

# Gametophyte features in a peculiar annual fern, *Anogramma leptophylla*

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*Anogramma leptophylla* is one of few homosporous ferns with ephemeral sporophytes and perennial gametophytic tubercles. This rare aspect of the life cycle has considerable bearing on the reproductive features of the gametophyte. Four populations in the Iberian Peninsula were selected to provide spores for laboratory cultures and to sample natural populations of gametophytes for comparative studies. Sexual expression and the role of the tubercle in the reproductive biology of this fern were studied. No significant differences were found between percentages of sexual versus asexual prothalli in natural populations or laboratory cultures, or between percentages of unisexual and bisexual prothalli. These results may indicate that sex in *A. leptophylla* is genetically determined instead of environmentally controlled as in most ferns. The presence of a gametophytic tubercle with nutrient reserves seems to be most favourable for the development of archegonia, and for the establishment of young sporophytes. This tubercle, which can resist stressful environmental conditions, allows for the development of new gametophytic outgrowths and of dormant embryos as soon as environmental conditions improve. This bryophyte-like behaviour gives this fern some advantages in colonisation and has probably contributed to its wide geographic range.

## Introduction

The homosporous leptosporangiate ferns are an important and diverse group of vascular plants. Their life cycle consists of two morphologically very different free-living and alternating phases: a haploid gametophyte and a diploid sporophyte. In most species, the sporophyte is a perennial plant with a well developed rhizome, roots, and leaves, while the gametophyte is a tiny plant with a thallose organization. However, there are a few ferns with short-lived sporophytes that complete their whole life in a single growing season, usu-

ally a few months. Some of these ferns produce perennial structures on their gametophytes. The genus *Anogramma* exhibits this pattern and in most of its species the sporophyte is annual, and the gametophyte persists by means of a tubercle (Tryon & Tryon 1982). The annual sporophyte and the perennial gametophyte give this fern a bryophyte-like life strategy (Proskauer 1964).

*Anogramma* includes about eight species that live in regions with alternating wet and dry seasons, mainly in the tropical areas of Mexico and Central and South America (Nakazato & Gastony 2003). However, *A. leptophylla* has one of



**Fig. 1.** Photograph of an *Anogramma leptophylla* prothallium with a well developed tubercule. Bar = 1 mm.

the widest ranges among all fern species (Christ 1910). It grows not only in the Neotropics, but also in the Mediterranean basin, Macaronesia, Atlantic Europe, tropical Africa, South Africa, India, Australia and New Zealand (Bostock *et al.* 1998). The wide distribution of this species may be due to its bryophyte-like life strategy, with a perennating gametophyte (Fig. 1) that behaves as in some hepatics (Proskauer 1964). *Anogramma leptophylla* prothalli were one time described by Taylor (1846) as a new species of liverwort, although the author hesitated about the genus in which to include it. In the Iberian peninsula, *A. leptophylla* grows in rocky habitats, mainly in wide cracks of siliceous rocks filled with soil where there are few or no competitors at all; it is found from the lowlands up to about 1000 m a.s.l., and under climates varying from typical oceanic to Mediterranean (Nogueira 1986, Salvo 1990).

The morphology and development of the prothalli of *Anogramma* species have been previously studied, and the prothallium of *A. leptophylla* is well known (Goebel 1889, Mehra & Sandu 1976, Pangua & Vega 1996, Hagemann 1997). Thus, our research focused on the reproductive biology of this fern.

Understanding sexual expression in the gametophyte generation is critical as this process ultimately determines the outcome of crossing events. This in turn influences the genetic structure of the sporophyte population. Contrary to many other plants where sexual expression is genetically controlled, in ferns environmen-

tal sex determination is the rule, which means that the sex of the gametophyte is determined, independently of its genotype, by several environmental factors (Korpelainen 1998, Juárez & Banks 1998). Factors affecting sexual expression in ferns may be: prothalli population density, nutrient availability, light, antheridiogens, weather conditions or water availability (Döpp 1950, Haig & Westoby 1988, Schneller *et al.* 1990, Korpelainen 1994, 1998, Yamane 1998, Page 2002, Huang *et al.* 2004, Kamachi *et al.* 2007, Schneller 2008, DeSoto *et al.* 2008). Also laboratory culture conditions might affect sexual expression and then laboratory data would not be an accurate reflection of what is occurring in nature (Ranker & Houston 2002).

