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# Fern feeding ecology of the Azores bullfinch, *Pyrrhula murina*

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Jaime A. Ramos (Universidade de Coimbra) e do Professor Doutor Luis García Quintanilla (Universidad Rey Juan Carlos, Madrid)

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**Abstract**

Ferns are an important component of many ecosystems and potentially provide an abundant food resource for consumers, but there are very few studies on the ecology of fern feeding by vertebrates. In this paper we examined fern selection, of both sporangia and leaves, by the Azores bullfinch, *Pyrrhula murina* an endemic bird of São Miguel Island, Azores. Nutrition of both spores and leaves (lipids, proteins, phenolics and caloric content) were compared between consumed and non-consumed fern species. Overall, from a variety of spores and leaf components, caloric and lipid contents were the most strongly correlated with bird preferences. In winter, the spores of consumed species (mostly *Woodwardia radicans* and *Culcita macrocarpa*) had a higher caloric, lipid and protein content than spores of little-and-non-consumed species (*Dryopteris* spp. and *Blechum spicant*). In late winter and spring, when spores were no longer available, the birds consumed young leaves of *Pteridium aquilinum* and *Osmunda regalis*, but those of *O. regalis,* with higher caloric and lipid contents, and lower phenolics, levels were preferred over *P. aquilinum*. Secondly, we studied the influence of altitude and canopy cover on spore maturation and timing of spore release in *Culcita macrocarpa* and *Woodwardia radicans* to understand when they are available for the Azores bullfinch. In addition, the factors affecting spore phenology traits have been poorly studied, contrasting with the abundant literature dealing with leaf expansion. We selected three sites at 400, 600 and 800 m and at each site, marked 12 mature individuals of each species. These sites were visited every 10 days to obtain a sample of spores and record whether sori were open. Sporangia were observed with light microscope to register: a) spores with perispore and completely fulfilled with protein and lipid droplets and b) spores with perispore and with some content inside (but not completely fulfilled). Spore maturation occurred before on *C. macrocarpa* than in *W. radicans*. Altitude had a significant effect in the maturation of spores, with maturation

occurring later from low to mid and high altitudes. Canopy cover had no significant effect on the maturation of spores. In both species spore release started in January and was gradual until the end of April, coincided with the wetmost period of the year. A gradual maturation and liberation of spores along an altitudinal gradient is important for the feeding of the Azores bullfinch in winter.

**Resumo**

Os fetos constituem um componente importante dos ecosistemas e potencialmente proporcionam abundantes fontes de alimento para animais, mas existem poucos estudos sobre a ecologia alimentar de fetos por vertebrados. Neste capitulo estudamos a selecção de esporangios e folhas de fetos pelo Priolo *Pyrrhula murina*, uma ave endemica da ilha de São Miguel, Açores. Os nutrientes dos esporos e folhas (lipidos, proteinas, fenóis e conteúdo calórico) foi comparada entre espécies de fetos consumidas e não consumidas. De uma grande variedade de componentes em esporos e folhas, o conteúdo calórico e de lípidos foram os mais associados à selecção de fetos pelo Priolo. No inverno os esporos das espécies consumidas (sobretudo *Woodwardia radicans* e *Culcita macrocarpa*) apresentam um conteúdo calórico, de lipidos e de proteinas mais alto do que aquele os dos esporos de espécies pouco consumidas ou não consumidas (*Dryopteris* spp. e *Blechum spicant*). No fim do inverno e na primavera, quando já não existem esporos disponíveis, as aves consumiram folhas novas de *Pteridium aquilinum* e *Osmunda regalis,* sendo estas últimas, com maior conteúdo calórico e de lípidos e menores níveis de fenóis, preferidas sobre *P. aquilinum*. Em segundo lugar estudamos a inluência da altitude e cobertura de vegetação de esporos de *C. macrocarpa* e *W. radicans*, de modo a compreender a disponibilidade deste recurso alimentar para o Priolo. Além disso, os factores que influenciam a fenologia de dos esporos têm sido pouco estudados, o que contrasta com toda a literatura que existe sobre expansão de folhas. Seleccionamos três locais a 400, 600 e 800 m e, em cada local, marcamos 12 individuos maturos de cada espécie. Estes locais foram visitados cada 10 dias para obter uma amostra de esporos e registrar se os sori estavam abertos. Os esporângios foram observados ao microscópio óptico para registrar: a) esporos com perisporio e completamente cheios com gotas proteicas e lipidicas e b) esporos com

perisporio e com algumas gotas, mas sem estarem completamente cheios. A maturação dos esporos ocorreu primeiro em *C. Macrocarpa* do que em *W. radicans*. A altitude influenciou significativamente a maturação dos esporos, e ocorreu progresivamente mais tarde desde zonas baixas até zonas altas. A cobertura da vegetação não influenciou a maturação dos esporos. Em ambas as espécies a libertação dos esporos começou em Janeiro e ocorreu gradualmente até o fim de Abril, coincidindo com o período máis húmido do ano. A maturação e libertação graduais dos esporos ao longo do gradiente de altitude é importante para fornecer alimento ao longo do Inverno para o Priolo.

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# Chapter I

## Chemical composition affects fern choice by the Azores bullfinch, *Pyrrhula murina*

# 1 – Introduction

The selection of food resources by herbivorous-granivorous animals is influenced by a variety of factors. Preference determines which available foods are consumed and is mainly influenced by food size and handling time (Diaz 1994), nutritional value (Schaefer et al. 2003) and secondary compounds such as phenolics. Ferns (Pteridophyta) are an important component of many ecosystems (Tryon 1986) and potentially provide an abundant food resource for consumers. Invertebrates such as gastropods and insects consume ferns to some extend but very few vertebrate species are known to consume ferns regularly. Some exceptions are found amongst birds such as the Takahē (*Notornis mantelli*) and the Kaka (*Nestor meridionalis*) in New Zealand (Mills et al, 1980; O´Donell and Dilks 1994), the Cantabrian Capercaillie (*Tetrao urogallus cantabricus*) in Spain (Rodriguez and Obeso 2000) and the Azores bullfinch or Priolo (*Pyrrhula murina*) in the Azores, Portugal (Ramos 1994). The reasons behind the ubiquitous low consumption of ferns by vertebrates are not clear but it has been suggested to be related to the high concentration of diverse biochemical defences on ferns (Seigler 1991; Moran 2004; Marrs and Watt 2006) and lignin contents (Cornelissen et al. 2004).

The critically endangered Azores bullfinch (IUCN 2005), is a bird endemic to the laurel forest of São Miguel Island, Azores, and ferns, both sporangia and leaves, form an important part of its diet (Ramos 1995). Overall the Azores bullfinch prefers seeds over fern sporangia and these over fern leaves (Ramos 1994; 1996). The main consumption period of fern sporangia occurs from December to April, the period of lowest temperature and lowest seed abundance, but sporangia of *O. regalis* are also taken in May-July. The main feeding period on fern leaves is April - June (Ramos 1994; 1995). Ramos (1995) described the seasonal pattern of fern feeding by the Azores Bullfinch and showed that sporangia of three species are consumed: *Woodwardia*

*radicans*, from October to March, with a peak in November-January, *Culcita macrocarpa*, from November to April, with a peak in January-March, and *Osmunda regalis*, in May-July. In respect to leaves, only young ones were consumed, especially from *P. aquilinum* and *O. regalis*, in April-June, but *O. regalis* seemed highly preferred to *P. aquilinum* because birds switched from *P. aquilinum* to *O. regalis* as soon as this last species became available (Ramos 1994). Since these studies were carried out, 14 km of new tracks were opened within the core area of the Azores bullfinch under the scope of a conservation project. Therefore, it is important to assess whether the patterns of fern consumption found by Ramos (1994; 1995) are the same within the larger area now accessible. The observations of Ramos (1995) raised several questions in relation to fern feeding behaviour by the Azores bullfinch: To what extent the Azores bullfinch consumes other fern species? Given that spores of both *W. radicans* and *C. macrocarpa* are apparently available at the same time, why does the foraging peak in *W. radicans* occurs before than that in *C. macrocarpa*? How important are leaves of other species to the Azores bullfinch and why is *O. regalis* preferred? Which fern species and leaf developmental stages are preferred and why? To answer these questions we made an extensive survey of fern sporangia and leaf feeding by the Azores bullfinch and analysed the variation in caloric, lipid, protein and phenolic content of consumed and non consumed species. We defined different developing stages of the leaves to investigate which ones are preferred because toughness increases as leaves age, and digestibility diminishes (Hill and Lucas 1996).

Birds are expected to maximize energy intake, therefore we evaluated the calorific content of sporangia and leaves of consumed and non consumed fern species to assess whether the Azores bullfinch selects ferns on the basis of its energetic content. Lipids are highly energetic and it is known that the European bullfinch (*Pyrrhula*

*pyrrhula*) selects fat rich ash (*Fraxinus* sp.) seeds in winter (Greig-Smith 1985). Therefore, particularly during winter, we expect that fern species high in lipids should be selected. The capacity of ferns to synthesize lipids varies among species and leaf developmental stage (Rozenstvet et al. 2001). In this study, analyses were carried out when sporangia were mature and when leaves were young and smooth, because these are the stages that are consumed by the Azores bullfinch (Ramos 1994; 1995).

Proteins are a limiting resource for birds, and vegetable food sources are generally poor protein sources (Izhaki 1993). We examined soluble protein content in ferns because previous studies with European bullfinches showed that it plays an important role in the selection of food sources (Summers and Jones 1976). Phenolics are regarded as key components for plant defence strategies and their importance in feeding ecology has received much attention (Rosenthal and Berenbaum 1991; Berenbaum 1995). Several studies revealed a negative correlation between phenolic content and herbivory (Jakubas et al. 1989; Snyder 1992), including in the European bullfinch (Wilson 1984; Greig-Smith and Wilson 1985). Phenolics precipitate proteins and thus may reduce protein availability by reducing protein digestibility and assimilation.

Here we studied the preference patterns for fern species and shifts in fern preference by the Azores bullfinch. Our specific objectives were: (1) to determine which fern species and which parts of these species, fertile leaves with sporangia or unfertile leaves, are selected by the Azores bullfinch on different periods, (2) to assess which developmental stages of fern leaves are preferred, and (3) to compare the caloric, lipid, protein and phenolics content of sporangia and leaves in consumed and non consumed fern species.

# 2 - Methods

## 2.1. Study area

The Azores bullfinch has always been confined to the Eastern part of the Island of São Miguel, Azores, Portugal. Its distribution is highly associated with the native laurel forest, composed of evergreen trees (e.g. *Ilex perado spp. azorica, Laurus azorica, Vaccinium cylindraceum*) between 350 - 900 m. This forest has 947 vascular plant species, 71 (7.5%) of which are ferns (Dias 1996). The understory is dominated by evergreen ferns (e.g. *C. macrocarpa*, *Dryopteris affinis*, *Dryopteris aemula*, *Dryopteris azorica*, *Pteris incompleta*, *W. radicans*) and winter deciduous ferns (e.g. *Blechnum spicant*, *O. regalis*, *P. aquilinum*) (Dias 1996). The area has been reduced and altered by plantations of Japanese red cedar (*Cryptomeria japonica)* and by the invasion of exotic plants, mainly Australian cheesewood (*Pittosporum undulatum)*, Kahili ginger (*Hedychium gardneranum)* and Lily-of-the-valley tree (*Clethra arborea)*. It is classified as a Special Protection Area (SPA) under the Natura 2000 network.

## 2.2. Observation of foraging birds

To evaluate the importance of ferns in the diet of the Azores bullfinch in relation to other food resources, we observed foraging birds throughout the study area from October 2006 to July 2007. Every time a bird was observed one feeding record was obtained according to the rule: one bird, one food, one record (Newton 1964). Observations were performed homogeneously from dawn to sunset by experienced observers and covered the whole SPA.

