

**Factors affecting lichen reproduction, a case study in the  
genus *Cladonia* (Lecanorales, Ascomycota)**

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**FACTORS AFFECTING LICHEN REPRODUCTION, A CASE STUDY IN THE GENUS *CLADONIA*  
(LECANORALES, ASCOMYCOTA)**

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## ABSTRACT

*Cladonia* is a genus of lichen-forming fungi whose ascospore discharge and germination patterns are still underexplored. We studied these behaviours in seven *Cladonia* spp. from two localities (southern England, central Spain) over one year.

We found seasonality in ascospore discharge and germination was present. Ascospore discharge and germination seemed to be more affected by climatic variables than by seasonal period. In general, peak ascospore discharge occurred in winter-spring and lowest rates occurred in summer-autumn across both localities.

Temperature, precipitation and humidity affected ascospore discharge. Temperature and precipitation affected germination rates.

We found no relationship between genetic variability and ascospore discharge or germination.

Keywords: seasonality – sporulation – germination

## INTRODUCTION

Lichens are traditionally considered symbiotic organisms, consisting of a fungal partner (mycobiont) and one or multiple photosynthesising partners (photobiont), usually an alga, cyanobacterium or both (Molina, Divakar & González, 2015). More recent research shows they are complex self-sustaining ecosystems containing additional microscopic organisms (Hawksworth & Grube, 2020).

Lichens reproduce asexually or sexually. Asexual reproduction occurs via vegetative propagules, such as isidia or soredia, or by thallus fragmentation, wherein both fungus and alga are dispersed together (Steinová et al., 2019). Sexual reproduction occurs only in the mycobiont via ascospores (meiotic spores) (Bowler & Rundel, 1975). Parasexual reproduction has also been suggested (Tripp & Lendemer, 2018). Different lichen species employ different combinations of reproduction strategies (Tripp, 2016).

Studies on the release of ascospores (sporulation), germination and their affecting factors date back to at least 1927 (Werner, 1927). Research since then shows sporulation occurring sporadically in some species, and continuously in others (Werner, 1927; Scott, 1959; Yamamoto et al., 1998). Intermittent sporulation is suggested to be driven by seasonal changes (Werner, 1927). Climatic variables, especially temperature and moisture (rainfall, relative humidity etc.) affect discharge and germination (Ahmadjian, 1965; Trail, 2007; Pyatt, 1973). Other factors such as winds, season (Clayden, 1997), light (Bailey & Garrett, 1968), and the effect of culture media composition on germination (Ahmadjian, 1964) have also been studied. No work has been carried out, to our knowledge, investigating genetic diversity as an affecting factor.

Understanding sexual reproduction in lichens would help us understand the biology of lichens better. It would also allow improvements in laboratory mycobiont cultivation, benefitting lichen consumption industries e.g. lichen-based pharmaceuticals (Molina et al., 2015). Whilst an effort has been made to understand these behaviours, with research advancing especially within the last 100

years, they are still understudied, including for members of the *Cladonia* genus. We aim to address that in this report.

*Cladonia* is a genus of lichen containing around 475 species, which have a sub-cosmopolitan distribution (Pino-Bodas & Stenroos, 2020). They often grow where vascular plants fail in harsh environmental conditions such as the taiga and tundra regions (Ahti & Oksanen, 1990; Zhurbenko & Pino-Bodas, 2017). They also grow in more tropical habitats (Ahti, 2000; Sangvichien, Hawksworth & Whalley, 2011). They are usually terricolous, but can grow on wood (Flakus et al., 2008). Crucially, they stabilise the soils, not only preventing erosion (Honegger, 2006), but allowing vascular plants to establish and plant communities to form (Ahti & Oksanen, 1990), subsequently providing the basis for whole ecological communities. Currently, ascospore sporulation and germination behaviours are mostly unknown in this genus.

The aims of this project are: (1) present an overview of the current knowledge on the discharge and germination of ascospores in lichen-forming fungi. (2) Study patterns of ascospore discharge and germination in the genus *Cladonia*, and answer the following questions. Is there seasonality in ascospore discharge and germination in *Cladonia*? Do all *Cladonia* species show the same physiological behaviour in the discharge and germination of ascospores? How do climatic factors affect sporulation and germination in *Cladonia*? Is there a relationship between a species' genetic diversity and the number of spores discharged or germinated?

Based on results of previous authors, we hypothesise: (1) there will be seasonality in ascospore discharge, and at least some degree of seasonality in germination. (2) These physiological behaviours will differ between species. Specifically, we predict seasonality will become more pronounced in species adapted to living in drier habitats. (3) Climatic variables (humidity, rainfall, temperature) will positively affect sporulation and germination. In temperature there may be a maximum cut-off point where sporulation and germination decline and/or cease. (4) Sporulation and germination may differ between species with higher and lower genetic diversity.

## MATERIALS AND METHODS

### *Lichen collection, isolation and culture*

Sampling was carried out in 2 localities: Aldeanueva de Barbarroya, Toledo, central Spain (39°42'01.7"N, 5°04'29.3"W); Thursley Natural Nature Reserve, Surrey, southern England (51°09'33.1"N, 0°42'46.7"W). Aldeanueva de Barbarroya has open holm-oak (*Quercus ilex*) wood vegetation, and quartzite rocks. It has a Mediterranean climate with hot, dry summers and mild, wet winters (IUCN, 2021). Thursley Nature Reserve is heathland with gritty and sandy soil. It has a temperate climate with warm summers, cold winters and consistent rainfall throughout the year (Kazemi & Mohorko, 2017).

Seven species were selected: four from Spain (*Cladonia dimorpha*, *C. foliacea*, *C. rangiformis* and *C. subburgida*) and three from England (*C. floerkeana*, *C. furcata* and *C. ramulosa*). Three thalli of each species were collected every two months from December 2018 to December 2019. Specimens were deposited at Royal Botanic Gardens, Kew (K-M).

Cultures were prepared the day after collection. Two apothecia per thallus were used, 252 cultures in total. We washed apothecia with 1ml of 0.1% of Tween 20 for 30 minutes, vortexing the tubes every 10 minutes. Then, we washed them twice with 1ml of MiliQ water for 30 minutes. Next, the apothecia were immersed in MiliQ water for 2 hours before removing residual water with filter paper. Cultures were prepared according to Ahmadjian's (1993) isolation and culture methods: a single apothecium was attached to the Petri dish lid using double-sided sticky tape. Malt Yeast extract agar medium was inserted into the Petri dish bottom. Petri dishes were inverted so ascospores would shoot upwards onto the medium. Apothecia were left for 24 hours to release their spores, after which any discharge was observed. If discharge had not occurred, they were left for an

additional 24 hours (48 hours total) and checked again. If ascospore discharge still hadn't occurred, we recorded no discharge (sporulation count = 0). After discharge, apothecia were removed, Petri dishes were sealed and were left at room temperature in the dark. After seven days we observed any germination using a Leica DMIL microscope. Ascospore discharge and germination were recorded from the space within a 1cm diameter circle to standardise measures.

Ten cultures became contaminated and no sporulation or germination data were retrieved from them. They were: six (of six) cultures in February in *C. foliacea*, then two from October and two from December in *C. ramulosa*.

Climatic data was collected from World Weather Online (2021) post-field collection, using the 'Historical Weather' feature. We retrieved data for the month of collection at the "Thursley, Surrey" weather station and the "Aldeanueva De Barbarroya, Castilla-La Mancha" station. See **Table 1** for our climatic variable measures.

**Table 1.** Climatic variable measures recorded for the month of collection for each collection.

<b>Variable</b>	<b>Monthly</b>
Average maximum temperature (°C)	
Average minimum temperature (°C)	
Average temperature (°C)	
Average humidity (%)	
Total rainfall (mm)	

### *Statistical analyses*

Two datasets, one per locality (England, Spain), were used to analyse seasonality in the two different climatic regions. We used a dataset for each species (seven additional datasets) to study the behaviours of each species. All statistical analyses were carried out in R Studio (RStudio Team, 2021).

- *Sporulation analyses*

We determined whether month (representing seasonality) was a significant factor in predicting sporulation for England's dataset, and for Spain's. Next, we wanted to see if monthly sporulation differed between species so we added species to each model in two-way regressions. Then, we analysed the effect of month in each species' dataset individually. A combination of linear regressions (LMs) and generalised linear models with negative binomial distribution (NBGLMs), using the 'glm.nb' function from the 'MASS' package (Venables & Ripley, 2002), were used. NBGLMs were appropriate for our datasets with severely skewed counts data (O'Hara & Kotze, 2010): Spain, *C. furcata* and *C. foliacea* datasets. LMs were used for more normally distributed datasets: England, *C. floerkeana*, *C. ramulosa*, *C. dimorpha*, *C. rangiformis* and *C. suburgida*. We selected for models using AIC model selection (**Appendix A1**). All explanatory variables were categorical, removing the requirement for linearity, and meaning that seasonality analyses compared mean sporulation each month to the base level's (February). We carried out post-hoc Tukey HSD tests to compare other combinations of months.