If climate is a factor determining sexual expression, the gametophyte populations of *A. leptophylla* growing in different climatic areas should show a different sexual expression. It has also been shown that gametophyte size is related to sexual expression in several ferns (Korpelainen 1994, Pangua *et al.* 1994, DeSoto *et al.* 2008). However, gametophyte size may be affected by environmental factors, e.g. antheridiogens, and a slower growth rate results in small, uniformly male prothalli (DeSoto *et al.* 2008).

The subterranean tubercule of *A. leptophylla* gametophytes has a function in nutrient accumulation (Goebel 1889, Mehra & Sandhu 1976). It is known that in many fern gametophytes sexual expression, especially the production of female gametangia that should provide support for the young sporophyte, is related to the amount of available nutrients.

Our research addressed the following questions regarding the gametophytes of *A. leptophylla*: (1) Is sexual expression determined by the different climatic conditions of the populations? (2) Are there any differences in sexual expression between natural gametophyte populations and cultivated ones? (3) Does gametophyte size and tubercule size affect sexual expression? (4) Do nutrient reserves affect sexual expression?

## Material and methods

We used six populations of *A. leptophylla* for this study. We selected four populations from

two different climates of the Iberian Peninsula (Table 1). Populations TUI and VAL were located in an area with a temperate oceanic climate, and populations TIE and TOR were located in areas with a Mediterranean climate (<http://www.ucm.es/info/cif>). We established the remaining two populations under laboratory conditions sowing spores obtained from one population from each climate regime, TOR and VAL, respectively.

For the laboratory cultures, we collected 20 to 25 fronds with mature but closed sporangia from each population. We let them dry out on sheets of smooth paper to allow spore release. Spores from individuals of the same population were pooled before sowing. Spore sowings were made in four replicates on Petri dishes, 5.5 cm in diameter, with nutritive media solidified with agar (Dyer 1979). After six weeks, 100 young prothalli per population were selected randomly among the four replicates. They were transplanted in groups of 25, regularly distributed, to plastic boxes, 10 × 10 cm, containing sterilized compost. Boxes were kept in a growing cabinet at 20 °C and a 16:8 hour photoperiod, at a light intensity of 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

After five months of culture, we randomly sampled 30 prothalli among all the replicates for each population. Each gametophyte was screened for gametangia and tubercles. Their amount of reserve nutrients (mainly starch) was roughly estimated studying transverse sections of the tubercles microscopically and classifying their content according to the following three

categories: (1) nutrient reserves absent; (2) nutrient reserves scarce, when only some cells in the central part of the tubercule section contained nutrient reserves, and (3) nutrient reserves abundant, when most cells showed reserve material. We used Lugol's solution to demonstrate the presence of starch. As size indicators, the diameter of the tubercles and the widest part of the prothalli were measured.

In order to compare the laboratory and field results, we studied the same parameters in thirty prothalli, randomly selected from each of the four wild populations (TUI, VAL, TIE and TOR).

We used SPSS (2006) for all statistical analyses. A chi-square ( $\chi^2$ ) test was used to explore the relationships between gametophyte sexual expression (presexual, male, female and bisexual) and gametophyte origin (temperate oceanic, Mediterranean, and cultivated), and with nutrient content (absent, scarce, or very abundant). A non-parametric Kruskal-Wallis test was performed to analyse differences between gametophyte and tubercule size according to sexual expression. This non-parametric test was carried out because the variables were not normally distributed.

## Results

Morphological development and sexual expression of the gametophytes were the same in both populations (VAL and TOR) studied in

**Table 1.** Acronyms and localities of the populations studied, with their germination percentage.

Population acronym	Locality	Mean annual rainfall (mm)	Mean annual temperature (°C)	Germination percentage
TIE	Spain, Ávila: La Adrada, river Tiétar valley, granite blocks, 500 m a.s.l., <i>S. Pajarón &amp; E. Pangua</i> , 10.III.2007	970	14.1	95
TOR	Spain, Madrid: Torrelodones, granite blocks, 850 m a.s.l., <i>S. Pajarón &amp; E. Pangua</i> , 18.III.2007	524	13.4	94
TUI	Spain, Pontevedra: Tui, granite walls, 100 m a.s.l., <i>S. Pajarón &amp; E. Pangua</i> , 4.III.2007	1496	14.2	95
VAL	Portugal, Minho: Valença do Minho, granite walls near the river, 80 m a.s.l., <i>S. Pajarón &amp; E. Pangua</i> , 3.III.2007	1480	14.5	98

the laboratory. The cells in the initial filament divided longitudinally forming a spatula-shaped structure with a lateral meristem. In some cases, several lobes and more than two meristematic zones were formed. In the laboratory cultures, antheridia developed first and were located at the base of the prothallium, where it was several cells thick. In a few prothalli, antheridia were also found on the flat thalloid part. Archegonia developed afterwards, and were embedded in the tubercle parenchyma, with the neck protruding. Then the gametophytes became bisexual. Eventually, several prothalli became female after antheridia discharged their spermatozoids.