## 2.3. Sporangia consumption

Two types of transects were carried out in order to determine sporangia consumption. Firstly, to determine which fern species are preferred, we performed 259

linear transects of 50 x 2 m covering the whole distribution area of the Azores bullfinch: 73 transects (28%) at low altitude (350-575 m), 110 (43%) at mid altitude (575-700 m) and 76 (29%) at high altitude (700-900 m). We counted all fern fertile leaves i.e. those with sori (clusters of sporangia), and recorded all obvious beak marks of the Azores bullfinch (hereafter named fern stripping). These are very characteristic and no other bird species in the area feeds on ferns (Ramos 1994; 1995). These transects were made between 7 January and 23 March 2007, which includes the main consumption period of fern sporangia (Ramos 1995). Secondly, to detect shifts in the preference within the two most consumed fern species (*W. radicans* and *C. macrocarpa*), we performed two sets of 150 x 2 m transects on the 30$^{th}$ of October and the 3$^{rd}$ of December to record fertile leaves (hereafter named leaves) with fern stripping in *W. radicans* and *C. macrocarpa*. These dates were chosen because Ramos (1995) detected a peak in the consumption of *W. radicans* in November, whereas *C. macrocarpa* was only taken from December onwards.

**2.4. Leaf consumption**

To evaluate the abundance and consumption of fern leaves, and to document the appearance of new leaves as well as shifts in their consumption, we marked 30 transects of 8 x 4 m between 16 and 23 March 2007. All ferns within each transect were individually marked, identified and their developmental stage recorded. We revisited all transects and marked new individuals every 15 days until 15 May 2007; on each visit the developmental stage of all marked individuals and the presence of fern stripping was recorded. We defined four phenological stages of leaf development: (1) Crozier: the initial uncoiling leaf with the petiole elongation. This stage has a characteristic "fiddle-head" shape due to faster growth of leaf base compared to the apex; (2) Expanding leaf:

the stage after crozier during rachis elongation and leaflet expansion; (3) Expanded leaf: the final overall expansion phase with all leaflets expanded; (4) Fertile leaf: this stage was recorded for *O. regalis* as this is the only fern species producing mature sporangia between March and May. In this species, sporangia form a tassel-like outgrowth at the apex of the leaf that matures after leaf expansion; mature sporangia can be recognized by turning green as the mature spores inside contain chlorophyll.

### 2.5. Physical and chemical determinations on sporangia and leaves

The length (mm) of sori was measured with callipers to 0.01 mm. The diameter or the longest axis, were measures for circular or linear sori, respectively. We carried out laboratorial determinations of caloric, lipid, protein and phenolic contents of spores and leaves. Analyses were conducted for spores of *W. radicans, C. macrocarpa*, *P. incompleta, Dryopteris* spp.*, B. spicant* and *O. regalis*, and for leaves of *P. aquilinum, O. regalis, C. macrocarpa*, *W. radicans*, *P. incompleta and Dryopteris* spp. Only mature sporangia and young leaves (expanding or recently expanded) were sampled as the Azores bullfinch only feeds on these parts of the ferns (Ramos 1994). Young leaves could be recognized by their soft lamina texture, due to the thin cuticle. Leaves and sporangia were frozen at -80ºC until analysis (1-4 months). Prior to analyses they were oven dried at 60ºC until weight stabilization (approx. 4 days). To determine the caloric content of spores and leaves, a small quantity of the samples (50 to 200 mg) was converted into pastilles. Each item was crushed to dust in a mortar, then introduced in a press and used to determine the caloric content of each species in a PARR 1425 calorimeter. Three pastilles of each sample were used and the mean caloric content calculated. Lipids were extracted and determined with a chloroform: methanol mixture according to Folch et al. (1957). Soluble proteins were measured with the Folin-

Ciocalteu reagent (Folin and Ciocalteu 1927) with the method developed by Lowry et al. (1951). For free phenolic content, samples were analysed using the method described by Julkunen-Titto (1985), based on the reduction of the phosphotungstic-phosphomolybdic (Folin and Denis 1912) present in the Folin-Ciocalteu reagent (Folin and Ciocalteu 1927). Both proteins and phenolics concentrations in all samples were determined from calibration curves of standards of bovine albumine (Armour and Company, Chicago) and gallic acid (Hagerman and Butler 1989) respectively. For each lipid, protein and phenol assay, 100 mg dry weight samples were used and results are given in mg/g dry weight of spores or leaves. For further information of the processes see Annex I.

**2.6. Statistical analyses**

We used Chi-square tests to: (1) investigate differences between the number of available and stripped fertile leaves of *W. radicans* and *C. macrocarpa* in October and December; (2) assess differences in the number of available and stripped fertile leaves among different species found from January to March; and (3) test differences in consumption among the four phenological stages of *O. regalis* between April and May.

Two Logistic Regression Models were built; (a) to predict the probability of observing stripped leaves of *W. radicans*, *C. macrocarpa* and *P. aquilinum* before 30 April; (b) to predict the stripping on *W. radicans*, *C. macrocarpa*, *P. aquilinum* and *O. regalis* after 30 April, when *O. regalis* leaves become available. The predicted variable was consumption (1) or no consumption (0) of a specific fern leaf and the predictors were fern species, phenological stage and date (all categorical). Hosmer and Lemeshow´s (2000) methods and model-building strategy were followed. Selection began with a univariate analyses to assess the relationship between each predictor

variable and the dependent variable. Any variable whose univariate test had a P-value smaller than 0.25 was a candidate for the multivariate model. The correlation among the candidate variables was assessed with a Spearman rank correlation test in order to avoid including in the model redundant variables ($r^2 > 0.6$; Whitehead 1998). All categorical variables were included in the model using reference cell coding which consists of defining a reference category for each variable, with which the other categories were compared. The importance of each variable in the multivariate model was verified using the Wald statistic for each variable and the Likelihood Ratio test between models, to evaluate if a model containing a certain variable was significantly better than a model without it. Finally, all interactions among the variables incorporated in the main effects model were included into the model one at a time. The Likelihood Ratio test and the Wald statistics were used to decide if any of the interactions was relevant for the model ($\alpha<0.05$). After obtaining a definitive model, the intercept coefficients of the predictors in the model, together with the Odds Ratio, were analysed to understand the nature of the relationship between each predictor and the outcome variable. The relative contribution of each variable to explain fern stripping was addressed using the Odds Ratio to compare the probability of having a fern stripped at each category with the reference level.

Statistical analyses were performed with Statistica 6.0 (StatSoft, Inc. 1984-2001).

# 3 - Results

### 3.1. Observation of foraging birds

A total of 1,671 feeding records of the Azores bullfinch were obtained during this study**.** We observed three main periods of fern consumption by the Azores bullfinch: (1) Sori of *W. radicans* and *C. macrocarpa* between October and April, (2) sori of *O. regalis* in May-June and (3) leaves of *P. aquilinum* and *O. regalis* in March-July (Table 1). Although sporangia and leaves of other fern species were available during these periods (see below) we did not observe any bird consuming them. The birds seem to feed first on sporangia of *W. radicans* (in October) and only in November they began feeding on sporangia of *C. macrocarpa*. We observed the same pattern in the end of the sporangia feeding period, as birds stop feeding on *W. radicans* in March and on *C. macrocarpa* in April (Table I).

**Table I**: Comparison of observations (%) of foraging birds (one bird, one food = one record) on ferns and other food types during the period of fern consumption, October-July, in 2006-2007.

| **Month** | **O** | **N** | **D** | **J** | **F** | **M** | **A** | **M** | **J** | **J** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Number of records** | 44 | 107 | 69 | 141 | 143 | 264 | 299 | 299 | 224 | 81 |
| **Herbaceous seeds** | 15.9 | 9.3 | | | | 1.5 | 1.0 | 66.9 | 89.3 | 66.7 |
| **Fleshy fruit seeds** | 75.0 | 32.7 | 2.9 | 2.8 | 2.1 | 2.3 | 1.0 | 2.7 | 0.9 | 18.5 |
| **Tree & shrub seeds** | | 21.5 | 59.4 | 74.5 | 23.1 | 4.2 | 2.3 | 2.0 | | |
| **Vegetative buds** | 4.5 | 3.7 | | | 0.7 | | 2.7 | 7.7 | 4.5 | 6.2 |
| **Flower buds** | | 1.9 | | 5.0 | 26.6 | 71.2 | 78.3 | 5.0 | 0.9 | 4.9 |
| **Insects** | | | | | | | | | 0.9 | |
| **Fern sporangia** | | | | | | | | | | |
| *Woodwardia radicans* | 4.5 | 22.4 | 14.5 | 9.2 | 27.3 | 2.3 | | | | |
| *Culcita macrocarpa* | | 8.4 | 23.2 | 8.5 | 20.3 | 11.4 | 3.7 | 0.3 | | |
| *Osmunda regalis* | | | | | | | | 4.0 | 1.3 | |
| **Fern leaves** | | | | | | | | | | |
| *Pteridium aquilinum* | | | | | | 7.2 | 10.7 | 11.4 | 0.9 | 3.7 |
| *Osmunda regalis* | | | | | | | 0.3 | | 1.3 | |

### 3.2. Sporangia consumption

We monitored the following fern species: *W. radicans*, *C. macrocarpa*, *Dryopteris* spp., *P. incompleta*, *B. spicant*, *Diplazium caudatum*, *Christella dentata*, *Stegnogramma pozoi*, *Adiantum hispidulum, Pityrogramma ebenea*, *Cyathea cooperii*

and *Dicksonia antarctica* with fertile leaves bearing sori available for the Azores bullfinch. All leaves were more abundant at low altitudes, except those of *C. macrocarpa and Dryopteris* spp. which were more abundant at mid altitudes (Figure 1a). Fern stripping was recorded on four species: *W. radicans*, *C. macrocarpa, P. incompleta* and *Dryopteris* spp., although the last two species had very few leaves displaying beak marks. Overall, consumption of fern sporangia decreased from low to high altitudes (Figure 1b).

Sporangia consumption differed significantly among fern species ($\chi^2_6$ = 2133.85, P<0.001, Figure 1). The chi-square showed that the number of stipped leaves in *W. radicans* and *C. macrocarpa* (1041 and 561, respectively) was much higher than what would be expected in the case of no preference (396.0 and 498.5 respectively). *Woodwardia radicans* was more abundant at low altitudes and *C.* macrocarpa at high altitudes (Figure 1a); They were more consumed at low and high altitudes, respectively (Figure 1b).

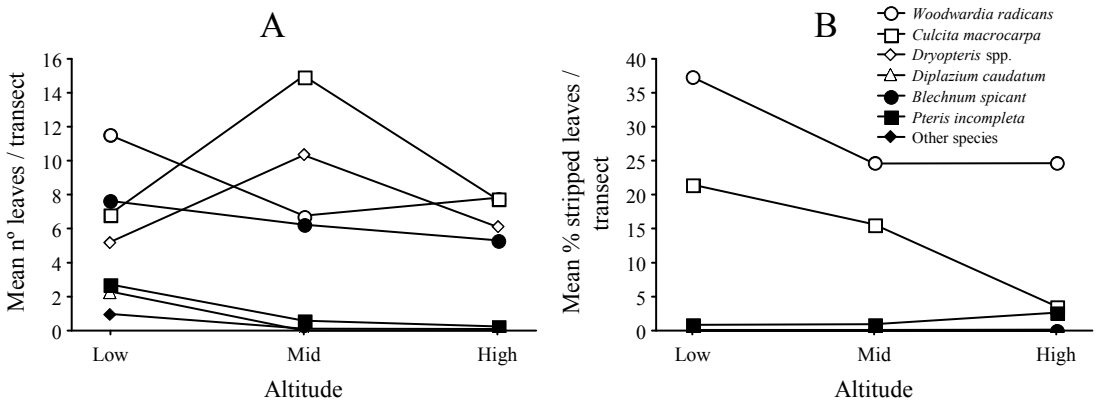



**Figure 1**: % of fertile leaves/ transect (A) and % of stripped fertile leaves (B) in relation to altitude. We measured fertile leaves as number of leaves with sori and consumption as the number of leaves with stripping marks. For graph clarity standard errors are not shown.

