 days with regard to the Spanish one. The first date of detection of each phenological stage was related to the amount of accumulated heat reached at that moment, in order to compare the two ponds and describe the life cycle of the species. Finally, accumulated sunlight in each latitude was considered as complementary in relation to accumulated heat to understand our data (Rey-Boissezon, 2014). We calculated the hours of sun received by the charophytes at each phenological stage by using the same biofix as in accumulated heat.

3. Results

3.1. Limnological features

Both studied ponds had similarities regarding their trophic status, with similar concentrations of TN and TP. HAI presented higher values of dissolved oxygen and it was slightly more alkaline than LNC. Regarding the conductivity of water, however,

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LNC –with a relevant sea influence- had higher values, mainly due to considerably greater chloride and sodium concentrations (Table I).

3.2. Daily mean temperature (DMT) dynamics and key dates

Air temperature dynamics differed at both geographical locations, generally with warmer values at the Spanish site throughout the year (Fig. 2A). With regard to the minimum DMT, HAI reached negative values (-0.1°C, December 2014), whereas LNC temperature was always above 7.0°C. For example, towards the end of the winter (January to March 2014), we found DMT values of 10-20°C in LNC, showing a high variability between subsequent days. Meanwhile, a gradual increase in air temperature was recorded in HAI from temperatures of 2-6°C, ending in spring with one of the highest values (28.3°C, June). The highest daily mean temperature (28.8°C) occurred in mid-July (Fig. 2A), being 0.7°C lower than the maximum found in LNC, which also occurred in mid-July and later in early August.

Water temperature dynamic resembled air temperature dynamic at both ponds (Fig. 2B, only shallowest and deepest water values from HAI are shown), with daily values in water fluctuating much less. Again, maximum DMT happened earlier in HAI. Swiss waters from HAI were warmer at all depths than deeper waters from LNC during all of June, with temperatures up to 25.5°C at HAI-50 cm (Fig 2B). Waters from LNC kept on warming in July, reaching their maximum DMT at LNC-15 cm during August (28.9°C). According to water temperature, the spring onset in HAI appeared 26 days later than in LNC, and the end of autumn was determined just 6 days earlier in LNC compared to HAI (Fig. 3).

3.3. Seasonal temperature

Air DMT from LNC was higher than HAI temperature in all seasons, with minimum differences in spring, and maximum ones in winter (Fig. 4A & 4C). At both ponds, water temperature fluctuated less than air temperature, with more similar values in water temperature between the two ponds (Fig. 4B & 4D). Comparing the temperature from the deeper area of the Spanish pond (LNC – 30 cm) with the shallowest site from the Swiss pond (HAI – 50 cm) -the most similar conditions between the two locations-, underwater DMT was higher at the Spanish site, with the largest differences in winter (+4.4°C) and autumn (+3.4°C), and more similar values in spring (+0.9°C) and summer (+1.4°C) (Fig. 4D).

3.4. Accumulated heat

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Underwater accumulated heat was higher in shallower than in deeper waters in both ponds (Fig. 5). The warmer climate of Spain implied a higher underwater accumulation of growing degree days than in Switzerland. Taking the 1 November 2013 as the starting date for shallower waters from LNC, there was already an accumulated heat of 443 GDD by the beginning of 2014. The GDD curves of this pond did not stabilize around a maximum, but reached 5317 GDD by the end of the year in LNC-15 cm. In HAI, however, accumulated heat reached 4012 GDD at a depth of 50 cm and remained stable during the final days of December due to temperatures that approached the low threshold used for calculation.

3.5. Reproductive phenology of Chara hispida

Only sterile charophytes were observed at the beginning of the study period in both ponds and at all depths, except for LNC-15 cm, where specimens already showed ripe gametangia (both antheridia and oogonia) at the end of January. With this exception, *C. hispida* sexually reproduced from spring to autumn in both ponds (Table II). Specimens took 2-3 months to produce the first gametangia and for them to mature in LNC, whereas they took 3-5 months in HAI. Detection of both sexual organs was not simultaneous in most of the sampled sites. In LNC-30 cm, for example, we observed the first ripe antheridia around 9 days earlier than the first ripe oogonia. At least 4-5 months were needed for the maturation of oospores in LNC (6 months in HAI) and, after 8-11 months, all specimens in both populations became sterile again. The end of the sexual reproductive season appeared one month later in the calendar in the LNC shallow water compared to the deeper site. Almost no effect of depth was observed in the reproductive phenology of the specimens from HAI.