The percentages of asexual and sexual gametophytes were different across natural populations, ranging from 4.2% to 13.3% for the asexuals, and from 86.7% to 95.8% for the sexuals. For the four field populations (Table 2), we found an overall percentage of 7.8% asexuals and 92.2% sexuals. In the laboratory cultures, we obtained similar results, with a mean of 9.2% asexual and 90.8% sexual prothalli. Male and bisexuals were well represented, but only a few females were observed (Table 2).

The analysis of the contingency table of asexual/sexual *vs.* cultures, Mediterranean climate/temperate oceanic climate did not show a significant difference, as indicated by the results of the chi-square test ( $\chi^2 = 0.76$ ,  $df = 2$ ,  $p > 0.1$ ).

The percentage of bisexual prothalli was higher than the percentages of unisexuals in the natural populations and laboratory cultures, 45% unisexual, 55% bisexual in the natural populations, and 39%, 61%, respectively, in the labora-

tory cultures. However these differences were not statistically significant ( $\chi^2 = 1.08$ ,  $df = 2$ ,  $p > 0.1$ ).

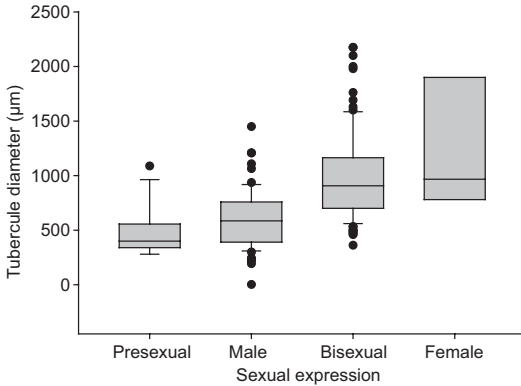
Finally, we found no differences in the number of male and female prothalli among the gametophytes from temperate oceanic climate, Mediterranean climate and the laboratory cultures ( $\chi^2 = 1.53$ ,  $df = 2$ ,  $p > 0.1$ ).

As there were no significant differences in the sexual expression of the populations from different origins and treatment, i.e. field and laboratory, relationships between the size of the tubercle and the prothalli, and sexual expression itself was analysed using data from all populations together. The size of the tubercles increased with age, thus following the ontogenetic sequence of gametangia (Fig. 2); female prothalli, the latest in this sequence, possessed the largest tubercles. The diameter of the green thalloid parts of the prothalli showed similar differences (Fig. 3), however, bisexual gametophytes were the widest and presexual ones the narrowest. The Kruskal-Wallis test indicated statistical significance of differences in the tubercle size ( $H = 59.061$ ,  $df = 3$ ,  $p < 0.001$ ), and prothallus size ( $H = 27.442$ ,  $df = 3$ ,  $p < 0.001$ ).

Food reserve accumulation was clearly related to sexual expression (Fig. 4). Sexual gametophytes showed a higher accumulation of food reserves than asexuals ( $\chi^2 = 29$ ,  $df = 2$ ,  $p < 0.001$ ), and bisexual gametophytes had a higher food content than unisexuals ( $\chi^2 = 44$ ,  $df = 2$ ,  $p < 0.001$ ). Presence of nutrient reserves also showed significant differences between archegoniate prothalli, (i.e. female and bisexuals), and male prothalli ( $\chi^2 = 49.974$ ,  $df = 1$ ,  $p < 0.001$ ).

**Table 2.** Percentages of sexual and asexual gametophytes and sexual expression in percentages of each sex from each population.  $n$  = number of individuals.