LM assumptions were verified using diagnostic residuals plots (Zuur et al., 2009). *C. ramulosa* and *C. dimorpha* datasets showed some heterogeneity in their variance. Their LMs were justified when considering our aim was to investigate any patterns in our data, not accurately predict sporulation, and the robustness of regression analyses (Knell, 2020). NBGLM errors and variance conformed to their distribution function.

GAM models were tested using the 'gam' function from the 'mgcv' package (Wood, 2011). However, they were not a good fit for our data; k-value was too low ( $p < 0.001$ ) even at maximum value. They were not used.

- *Germination analyses*



To determine whether there was seasonality in germination, we carried out Kruskal-Wallis analyses (suitable for our heteroscedastic, non-normal errors datasets) on all datasets except *C. foliacea*, *C. rangiformis* and *C. suburgida* which had insufficient data to detect any seasonal patterns.

- *Climatic data analyses*

Because results of seasonality in sporulation and germination were significant, effects of climatic variables on sporulation and germination were studied. To measure the effect of our climatic variables (**Table 1**) on sporulation, we used a negative binomial GLM (selected for using AIC) on all the data as one dataset. To measure the effect on germination, we used a multiple linear regression with an arcsine square root transformation on the germination data, which was appropriate for our skewed percentage data (Sokal & Rohlf, 1981). Homogeneous variance was satisfied. Though errors were negatively skewed, we determined multiple LM was appropriate for the same reasons as above.

In order to determine how much variation in sporulation count was explained by our climatic variables, redundancy analyses (RDA) were carried out using the 'rda()' function from the 'vegan' package (Oksanen et al., 2020).

- *Genetic sequencing data collection & analyses*

All ITS rDNA sequences available from GenBank, in addition to unpublished sequences from Pino-Bodas (in prep), for our selected *Cladonia* species were aligned. The DNA extraction, PCR and sequencing protocols used to obtain these sequences are described in Pino-Bodas et al. (2020). One alignment per species was generated, except for *C. floerkeana* because only four sequences were available. Sequences were aligned using the online version of MAFFT (Katoh & Standley, 2013). Then, alignments were visualised in BioEdit (Hall, 1999). Nucleotide diversity (Pi), number of haplotypes and haplotype diversity were calculated in DnaSP 6 (Rozas et al., 2017).

## RESULTS

All available literature on seasonality in ascospore discharge and germination is summarised in **Table 2.**

**Table 2.** Summary of the current literature on lichen-forming fungi and whether seasonality was observed or not (Y/N) in sporulation and germination. See **Appendix B** for additional taxonomic information.

Species	Family	Geographical region/climate	Seasonality in sporulation	Seasonality in germination	Reference
1	Teloschistaceae	-	Y	-	Werner, 1927 (as cited by Scott, 1959)
1	Peltigeraceae	Scotland Temperate	N	N	Scott, 1959
1	Lecanoraceae	England Temperate	N	-	Bailey & Garrett, 1968
13 genera	Baeomycetaceae		Y	Y	
13 spp.	Caliciaceae		Y	Y	
total	Lecanoraceae		Y	Y	
	Ramalinaceae		Y	Y	
	Lecideaceae		Y	Y	
	Graphidaceae	Wales	Y	Y	
	Ochrolechiaceae	Temperate	Y	Y	Pyatt, 1969
	Pertusariaceae		Y	Y	
	Teloschistaceae		Y	Y	

8 genera	Acarosporaceae		N		
14 spp.	Arthoniaceae		N		
total	Opegraphaceae	British Isles	N		
	Caliciaceae	Temperate	N	-	Garrett, 1971
	Ramalinaceae		N		
	Lecanoraceae		Y + N		
	Teloschistaceae		N		
1	Teloschistaceae	Oregon, USA Temperate-oceanic	Y	-	Ostrofsky & Denison, 1980
1	Rhizocarpaceae	Wales Temperate	Y	-	Clayden, 1997

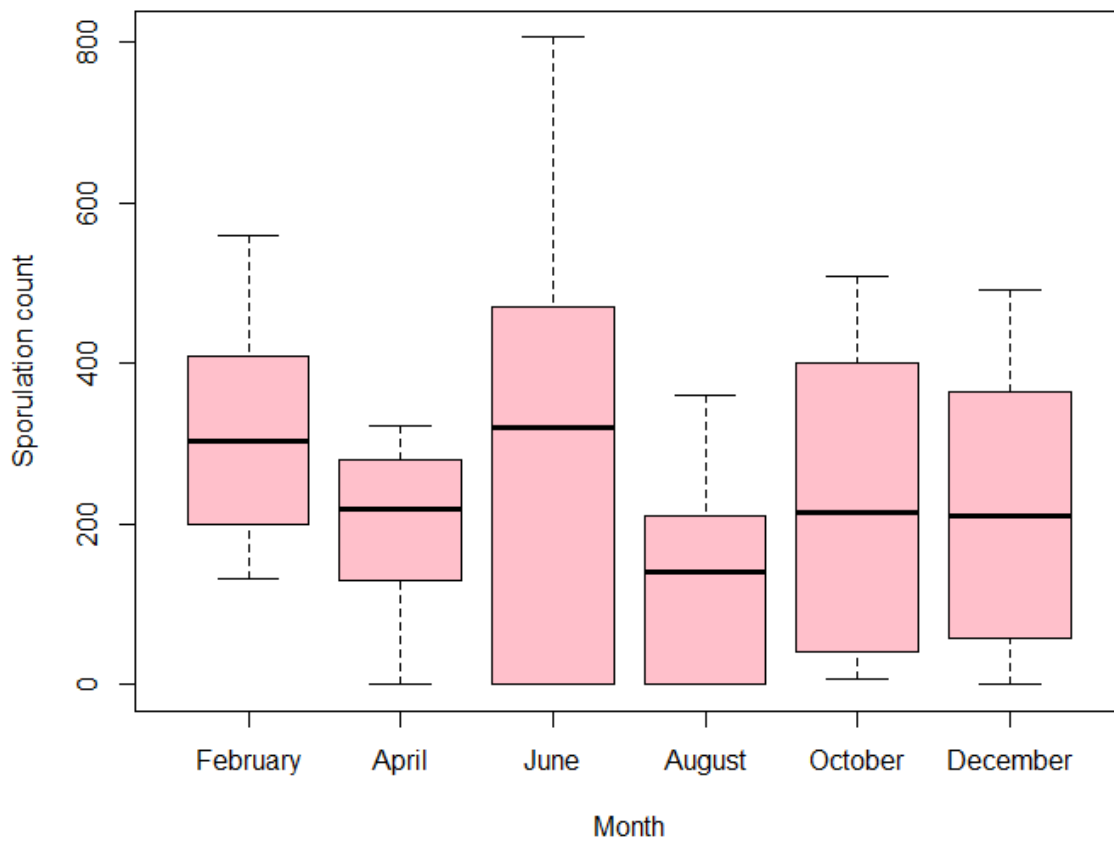
15 genera	Arthoniaceae		Y		
18 spp.	Caliciaceae		Y		
total	Lecanoraceae		Y		
	Parmeliaceae		Y		
	Ramalinaceae		Y		
	Stereocaulaceae		Y		
	Lecideaceae	Japan	Y	-	Yamamoto et al., 1998
	Graphidaceae	Temperate	N		
	Peltigeraceae		Y		
	Pertusariaceae		Y		
	Sarrameanaceae		Y		
	Megalosporaceae		Y		
1 genus		Oregon, USA			
2 spp. total	Lobariaceae	Temperate-oceanic	N	-	Denison, 2003
3 genera	Trypetheliaceae	Thailand, Vietnam,	N	N	
4 spp. total	Graphidaceae	Cambodia	N	N	Sangvichien et al., 2011
	Cladoniaceae	Tropical	Y	Y	