Transects to record fern stripping revealed that, at the end of October, birds fed more on *W. radicans* than on *C. macrocarpa* (Table II), a pattern confirmed by the observations of foraging birds (Table I). In December there was no significant difference in the presence of fern stripping marks on both species (Table II), again a pattern confirmed by the feeding records (Table I).

**Table II**: Number of leaves of *W. radicans* and *C. macrocarpa* with sori and % of leaves with fern stripping along two transects, 150m length and 2 m width, made in October and December 2006.

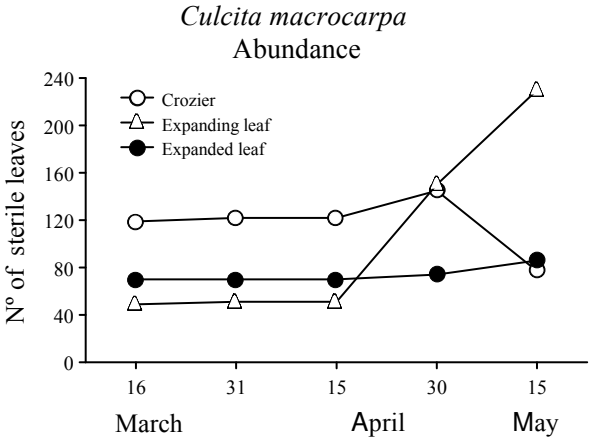
| | 30 October | | 03 December | |
|---|---|---|---|---|
| | No. of fertile leaves | % leaves with fern stripping | No. of fertile leaves | % leaves with fern stripping |
| ***Woodwardia radicans*** | 53 | 28 | 40 | 80 |
| ***Culcita macrocarpa*** | 41 | 2 | 40 | 60 |
| $\chi^2_1$ (Yates correction) | 9.19, P < 0.001 | | 2.92 , P > 0.05 | |

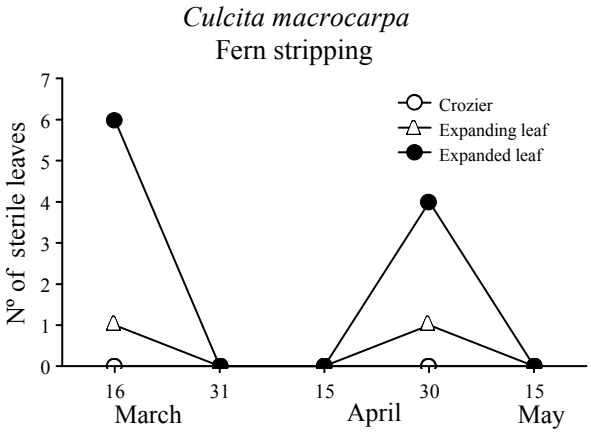
### 3.3. Leaf consumption

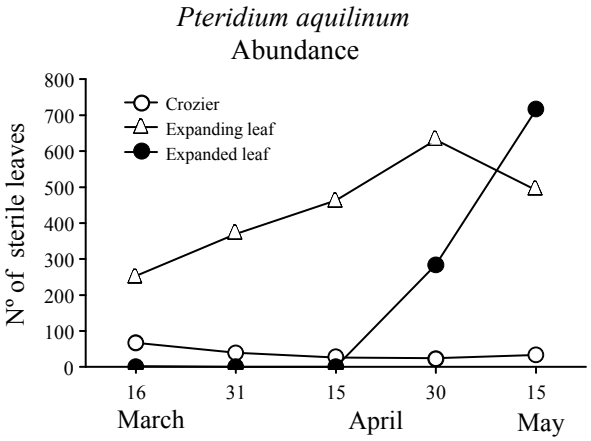
Figure 2 compares the abundance of leaves per species with the number of leaves exhibiting stripping marks, from mid March to mid May. From all species present in transects only *P. aquilinum* and *O. regalis* exhibited conspicuous fern stripping by the Azores bullfinch in this period (Figure 2). New *P. aquilinum* leaves become available in late March when the Azores bullfinch was observed feeding on them. The birds consumed very young leaves, leaving conspicuous bill marks in the lamina of expanding leaves; petioles of croziers were also broken and chewed (authors personal observations) *W. radicans* and *C. macrocarpa* exhibited stripping signs only on up to four and seven leaves, respectively, and these were never very conspicuous, suggesting that the birds only tried these foods. Moreover, during the period of these transects, birds were never observed foraging on leaves of either of these species (Table 1). Despite the high availability of *P. aquilinum* leaves in mid May, there was a marked

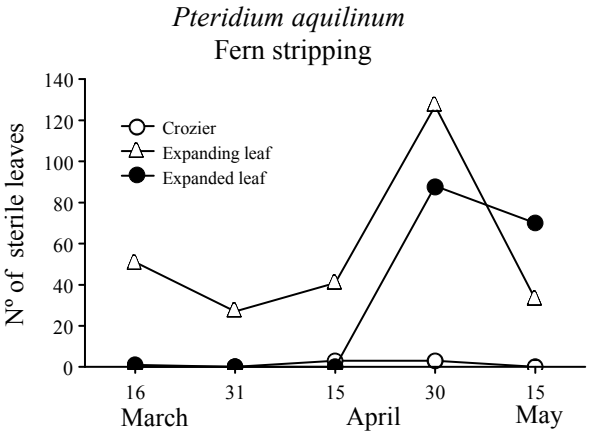
decrease in the proportion of leaves with fern stripping at this time (Figure 2). This decrease coincided with a noticeable increase in fern stripping on leaves of *O. regalis*, clearly suggesting a diet shift by the Azores bullfinch (Figure 2). As there was no fern stripping on *Dryopteris spp.*, *P. incompleta* and *B. spicant* until 15 April we did not mark more leaves of these species after this visit; we continued however to check marked leaves until mid May but we did not find any evidence of consumption.

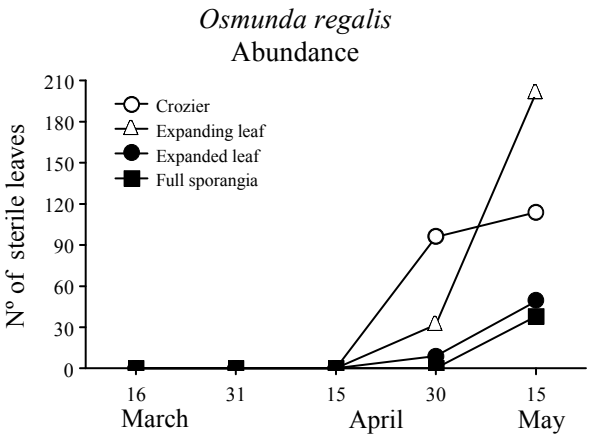
The Azores bullfinch started to feed on sporangia of *O. regalis* as soon as these became available (early May; Figure 2). A Chi-square analysis showed significant differences in the consumption of the four phenological stages (crozier, expanding leaf, expanded leaf and full sporangia) of *O. regalis* from 30 April to 15 May ( $\chi^2_3$ = 93.64, P < 0.001). Sporangia were clearly preferred (observed value = 18, expected value = 7.2) over leaves and expanding leaves were preferred (observed value = 76, expected value = 44.1) over expanded leaves and crozier (Figure 2).

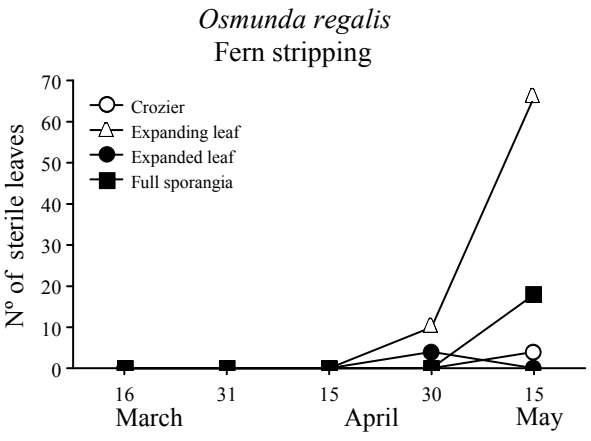

*Culcita macrocarpa*
Abundance
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
0
40
80
120
160
200
240
16
31
15
30
15
March
April
May


*Culcita macrocarpa*
Fern stripping
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
0
1
2
3
4
5
6
7
16
31
15
30
15
March
April
May


*Pteridium aquilinum*
Abundance
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
0
100
200
300
400
500
600
700
800
16
31
15
30
15
March
April
May


*Pteridium aquilinum*
Fern stripping
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
0
20
40
60
80
100
120
140
16
31
15
30
15
March
April
May


*Osmunda regalis*
Abundance
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
Full sporangia
0
30
60
90
120
150
180
210
16
31
15
30
15
March
April
May


*Osmunda regalis*
Fern stripping
Nº of sterile leaves
Crozier
Expanding leaf
Expanded leaf
Full sporangia
0
10
20
30
40
50
60
70
16
31
15
30
15
March
April
May

