



## Is gender expression genetically fixed in ferns? A study of *Culcita macrocarpa*

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### Abstract

An individual's sex can be genetically fixed at conception (genetic sex determination, GSD) or decided afterwards according to its immediate environment (environmental sex determination, ESD). When environments differentially influence male and female performance, ESD might evolve. Homosporous ferns are the only plant group in which ESD is predominant. Several culture studies have found that *Culcita macrocarpa* is first male and then hermaphrodite, even in conditions supposed to be optimal for growth. This gender sequence is fixed under a combination of two light levels and three nutrient levels. At low light level gametophytes grew slowly, irrespective of nutrient level, and most became male. In addition, there were some hermaphrodites but no females. At high light level gametophytes showed faster growth, especially with mid and high nutrient levels, and although most gametophytes became male, some were females. These results are thus in agreement with the ESD theoretical framework. It can be concluded that any realistic assessment of gender sequence in fern gametophytes must consider a broad range of culture conditions.

**Keywords:** Environmental sex determination, Homosporous fern, *Culcita macrocarpa*, hermaphrodite, gender expression.

### INTRODUCTION

An individual's sex can be genetically fixed at conception (genetic sex determination, GSD) or decided afterwards according to its immediate environment (environmental sex determination, ESD). The mechanism of sex determination allows the gametophyte to determine its sex by sensing its environment rather than by genetic pre-determination. When environments differentially influence male and female performance, ESD might evolve. Organisms may experience variation in conditions like temperature or nutrient availability during development, which can influence their performance as adults. Although certain environments will be more favourable, one sex may gain relatively more by developing in these conditions, whereas the other sex may lose less by developing in unfavourable circumstances. When the differential fitness effects are sufficiently strong, environmental conditions could become used as cues or sex determination. This possibility is an important ingredient in the traditional explanation for the occurrence of ESD, originally put forward by Charnov and Bull (1977).

Environmental sex determination is observed in some plants (Freeman *et al.*, 1980), but homosporous ferns are the only plant group in which ESD is predominant. Gametophytes of many homosporous ferns tend to become male under poor growing conditions, such as poor light level and quality (Guillon and Fievet, 2003), low nutrient availability (Korpelainen, 1994; Ghosh *et al.* 2012). Conversely, gametophytes normally become female under rich growing conditions. However, in many species actual gender is conditional on environmental conditions. Environmental sex determination is a form of phenotypic plasticity, by which individuals produce either female, male, or both sex organs depending largely upon environmental circumstances (Leimar *et al.*, 2004).

Carbohydrates are known to perform important regulatory functions in the plant life cycle, including photosynthesis (Halford and Paul, 2003) and carbohydrate partitioning (Rook and Bevan, 2003) mechanisms by using different sugars act to influence gene expression and ultimately plant development (Gibson, 2005). Nutrients as well as

other factors are implicated in the growth and development of gametophytes and sporophytes (Fernández *et al.*, 1999).

Light is well known to be the most influential of the various environmental factors that control the developmental processes of ferns, and an enormous amount of work on photomorphogenesis of ferns, especially in their haploide generation, has been carried out (Miller 1968; Dyer 1979; Furuya, 1985). For a fern gametophyte, light is an important environmental signal that guides development and photosynthetic efficiency (Raghavan 1989; Kamachi *et al.*, 2007).

*Culcita macrocarpa* C. Presl is the only European member of the family Dicksoniaceae. This fern is located in northern coastal regions of Spain, and in the Azores, Madeira and Canary Islands. The ideal habitat for this species is mature riparian woodland on north-facing slopes of enclosed valleys, close to the coast (Amigo and Norman, 1995). This species typically show strong clonal growth leading to patch formation. The disjunct distributions increase the probability that populations will be affected by genetic drift (e.g. Young *et al.* 1996).

The present study investigated that the environmental sex determination of *Culcita macrocarpa* under a wide range of nutrient and light conditions. Other environmental factors affecting gender in ferns, such as nutrients, have received very little attention in most reviews of sex expression in fern gametophytes (Cousens 1988). The gametophyte of *C. macrocarpa* has been studied previously by Stokey (1930), Rezende-Pinto (1943), Mukherjee and Sen (1986). Although these studies described the sequence of appearance of gametangia, their central focus was on morphology. The only study addressing sexual expression in ferns from an ESD perspective is Guillon & Fievet (2003) who analysed the effect of light on horsetail gametophytes. DeSoto *et al.*, (2008) and Ghosh L. (2009) fruitfully studied sexual expression in fern gametophytes within the ESD theoretical framework. According to ESD theory and previous empirical evidence, an association between stressing conditions (high light, low nutrients), small size and male gender were expected.