### *Sporulation in England*

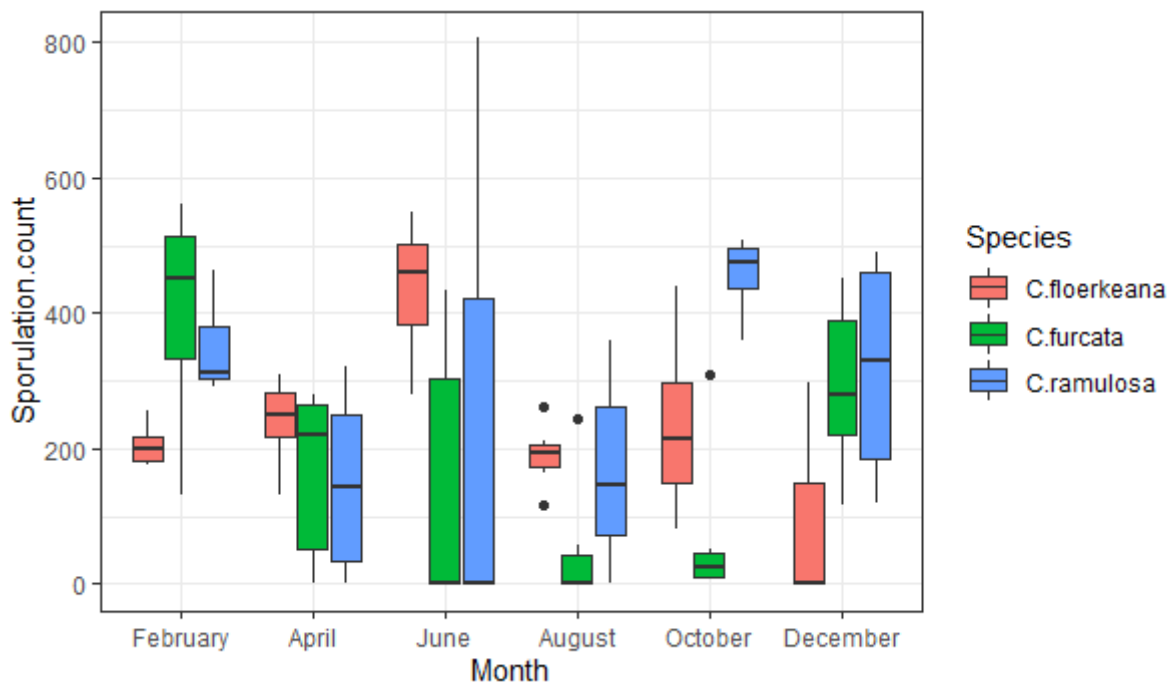
Month was a significant predictor of sporulation ( $F_{5, 98} = 2.448$ ,  $p = 0.039$ ). Peak sporulation occurred in February (mean = 319 ascospores, standard deviation, SD, = 128.901), and lowest was in August (mean = 136, SD = 114). Mean sporulation in August ( $p = 0.002$ ) and April ( $p = 0.022$ ) were both significantly lower than in February. No other pairwise comparisons were significant. **Fig. 1A,**

### **Appendix A1.**

1A)



1B)

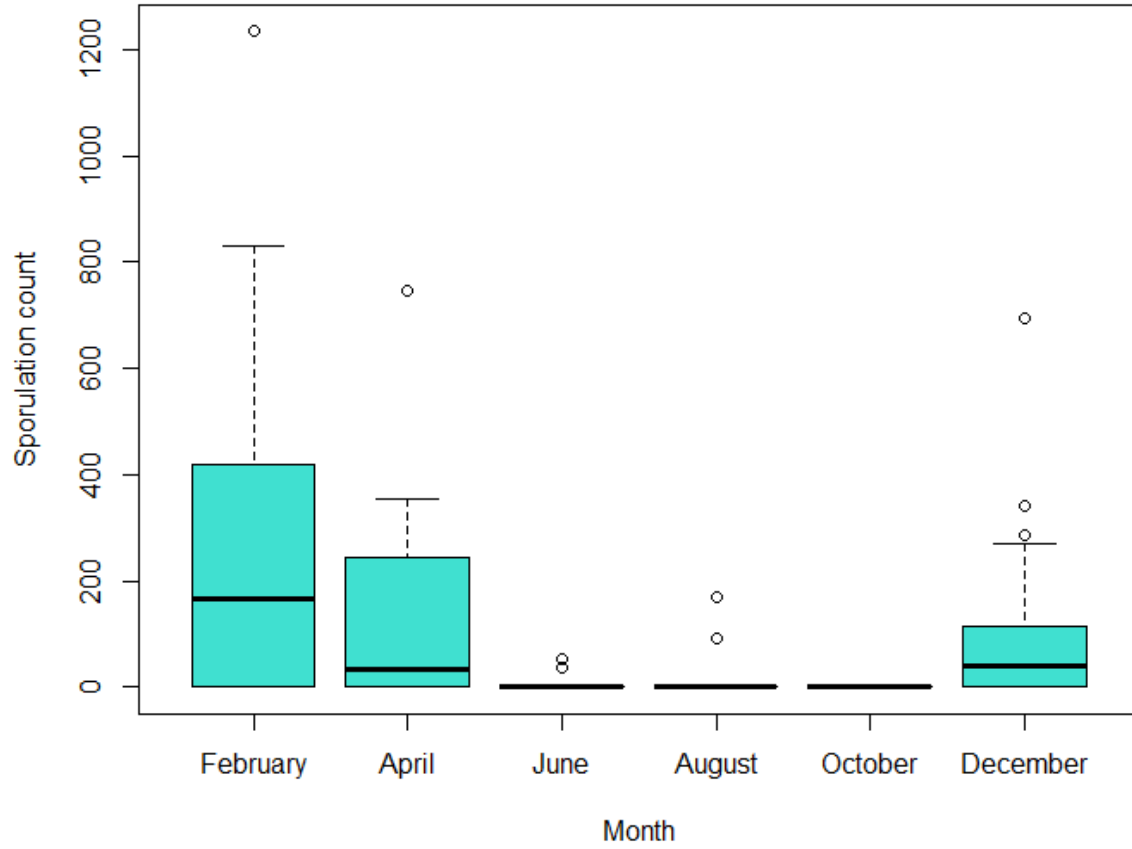


**Figure 1.** Ascospores discharged every two months by three species of *Cladonia* from Thursley, UK, overall (1A) and for each species (1B) during 2019.

#### *Sporulation in Spain*

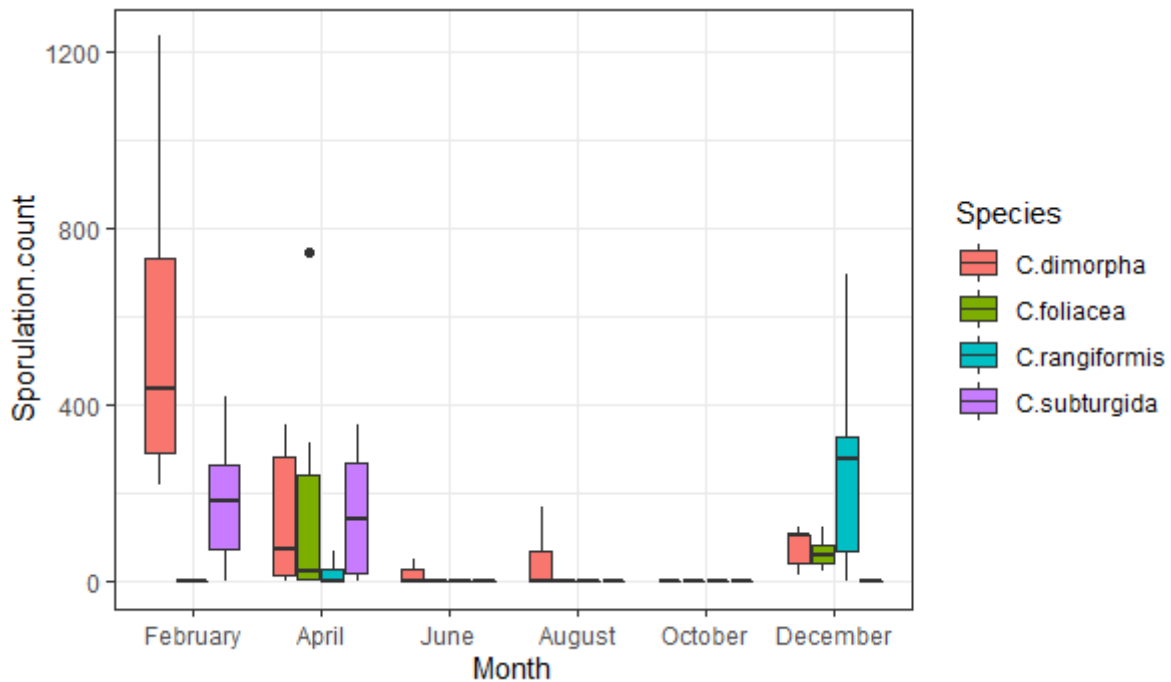
Month was a significant predictor of sporulation ( $df = 5$ , Dev. = 53.613, Resid.  $df = 132$ , Resid. Dev = 86.907,  $p < 0.001$ ). Peak sporulation occurred in February (mean = 251 ascospores, SD = 333.846), lowest was in October (mean = 0, SD = 0). Mean sporulation was significantly lower in June ( $p < 0.001$ ), August ( $p < 0.001$ ), October ( $p < 0.001$ ) and December ( $p = 0.038$ ) than in February. No other comparisons were significant. **Fig. 2A, Appendix A1.**

2A)





2B)



**Figure 2.** Ascospores discharged every two months by four species of *Cladonia* from Toledo, Spain, overall (2A) and for each species (2B) from December 2018 – October 2019.

#### *Sporulation in each species*

In England overall, month determined sporulation ( $F_{5, 96} = 2.483$ ,  $p = 0.037$ ), but species did not ( $F_{2, 96} = 1.699$ ,  $p = 0.1883$ ). This indicates that the three species, *C. floerkeana*, *C. furcata* and *C. ramulosa*, have similar monthly sporulation behaviours. In each individual species' model, the main effect of month on sporulation was significant in *C. floerkeana* ( $F_{5, 30} = 9.330$ ,  $p < 0.001$ ), but not in *C. furcata* ( $df = 5$ , Dev. = 5.393, Resid.  $df = 30$ , Resid. Dev = 41.740,  $p = 0.370$ ) or *C. ramulosa* ( $F_{5, 26} = 1.833$ ,  $p = 0.141$ ).