C. hispida sexually reproduced in a wide range (10-25°C) of water daily mean temperature (DMT). Gametangia maturation took place at between 10°C and 18°C DMT, while oospore maturation happened at between 14°C and 25°C. All events in the Swiss *C. hispida* population started when DMT was higher than in the Spanish pond. High temperatures during summer did not stop the generation of new gametangia in the newly grown parts of the charophytes in either LNC or HAI.

Considering the day of the year when a certain reproductive stage was detected, charophytes from HAI were delayed by 40-43 days with respect to those from LNC (Table II). In the Spanish pond, production and maturation of the first gametangia took about 400-600 GDD, maturation of the first oospores needed at least 1000-1300 GDD and, after 3300-4400 GDD, all specimens were sterile. However, charophytes from the Swiss pond needed higher values of accumulated heat to

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produce and ripen gametangia and oospores (400-800 GDD and 1400 GDD, respectively), whereas lower values were needed to revert back to a sterile state after the reproductive period (3200-3400 GDD) (Table II, Fig. 6). Regarding light energy, all phenological events generally needed more accumulated hours of sun in the Swiss population (Table II): about 500 h to produce and 650 h to ripen gametangia, and about 1000 h to ripen oospores. In the Spanish pond, however, we detected the first gametangia after approximately 325 h of sun, the first ripe gametangia after 500 h and the first oospores after 900 h.

4. Discussion

The two compared populations came from ponds with similar trophic status, but differed in the solute composition of the water, reflected in their different values of conductivity. The growth in brackish (LNC) or freshwater (HAI) conditions may influence the fertility of *C. hispida* as described for other charophytes (Soulié-Märsche, 2008), although we do not consider water chemistry –at least the differences between the two studied ponds- to be determinant for the first appearance of sexual organs. It appears, however, to be significant for the calcification of oospores into gyrogonites, as we observed differences in the presence of calcified propagules between both populations: slightly calcified oospores in LNC, versus hardly calcified gyrogonites in HAI. Higher availability of bicarbonates in the water of the Swiss pond could explain this observation.

4.1. Time for sex: effects of latitude and depth

Overall, both studied populations produced and ripened sexual organs from spring to autumn, over approximately 6 months, agreeing with the period described in another study for *Chara* sp. (Vromans et al., 2013). However, the different geographical position of both studied sites resulted in a delay of 40-43 days in the sexual reproduction of *C. hispida* from the Swiss population with regard to the Spanish one, in accordance with that forecasted by Hopkins' Bioclimatic Law (Hopkins, 1918). Although regional and local conditions can influence the observed outcome, our results indicate that this law might currently be used to approximately predict *C. hispida* phenology throughout Europe. It should be noted that ripe antheridia appeared before ripe oogonia throughout the growing season, as previously noticed in Calero et al. (2015) for the Spanish population. In fact, protandry is a largely described phenomenon for charophytes (Guerlesquin, 1987). In this study, the detected time lag between the two phenological stages seems to be

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determined by the sampling period. In other Swiss populations of *C. hispida*, however, we have observed a time lag of approximately just one week (Auderset Joye & Rey-Boissezon, unpublished results).

The phenology of the studied Spanish population differed remarkably as a function of the depth where the specimens grew. The reproductive season (6 months) in deeper water started in March, whereas the conditions in shallower water advanced reproductive events, already started in winter. In fact, the specimens from shallower water produced at least some gametangia, particularly antheridia, in every month of the year. This advanced reproductive onset, as well as the later end of the reproductive period in shallower water, lengthened the reproductive season (8 months) compared to deeper growing populations. Several studies have shown earlier onset dates and the lengthening of the season as consequences of climate change, and its associated warming conditions, in several populations and communities (Fitchett et al., 2015), indicating their potential effect on a better fitness and survival for some species (Forrest & Miller-Rushing, 2010). In shallower water, in spite of the occurrence of gametangia most of the year, spring concentrated the highest frequencies of fertile specimens, which presented a higher production of sexual organs than specimens growing in deeper waters (Calero & Rodrigo, unpublished results). Other studies also reported increased sexual reproduction rates for charophytes in shallower depths (Asaeda et al., 2007).