Although reference to such environmental influences on gametophyte gender are scattered in the literature on gametophyte biology, it is only recently that an ESD-based interpretation has gained momentum. In the present paper, the effects of nutrient and light as double factors on gender expression and gametophyte size of *C. Macrocarpa* were assessed.

## METHODS

*C. macrocarpa* gametophytes were cultured under varying nutrient and light conditions. Spores were sown under three different nutrient levels (Dyer agar diluted 0, 100 and 10000 times, respectively) and two different light levels (60 and 5.5 PARS). Seven weeks after sowing, 200 gametophytes per nutrient and light level combination were transplanted into individual cell under the same nutrient and light conditions for germination. Cultures were incubated in a phytothron with a 14h photoperiod and alternating 20<sup>0</sup>C-light/15<sup>0</sup>C-darkness temperatures.

To elucidate patterns of gender expression in gametophytes, 100 gametophytes per nutrient and light combination were randomly sampled. Gametophytes were stained with acetocarmine-chloral hydrate after 19 and 27 weeks of sowing and their sex and number of gametangia were determined under a light microscope. To determine gametophyte size, all individuals were scanned and their area was measured with the program *Image* (Abramoff *et al.* 2004).

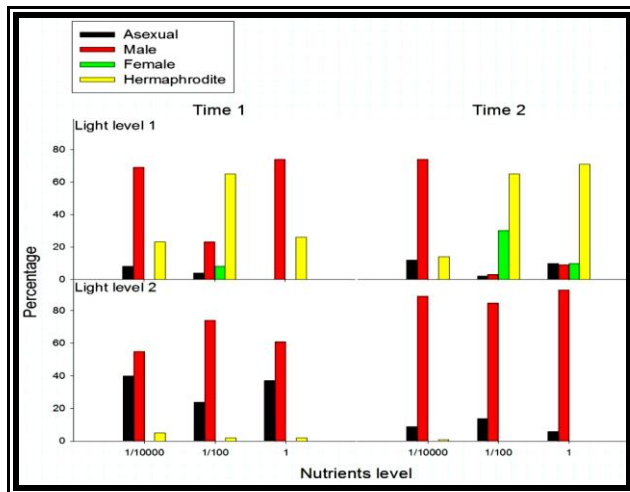
The effects of nutrient and light on gametophyte gender and size were separately analysed by fitting generalized linear models (GLMs) using the GENMOD procedure of SAS 9.0 (SAS Institute 2002). Models included the effects of treatment level, growth chamber and harvest time nested into chamber; all of these were considered as fixed effects. Growth chamber was treated as a fixed effect due to the complex computational analysis for a multinomial distribution of response variable with random predictors. To consider as a fixed effect one that is random is more desirable than transforming the variable distribution and should not affect our conclusions. Gender was considered as a fixed effect in the models for size.

## RESULTS

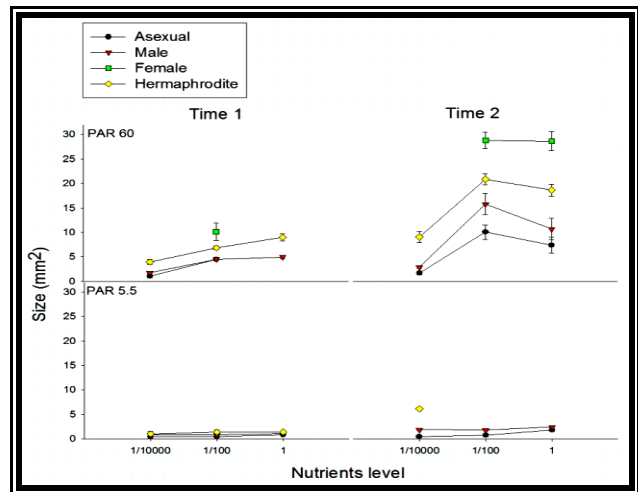
Gender was significantly affected by different nutrient and light levels. At low light level gametophytes grew slowly, irrespective of nutrient level, and most became male. In addition, there were some hermaphrodites but no females. At high light level gametophytes showed faster growth, especially with mid and high nutrient levels, and although most gametophytes became male, some were females. In the first time, laboratory populations were generally mostly antheridiate and bisexuals but in the second time antheridiate with archegoniate and bisexuals (Fig.1).