In *C. floerkeana*, peak sporulation occurred in June (mean = 437, SD = 100), lowest was in December (mean = 83, SD = 132). Mean sporulation in June was significantly higher than in February ( $p = 0.029$ ), April ( $p = 0.012$ ), August ( $p = 0.001$ ), October ( $p = 0.008$ ) and December ( $p < 0.001$ ).

In *C. furcata*, peak sporulation occurred in February (mean = 404 ascospores, SD = 160.784), lowest was in August (mean = 50, SD = 98). Mean sporulation in August ( $p = 0.047$ ) and October ( $p = 0.047$ ) were significantly lower than in February.

In *C. ramulosa*, no two months' mean sporulation differed significantly from each other. **Fig. 1B, Appendix A1.** No other comparisons were significant in any model.

In Spain overall, month determined sporulation ( $F_{5, 129} = 8.191, p < 0.001$ ), while species had a borderline main effect ( $F_{3, 129} = 2.637, p = 0.052$ ). *C. dimorpha* had a significantly higher mean sporulation than *C. rangiformis* ( $p < 0.001$ ) and *C. suburgida* ( $p < 0.001$ ) in February. We found no further significant differences between species in any month. In each individual species' model, month was a significant predicting factor: *C. dimorpha* ( $F_{5, 30} = 8.723, p < 0.001$ ), *C. foliacea* ( $df = 4, Dev. = 81.552, Resid. df = 25, Resid. Dev = 14.655, p < 0.001$ ), *C. rangiformis* ( $F_{5, 30} = 6.145, p < 0.001$ ) and *C. suburgida* ( $F_{5, 30} = 6.034, p < 0.001$ ).

In *C. dimorpha*, peak sporulation occurred in February (mean = 567, SD = 393), lowest was in October (mean = 0, SD = 0). Mean sporulation in all other months were significantly lower than in February ( $p < 0.001$  for all).

In *C. foliacea*, peak sporulation occurred in April (mean = 185 ascospores, SD = 300). Lowest occurred in June, August and October (mean = 0, SD = 0 for all). The NBGLM showed these values were not significantly different from 0 ascospores ( $p = 0.998$ ).

In *C. rangiformis*, December had peak sporulation (mean = 266, SD = 258), and was significantly higher than mean sporulation in February, June, August, October (mean = 0, SD = 0,  $p = 0.002$  for all), which were the joint-lowest months. December's mean sporulation was higher than April's ( $p = 0.004$ ).

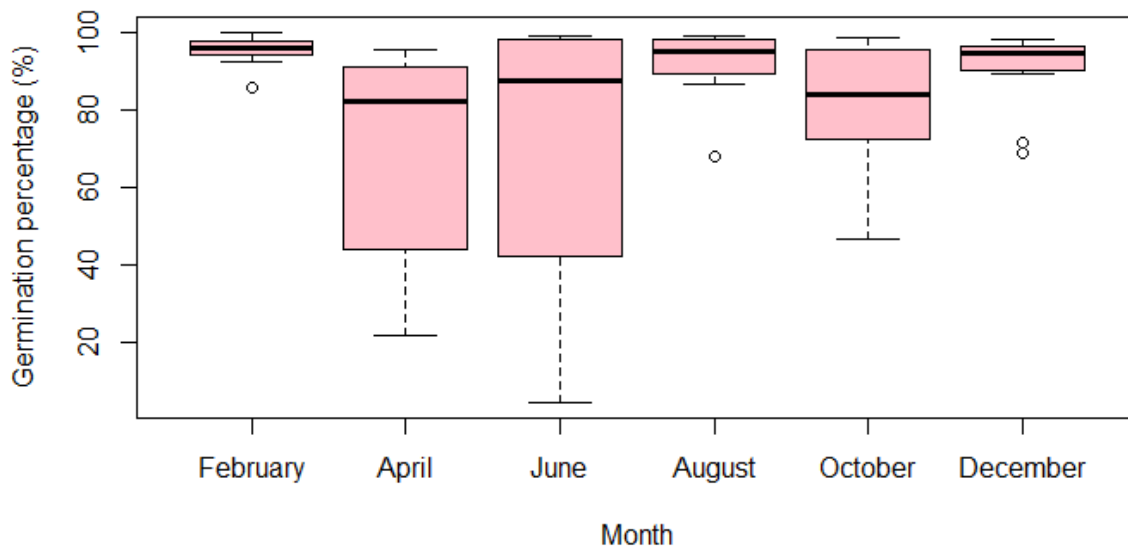
In *C. suburgida*, February had peak sporulation (mean = 187, SD = 156), and was significantly higher than the mean sporulation in June, August, October and December ( $p < 0.001$  for all), which were

the joint-lowest months (mean = 0, SD = 0 for all). Mean sporulation in April was borderline significantly higher than in June, August, October ( $p = 0.051$  for all). **Fig. 2B, Appendix A1.** No other comparisons were significant in any model.

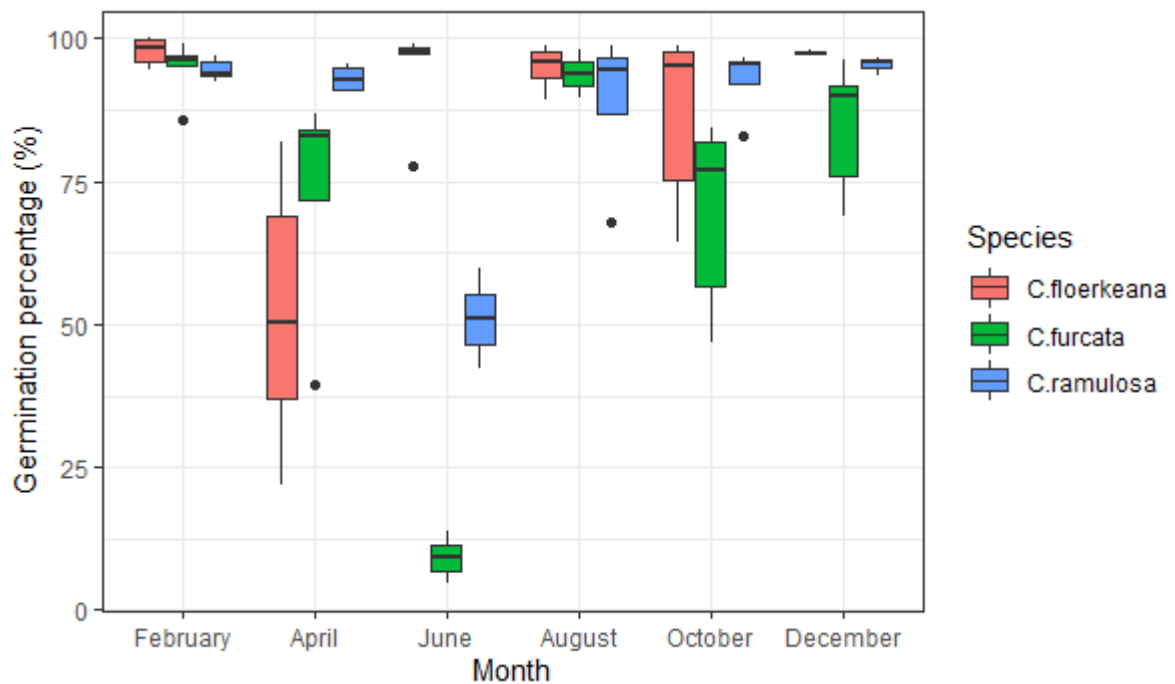
### *Germination*

In England overall, germination rates varied between months ( $H(5) = 19.502, p = 0.002$ ). Germination occurred every month, with peak in February (mean = 95.7%, SD = 3.4) and lowest rate in June (mean = 68.8%, SD = 37.0). **Fig. 3A & Appendix C1.**

**3A)**



3B)