In the Swiss pond, *C. hispida* started producing gametangia later (April) than in lower latitudes, although it took 6 months to achieve the whole reproductive cycle, comparable to the deeper site in LNC. The first ripe gametangia in deeper water was delayed by about 1-2 weeks compared to shallower water (<250 cm). As the season progressed, however, *C. hispida* shoots grew towards the surface and were consequently less affected by depth.

4.2. Heat for sex: effects of temperature and GDD

The *C. hispida* population from the Spanish pond produced its first sexual organs at a daily mean water temperature (DMT) of approximately 10°C, a lower value than that of the Swiss population (HAI). In HAI, specimens started producing gametangia at around 15°C, the minimum temperature typically described in the literature for charophyte reproduction (Guerlesquin, 1987). These reproductive events in HAI happened when water DMT increase was maximal, thus at the spring onset according to our curve fitting analysis. However, *C. hispida* from LNC already had gametangia and started ripening them at the spring onset key date for water

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temperature. This difference in the biological responses to the key dates suggests that DMT is not the best predictor for phenological events, and that other factors are triggering reproductive events in *C. hispida*.

Different field experiments with charophytes have shown that *Chara* sexually reproduces more in spring and summer than in autumn (Bonis et al., 1993; Warwick & Brock, 2003). So, seasonal temperature may be useful to understand the environmental requirements of the species. However, underwater Spanish and Swiss temperatures differed by less than 1°C on average during the spring of 2014 (by comparing the two sites with more similar conditions), so neither does this metric seem relevant when studying the spring phenology of these populations.

Growing degree day models have proved to be highly accurate at predicting the phenology of a given species (Pau et al., 2011) through the description of the heat range needed to develop to the next stage in its life cycle (Miller et al., 2001). Very few publications have applied the GDD approach to charophytes. Rey-Boissezon (2014) showed the importance of accumulated heat to explain the sterility or fertility of *Nitellopsis obtusa* specimens, whereas Calero et al. (2015) described how different species of charophytes, even from the same particular aquatic ecosystem, have distinct heat requirements in order to become fertile. Although there is no information about GDD requirements for *C. hispida* in the literature, a study in a pond close to HAI in 2012 showed similar GDD values for each phenophase (Ben Meftah, 2014). In the current study, the growing degree day model proved to be valid for the first detection of unripe (409-420 GDD) and ripe gametangia (601-772 GDD), as *C. hispida* from both ponds showed close values. For the shallower population from the Spanish pond, accumulated heat during the previous autumn and winter explained the advanced reproductive onset. The beginning of oospore maturation, however, needed more heat in HAI than in LNC, and the heat accumulation difference also seemed sufficient to lengthen the reproductive season in the lower latitude by two months. Trudgill et al. (2005) showed that increasing latitude decreases the low threshold of temperature for a species, and increases its GDD requirements. In the current comparison, the first statement is not supported since the daily mean temperature at which the gametangia appeared and matured was higher in the Swiss pond. The second statement is fulfilled as GDD values for the maturation of gametangia and oospores tended to be higher in the pond located at a higher latitude (HAI). Further studies will allow us to better determine the heat ranges for reproductive events on charophytes. However, our results suggest that

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heat accumulation alone is insufficient to explain all the differences observed. Other factors, such as light, surely affect the reproductive timing of the species.

4.3. Other factors: effect of light

In HAI, where daily temperature was almost the same throughout the water column, the higher values of light received in shallower water also contributed to the different timing of phenological events when comparing shallow and deeper populations. Casanova (1994) found that transferring *Chara australis* from a depth of 105 cm to 54 cm, and therefore receiving higher solar radiation, increased the amount of reproductive specimens. The development of gametangia in charophytes is related to the storage of photoassimilates in vacuoles (Kirst et al., 1988). Besides, the physiological activities of hormones that largely depend on light (such as auxins and gibberellins) also produce photoreactions on gamentangia (Maszewski, 1980). Experiments with *Chara vulgaris* have shown how higher irradiances and longer days produced an early reproduction onset (Wang et al., 2008). Sato et al. (2014), however, described that shoots of *Chara braunii* had their best reproductive fitness under low-light conditions (10 µmol m^{-2} s^{-1}), whereas they did not form gametangia under, what they called, high-light conditions (70 µmol m^{-2} s^{-1}). Although there are no *in situ* data for the period of this study in the Spanish pond, Sato's values of irradiance are far from the up to 200 µmol m^{-2} s^{-1} of underwater radiation measured in further periods at 40 cm from the water surface in the deeper area of LNC. As for accumulated heat, light requirements for gametangia development seem to be species-specific in charophytes.