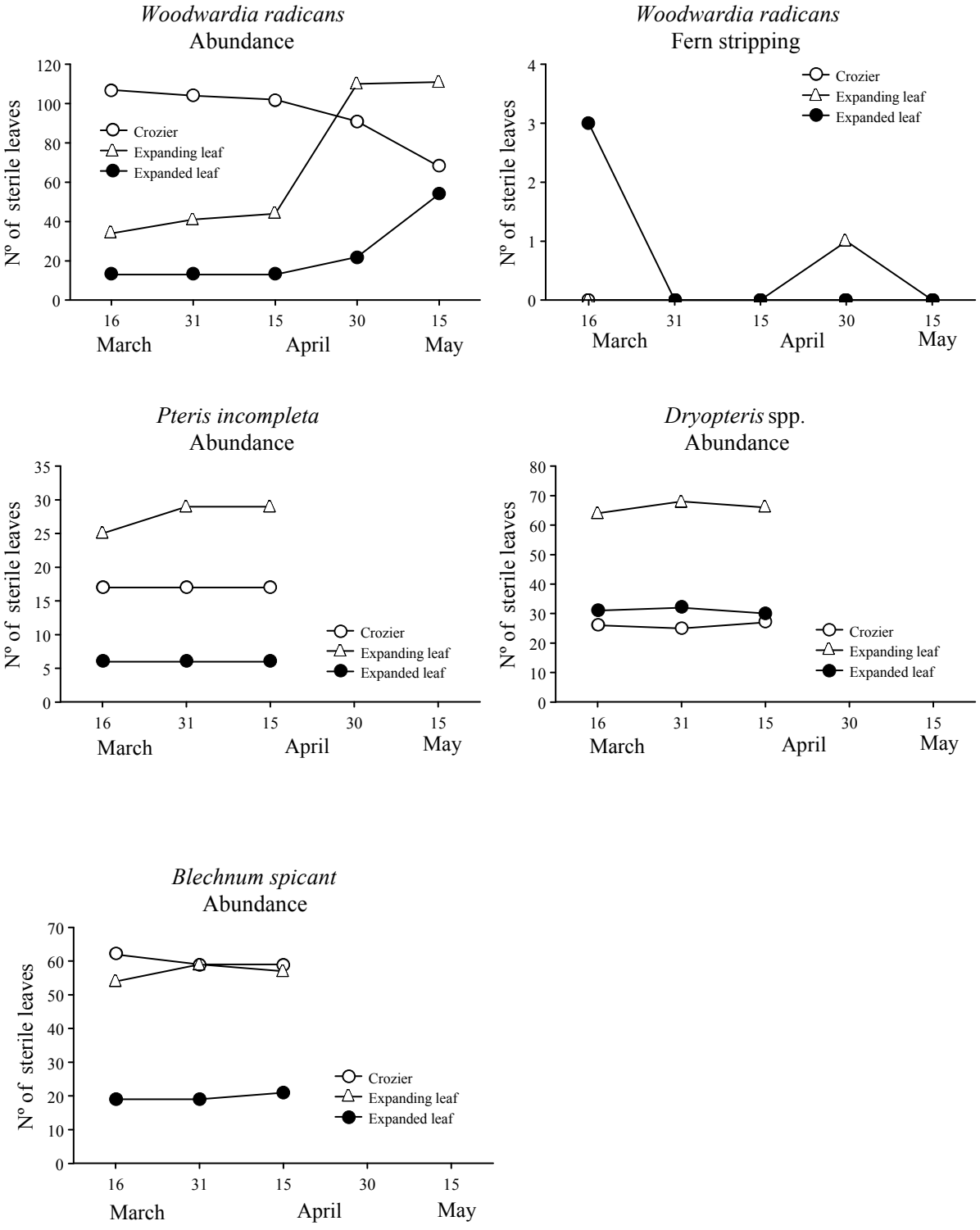



**Figure 2**: Abundance and fern stripping on leaves from 16 March to 15 May 2007 along 30 transects repeated every two weeks. Abundance was measured as the number of leaves and fern stripping as the number of leaves with beak marks for the first visit or leaves that had new beak marks, i.e. in the previous 15 days, for next visits. Three phenological stages were differentiated, crozier (fern leaves that roll out from the base to the end of the leaf. Expanding leaf (the lamina is developing) and expanded leaf (full expanded lamina) and, for *O. regalis* there was a fourth category, full sporangia (fertile leaves). For *Dryopteris spp.*, *P. incompleta* and *B. spicant* no more leaves were marked after 15 April as no consumption was recorded until

that moment although marked leaves were observed until mid May but consumption was not recorded. Note different scales on Y axis.

The Logistic Regression Model for the presence of fern stripping on *C. macrocarpa*, *W. radicans* and *P. aquilinum* for the period 16 March – 15 May predicted 92.1% of the cases correctly (Table III). The non significant Hosmer & Lemeshow test indicates that the model fitted the data well (Table IIIa). According to the sign of the coefficients and the Odds Ratio all species were poorly consumed except *P. aquilinum*. The probability of observing leaves of *P. aquilinum* with fern stripping was 22 times higher than the probability of observing fern stripping on *W. radicans* (the least consumed and reference species, Table IIIa). Expanded leaves were the most consumed phenological stage and crozier was the least consumed. No interaction contributed significantly to improve the fit of the model.

**Table III**: (a) Logistic Regression model to predict the probability of leaves with fern stripping using three explanatory variables: fern species, date and phenological stage (N=5814 leaves). (b) Logistic Regression model to predict the probability of fern stripping on leaves of *W. radicans*, *C. macrocarpa*, *P. aquilinum* and *O. regalis* using the same three explanatory variables as A uses (N = 3899 leaves). Given are the Coefficients of the variables ± SE, Wald statistic and its P-values, and estimated Odds ratio (OR).

**a**

| | **Coefficient** | **Wald** | **P** | **OR** |
|---|---|---|---|---|
| **Fern species** | | | | |
| *Woodwardia radicans* | | 117.79 | <0.001 | |
| *Culcita macrocarpa* | 0.34 ± 0.58 | 0.35 | 0.555 | 1.41 |
| *Pteridium aquilinum* | 3.09 ± 0.51 | 37.12 | <0.001 | 22.05 |
| **Phenological stage** | | | | |
| Expanded leaf | | 48.64 | <0.001 | |
| Crozier | -2.55 ± 0.44 | 34.10 | <0.001 | 0.08 |
| Expanding | -0.64 ± 0.13 | 25.53 | <0.001 | 0.53 |
| **Date** | | | | |
| 15 May 2007 | | 151.70 | <0.001 | |
| 16 March 2007 | 1.52 ± 0.20 | 60.55 | <0.001 | 4.57 |
| 31 March 2007 | 0.21 ± 0.24 | 0.74 | 0.391 | 1.23 |
| 15 April 2007 | 0.51 ± 0.21 | 6.04 | 0.014 | 1.66 |
| 30 April 2007 | 1.42 ± 0.14 | 110.88 | <0.001 | 4.15 |
| Constant | 5.27 ± 0.52 | 104.37 | <0.001 | 0.01 |
| Hosmer & Lemeshow test | $\chi^2_8$ = 9.96, P=0.27 | | | |
| % Cases classified correctly | 92.1% | | | |

**b**

| | Coefficient | Wald | P | OR |
|---|---|---|---|---|
| **Fern species** | | | | |
| *Osmunda regalis* | | 163.78 | <0.001 | |
| *Culcita macrocarpa* | -4.50 ± 0.47 | 90.52 | <0.001 | 0.01 |
| *Pteridium aquilinum* | -1.54 ± 0.16 | 96.64 | <0.001 | 0.21 |
| *Woodwardia radicans* | -5.51 ± 1.01 | 29.59 | <0.001 | 0.01 |
| **Phenological stage** | | | | |
| Expanded leaf | | 74.62 | <0.001 | |
| Crozier | -3.57 ± 0.42 | 71.92 | <0.001 | 0.03 |
| Expanding | -0.44 ± 0.12 | 13.84 | <0.001 | 0.64 |
| **Date** | | | | |
| 15 May 2007 | | | | |
| 30 April 2007 | 1.15 ± 0.12 | 90.18 | <0.001 | 3.15 |
| Constant | -0.55 ± 0.16 | 12.55 | <0.001 | 0.56 |
| Hosmer & Lemeshow test | $\chi^2_6$ = 4.57, P=0.60 | | | |
| % Cases classified correctly | 88.7% | | | |

Because *O. regalis* leaves developed only in late April the models were ran for the period 30 April - 15 May including *O. regalis*. This second model predicted 88.7% of the responses correctly and fitted the data equally well (Hosmer and Lemeshow test: $\chi^2_8$ = 4.57, P = 0.60, Table IIIb). According to the sign of the coefficients and the Odds Ratio of each of the three variables we concluded that: (1) The fern stripping of leaves was much higher in *O. regalis* (the reference species) than in all other species, (2) Fern stripping was much lower on crozier than on expanded leaves (reference), and the probability of fern stripping on expanding leaves was about half of that on expanded

leaves (Table IIIb). The interaction between phenological stage and date was not included in the final model because it did not contribute significantly to explain fern stripping.

### 3.4. Physical and chemical determinations on sporangia and leaves

Sori size did not varied significantly between consumed and non consumed species. Species such as *B. spicant* with large sori (12.4 ± 3.9 mm; Table IV) were never consumed by birds, *P. incompleta*, with the largest sori (14.3 ± 4.4 mm) and *Dryopteris spp*. with the smallest (0.9 ± 0.1 mm) were much less consumed than *W. radicans* and *C. macrocarpa* with intermediate sori size (4.8 ± 1.4 mm and 2.9 ± 0.4 mm respectively). Based on our results we cannot conclude on the existence of selection based on sori size.

The caloric content of consumed species was highest for sporangia of *P. incompleta* and lowest for *P. aquilinum* leaves (Table IV). Caloric content of *B. spicant* spores, a non consumed species, was 20.36 ± 0.07 KJ/g, which is higher than *C. macrocarpa* spores (19.09 ± 0.56 KJ/g), one of the most consumed species (Table 4). The very little consumed sporangia of *Dryopteris* spp. had the lowest energetic content (16.88 ± 1.93 KJ/g; Table IV). Regarding leaves, the calorific content of the most consumed species, *O. regalis*, with 21.49 ± 0.34 KJ/g, was higher than that of *P. aquilinum*, the second most consumed species (18.37 ± 0.62 KJ/g). For the other consumed and non consumed species contents varied between 20.74 ± 0.50 KJ/g for *C. macrocarpa* (leaves little consumed) and 18.28 ± 0.31 KJ/g for *P. incompleta* (leaves not consumed; Table IV).

Lipid content was higher in spores than in leaves (Table IV). Consumed species had more lipids than non-consumed species for both spores and leaves (Table IV). Protein analyses indicate that leaves have relatively higher proportions of available

proteins than spores. Protein content of consumed spores (*W. radicans* and *C. macrocarpa*) was much higher than that from very little or non consumed species (*Dryopteris* spp. and *B. spicant*; Table IV). On the other hand, and contrary to our expectations, the protein content of leaves from species that were little consumed was much higher than that of consumed species. Phenolic content was higher in leaves than in spores but there was no clear pattern between consumed and non consumed species (Table IV). Noticeably, leaves of *O. regalis*, which were preferred over those of *P. aquilinum*, contained lower levels of phenolics. Sporangia of *O. regalis*, available and consumed in May-July (outside the main sporangia feeding season), had a higher lipid content than leaves (Table IV), which could explain preference for sporangia over leaves.

**Table IV**: Comparison of sori length (N = 10), caloric content, and composition of spores and leaves between consumed and non consumed fern species by the Azores bullfinch. Only abundant species for each of the main period of sporangia feeding (November-March) and leaf feeding (April-June) were considered. Results are mean ± SD.

| | | | | Caloric content | | Lipids | | | Proteins | | | Phenolics | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Species | Sori length (mm) | Sampling date | KJ/g | Sampling date | N | mg/g | Sampling date | N | mg/g | Sampling date | N | mg/g |
| **Winter Spores** | **Consumed** | *Woodwardia radicans* | 4.8 ± 1.4 | 13 April 06 | 21.86 ± 0.41 | 12 Oct 07 | 2 | 185.00 ± 8.49 | 07 Dec 06 | 2 | 1.65 ± 0.02 | 07 Dec 06 | 2 | 1.21 ± 0.03 |
| | | *Culcita macrocarpa* | 2.9 ± 0.4 | 13 Ago 06 | 19.09 ± 0.56 | 12 Oct 07 | 4 | 274.25 ± 26.06 | 07 Dec 06 | 2 | 2.27 ± 0.02 | 07 Dec 06 | 2 | 0.66 ± 0.02 |
| | | *Pteris incompleta* | 14.3 ± 4.4 | 10 Nov 07 | 27.86 ± 0.13 | 16 Mar 07 | 2 | 257.50 ± 7.78 | 16 Mar 07 | 2 | 0.72 ± 0,08 | 16 Mar 07 | 2 | 1.10 ± 0.03 |
| | **Little consumed** | *Dryopteris* spp. | 0.9 ± 0.1 | 29 Oct 06 | 16.88 ± 1.93 | 10 Nov 07 | 2 | 174.50 ± 10.61 | 10 Nov 07 | 3 | 0.74 ± 0.04 | 10 Nov 07 | 3 | 0.63 ± 0.04 |
| | **Non consumed** | *Blechnum spicant* | 12.4 ± 3.9 | 10 Nov 07 | 20.36 ± 0.07 | 10 Nov 07 | 2 | 167.50 ± 21.92 | 10 Nov 07 | 3 | 1.41 ± 0.27 | 10 Nov 07 | 3 | 0.39 ± 0.05 |
| **Spring spores** | **Consumed** | *Osmunda regalis* | | 08 May 07 | 19.46 ± 0.05 | 08 May 07 | 2 | 42.00 ± 7.07 | 08 May 07 | 2 | 3.69 ± 0.05 | 08 May 07 | 2 | 1.89 ± 0.02 |
| **Leaves** | **Consumed** | *Pteridium aquilinum* | | 12 April 06 | 18.37 ± 0.62 | 12 April 07 | 2 | 36.50 ± 13.44 | 12 April 06 | 10 | 9.03 ± 0.41 | 12 April 07 | 2 | 1.92 ± 0.04 |
| | | *Osmunda regalis* | | 08 May 07 | 21.49 ± 0.34 | 08 May 07 | 3 | 36.00 ± 10.15 | 08 May 07 | 10 | 5.45 ± 0.33 | 08 May 07 | 2 | 1.96 ± 0.05 |
| | **Little consumed** | *Woodwardia radicans* | | 12 April 07 | 19.48 ± 0.40 | 12 April 07 | 2 | 16.00 ± 18.38 | 12 April 07 | 10 | 42.87 ± 0.79 | 12 April 07 | 3 | 2.19 ± 0.21 |
| | | *Culcita macrocarpa* | | 12 April 07 | 20.74 ± 0.50 | 12 April 07 | 2 | 34.00 ± 1.41 | 12 April 07 | 5 | 37.57 ± 1.38 | 12 April 07 | 3 | 2.17 ± 0.06 |
| | **Non consumed** | *Pteris incompleta* | | 12 April 06 | 18.28 ± 0.31 | 12 April 07 | 2 | 31.00 ± 5.66 | 12 April 07 | 10 | 2.37 ± 0.16 | 12 April 07 | 3 | 2.18 ± 0.05 |
| | | *Dryopteris* spp. | | 12 April 06 | 20.15 ± 0.28 | 12 April 07 | 2 | 12.00 ± 5.66 | 12 April 07 | 10 | 1.37 ± 0.10 | 12 April 07 | 3 | 1.92 ± 0.16 |