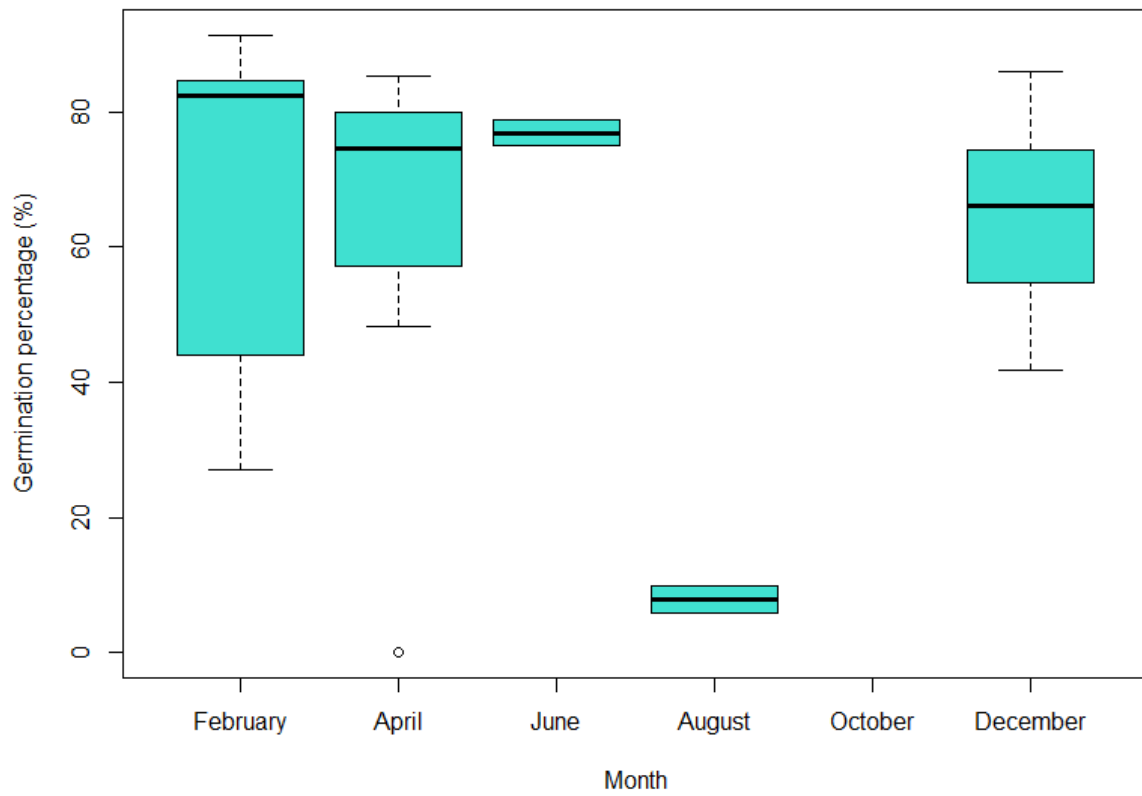
**Figure 3.** Ascospore germination rate by three species of *Cladonia* from Thursley, UK, overall (3A) and for each species (3B) during 2019.

In Spain overall, germination did not vary significantly between months ( $H(4) = 6.181, p = 0.186$ ).

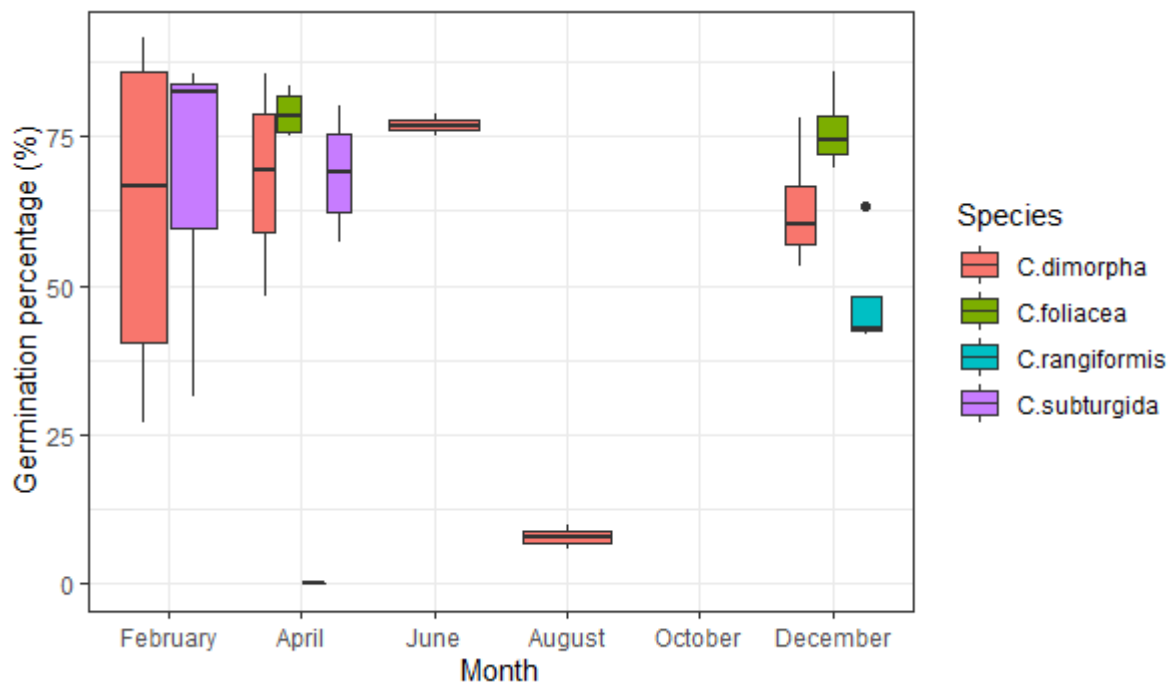
Germination occurred every month but not in every species. Peak germination occurred in June (mean = 76.9%, SD = 2.7), lowest rates occurred in August (mean = 7.9%, SD = 2.8). **Fig. 4A.** &

**Appendix C1.**

4A)



4B)



**Figure 4.** Ascospore germination rate by four species of *Cladonia* from Toledo, Spain, overall (4A) and for each species (4B) from December 2018 – October 2019.

In *C. floerkeana*, germination differed significantly between months ( $H(5) = 15.029$ ,  $p = 0.010$ ). Peak occurred in February (mean = 97.7%, SD = 2.3), lowest was in April (mean = 52.0%, SD = 22.9). In *C. furcata*, germination differed significantly between months ( $H(5) = 16.805$ ,  $p = 0.05$ ). Peak occurred in February (mean = 94.9%, SD = 4.8), lowest was in June (mean = 9.2%, SD = 6.5). In *C. ramulosa*, germination did not differ significantly between months ( $H(5) = 6.457$ ,  $p = 0.264$ ). Peak was in December (mean = 95.4%, SD = 1.4), lowest was in June (mean = 50.9%, SD = 12.4). **Fig. 3B &**

**Appendix C1.**

In *C. dimorpha*, germination did not differ significantly between months ( $H(4) = 5.991$ ,  $p = 0.199$ ). Peak was in June (mean = 76.9%, SD = 2.7), lowest was in August (mean = 7.9%, SD = 2.8). **Fig. 4B &**

**Appendix C1.**

*Effect of climatic variables on sporulation and germination*

Sporulation increased with average maximum temperature and with humidity, and decreased with average temperature and rainfall. It was not related to average minimum temperature (**Table 3**).

Germination decreased with average maximum temperature and with rainfall, and increased with average minimum temperature. It was not related to average temperature or humidity (**Table 4**).

**Table 3.** Results of the NBGLM on effects of climatic variables on sporulation. Estimates are estimated negative binomial regression coefficients (log of expected counts).

	Estimate	Standard Error	<i>p</i> -value	Significance
(Intercept)	0.044245	2.353964	0.985	

<b>Average maximum temperature</b>	<b>0.303745</b>	<b>0.107307</b>	<b>0.00465</b>	<b>**</b>
<b>Average minimum temperature</b>	0.106618	0.102015	0.29597	
<b>Average temperature</b>	<b>-0.51049</b>	<b>0.113373</b>	<b>6.71E-06</b>	<b>***</b>
<b>Average humidity</b>	<b>0.09953</b>	<b>0.023171</b>	<b>1.74E-05</b>	<b>***</b>
<b>Total rainfall</b>	<b>-0.01954</b>	<b>0.004057</b>	<b>1.47E-06</b>	<b>***</b>

Significance codes: <0.000 '\*\*\*', 0.001 '\*\*', 0.01 '\*', 0.05 '.', 0.1-1 ''

**Table 4.** Results of the regression on effects of climatic variables on germination. Estimate coefficients are the arcsine square root of expected counts.

	<b>Estimate</b>	<b>Standard Error</b>	<b>p-value</b>	<b>Significance</b>
<b>(Intercept)</b>	<b>1.455854</b>	<b>0.4387635</b>	<b>0.001195</b>	<b>**</b>
<b>Average maximum temperature</b>	<b>-0.07262</b>	<b>0.0204715</b>	<b>0.000553</b>	<b>***</b>
<b>Average minimum temperature</b>	<b>0.072554</b>	<b>0.01672</b>	<b>2.97E-05</b>	<b>***</b>
<b>Average temperature</b>	0.001864	0.0162455	9.09E-01	
<b>Average humidity</b>	0.004104	0.0043113	3.43E-01	
<b>Total rainfall</b>	<b>-0.00195</b>	<b>0.0008681</b>	<b>2.66E-02</b>	<b>*</b>

Significance codes: <0.000 '\*\*\*', 0.001 '\*\*', 0.01 '\*', 0.05 '.', 0.1-1 ''

The variation in sporulation is better explained by a combination of all climatic variables together (20.4%), rather than any one variable on their own. Humidity had the highest significant conditional effect, followed by temperature. 79.6% of the variance in sporulation was not explained by our model, indicating other factors have more of an effect than our climatic variables. **Table 5.**

**Table 5.** Results of redundancy analysis (RDA) of the significant monthly climatic variables on sporulation. Conditional = effects of that variable controlling for shared effects with all other variables. Simple = unique effects of that variable.