Light differences due to latitude may also be affecting the reproductive onset of *C. hispida*. At the location of the Spanish pond, approximately 2700 hours of sunlight were accumulated over 2014 (ECA dataset, Klein Tank et al., 2002), almost 1000 hours more than at the Swiss location (1860 h; MétéoSuisse, 2015). As for accumulated heat, phenological events needed less sunlight at the lower latitude.

Studies in plant development have shown great molecular interaction between light and temperature signalling pathways for flowering (Franklin, 2009), indicating that both factors act together to trigger reproductive processes. Disentangling the effect of both factors on the reproduction of charophytes is complex and controversial (Rey-Boissezon, 2014). Since each species adapts and acclimatizes to different environmental conditions, further case studies and complementary laboratory experiments are needed to understand the synergistic role of light and temperature on the phenology of *C. hispida* and this macrophyte group.

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4.4. Final remarks and conclusions

This study represents the first attempt to compare geographical differences of charophyte phenology. The effect of geography is important to understand the potential effect of future increases in temperatures at different latitudes, particularly higher ones. The current global warming trend is causing an advance of the spring onset, and this process is latitude dependent (Parmesan, 2007). Thus, climate change could be affecting the fulfilment of Hopkins' Law. This study was exploratory and, therefore, comparatively long-term, inter-site and multi-latitude studies are needed to accurately define the interactive influences of all environmental factors (particularly temperature and solar radiation) on charophyte phenology and to provide a deeper understanding of the suitability of the application of Hopkins' Law for aquatic ecosystems. For the moment, with the data obtained in this study, we can say that (i) temperature is one of the most important drivers of charophyte reproductive phenology, and (ii) accumulated heat (GDD) rather than daily mean temperature has to be known to predict phenological events.

If the foreseeable warming in Switzerland continues (an approximate increase of 3-5°C in air temperature by the end of the century -CH2011, 2011-, the same difference currently measured between the studied Spanish and Swiss ponds), we can foresee that the sexual events of *C. hispida* would be advanced by more than one month, considerably lengthening the reproductive season of the macrophyte species.

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SECOE (*Service de l'Ecologie de l'Eau - Etat de Genève*) measured part of the water parameters from the Swiss pond. English was edited by Daniel Sheerin (Online English S.C.).

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Figure Captions

Figure 1. Location within the European continent of the two studied ponds: the Spanish pond, *Llacuna Nova del Canyar* (LNC), and the Swiss one, *Étang Robert Hainard* (HAI). The aerial photographs show the location of the temperature probes in each case (white stars) and the depth of the site where they are located. The attached table shows some basic information about the two ponds (geographical coordinates, area, etc.).

Figure 2. Air (**A**) and underwater (**B**) daily mean temperature dynamics, at different depths from the water surface, from *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland) over 2014.

Figure 3. Daily mean (dots), bell-shaped model fitted (grey line) and derivate function (black line) from water temperature from *Llacuna Nova del Canyar* (LNC, Spain, **A**) and *Étang Robert Hainard* (HAI, Switzerland, **B**) over 2014. All variables were standardized. Vertical lines indicate the key-dates for temperature: spring onset (S), maximum temperature (Mx) and end of autumn (A). The represented model in each case is the best fitted one (more details in 2.3. *Calculations and statistics related to temperature data*): the Fourier model of 2 terms for LNC - 30 cm and the Weibull model of 6 parameters for HAI - 50 cm.

Figure 4. Air (**A**) and underwater (**B**) daily mean temperature (DMT) distribution by seasons from *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland) over 2014. The bottom part of the figure shows the DMT difference between LNC and HAI for air (**C**) and underwater (**D**) DMT. This Tukey Mean-Difference plot uses two directly related variables (x= HAI DMT, y=LNC DMT) and shows the difference (y_i-x_i) on the vertical axis, against the average ((y_i+x_i)/2) on the horizontal one. Winter (Win): 1 January-31 March; Spring (Spr): 1 April-30 June; Summer (Sum): 1 July-31 September; Autumn (Aut): 1 October-31 December.

Figure 5. Accumulated Growing Degree Days (GDD) over 2014 in *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland) at different depths from the water surface (more details about calculations in *2.3. Calculations and statistics related to temperature data*). The graph at the top left shows accumulated GDD for LNC, with 1 January as the biofix date in all cases for a better comparison between depths.