Figure 3 shows the analyses of protein and phenolic content in spores of *W. radicans* and *C. macrocarpa* throughout the winter. Data revealed that *W. radicans* had higher protein content than *C. macrocarpa* in October. There was a general decrease in protein content for both species through the winter. In October, the pattern was the opposite for phenolics, which were higher in *C. macrocarpa* than in *W. radicans*. From November onwards phenolics were always higher in *W. radicans* than in *C. macrocarpa.* Thereafter, the phenolic content for *C. macrocarpa* stabilized but that of *W. radicans* continued to increase.

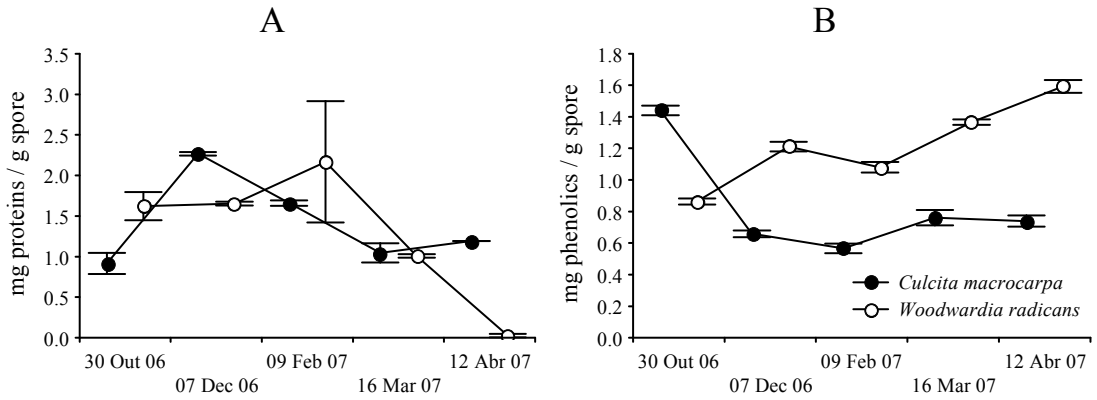



**Figure 3**: Mean ± SD of proteins (A) and phenolics (B) of *C. macrocarpa* and *W. radicans* spores during the period of fern consumption. For each period two replicates of 100 mg dry weight were analyzed.

# 4 – Discussion

In this study we evaluated fern stripping within the whole distribution area of the Azores bullfinch and showed that: (1) During winter, sporangia of *W. radicans* and *C. macrocarpa* are highly preferred over those of all other fern species available. (2) In spring, leaves of *O. regalis* are preferred over those of *P. aquilinum*. (3) Regarding the consumption of the main fern species this study confirms earlier observations by Ramos (1994; 1996). We observed obvious beak marks of Azores bullfinch on fertile leaves of *P. incompleta* and *Dryopteris* spp. which was not detected in foraging observations made in the 1990's, however the consumption of this species was marginal. *P. incompleta* increased as a consequence of the removal of exotic plants, which began in 2004 (authors personal observations) as part of an ongoing habitat restoration project. This increase in abundance is likely to have translated in an increase of consumption by the Azores bullfinch. However, it was much less abundant than *W. radicans* and *C. macrocarpa*, and occurred mainly in dense forest, which may explain the lack of feeding records in the 1990's and in the present study. When young leaves of *P. aquilinum* and *O. regalis* become available, in mid March and mid April, respectively, the Azores bullfinch began feeding on them as most sporangia of *W. radicans* and *C. macrocarpa* had already released their spores. The evaluation of fern consumption based on observations of foraging birds suggests that *O. regalis* is of little importance for the Azores bullfinch. This is probably because new fronds of *O. regalis* appear in fairly dense forest, where birds are more difficult to observe, given that our transects and faecal analysis conducted by Ramos (1995) demonstrated that *O. regalis* is important in the diet of the Azores bullfinch between April and June.

Ramos (1996) showed that the Azores bullfinch selected the longest sori of *W. radicans*. Our interspecific comparison shows that nutrient content is more important than sori size in explaining preference for fern sporangia. Birds' feeding preferences

have usually been analysed within the framework of optimal diet models. These models predict that birds should choose species that provide maximum energy intake per unit foraging time (Stephens and Krebs 1996; Sih and Christensen 2001). Comparing the mean caloric content of sporangia from consumed species (mean = 21.42 KJ/g, range = 16.88 to 27.86 KJ/g) with the caloric content of seeds eaten by the Azores bullfinch in other seasons (unpublished data), which values range from 15.58 KJ/g (*Rumex conglomeratus*) to 27.12 KJ/g (*Leycesteria formosa*), we can conclude that caloric content of spores is relatively high. The caloric content of leaves varied from 18.37 KJ/g (*P. aquilinum*) to 21.49 KJ/g (*O. regalis*), which are comparatively higher than the 17.10 KJ/g for the fern *Polystichum sp.*, consumed by Cantabrian capercaillie (Rodriguez and Obeso 2000). The caloric content of leaves is lower than that of sporangia but relatively similar to that of other foods highly consumed by the Azores bullfinch such as flower buds of *Ilex perado* (21.78 KJ/g). Although seeds should be more rewarding per unit handling time because of their larger mass, this comparison shows that fern sporangia can be an important source of energy that may have been overlooked in traditional ecological studies.

Regarding sporangia, the most consumed species (*W. radicans*, *C. macrocarpa and P. incompleta*) had a higher lipid content than that of little and non consumed fern species. Many lipid drops were present inside the spores during the main feeding period on sporangia. The importance of lipids for the Azores bullfinch is reflected in their extensive consumption of lipid rich spores and leaves, which may be explained by the fact that birds will easily meet their metabolic requirements of thermoregulation using lipids (Mills et al. 1980) rather than other nutrients. The fact that birds switched rapidly from *P. aquilinum* to *O. regalis* as soon as the latter became available is likely to be a consequence of a higher calorific and a lower phenolic content in *O. regalis* than in *P.*

*aquilinum*. As expected, the lipid content of *P. aquilinum* and *O. regalis* leaves were higher than that of non consumed species. The carcinogenicity of the vegetative tissues of *P. aquilinum* has long been established (Moran 2004). Both vegetative tissues and spores of *P. aquilinum* can damage the DNA of consumers, whereas *O. regalis* cannot (Simán et al. 2000). This may also contribute to explain why the Azores bullfinch significantly decreased feeding on leaves of *P. aquilinum* as soon as *O. regalis* leaves became available.

Proteins are important for birds during the autumn and winter as they are finishing a post-breeding moult and proteins are also required to maintain body weight (Brice and Grau 1991), particularly for growth of muscles and tissues in first year birds (Zanotto and Bicudo 2005). Protein content in sporangia of consumed species was higher than that of little consumed or non consumed species. Contrarily to the results regarding sporangia, leaves of consumed species, *P. aquilinum* and *O. regalis,* (with higher lipid content), had lower protein content than leaves of little consumed species, *W. radicans* and *C. macrocarpa.* This fact supports the hypothesis that during late winter, when Azores bullfinch needs to maximize energy intake, lipids are more important than proteins (Stiles 1992; 1993).

The seasonal variation in phenolic content might contribute to understand differences in fern foraging patterns by the Azores bullfinch despite the fact that high phenolic levels in *C. arborea* (Ramos 1996) did not inhibit the Azores bullfinch from taking seeds in October-December. Our feeding records and fern stripping transects indicated an apparent preference for *W. radicans* over *C. macrocarpa* in October. In this month, *W. radicans* had a lower phenolic content than *C. macrocarpa* and a higher protein content which probably explains bird choice. At high altitudes other food sources, such as flower buds *of Ilex perado* spp. *azorica*, are available from March

onwards (Ramos 1995), which may explain why *C. macrocarpa* was more consumed than *W. radicans* in the end of winter. As the birds move to higher altitudes (where *C. macrocarpa* is more abundant than *W. radicans*) to feed on flower buds they may take also *C. macrocarpa* as a secondary food source (Ramos 1996). Levels of soluble proteins in *C. macrocarpa* increased from November onwards and levels of phenolics decreased, which coincided with the timing of intense feeding on *C. macrocarpa*. The low number of fern stripping marks on croziers may be due to high concentration of phenolics in this leaf stage (Marrs and Watt 2006), which will increase their defence to herbivory. High levels of phenolics can lead to low palatability and depressed growth rates, although it is still unknown what clues animals use to avoid astringent food (Mole and Waterman 1987b; Bernays et al. 1989).

The amount of lignin, an important component of the plant cell wall, determines to a great extent leaf digestibility (Cornelissen et al. 2004). In most cases, digestibility decreases as leaves age as a consequence of legnification (Lowman and Box 1983; Hill and Lucas 1996), which may contribute to explain why old fern leaves were never consumed by the Azores bullfinch. Digestibility of fern leaves from several species consumed by the Roosevelt elk (*Cervus elaphus roosevelti*) ranged from 23 to 46%, whereas that of grasses ranged from 55 to 76% (Hutchins 2006), meaning that fern leaves are less suitable for herbivores than grasses.

This is the first study examining in detail the selection of fern sporangia and leaves by a vertebrate species and describes how important ferns are for the Azores bullfinch. We can conclude that during winter sporangia of *W. radicans* and *C. macrocarpa* were preferred than those of other species; only *P. incompleta* and *Dryopteris spp.* were also consumed, but rates were very low, especially for *Dryopteris spp.*. In early spring *P. aquilinum* and *O. regalis* leaves were consumed. Expanded

leaves were preferred over other developmental stages except for *O. regalis*, in which sporangia were preferred (probably due to their higher lipid content), followed by expanding leaf. Lipid and caloric content of fern leaves were lower than those of fern spores, however, contrarily to our expectations, protein content of leaves was much higher than that in sporangia. The consumption of leaves in early spring may enable birds to survive until better foods became available. The selection of ferns containing the highest caloric, protein and lipid contents by the Azores bullfinch indicates that birds are actively selecting the most energetic food items. Amongst nutrients, lipids were the most important in explaining fern preferences as predicted by the optimal dietary and nutrient regulation theories (Sih and Christensen 2001).