Explanatory variable	Adjusted R Squared value	Df	F-value	P-value	Significance
All variables	0.204	4, 237	16.417	0.001	***
Temperature (conditional)	0.136	2, 239	19.895	0.001	***
Precipitation (conditional)	-0.002	1, 240	0.625	0.417	
Humidity (conditional)	0.187	1, 240	56.444	0.001	***
Temperature (simple)	-0.006	2, 237	0.073	0.926	
Precipitation (simple)	0.012	1, 237	4.472	0.040	*
Humidity (simple)	0.068	1, 237	21.236	0.001	***

Significance codes: <0.000 '\*\*\*', 0.001 '\*\*', 0.01 '\*', 0.05 '.', 0.1-1 ''

#### *Genetic variation of the species*

Genetic analyses revealed four of the six *Cladonia* species we had data for were consistently within the top three highest scores of genetic diversity: *C. dimorpha*; *C. furcata*; *C. foliacea*; *C. rangiformis* – whilst *C. subturgida* had the lowest genetic diversity across all measurements. **Table 6.**



**Table 6.** Results of genetic variance analyses for each *Cladonia* species, based on ITS rDNA sequences. Number of ITS sequences available for each species (*N*), the number of haplotypes (*h*), the haplotype diversity (*Hd*) and the nucleotide diversity (*Pi*).

<b>Species</b>	<b><i>N</i></b>	<b><i>h</i></b>	<b><i>Hd</i></b>	<b><i>Pi</i></b>	
<i>C. dimorpha</i>		25	13	0.897	0.01547
<i>C. foliacea</i>		45	22	0.953	0.00753
<i>C. furcata</i>		47	25	0.952	0.01428
<i>C. ramulosa</i>		48	15	0.795	0.00443
<i>C. rangiformis</i>		152	21	0.749	0.01314
<i>C. suburgida</i>		48	11	0.498	0.00123

## DISCUSSION

### *Seasonality in sporulation/germination in lichen-forming fungi*

We summarised the current knowledge of discharge and germination of ascospores in lichen-forming fungi with regards to seasonality. In temperate zones, lichen-forming fungi displayed both seasonality and non-seasonality in sporulating and germination behaviours (**Table 2**). In tropical climates, seasonality and non-seasonality were also observed (**Table 2**). Of the families that showed seasonality (**Table 2**), most of them discharged more ascospores during the winter-spring months, and less during the summer (Werner, 1927; Pyatt, 1969; Garrett, 1971; Ostrofsky & Denison, 1980; Yamamoto et al., 1998; Clayden, 1997; Sangvichien et al., 2011), though some families, Lecanoraceae, Ramalinaceae, Graphidaceae, Rhizocarpaceae, displayed multiple peaks (Pyatt, 1969; Clayden, 1997), and exact rates of sporulation were dependent on species. In those studies that observed seasonality in germination, rates were similar to sporulation; in general, germination was higher in winter-spring and lower, commonly none, in summer (Pyatt, 1969; Sangvichien et al., 2011).

In the studies based on numerous species, it seemed each species had optimum sporulating and germinating temperatures (which sometimes differed from each other), with rates decreasing either side of those optimums until maximum and minimum limits were reached and neither activity occurred. For example, Pyatt (1969) found no Welsh species germinated at a low of 6°C, *Xanthoria polycarpa* did not sporulate at highs of 25°C or 30°C (Ostrofsky & Denison, 1980), and no east Asian species sporulated at 45°C (Sangvichien et al., 2011). We conclude species adapted to hotter climates have higher optimum sporulation and germination temperatures, whilst species living in cooler climates have lower optimum temperatures.

Studies showed some species had seasonal sporulation while others from the same family sporulated year-round, e.g., Teloschistaceae (Garrett, 1971; Pyatt, 1969). Other literature supports that even within the same genus, different reproductive strategies can occur (Alors et al., 2019). One suggestion is that seasonality in sporulation is more related to growth form than to phylogenetic relationships between species. Most non-seasonal species from the studies (**Appendix B**) were crustose. Sangvichien et al. (2011) found crustose species sporulated and germinated at higher rates than foliose, fruticose or pendent species. We suggest these non-seasonal crustose species were less affected by seasonal changes due to their flat shape. The smaller surface areas would minimise water loss by evaporation, thus in hotter temperatures they retain more water than fruticose (or other) species, potentially aiding sporulation. More study about the seasonality in sporulation of lichens with different growth forms should be conducted.

#### *Seasonality in sporulation/germination in the genus Cladonia*

Our study provides new insight into the sporulation and germination of ascospores in the genus *Cladonia*. Although other studies have included *Cladonia*, only few species were studied – our study is more extensive and up to date. We add novel understanding of sporulation and germination behaviours of *Cladonia* spp. from Mediterranean climates.

Our results give evidence for seasonality in the discharge and germination of ascospores in *Cladonia*; more ascospores were discharged and more ascospores germinated, in general, in the winter-spring, whilst rates dropped in summer-autumn. This supports our hypotheses that seasonality is present, and is congruent with results found in other *Cladonia* species (Sangvichien et al., 2011). Also in line with our predictions, we found these seasonality effects become more pronounced in species from a drier habitat. Species from Spain had dramatically lower sporulation and germination rates over the summer-autumn, often discharging zero spores during this time, whereas although rates in species

from England decreased over summer (with the exception of a second peak in June, which we suggest is partly-explained by the dramatic increase in rainfall, **Appendix D3**), sporulation and germination still occurred in this season. More pronounced seasonality was expected in species from Spain because of the comparatively hotter, drier conditions during the summer. These are less optimal conditions for fungi, especially given that asci require water to increase their turgidity, which is essential to shoot out ascospores with enough pressure (Trail, 2007). On the other hand, the non-seasonality in *C. furcata* and *C. ramulosa* from England is justified because England has no dry season; it is humid and wet year-round (Met Office UK, 2021). These moist conditions are more favourable for fungi (Scott, 1959). However, why did some species show seasonality (*C. floerkeana*, *C. furcata* in germination), when other species from the same locality did not (*C. furcata* in sporulation, *C. ramulosa*)? We propose this is due to between-species differences in sensitivity to climatic factors, such as varying degrees of desiccation tolerance (Crittenden, Hawksworth & Campbell, 1995; Kappen, 1973) and heat tolerance. We suggest seasonal species are more sensitive to climatic conditions, and/or non-seasonal species are more resistant. Additionally, *C. dimorpha* sporulated in summer months when other co-habiting species did not, suggesting this species is well adapted to high temperatures and desiccation. In fact, this species is restricted to Mediterranean regions (Ahti & Hammer, 2002; Burgaz et al., 2017). This shows sporulation and germination behaviours differ between species, supporting our hypotheses, and complimenting current research (Pyatt, 1969; Garrett, 1971).

We did not have statistical evidence proving germination was seasonal in species from Spain, but our data suggest there was; *C. dimorpha* had high germination rates year-round except for August. We put statistical non-significance partly down to small sample size. Our results are inconclusive. Future study should investigate seasonality in germination in species from Mediterranean climates.

Sporulation was affected by temperature, humidity and rainfall. All three variables were inextricably linked together, supporting existing research (Clayden, 1997). Germination was affected by

temperature and rainfall. This supports our hypothesis that climatic variables affect sporulation and germination. Our results of increased sporulation with humidity are consistent with previous research (Garrett, 1971), however our result of decreased sporulation with rainfall contrasts previous research (Pyatt, 1969; Ostrofsky & Denison, 1980) and our hypotheses, as does our result of decreased germination rates with rainfall (Pyatt, 1969). The nature of the relationship between temperature and sporulation and germination was less clear in our data, and does not support our hypotheses. We can only conclude temperature affects sporulation and germination, not how it does so. Changes in sporulation/germination could also be driven by other climatic variables which were beyond the scope of our study, such as dew, frost and fog (Verseghy, 1965; Rundel, 1978).

#### *Genetic variation, sporulation and germination*

There was little evidence for a relationship between genetic diversity and sporulation or germination. *C. dimorpha* had high genetic diversity (highest haplotype diversity) and highest ascospore discharge but only in February, and it did not have highest germination rates. This one finding cannot be generalised to the species as a whole. Other species with highest genetic diversity values did not have highest sporulation or germination rates. Contrastingly, the least genetically diverse species, *C. subturgida*, had lowest sporulation from June to December, supporting a potential relationship. However, other species with joint-lowest sporulation at this time did not have lowest genetic diversity. Our results cannot prove a relationship between genetic diversity and sporulation or germination. This may be because many species of *Cladonia* reproduce mainly through vegetative propagules (Ahti & Hammer, 2002), and species which reproduce vegetatively are expected to have lower genetic diversity than species which reproduce sexually (Otálora et al., 2013). We did not include the role of the photobiont or geographic factors in this study, but they affect genetic diversity in the mycobiont (Lagostina et al., 2020; Steinová et al., 2019). Our results

are far from conclusive and much more work in this area is needed to disprove or prove any relationship.