Population	$n$	Asexual	Sexual	Male	Female	Bisexual
<b>Field</b>						
TUI	30	7	93	50	0	50
VAL	24	4	96	22	9	69
TOR	32	6	94	30	10	60
TIE	30	13	87	54	4	42
<b>All field populations</b>	116	8	92	39	6	55
<b>Laboratory</b>						
VAL	34	3	97	33	0	67
TOR	31	16	84	42	4	54
<b>All lab populations</b>	65	9	91	37	2	61



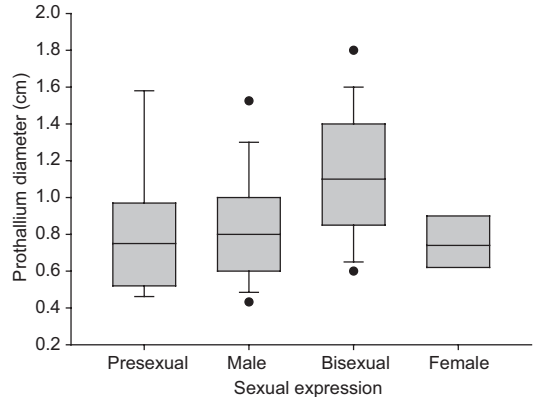
**Fig. 2.** Box-and-whisker plot of tubercule size at the presexual, male, female and bisexual stages, for all populations. Medians are indicated by horizontal lines within boxes, boxes are 75th and 25th percentiles, whiskers are 10th and 90th percentiles, black dots indicate outliers.

When studying the tubercule sections to quantify nutrient reserves, we observed associated fungi similar to mycorrhizae. These mycorrhizal-like fungi appeared only in samples from natural populations, and were absent from tubercules of prothalli grown in the laboratory.

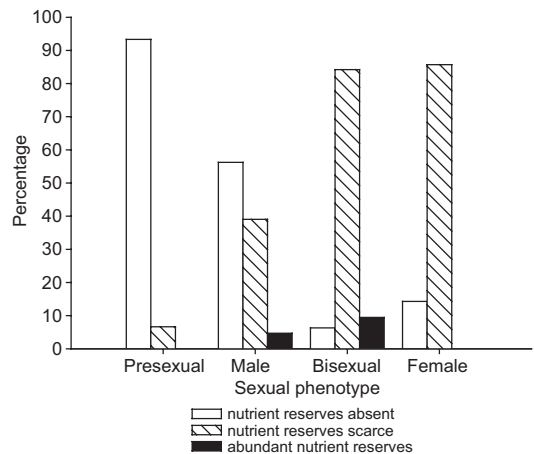
## Discussion

The morphology and development of the gametophytes grown in the laboratory, as well as the position and the morphology of antheridia and archegonia, was the same as described by Mehra and Sandhu (1976), Cheema (1980), Pinel (1996), and Pangua and Vega (1996).

Our results showed that sexual expression was not related to the geographic locality and thus not affected by the different climates in which the gametophytes developed. This was also true for the standard conditions used in the laboratory cultures when compared with naturally grown ones. Bisexual gametophytes were more abundant than unisexual ones, both in natural conditions and in the laboratory cultures. This sexual behaviour contrasts with what was observed in other ferns such as *Blechnum spicant*, *Athyrium filix-femina* and *Sadleria* spp. (Cousens 1979, 1981, Schneller 1979, Ranker & Houston 2002), where unisexual gametophytes prevail over bisexual ones, e.g., 90% were uni-



**Fig. 3.** Box-and-whisker plot of the prothallium diameter at the presexual, male, female and bisexual stages, for all populations. Medians are indicated by horizontal lines within boxes, boxes are 75th and 25th percentiles, whiskers are 10th and 90th percentiles, black dots indicate outliers.



**Fig. 4.** Percentages of different nutrient reserve classes at the different sexual stages (presexual, male, female and bisexual).

sexual in *Sadleria*, both in the field and in the laboratory (Ranker & Houston 2002). When comparing the proportion of female and male prothalli among the unisexuals, there were no differences between the results obtained in the laboratory cultures and in the field. In natural populations of gametophytes of *Athyrium* and *Sadleria* the number of males was higher than in the laboratory (Schneller 1979, Ranker & Houston 2002). These differences were explained by the diluted effect of antheridiogen that resulted from growth on natural soil as compared with

artificial growing medium like mineral agar in the laboratory. Moreover, the age structure of the populations also explained differences in the effects of antheridiogen. In the laboratory cultures, populations are established by a single spore sowing and all gametophytes reach the antheridiogen producing state more or less at the same time. Natural populations however would be mixed-aged, and antheridiogen-producing gametophytes would affect younger ones that would produce antheridia (Ranker & Houston 2002, García-Gómez *et al.* 2010). The high percentage of bisexual gametophytes in *A. leptophylla* might suggest a high potential for intragametophytic selfing, although this type of fertilization is not obligate in bisexual prothalli (Cousens 1975, Quintanilla *et al.* 2005). Moreover, the presence of a relatively high proportion of males and the fact that they are protandrous, might favour intergametophytic fertilizations (Cousens 1975, Pangua *et al.* 1994). An intragametophytic selfing reproductive system would lead to genetically uniform populations with an excess of homozygous sporophytes, while intergametophytic crossings would lead to more genetically variable populations. Preliminary isozyme data of the genetic structure of these populations (own unpubl. data) suggests that intragametophytic selfing is rare.