In all respects, female gametophytes were much larger, than other types of gametophytes. Hermaphroditic gametophytes were larger than male gametophytes, which

were larger than asexual gametophytes. The size of females and bisexuals increased with increasing nutrient and light level over time (Fig.2).



**Figure 1.** Relative frequencies of gametophyte genders at the two light levels and three nutrient levels



**Figure 2.** Gametophyte size at the two light levels and three nutrient levels.

**Table 1.** Summary of GLMs for the effects of nutrients and light on gender and size of *C. macrocarpa* gametophytes.

Experiment	Variable	Effect in model	Df	G <sup>2</sup>	P
Gender		Nutrients (N)	2	4109	<0.0001
		Chamber (C)	1	1.35	0.2382
		Time (T) (Chamber)	3	222.86	<0.0001
		Nutrient × Time (Chamber)	11	57.38	<0.0001
		Nutrients (N)	2	388.85	<0.0001
Nutrients		Gender	3	43.21	<0.0001
		Chamber	1	6.83	0.0091
		Size	3	354.58	<0.0001
Size		Time (Chamber)	3	354.58	<0.0001
		Nutrient × Time (Chamber)	11	228.76	<0.0001
		Gender × Time (Chamber)	15	37.06	0.0004
		Light	1	14.89	0.0031
		Chamber	1	0.01	0.9037
Light		Time (Chamber)	3	135.86	<0.0001
		Light × time (Chamber)	7	20.12	0.1678
		Light	1	229.74	<0.0001
		Gender	3	59.55	<0.0001
		Chamber	1	2.37	0.1222
Size		Time (Chamber)	3	35.34	<0.0001
		Light × time (Chamber)	7	184.69	<0.0001
		Gender × Time (Chamber)	15	28.85	0.0126

Size was significantly affected by all main effects and the interactions light x time and gender x time (Table1). The size ranking of genders was similar to that in the nutrient experiment: male < bisexual < female after 19 and 27 weeks. The gametangial sequence in the all light levels was not as clear as in the other diploids and requires further investigation. All types of prothalli-asexual, male and bisexual appeared at all the time but female prothalli were zero in proportion under low light level after 27 wks.

## DISCUSSION

The study was performed to determine whether nutrition or photomorphogenetically active light affects sex determinations in the gametophytes of *Culcita macrocarpa*. In the present study, gametophytes of *C. macrocarpa* were initially male and subsequently hermaphrodite under the different nutritions with different light level. The data obtained, their sex determination is protandry which showed the agreement with previous research (Quintanilla *et al.*, 2005). When sex depends on environment rather than genotype, the sex expressed under relatively unfavorable conditions will be more abundant. Typically, individuals that are small or in relatively unfavorable circumstances will express themselves as a particular sex, whereas those in favorable conditions will be of the opposite sex (Charnov, 1982). In this study under favorable condition female gametophytes developed whereas male gametophytes developed in all conditions.

The following ontogenetic sequence have been found in this experiment. Under all growth conditions (high and low nutrient and light level), growth prospects were good and gametophytes matured sexually at a small size, turning into males which is contrary with *Woodwardia radicans* (DeSoto L *et al.*, 2008), shows great similarity in various life history characteristics (Quintanilla *et al.*, 2000). But after 27 weeks male gametophytes are similar more or less but asexual gametophytes decreased and turn into females or bisexuals. In that time under good growth conditions gametophytes matured sexually at a relatively large size and developed into females. Because after 19 weeks under good growth condition male and bisexual gametophytes developed but at the time of further growth when the number of male gametophytes were more or less similar whereas female and bisexual gametophytes increased. So here the interpretation of bisexual and females as deriving mostly from asexual prothalli is based on the intermediate number of bisexuals in this experiment.

Environmental sex determination (ESD), where an individual's sex is determined in response to an environmental cue. Theoretical work has suggested that ESD is favoured by natural selection if the environment experienced during development is variable and exerts a gender dependent influence on fitness (Bull, 1983). In case of *G. duebeni*, under the influence of photoperiod, most of the young born early in the year become male, those born toward the end of the year mostly become female (Naylor *et al.*, 1988b; Mc Cabe and Dunn, 1997).

In this study indicates that males benefit more than females. By becoming male they are maximising the fitness gain associated with their large number. Large number is more important for the success fertilization of males than of females. Bull and Charnov (1988) predicted that ESD should be selected when the environment into which an individual is born has a differential effect on male and female fitness. This could have biased the results if there will be an affect of male number on such in future research. This is an interesting result and may, in fact, provide further support for adaptive explanations of ESD if very large number of males represents themselves with a few females from asexual gametophytes at a time under ESD.