We conclude there is seasonality in the discharge and germination of ascospores in *Cladonia* from temperate and Mediterranean climates. These behaviours differ between species, including within the same locality. Seasonal effects are more pronounced in species from a Mediterranean climate than from a temperate climate. We prove temperature, humidity and precipitation affect sporulation and germination, adding to this robust finding from literature. No clear patterns between genetic variation and sporulation or germination could be discerned.

Studying sexual reproductive behaviours in lichen-forming fungi is essential to further understand the biology lichens. Implications also include better mycobiont culturing in laboratories, which could be used in the commercial lichen consumption industry.

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## Appendix A: Sporulation data model selection and summarised data

**Table A1.** AIC scores for each sporulation data regression model with different distribution types.

The models with the lowest AIC score were selected for each of our final regression analyses. They are highlighted in green below.

Dataset	Explanatory variables	LM	GLM with Poisson	GLM with NegBin
		England	Month	1374.4460
Spain	Month	1797.7500	18816.0000	773.2400
England	Month + Species	1374.8290	16500.0000	16500.0000
Spain	Month + Species	1795.5360	16630.0000	16620.0000
<i>C. floerkeana</i>	Month	435.8190	2017.4000	470.1600
<i>C. furcata</i>	Month	468.9746	5023.2000	412.4400
<i>C. ramulosa</i>	Month	435.2265	5210.4000	5211.7000
<i>C. dimorpha</i>	Month	482.6727	3469.6000	3470.8000
<i>C. foliacea</i>	Month	386.0671	2377.5000	143.5100
<i>C. rangiformis</i>	Month	445.3307	1865.4000	1867.2000
<i>C. subturgida</i>	Month	432.3068	1813.8000	1815.6000

**Output A1.** Mean sporulation and standard deviation (SD) for each species, each month, and for each locality. Values are rounded to the nearest integer.

	February	April	June	August	October	December
<b>England</b>	319	185	268	136	228	220
	SD = 129	SD = 116	SD = 268	SD = 114	SD = 116	SD = 173
<b><i>C. floerkeana</i></b>	206	241	437	190	234	83
	SD = 30	SD = 64	SD = 100	SD = 48	SD = 131	SD = 132
<b><i>C. furcata</i></b>	404	166	140	50	71	293
	SD = 161	SD = 131	SD = 217	SD = 98	SD = 118	SD = 127
<b><i>C. ramulosa</i></b>	347	149	228	167	455	318
	SD = 69	SD = 136	SD = 361	SD = 138	SD = 67	SD = 181
<b>Spain</b>	251	125	4	11	0	103
	SD = 334	SD = 187	SD = 13	SD = 38	SD = 0	SD = 159
<b><i>C. dimorpha</i></b>	567	142	15	43	0	80
	SD = 393	SD = 166	SD = 23	SD = 71	SD = 0	SD = 47

<b><i>C. foliacea</i></b>	NA	185	0	0	0	67
		SD = 300	SD = 0	SD = 0	SD = 0	SD = 38
<b><i>C. rangiformis</i></b>	0	18	0	0	0	266
	SD = 0	SD = 30	SD = 0	SD = 0	SD = 0	SD = 258
<b><i>C. subturgida</i></b>	187	155	0	0	0	0
	SD = 156	SD = 150	SD = 0	SD = 0	SD = 0	SD = 0



**Appendix B:** Additional taxonomic information of the lichen-forming fungi studied in literature for their seasonality/non-seasonality in sporulation and germination

<b>Species</b>	<b>Family</b>	<b>Order</b>	<b>Reference</b>
<i>Xanthoria parietina</i>	Teloschistaceae	Teloschitales	Werner, 1927 (as cited by Scott, 1959)
<i>Peltigera praetextata</i>	Peltigeraceae	Peltigerales	Scott, 1959
<i>Lecanora conizaeoides</i>	Lecanoraceae	Lecanorales	Bailey & Garrett, 1968
<i>Baeomyces rufus</i>	Baeomycetaceae	Baeomycetales	
<i>Buellia canescens</i>	Caliciaceae	Caliciales	
<i>Lecanora campestris</i>	Lecanoraceae	Lecanorales	
<i>Lecanora conizaeoides</i>	Lecanoraceae	Lecanorales	
<i>Lecanora atra</i>	Lecanoraceae	Lecanorales	
<i>Toninia coeruleonigricans</i>	Ramalinaceae	Lecanorales	
<i>Lecidea limitata</i>	Lecideaceae	Lecidales	Pyatt, 1969
<i>Lecidea macrocarpa</i>	Lecideaceae	Lecidales	
<i>Graphis sp.</i>	Graphidaceae	Ostropales	
<i>Ochrolechia parella</i>	Ochrolechiaceae	Pertusariales	
<i>Pertusaria pertusa</i>	Pertusariaceae	Pertusariales	
<i>Xanthoria parietina</i>	Teloschistaceae	Teloschitales	
<i>Caloplaca heppiana</i>	Teloschistaceae	Teloschitales	
<i>Sarcogyne regularis</i>	Acarosporaceae	Acarosporales	
<i>Arthonia impolita</i>	Arthoniaceae	Arthoniales	
<i>Arthonia radiata</i>	Arthoniaceae	Arthoniales	
<i>Opegrapha atra</i>	Opegraphaceae	Arthoniales	
<i>Buellia punctata</i>	Caliciaceae	Caliciales	
<i>Bacidia rubella</i>	Ramalinaceae	Lecanorales	
<i>Lecanora carpinea</i>	Lecanoraceae	Lecanorales	Garrett, 1971
<i>Lecanora atra</i>	Lecanoraceae	Lecanorales	
<i>Lecanora campestris</i>	Lecanoraceae	Lecanorales	
<i>Lecanora chlarotera</i>	Lecanoraceae	Lecanorales	
<i>Lecanora dispersa</i>	Lecanoraceae	Lecanorales	
<i>Lecanora muralis</i>	Lecanoraceae	Lecanorales	
<i>Caloplaca aurantia</i>	Teloschistaceae	Teloschitales	
<i>Xanthoria parietina</i>	Teloschistaceae	Teloschitales	

<i>Xanthoria polycarpa</i>	Teloschistaceae	Teloschitales	Ostrofsky & Denison, 1980
<i>Rhizocarpon lecanorinum</i>	Rhizocarpaceae	Rhizocarpales	Clayden, 1997
<i>Arthonia cinnabarina</i>	Arthoniaceae	Arthoniales	
<i>Buellia stellulata</i>	Caliciaceae	Caliciales	
<i>Pyxine endochrysin</i>	Caliciaceae	Caliciales	
<i>Lecanora megalocheila</i>	Lecanoraceae	Lecanorales	
<i>Lecanora subimergens</i>	Lecanoraceae	Lecanorales	
<i>Myelochroa entotheiochroa</i>	Parmeliaceae	Lecanorales	
<i>Parmelia laevior</i>	Parmeliaceae	Lecanorales	
<i>Ramalina crassa</i>	Ramalinaceae	Lecanorales	
<i>Stereocaulon commixtum</i>	Stereocaulaceae	Lecanorales	Yamamoto et al., 1998
<i>Porpidia albocaerulescens</i>	Lecideaceae	Lecidales	
<i>Graphina soozana</i>	Graphidaceae	Ostropales	
<i>Graphis cervina</i>	Graphidaceae	Ostropales	
<i>Graphis tenella</i>	Graphidaceae	Ostropales	
<i>Peltigera praetextata</i>	Peltigeraceae	Peltigerales	
<i>Peltigera pruinosa</i>	Peltigeraceae	Peltigerales	
<i>Ochrolechia parellula</i>	Pertusariaceae	Pertusariales	
<i>Loxospora ochrophaea</i>	Sarrameanaceae	Sarrameanales	
<i>Megalospora tuberculosa</i>	Megalosporaceae	Teloschitales	
<i>Lobaria oregana</i>	Lobariaceae	Peltigerales	
<i>L. pulmonaria</i>	Lobariaceae	Peltigerales	Denison, 2003
<i>Trypethelium eluteriae</i>	Trypetheliaceae	Trypetheliales	
<i>Graphis elegans</i>	Graphidaceae	Ostropales	Sangvichien et al., 2011
<i>G. rigidula</i>	Graphidaceae	Ostropales	
<i>Cladonia submultiformis</i>	Cladoniaceae	Lecanorales	

## Appendix C: Germination data

**Output C1.** Mean germination percentage (%) and standard deviation (SD) for each species, each month and for each locality, to 1 decimal place. NA indicates no spores were released in the first place, therefore germination percentage could not be observed.