If sexual expression in the *Anogramma* gametophytes is not affected by the different environmental conditions in which they develop, it could be asked if these gametophytes are an exception to the environmental sex determination that occurs in most ferns (Korpelainen 1998), i.e., whether the ontogenetic development of the gametophyte is influenced by environmental factors, or instead is fixed and under genetic control.

The three sexual kinds of gametophytes coexist within populations, and their proportions are constant among all populations. Predominance of bisexual prothalli, considering that the sampling was made at the beginning of the growing season in early spring, may represent optimal conditions for fertilization and completion of the life cycle, faster than in most ferns (Page 2002).

Therefore, it should be investigated if unfavourable conditions change the sexual expres-

sion in this species, and cause an increase in the amount of female gametophytes. However, this would be different from what happens in most ferns, in which unfavourable conditions usually lead to the development of antheridia (Chiou *et al.* 1998, Korpelainen 1998, Greer & McCarthy 1999, Schneller 2008 and references therein). The prediction of female gender expression under favourable conditions and male expression under poor growth conditions (Haig & Westoby 1988), based on a high cost of female reproduction might not occur in *Anogramma* because of the possession of the tubercule and its nutrient reserves. It is known that tubercules act as dormant embryo refuges, and they may as well regenerate the green thalloid part of the gametophyte, or form vegetative gemmae (Mehra & Sandhu 1976, Baroutsis 1976, Nakazato & Gastony 2003).

Archegoniate prothalli have the largest surface areas and the largest tubercules. These data agree with the size-advantage hypothesis that is related to the higher cost of female production (Ghiselin 1969, Haig & Westoby 1988), and to observations in other studies about factors other than antheridiogens affecting the sexual determination of gametophytes. In these cases, relatively large gametophytes tend to develop as females or bisexuals, while the relatively small ones tend to be male or asexual (Huang *et al.* 2004, DeSoto *et al.* 2008).

The presence of mycorrhizal-like fungi in the subterranean part of the gametophytes has been described in several genera of lycophytes, such as *Lycopodiella*, *Lycopodium*, and *Huperzia* (Whittier 1998, Whittier & Carter 2007, Winter & Friedman 2008). These mycorrhizal-like fungus associations guarantee the germination of spores and the establishment of the young gametophyte in the dark. In *A. leptophylla*, there are also indications of mycorrhizal-like fungus associations (Mehra & Sandhu 1976, Pinel 1996, personal data), but there is no evidence of germination in the dark, and the perennial tubercule might have a role as a nutrient reservoir (Nayar & Kaur 1971). The effects of the fungus on the tubercule are not clear. In the laboratory cultures, where the mycorrhizal-like fungi were absent, no sporophytes were observed during the experiment. In the wild, where the mycorrhizal-

like fungi were present, sporophytes in various developmental phases were frequently observed.

As for the amount of nutrient reserves, our results showed significant differences among prothalli with different sexual expressions. Archegoniate prothalli, i.e. females and bisexuals, had accumulated a higher amount of nutrient reserves, starch, and lipids (Pinel 1996), than males and asexuals. This suggests that reserves accumulate as the tubercle develops and gametangia appear. The highest accumulation is reached when archegonia are present, i.e., when fertilization should occur, followed by the development of a young sporophyte. Thus, nutrient reserves would be directly related to reproductive success.

At the beginning of spring it is easy to find large populations of gametophytes of *A. leptophylla* that decrease during late spring and summer when competitors such as mosses, liverworts, or seed plants appear in the same habitat. The tubercle that remains in a resting state might facilitate the regeneration of the photosynthetic part of the prothallium at the beginning of the favourable season. This competitive advantage of *A. leptophylla* allows for fast development of its ephemeral sporophytes. In laboratory studies Molnár *et al.* (2008) found dormant tubercles that were able to survive for 2.5 years in drought conditions, and of which 5% quickly developed sporophytes after watering. This strategy increases the capacity to have short life cycles, as in mosses or small ephemeral flowering plants (Raven 1985, Richardson 1992, Page 2002). This gives *A. leptophylla* its bryophyte-like life-style and, as a consequence, its advantage in colonization that might explain its wide geographic range.

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