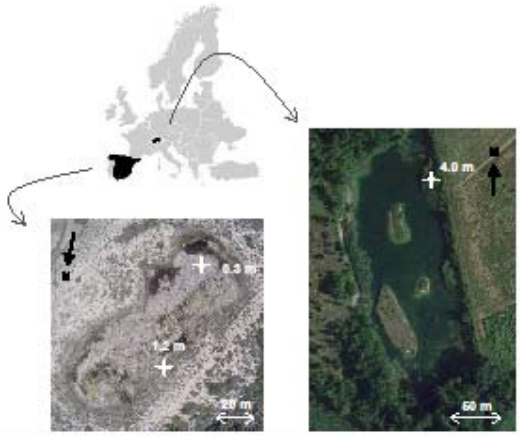
Figure 6. Summary life history of *Chara hispida* according to both studied populations (see differences in *3.5. Reproductive phenology of* Chara hispida).

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The approximate ranges of days and accumulated heat needed to reach each phenological stage are indicated. Photographs of sterile specimens allow comparison of the general morphology differences between the population from *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland).

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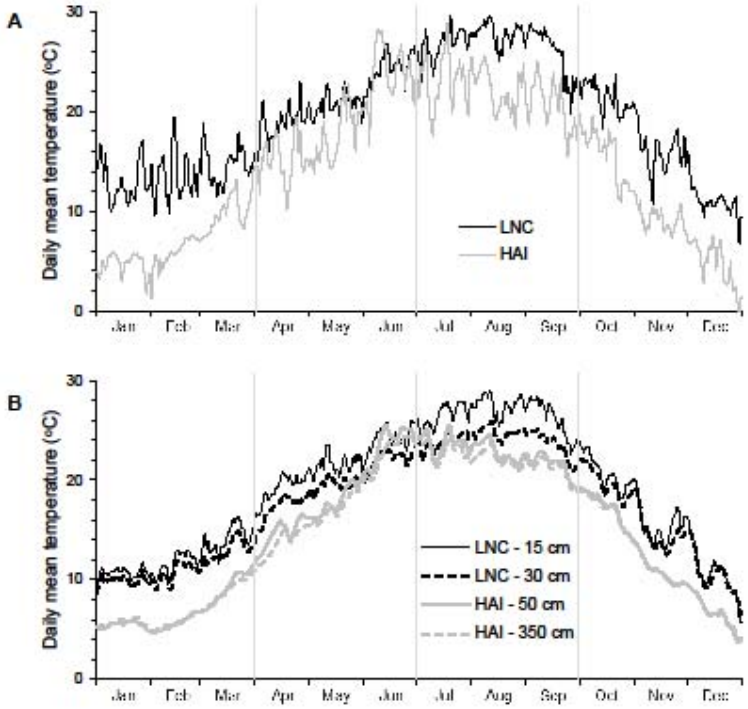
Figure 1



Features \ Pond	LNC *Llacuna Nova del Canyar*	HAI *Étang Robert Hainard*
Coordinates		
Latitude	39° 19' 40.6" N	46° 10' 54.5" N
Longitud	0° 18' 15.5" W	6° 01' 31.1" E
Elevation	3 m a.s.l.	350 m a.s.l.
Area	5900 m²	17000 m²
Maximum depth	1.5 m	5.2 m
Water catchment	Rainfall, groundwater, and saline spray	Groundwater and rainfall
Water level change in 2014	30 cm	100 cm
Emergent vegetation	High density	Scarce. Two islands with reeds
Submerged vegetation	4 sp. of charophytes	6 sp. of charophytes

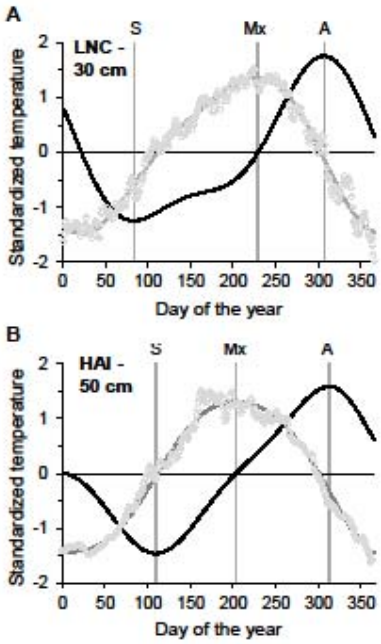
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Figure 2