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# Chapter II

# Spore maturation and release of two evergreen Macaronesian ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an altitudinal gradient

# 6 - Introduction

Spores, the first cells of the gametophyte generation, play a critical role on fern biology. Spore release is a prerequisite for the establishment of new gametophytes and sporophytes and is the vehicle for gene flow between fern populations (Haufler 2002), similarly to seeds in vascular plants. Ferns produce large amounts of spores, which enables them to disperse to wider areas than that occupied by the sporophytes (Page, 1979). Therefore, spore release and germination may be the first stage limiting the distribution and abundance of ferns. Surprisingly, the factors affecting spore phenology have been poorly studied, contrasting with the abundant literature on leaf expansion (Willmot, 1989; Johnson-Groh & Lee, 2002; Schmitt & Windisch, 2006). Moreover, studies on spore maturation and release have focused mainly on cold-temperate species (e.g. Peck et al., 1990, Bauer et al., 1991).

*Culcita macrocarpa* is the only Dicksoniaceae present in Europe, and *Woodwardia radicans* together with *Blechnum spicant* the only Blechnaceae. They occur in a warm-temperate range that extends discontinuously through Macaronesia (Azores, Madeira and Canary Islands), the Atlantic coast of the Iberian Peninsula and, in case of *W. radicans*, some locations in the Mediterranean region. Both species are considered relicts of the tropical flora that covered the Mediterranean area during the Tertiary period (Pichi-Sermolli, 1979, 1988) and are presently included in several lists of threatened species (e.g. Ormonde, 1990; Cellinese, 1996; Bañares et al., 2003) and in the Annex II of the EU Habitats Directive. Disregarding this importance there is little knowledge on their life history traits, which difficult their conservation.

Apart from their biogeographical affinities, *C. macrocarpa* and *W. radicans* share the same life-form, with large shoots above ground and evergreen leaves that can reach more than two meters long, which makes them two of the biggest ferns in Europe (Flora Europea). Previous studies on leaf phenology of *C. macrocarpa* and *W. radicans*

were carried out in northwest Iberian Peninsula, the northernmost limit of their range (Quintanilla). Spore release for both species occurred around the spring equinox, with small differences between populations and between years. Small inter-population variation was presumably due to the narrow altitudinal gradient between populations, of only about 200 m (Quintanilla). *C. macrocarpa* and *W. radicans* are very abundant in the Azores (Dias, 1996), the wettest and northernmost Macaronesian archipelago, where they occur along a large altitudinal gradient: 300 -1000 m for *C. macrocarpa*, and 50 - 950 m for *W. radicans* (Schäfer, 2002). Therefore, Azorean populations are a suitable model to study the effects of altitude-correlated environmental factors on spore maturation and release. Additionally the sporangia of these two species are important food resources for the critically endangered Azores bullfinch (*Pyrrhula murina*) with a population of less than 400 individuals (SPEA, 2007) and restricted to the laurel forest in the east of São Miguel Island (Ramos 1994, 1996a). Sporangia of *W. radicans* and *C. macrocarpa* seem to be taken only when they contain full mature spores rich in lipids and therefore highly energetic (Arosa, 2008). Once the indusium and sporangia open, spores are released leaving only sporangia cell walls with negligible nutritional value (author's unpublished data). Because sporangia have no nutritional value after spore release, the time of maturation and spore release is crucial to the feeding ecology of the Azores bullfinch. In particular, it is important to know: (1) the time of occurrence of full mature spores and whether this is influenced by altitude and canopy cover, and (2) whether spore liberation is synchronized or gradual over time, providing a reliable food source over a long time period. This study examines the influence of environmental variables, especially altitude and vegetation cover, on *C. macrocarpa* and *W. radicans* spore maturation and release. We hypothesized that spore maturation and release should

occur earlier at lower altitudes, because of higher temperature and insulation and lower relative humidity.

# 7- Methods

### 7.1. Study species

*C. macrocarpa* has triangular leaves, four-five pinnate and sori (i.e. clusters of sporangia) are reniform and marginal. Leaves of *W. radicans* are ovate-lanceolate and two-pinnate. Sori are oblong and arranged in two parallel rows close to the central vein. Both species have an indusium that completely encloses the sori from the end of leaf expansion until spore release. Leaves of both ferns are arranged in crowns at the apex of shoots that grow horizontally along the surface of the substrate. *C. macrocarpa* and *W. radicans* can also reproduce vegetatively by stem fragmentation or budding near leaf apex, respectively.

### 7.2. Study area and study populations

The study was carried out in *Serra da Tronqueira*, São Miguel Island, archipelago of the Azores (37º47'N, 25º13'W). This area is a steep volcanic range with oceanic climate (Silva, 2003). Temperatures are mild throughout the year (mean annual temperature 17º C at sea level) and there is no frost. Rainfall increases with altitude varying from 1500 to 3000 mm. The canopy of the natural laurel forest is dominated by evergreen trees and shrubs (*Laurus azorica*, *Prunus lusitanica* spp. *azorica, Erica azorica*, *Vaccinium cylindraceum*, *Juniperus brevifolia, Ilex perado* spp. *azorica, Viburnum tinus* spp. *subcordatum, Frangula azorica and Myrsine africana)*. Most of the original forest has been replaced with C*ryptomeria japonica* plantations (300 - 900 m) and the remaining patches are invaded by alien species: *Hedychium gardneranum* (0 - 950 m), *Clethra arborea* (500 - 900 m) and *Pittosporum undulatum* (50 - 650 m) (Schäfer, 2002). Ferns are absent or rare in patches of dense and homogenous exotic vegetation. To study microclimate and spore phenology traits, we selected three sites with both *C. macrocarpa* and *W. radicans* at 400, 600 and 800 m (hereafter referred to

as low, mid and high altitude) coinciding with the altitudinal distribution of the Azores bullfinch. At each altitude 12 mature individuals of each species, i.e. with at least one fertile leaf, were tagged, yielding to a total of 72 marked ferns (12 individuals × 3 populations × 2 species).

### 7.3. Microclimatic study

Temperature and relative humidity measures were obtained with three thermohigrometers placed at each altitude (400, 600 and 800 m). The full period of this study was divided in two sampling periods for analysis regarding fern phenology: March – April 2007 and October 2007- April 2008 and records were measured every hour. All termohigrometers (HOBO Pro v2 logger, Onset Computer Corporation) were placed consistently 1.5 m above ground under similar exposition conditions.

To determine canopy cover, hemispherical photographs at 1.30 m over each tagged individual fern were taken using a digital camera (Nikon CoolPix 995, Nikon, Japan) with a Fish Eye Converter (Nikon FC-E8, Nikon, Japan). They were orientated to the magnetic north and horizontally located using a level (Valladares, 2006). Images were processed with Gap Light Analizer 2.0 (Forest renewal BC, Canada). The variable gathered was % of canopy cover; this value was obtained as 100 minus % canopy openness (the percentage of open sky seen from beneath a forest canopy). Analysis of variance (ANOVA) was carried out after arcsine transformations of the % of canopy cover to determine whether significant differences in cover existed among altitudes for both fern species.

### 7.4. Spore phenology

From 10 November 2006 to 15 May 2007, the six study populations (see above) were visited every 10 days to assess whether spore maturation and release differs seasonally with altitude. At the base of a fertile pinna of each individual (12 individuals/population), two opposite pinnules were marked, one to study spore maturation and the other to study spore release. Maturation was studied by collecting 6 sori per pinnule in each visit. Sori were stored in Eppendorfs to keep sporangia hydrated and avoid spore release. In the laboratory, sporangia were opened with a lancet and their content was observed with a light microscope. The presence/absence of perispore and spore content was determined in four random samples of 100 spores per individual until 9 March 2007 (corresponding to the period before 50% of the sori of each individual opened). We sorted spores into four morphological groups: (a) Full mature spores, i.e. those with perispore and completely fulfilled with protein and lipid drops (completely yellow); (b) half-mature spores, i.e. those with perispore and with some filling but not completely fulfilled; (c) immature spores, i.e. those without perispore, and (d) abortive spores. Groups a, b and c were used to assess the progress of maturation. Spores were considered aborted when they lacked protoplast or were collapsed. They were counted because they have no nutritional value and thus may affect Azores bullfinch sustenance.

Spore release was studied as per Quintanilla to allow comparison between the Azorean and the Galician populations. The number of sori that had released spores, i.e. with open indusium was counted on both species. Date of spore release was defined as the day on which more than half of sori on marked pinnules had released spores.

To determine whether maturation and spore release differed between species and altitudes we fitted Generalized Linear Models (GzLMs) to the data using SAS procedure GENMOD (SAS Institute 2002). The timing of spore maturation was

measured in two ways: (1) number of days to get 50% of full mature spores, and (2) number of days to get 90% of full mature plus half-mature spores, in both cases day zero was defined as 1 January 2006. Date of spore release was measured as the number of days (after 1 January 2007) to get 50% of the marked leaves with open sori. The explanatory variables considered in this analysis were fern species (*C. macrocarpa* and *W. radicans*) and altitude (low, mid and high); canopy cover was included as a covariate. We used a Poisson distribution with log link function because variables departed from a normal distribution.

### 7.5. Mature spores and germination

The relationship between morphological characters of spores and their ability to germinate was analyzed with laboratorial germination trials to assess if there is any difference on the germination efficiency between full mature and half-mature spores. Ferns typically show a progressive maturation with both mature and immature sporangia in the same sorus (Eames, 1936). A full mature spore presents perispore and is full of content inside, i.e. with protein granules and lipid droplets (Raghavan, 1989). On 9 February 2007 fertile pinnules of the tagged individuals at mid altitude (12 individuals/species) were collected, and spores from these pinnules were sown on mineral agar (Dyer, 1979) in plastic Petri dishes subsequently sealed with parafilm. For each individual, four Petri dishes (replicates) were incubated. Dishes were put into a growth chamber (20 ºC-light/15 ºC-dark temperatures, 14h photoperiod, PAR 35 µmol $m^{-2}$ $s^{-1}$). Germination rate was assessed six weeks after sowing, counting 100 spores per dish. The criteria used to access germination were the protrusion of the rhizoid initial out of the spore coat (Turnwald et al., 1999). The relationship between the percent of spores completely fulfilled observed at the microscope and % of spores that germinated

(arcsine transformed data) was explored with Pearson correlation coefficients (Zar, 1996).

# 8- Results

### 8.1. Microclimatic study

As expected, temperature decreased and humidity increased with altitude (Table V). From these two periods, April-March 2007 and October 2007-April 2008, mean temperatures ranged from 12.9-14 ºC to 9.8-11.2 ºC at low and high altitudes respectively and relative humidity was minimum (88.7-91.4 %) at low altitudes and maximum (97.9-98.9 %) ate high altitudes.

**Table V**: Temperature and relative humidity (mean ± SD) for three different altitudes. Values were recorded hourly from 16th March to 30th April 2007 and 1st October to 4th April 2008, with three thermohigrometers placed 1.5 m above ground at each altitudes.

| Altitude | Date | Temperature (ºC) | Relative humidity (%) |
|---|---|---|---|
| **Low** | March-April 2007 | 12.9 ± 3.3 | 88.7 ± 11.0 |
| | October 2007-April 2008 | 14.0 ± 3.2 | 91.4 ± 9.7 |
| **Mid** | March-April 2007 | 11.0 ± 3.1 | 93.5 ± 8.3 |
| | October 2007-April 2008 | 12.0 ± 3.0 | 96.2 ± 6.9 |
| **High** | March-April 2007 | 9.8 ± 2.1 | 97.9 ± 3.4 |
| | October 2007-April 2008 | 11.2 ± 2.5 | 98.9 ± 3.1 |

The height and structure of the dominant tree species at each altitude did not allow the same quantity of light to reach the understory, although our results showed a medium-high canopy cover for the three sites with mean values ranging from 61.45% at mid altitude to 84.28% at high altitude. At high altitude, canopy cover was homogeneous for both species, because vegetation was mostly *C. japonica* and *Pinus nigra* plantations (Table VI). In our study, native vegetation (cleared recently from exotic species) was more abundant at mid altitudes, whereas *P. undulatum* was the most abundant species at low altitudes. Results from ANOVA showed that canopy cover above both *C. macrocarpa* and *W. radicans* differed significantly among low, mid and high altitude populations (Table VI), and the Tukey test revealed that *C. macrocarpa* at

mid altitudes differed from that at both low and high altitudes; and that *W. radicans* at high altitudes differed from that at low and mid altitudes.