For *Culcita macrocarpa*, a homosporous fern with ESD, it has been proposed that this flexible sex determination system is adaptive because males gain more from large size. Under ESD, young which are born earlier in the season become mostly males and, experiencing longer to grow, are therefore larger at breeding than females which

are born later in the season. The gametophytes of homosporous ferns possess the ability to be either unisexual (antheridiate or archegoniate) or bisexual. In addition, they may undergo developmental sequences involving changes in sexuality over time due to genetic or environmental factors (Klekowski, 1969a; Greer and McCarthy, 1999; Ranker and Houston, 2002).

At low light level gametophytes grew slowly, irrespective of nutrient level, and most became male, there were some hermaphrodites but no females but under high light level gametophytes showed faster growth, especially with mid and high nutrient levels. There was a high proportion of male next bisexual and a few proportions of females after 19 weeks. There is evidence that homosporous ferns actually show environmental sex determination, i.e. the development of female, male or both sex organs largely depends on environmental conditions (Korpelainen, 1998). Mainly based on a high cost of female production, Haig and Westoby (1988) predicted female gender expression under favourable growth conditions and male expression under poor growth conditions for homosporous fern gametophytes. Here the strong prediction is that the sex expression is not dependent only due to stress of population resources such as nutrition, light, temperature, density etc but also it is biased for time during development. Gametophyte growth and sexual expression are affected by many factors, including spore size, germination time, nutrition source, growth density, light, temperature and antheridiogen (Schedlbauer, 1976; Nester and Schedlbauer, 1981).

Initially, gametophytes became male, significantly smaller and more slender. Male and bisexual gametophytes dominated in populations of all light and nutrition level respectively. At high light and nutrition level female gametophytes were produced. Female gametophytes were larger than gametophytes of all other types. Hermaphroditic gametophytes were larger than male gametophytes, which were larger than asexual gametophytes (Yao-Moan Huang *et al.*, 2004). This was true of *C. microcarpa* gametophytes. In this observation, light affected gametophyte size and shape, and size was closely related to gametophyte gender with time and environmental factors. When gametophytes are sparse, each gametophyte can obtain more resources, those gametophytes are female.

Sex expression may be labile during the reproductive lifespan- sequential hermaphroditism (Charnov, 1982). In this experiment we have seen a high proportion of male prothalli produced at all time, with the remainder being bisexual and female prothalli produced spending more

time. According to theory, the relationship between plant size (growth rate) and gender expression may be adaptive because of the different costs of male and female functions. Female function demands a higher energetic investment and a longer time commitment (Ghiselin, 1969). Female reproduction is more costly because successful sporophytes develop only on large gametophytes (Sakamaki and Ino 1999). There was a clear relationship between size and sexual expression, with males being smaller than females in all treatments. In other words slow growth favored maleness, whereas fast growth favored femaleness, irrespective of the presence or absence of antheridiogen (Korpelainen, 1994).

In crowded populations antheridia could be a consequence of competition for light and resources as suggested by Willson (1981). It could be an advantage for a spore to respond to a signal from a male and become a female. Sperms that are produced in the range of their activity may reach the egg. More antheridia could be a mechanism to guarantee dense gametophyte populations with a small distance between the different individuals necessary for outbreeding (Schneller *et al.*, 1990). From an ecological perspective, excessive antheridia production may influence the diversity of habitats in which *Culcita macrocarpa* may survive.

Antheridia can promote out crossing by facilitating dioecy, thereby providing a greater pool of genetic variation for breeding populations of gametophytes (Naf, Nakanishi and Endo, 1975). However, self-regulation of gender in gametophyte communities is also possible. Gender could be a function of developmental stage. If so, there should be no relationship between size hierarchy and gender. Expression of the male gender in small gametophytes should occur independently of the presence of female gametophytes in a community. Maleness in small gametophytes correlated positively with increases in size hierarchies and the presence of female gametophytes (Hamilton and Lloyd, 1991). Antheridia influence on reproductive biology within species, antheridia influence between species and its role in hybridization, ecological and evolutionary aspects of different groups of ferns.

## CONCLUSION

In the present study, gametophytes of *C. macrocarpa* were initially male and subsequently hermaphrodite under the different nutritions with different light level. The data obtained, their sex determination is protandry which showed the agreement with previous research

(Quintanilla *et al.*, 2005). Both conditions are favourable for developing of male prothalli but only good environment is favourable for female gametophyte growth which are thus in agreement with the ESD theoretical framework. It can be concluded that any realistic assessment of gender sequence in fern gametophytes must consider a broad range of culture conditions.

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