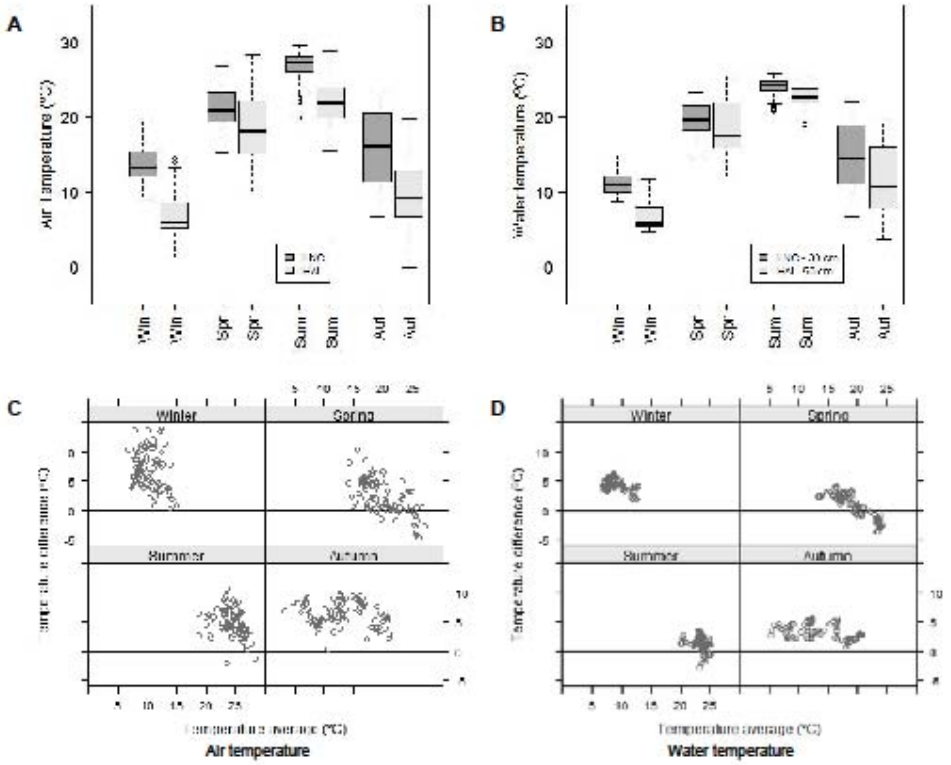
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Figure 3



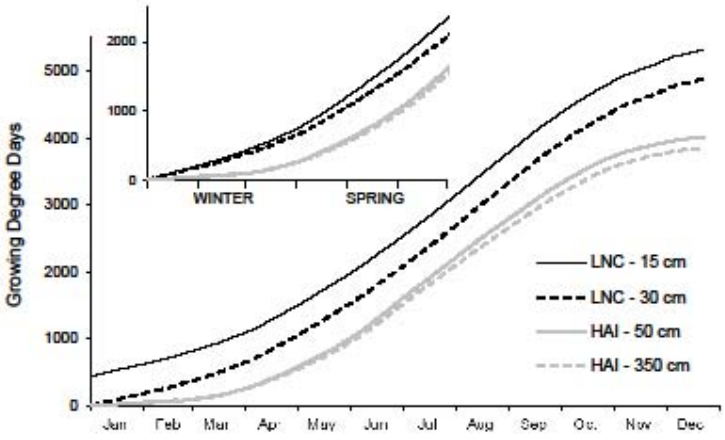
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Figure 4