**Table VI**: Analysis of variance comparing canopy cover of *C. macrocarpa* e *W. radicans* (mean % ± SD) at three altitudes. Results were obtained from hemispherical photography analysed with Gap Light Analyzer. Lines with different letters indicate significant differences (Tukey test).

| Species | Altitude | | | ANOVA |
|---|---|---|---|---|
| | Low | Mid | High | |
| ***C. macrocarpa*** | 72.8 ± 15.31[a] | 61.4 ± 9.46[b] | 82.0 ± 4.85[a] | $F_{2.33}$=11.06, p< 0.001 |
| ***W. radicans*** | 68.8 ± 10.75[b] | 63.7 ± 11.51[b] | 84.3 ± 3.93[a] | $F_{2.26}$=16.51, p< 0.001 |

## 8.2. Spore phenology

The percentage of full mature *C. macrocarpa* spores was approximately constant over time while *W. radicans* spores experiment a period of accelerated full maturation in December and January, particularly at high altitudes (Figure 4). The highest percentaje of full mature plus half-mature spores was obtained for *C. macrocarpa* (100 %), not always reaching 100% for *W. radicans* (Figure 5).

The results of the GzLM (Table VII) show that species, altitude and the interaction between them all had a significant effect on the number of days needed to obtain 50% of full mature spores, which occurred later with increasing altitude. Canopy cover had no significant effect in the occurrence of full mature spores. The number of days to obtain 90% of full mature plus half-mature spores was only significantly influenced by species (Table VII).

There were low percentages of abortive spores for both species, never higher than 12%. For *C. macrocarpa* mean (%) ± SD were 4.5 ± 2.9, 8.1 ± 3.3, 4.9 ± 2.5 for low, mid and high altitudes, respectively. For *W. radicans* values obtained at the three different altitudes were very similar: 5.3 ± 1.6, 5.6 ± 4.3, 6.1 ± 2.0, respectively.

Spore release for both species, started in January and finished in late April (Figure 6). Spore release was gradual, even in a single leaf, and highly synchronous within populations, demonstrated by the small standard deviations. For *W. radicans* at high altitudes within population standard deviations were higher, which means less synchrony. Altitude was the only measured variable that significantly influenced the sori open date (Table VII).

**Table VII**: Results of GzLM Models for the no. of days needed to obtain 50% of full mature spores (i.e. with perispore and completely fulfilled), the no. of days needed to obtain 90% of full plus half-mature spores (i.e. with perispore and partially filled inside) and sori release (i.e. date when 50% of the sori released spores) for *C. macrocarpa* and *W. radicans* at three different altitudes. Significant effects are indicated in bold. (sp = species, alt = altitude)

| Variable | Effect | DF | F | P |
|---|---|---|---|---|
| **Full mature** | sp | 1.53 | 4.18 | **0.041** |
| | alt | 2.53 | 7.08 | **0.0289** |
| | cover | 1.53 | 1.85 | 0.1735 |
| | sp*alt | 2.53 | 10.25 | **0.0059** |
| **Full plus half-mature** | sp | 1.58 | 12.16 | **0.0005** |
| | alt | 2.58 | 5.28 | 0.071 |
| | cover | 1.58 | 0.49 | 0.4834 |
| | sp*alt | 2.58 | 4.86 | 0.0881 |
| **Spore release** | sp | 1.51 | 0.66 | 0.4157 |
| | alt | 2.51 | 6.11 | **0.0472** |
| | cover | 1.51 | 0.38 | 0.5363 |
| | sp*alt | 2.51 | 0.06 | 0.9682 |

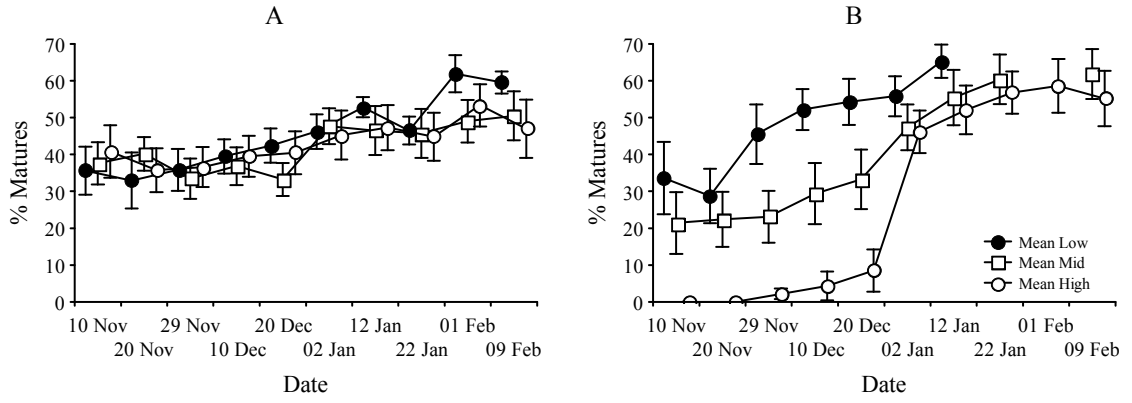


**Figure 4**: Percentage of full mature spores (with perispore and completely fulfilled inside) of C. macrocarpa (A) and W. radicans (B) along the season from microscope observations. Results are proportion of fulfilled spores in relation to the total (spores not fulfilled, without perispore and abortives were not included). For each altitude, species and date a sample of 12 permanent marked individuals were analyzed. Data are mean ± SE.

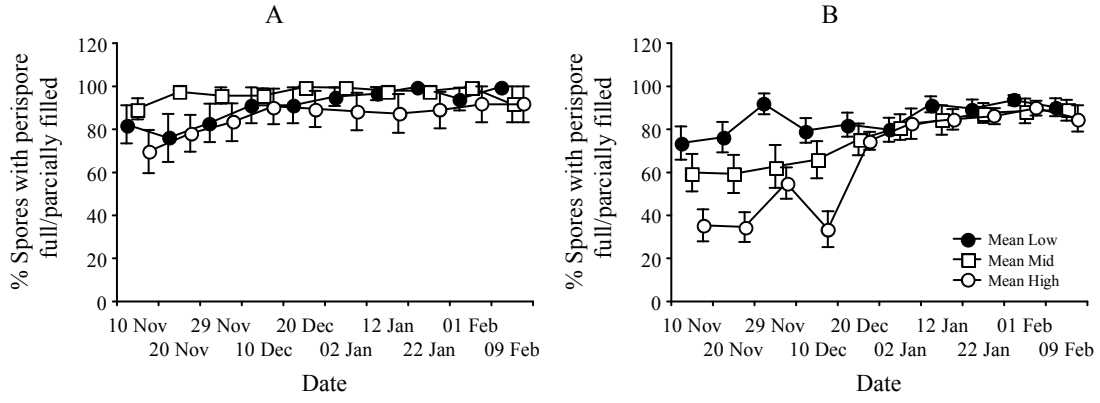


**Figure 5**: Percentage of full mature plus half-mature spores of C. macrocarpa (A) and W. radicans (B) along the season. Results are the proportion of spores from microscope observations (spores without perispore were not included). For each altitude, species and date 12 permanently marked individuals were analyzed. Data are mean ± SE.

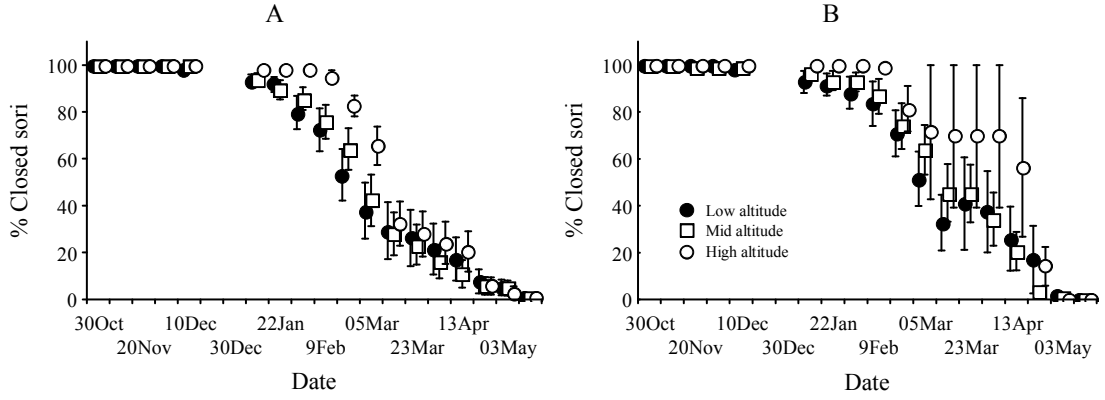


**Figure 6**: Spore release date for *C. macrocarpa* (A) and *W. radicans* (B) from 30 October 2006 to 15 May 2007. Results are Mean ± SE. N = 12 for each altitude.

### 8.3. Mature spores and germination

The correlation between the proportion of germinated spores and the proportion of full mature spores was significant for *C. macrocarpa* (r = 0.67, p = 0.017) and close to significant for *W. radicans* (r =0.52, p=0.085; Figure 7). The correlation between the proportion of germinated spores and the proportion of full mature plus half-mature spores was almost significant for *C. macrocarpa* (r=0.49, p = 0.102) but not for *W. radicans* (r = 0.22, p = 0.486). These results suggest that partially developed spores have a lower germination capacity than fully mature spores.

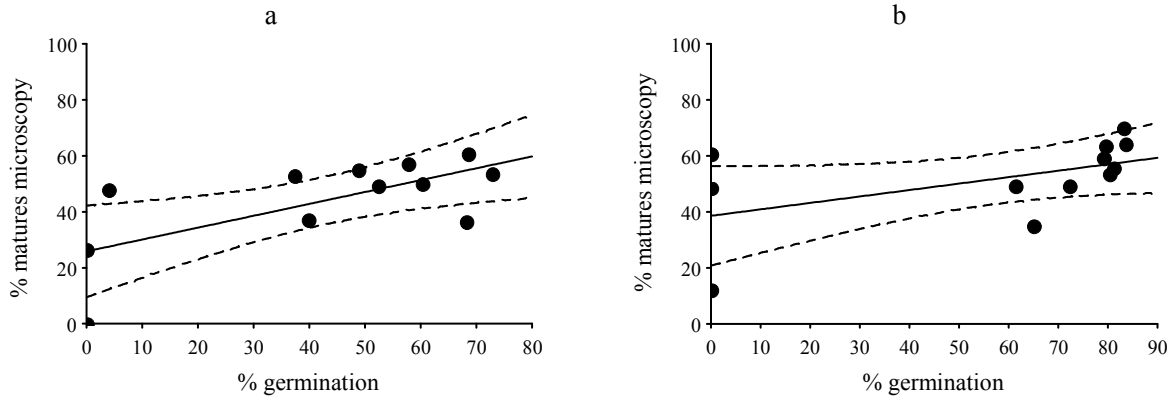


**Figure 7**: Correlations between the proportion of full mature spores (completely yellow and fulfilled with proteins and lipid drops) and the proportion of germinated spores for both C. macrocarpa (a) and W. radicans (b). Graphs show regression lines with 95% confidence limits. Equation for a is y = 25.870 + 0.42376x and for b is y = 38.613 + 0.2303x.