	February	April	June	August	October	December
<b>England</b>	95.7	69.8	68.8	92.5	81.8	90.5
	SD = 3.4	SD = 25.2	SD = 37.0	SD = 8.4	SD = 16.9	SD = 9.8
<b><i>C. floerkeana</i></b>	97.7	52.0	94.7	95.1	86.7	97.5
	SD = 2.3	SD = 22.9	SD = 8.3	SD = 3.7	SD = 15.8	SD = 0.7
<b><i>C. furcata</i></b>	94.9	73.1	9.2	93.7	69.8	84.9
	SD = 4.8	SD = 22.4	SD = 6.5	SD = 6.0	SD = 16.8	SD = 11.6
<b><i>C. ramulosa</i></b>	94.5	93.0	50.9	88.9	92.6	95.4
	SD = 1.8	SD = 2.4	SD = 12.4	SD = 12.6	SD = 6.4	SD = 1.4
<b>Spain</b>	65.3	61.7	76.9	7.9	NA	64.0
	SD = 24.8	SD = 28.2	SD = 2.7	SD = 2.8		SD = 13.8
<b><i>C. dimorpha</i></b>	62.6	68.2	76.9	7.9	NA	62.8
	SD = 27.9	SD = 16.2	SD = 2.7	SD = 2.8		SD = 9.2
<b><i>C. foliacea</i></b>	NA	78.9	NA	NA	NA	75.9
		SD = 4.1				SD = 5.9
<b><i>C. rangiformis</i></b>	NA	0.0	NA	NA	NA	47.8
		SD = 0.0				SD = 10.5
<b><i>C. suburgida</i></b>	68.6	68.9	NA	NA	NA	NA
	SD = 23.4	SD = 10.2				

## Appendix D: Climatic data

**Table D1.** Thursley, Surrey, England's climatic data from January 2019 to December 2019. Variables include: maximum monthly temperature (T Max (°C)); average monthly temperature (T Avg (°C)); minimum monthly average temperature (T Min (°C)); Humidity (%); total monthly rainfall (mm).

Country	Month	Year	T Max (°C)	T Avg (°C)	T Min (°C)	Humidity (%)	Rainfall (mm)
England	January	2019	6	4	2	85	32.5
England	February	2019	10	8	6	44	50.3
England	March	2019	11	9	6	82	92.6
England	April	2019	14	11	6	78	43.8
England	May	2019	15	13	7	82	93.1
England	June	2019	18	16	11	85	174
England	July	2019	21	19	14	79	66.6
England	August	2019	21	19	14	76	57.1
England	September	2019	19	16	11	77	90.8
England	October	2019	13	12	9	85	107.1
England	November	2019	9	8	5	88	136.4
England	December	2019	8	7	5	88	118.9

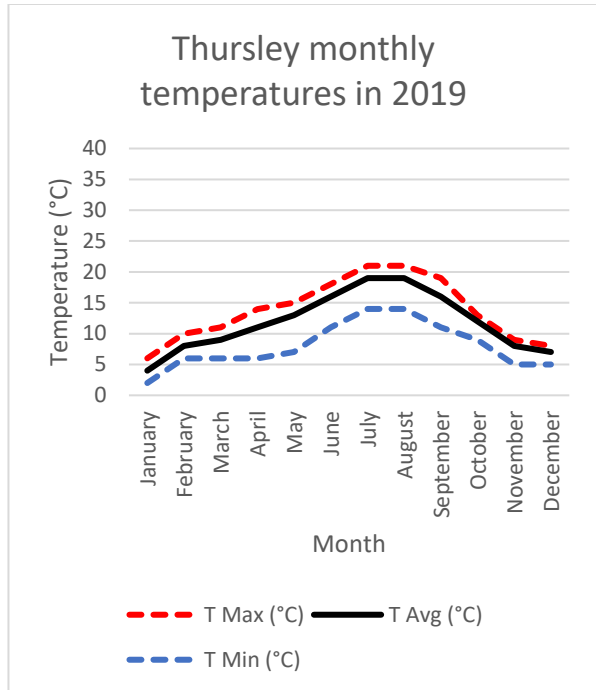
**Table D2.** Aldeanueva de Barbarroya, Toledo, Spain's climatic data from December 2018 to November 2019. Variables include: maximum monthly temperature (T Max (°C)); average monthly temperature (T Avg (°C)); minimum monthly average temperature (T Min (°C)); Humidity (%); total monthly rainfall (mm).

Country	Month	Year	T Max (°C)	T Avg (°C)	T Min (°C)	Humidity (%)	Rainfall (mm)
Spain	December	2018	14	10	6	63	12.7
Spain	January	2019	13	8	4	61	12
Spain	February	2019	16	11	5	55	9.9
Spain	March	2019	18	14	7	54	27.1
Spain	April	2019	19	15	7	63	75.8
Spain	May	2019	27	22	13	44	3
Spain	June	2019	30	25	15	36	0.5
Spain	July	2019	34	29	20	34	15.9
Spain	August	2019	33	28	19	36	27.3
Spain	September	2019	28	24	17	46	68.2

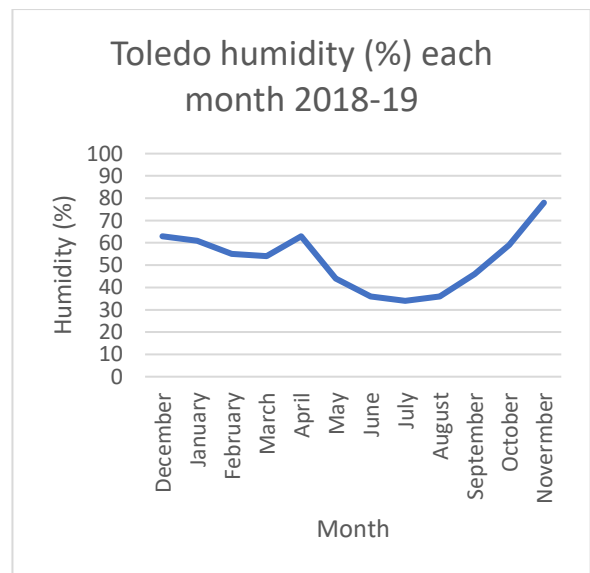
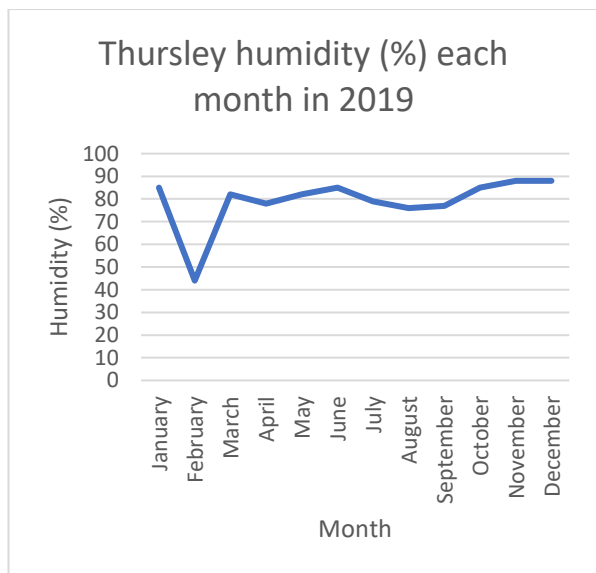
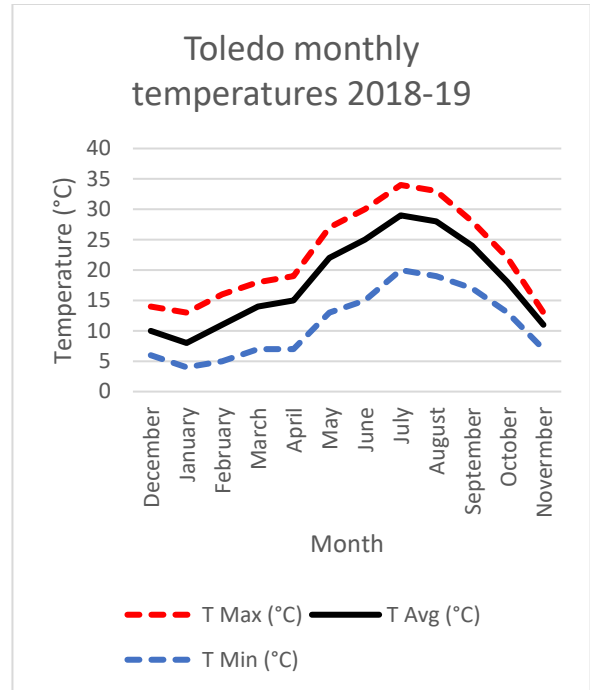
Spain	October	2019	22	18	13	59	50.2
Spain	November	2019	13	11	7	78	74.3

**Figure D3.** Climatic data of Surrey (England) in 2019 and Toledo (Spain) from December 2018 – 2019 for each month. Months shown are the months for which lichens were collected from their respective localities.

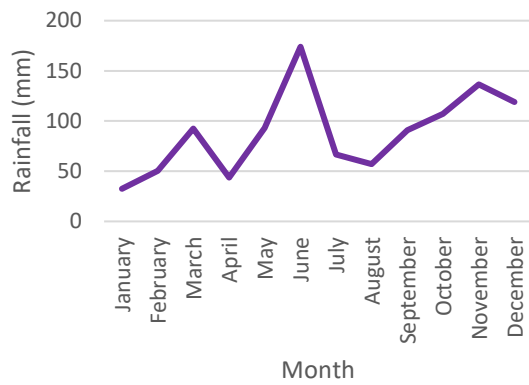
**England**



**Spain**



### Thursley rainfall (mm) each month in 2019



### Toldeo rainfall (mm) each month 2018-19