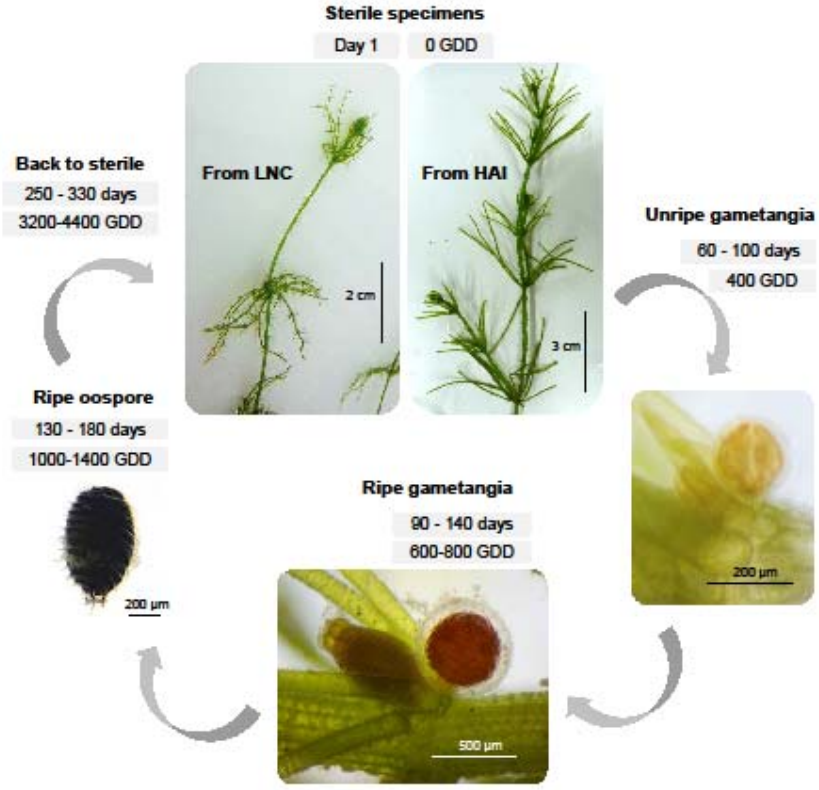
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Figure 5



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Figure 6



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Table I. Basic physical and chemical features of water from *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland) in 2014. Means (minimum-maximum) from monthly samplings are indicated for most variables. In LNC total nitrogen/total phosphorus are single data from March and solute composition of water from January (no more data available). n.d.: no data.

	pH	Conductivity	Dissolved oxygen		Total nitrogen	Total phosphorus
		(µS/cm)	(mg/L)	(Saturation %)	(mg/L)	(mg/L)
LNC, **Spain**	7.4	3435	5.2	60	1.0	0.010
	(7.2-7.5)	(2940-4070)	(2.6-8.1)	(29-92)		
HAI, **Switzerland**	8.2	475	10.1	108	2.0	0.013
	(8.0-8.3)	(398-557)	(8.5-12.4)	(77-143)	(1.0-3.3)	(0.010-0.021)

	HCO_3^-	Cl^-	SO_4^{2-}	Na^+	K^+	Ca^{2+}	Mg^{2+}
	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)	(mg/L)
LNC, **Spain**	181	639	29.1	365	n.d.	107	57.7
HAI, **Switzerland**	231	18.4	42.9	9.6	1.7	78	15.0

Table II. First detection of the main phenological stages of *Chara hispida* at different depths in *Llacuna Nova del Canyar* (LNC, Spain) and *Étang Robert Hainard* (HAI, Switzerland) during 2014. The date, the day of the year (taking the 1 January 2014 as day 1, except for LNC-15 cm where the 1 November 2013 is day 1), the accumulated heat (in Growing Degree Days, GDD), the daily mean temperature (DMT, °C) and accumulated sunlight (Sun, in hours of sun) reached at each moment are indicated. *: there were already unripe gametangia in January when the first observations were carried out. -: no sampling at those depths.

	First unripe gametangia					**First ripe gametangia**					**First ripe oospores**					**Back to 100% sterile specimens after reproduction**				
Depth	Date	Day	GDD	DMT (°C)	Sun (h)	Date	Day	GDD	DMT (°C)	Sun (h)	Date	Day	GDD	DMT (°C)	Sun (h)	Date	Day	GDD	DMT (°C)	Sun (h)
LNC (Spain)																				
15 cm	*	*	*	*	*	31 Jan	92	617	10.3	513	26 Mar	146	1034	14.0	864	30 Sep	334	4350	24.3	2502
30 cm	5 Mar	64	409	11.7	323	26 Mar	85	601	13.0	494	15 May	135	1277	19.9	908	2 Sep	245	3374	24.6	1925
HAI (Switzerland)																				
50 cm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2 Oct	275	3350	19.2	1614
150 cm	17 Apr	107	420	15.3	503	6 May	126	636	16.0	577	23 Jun	174	1421	25.0	984	2 Oct	275	3295	19.1	1614
250 cm	-	-	-	-	-	19 May	139	772	17.1	655	23 Jun	174	1372	24.5	984	-	-	-	-	-
350 cm	17 Apr	107	401	14.9	503	-	-	-	-	-	-	-	-	-	-	2 Oct	275	3199	18.9	1614