# 9- Discussion

As expected the maturation and spore release of both *C. macrocarpa* and *W. radicans* in São Miguel, Azores, was largely influenced by altitude. This effect of altitude is mediated by differences in humidity and temperature. The idea that humidity is important for spore maturation and spore release was assessed by Muller (1992), which showed that some fern species withered prematurely and did not set spores due to a severe spring drought. In general ferns need humidity to germinate and develop (Page, 2002; Murphy and Rumsey, 2005). Chiou et al. (2001) observed that spore maturation and release of *Cibotium taiwanense* were affected by temperature, occurring earlier under higher temperatures. Our results coincided with both patterns described.

Maturation was attained before on *C. macrocarpa* than on *W. radicans*, but both happened in January and February, the wettest period of the year in the Azores. The accumulation of protein and lipid drops inside the spore occurred mainly from December to February, and this accumulation occurred gradually after the formation of the perispore. Gradual spore maturation enables the fern to produce fresh spores until total depletion (McHaffie, 2005) and increases the probability that part of the spores will germinate and develop, because during such an extended period at least some released spores will encounter adequate conditions (Ranal, 1995). The gradual spore release over a period of time may be adaptative, ensuring higher chances of dispersal into new exposed microhabitats (Ranal, 1995), formed for example by the removal of invasive species in our study area.

Both *C. macrocarpa* and *W. radicans* were fairly synchronous in the sori opening period and spore release which, similarly to spore maturation, coincided with the wettest period of the year. Quintanilla (2000) showed that *C. macrocarpa* has lower optimal germination temperatures (15-20ºC) than *W. radicans* (25ºC), which could explain the earlier spore release in *C. macrocarpa*. Significant differences in spore

release in relation to altitude are related with lower humidity and higher temperatures at low altitudes, which explain earlier sporangia opening (Ranal, 1995). Not all spores were full mature at the end of the maturation period. This is a disadvantage for ferns, because germination rates will be lower and also for the Azores bullfinch since unfulfilled spores are less nutritious (Arosa, 2008).

The spore release for *C. macrocarpa* and *W. radicans* in Galicia, Spain occurred around the spring equinox (Quintanilla), which is later than in our study area. Galicia is the northernmost distribution area of *C. macrocarpa* and *W. radicans,* where weather conditions are characterized by low thermal amplitude and high precipitation and humidity. The annual precipitation in Galicia is 2440 mm, almost twice higher than in São Miguel (1311 mm). The mean annual temperature in Galicia ranged from 8.5 ºC to 19.8 ºC and in São Miguel from 11.5 ºC to 26 ºC. This comparison suggests that higher temperature and lower humidity will favour an earlier spore release.

The development of *Matteuccia struthiopteris* fertile fronds in Norway ceased during long dry and warm periods (Odland, 1995). Ranal (1995) studied the phenology of several fern species that produce fertile leaves between October-November, (*Microgramma lindbergii*, *M. squamulosa*, *Adiantopsis radiata*, *Polypodium latipes* and *Pteris denticulata*) in Brazil, and found that spore release also occurred gradually during the wettest period (February-April). However, other species in the same area that produce fertile leaves in January (*Polipodium hirsutissimun*, *P. pleopeltifolium* and *P. polypolioides*), released spores during the warmest period (April-August). These studies suggest that spore production always coincide with the wettest period while release may coincide with the wettest or the warmest period. For ferns growing in Taiwan most spores mature during the warm season (May to October, which the mean temperature of each month was higher than 20ºC) and few during the cold season (Chow et al., 2001).

Therefore, there is no clear climatic pattern for fern spore maturity and release as they appear to be influenced by different conditions, probably reflecting adaptations to local environmental conditions.

According to Wardlaw (1962) and Wardlaw and Sharma (1963) light intensity and duration of exposure to light is not generally important for ferns. However, leaves of *Pteridium aquilinum* and *Polypodium latipes* significantly increasing spore production as a response to light intensity (Conway, 1957, Dring, 1965 and Ranal, 1995) while leaves are sterile in dense canopy. There is a tendency for ferns on exposed sites to mature and release spores earlier than ferns in close forest (Sato, 1985), as open areas allow the light to go through while dense canopy intercepts most light. In our study however, spore maturation and release were not influenced by cover, presumably because our canopy cover values were all very high. Canopy cover was lower in mid altitudes for both species, which was a result of removal of exotic vegetation that was carried out by an ongoing conservation project in the area.

A gradual maturation and release of spores along an altitudinal gradient is important for the feeding of Azores bullfinch in winter. Hew and Wong (1974) suggest that a late release of spores may allow for a more seasonal input of energy for spore production, a larger total production of spores and/or more stored energy per spore. We can envisage that bird distribution should be progressively pushed up to higher altitudes along the season following spore development. Given that ferns constitute the main native food resource in winter for the Azores bullfinch, it is crucial to maintain abundant fern populations along this gradient. These fern populations are particularly important until early April when other food sources, such as flower buds of *Ilex perado* spp. *azorica* become available (Ramos, 1995, 1996b). Low percentages of abortive spores are also a positive aspect for the Azores bullfinch survival as they are empty.

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Annex I - **Chemical determinations on sporangia and leaves**

## I.1. Sample preparation

Only young fronds, i.e. expanding or recently expanded, were sampled as the Azores bullfinch only feeds on fronds at these stages (Ramos, 1994). Young fronds could be recognized by its soft lamina texture, due to the thin cuticle. Leaves and spores were frozen at -80ºC until analysis (1-4 months). Prior to analyses they were oven dried at 60ºC until weight stabilization (aprox. 4 days). For each phenol, lipid or protein assay, 100 mg dry weight samples were used. Analyses were conducted for *C. macrocarpa*, *W. radicans, P. incompleta, D. and B. spicant* spores and *W. radicans*, *C. macrocarpa*, *P. aquilinum, O. regalis, P. incompleta and Dryopteris* spp. fronds.

## I.2. Caloric content

To determine the caloric content of spores and fronds, items were thawed and dried at 60ºC. Items were weighed daily until a constant value was obtained, and then each item was crushed to dust in a mortar. A small quantity of the samples (0.058 to 0.200 g) were converted into pastilles in a press and used to determine the caloric content of each species in a PARR 1425 calorimeter. Three pastilles of each sample were made and the mean calculated. This calorimeter output is in cal/g dry weight (Fraschetti *et al.* 1994). The value obtained was converted to Joules per gram of dry weight (J/g dry weight).

## I.3. Lipids

Reagents: Chloroform-methanol mixture 2:1 by volume. Pure solvents upper phase and pure solvents lower phase: Chloroform, methanol, and water are mixed in a separatory funnel in the proportions 8:4:3 by volume. When the mixture is allowed to stand, a biphasic system is obtained. The two phases are collected separately and stored

in glass bottles. It has been found that the approximate proportions of chloroform, methanol and water in the upper phase are 3:48:47 by volume. In the lower phase, the respective proportions are 86:14:1. Either of the phases may be prepared directly by making use of the above proportions (Folch *et al*., 1956). Pure solvent upper phase containing 0.02 % $CaCl_2$. This solution was prepared shaking the appropriate amount of salt with pure solvents upper phase in a glass-stoppered vessel until solution is complete.

Lipids were extracted and determined according to Folch (1956). Samples are homogenized with 2:1 chloroform-methanol mixture (v/v) to a final dilution of 20-fold the volume of the sample. The homogenization of samples will be carried out in an mortar because other methods like homogenization with a Polytron or with a Potter-Elvehjem were not suitable for fern spores, because it is few quantity, volumes used are so low and as the spores walls are so hard there is no chance of breaking them with those methods, a lot of time would be necessary with the warming of the samples associated that can damage spore content properties, so mechanical homogenization will be done. The homogenate was centrifuged, either filtration with fat free paper can be done but it was decided to centrifuge because there would be no chance of losing volume and material as the working homogenate is so low. Also centrifugation can be used in preference to filtration as a means of obtaining a clear extract. Centrifugation of the homogenate itself is unsatisfactory because the specific gravity of the solvent mixture is too close to the density of the suspended material. Therefore, if centrifugation is to be used, it is necessary to lower the specific gravity of the homogenate by the addition of methanol. Usually the addition of 0.2 ml its volume of methanol suffices for the purpose. The amount of methanol added was 0.4 ml because the total volume for the homogenization was 2 ml (100 mg of sample). It is important at the end of the

procedure and before drying the sample that twice as much chloroform must also be added and the amount of water adjusted accordingly (8:4:3). After centrifugation a pellet appears and it has to be separated from the crude extract. The crude extract has to be moved to a test tube and it has to be washed. The crude extract is mixed with 0.2 ml its volume of distilled water (it can be used an adequate salt solution) and the mixture is allowed to separate into two phases by centrifugation (4500 $m^{-1}$, 3371 g for 10 minutes with 4 minutes of deceleration). As much of the upper phase as possible is removed by siphoning, and removal of its solutes is completed by rinsing the interface three times with small amounts (three times 0.20 ml) of pure solvents upper phase in such a way as not to disturb the lower phase. Finally, the lower phase and remaining rinsing fluid are made into one phase by the addition of methanol (one time 0.20 ml), and the resulting solution is diluted to the desired final volume (2 ml) by the addition of 2:1 chloroform-methanol mixture. To determine final lipids samples were weighted after been for 6 hours drying in the OTE, or until weight stabilization.

**I.4. Proteins**

Reagents: Reagent A: 2% $Na_2CO_3$ in 0.10 N NaOH. Reagent B: 0.5% CuSo4.5$H_2O$ in 1% of citrate tri-sodium. Reagent C: Alkaline copper solution. 50 ml of reagent A mixed with 1 ml of reagent B. Discarded after one day. Reagent D: Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) with 4% NaOH 2:1 by volume.

Proteins were measured with the Folin phenol reagent method (Lowry *et al.*, 1951). Homogenization of spores and leaves was done with a mortar and pestle in 5 ml of 50% (aqueous) methanol and these samples were stored at 4ºC for two hours. After storage trichloroacetic acid was added to precipitate proteins. Then, samples were centrifuged (4500 $m^{-1}$, 3371 g for 10 minutes with 4 minutes of deceleration) and the

pellet was resuspended in 5 ml of water (same volume as initial preparation) after mixing in the vortex again a new centrifugation was done to keep a clear extract. From this extract 1 ml was taken and then the Lowry method started, 5 ml of reagent C were mixed for each sample and was stored in an hot bath (30ºC) for 10 minutes after that reagent D was added, was kept for 30 minutes at the same temperature. Then absorbance was measured at 500nm.

Concentrations of proteins in all samples were determined from calibration curves using BSA (Armour and Company, Chicago) as standard. This standard showed an almost linear relationship between absorbance and standard concentration ($r^2$ = 0.9981).

**I.5. Phenolics**

Reagents: 50% (aqueous) methanol. Folin-Ciocalteau reagent (Folin and Ciocalteu, 1927) (50% diluted in water). $Na_2 CO_3$ saturated. Water.

Samples were analysed using an adaptation of the method described by Julkunen-Titto (1985). This method is based in the reduction of the phosphotungstic-phosphomolybdic (Folin and Denis, 1912) present in the Folin-Ciocalteu reagent. After homogenizing samples with a mortar and pestle in 5 ml 50% (aqueous) methanol, they were stored at 4ºC for two hours. Then samples were centrifugated (4500 $m^{-1}$, 3371 g for 10 minutes with 4 minutes of deceleration) and 1.5 ml of extract was taken, 100 µl of Folin reagent (50% diluted in water) was added. After keeping for 5 minutes at room temperature 200 µl of $Na_2CO_3$ saturated was added and incubated during 30 minutes at 40ºC. At the end 5 ml of water was added and absorbance measured at 765 nm against water blank.

The concentrations of phenolics in all samples were determined from calibration curves using gallic acid as standard (Hagerman & Butler, 1989). This standard showed an almost linear relationship between absorbance and standard concentration ($r^2$ = 0.9618).