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Research Article

Linking *Azolla filiculoides* invasion to increased winter temperatures in the Doñana marshland (SW Spain)

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Abstract

Unravelling how a multiplicity of global change factors might influence the expansion of alien plants is a major goal of invasion biology. We explored the association of climatic conditions (i.e. annual rainfall and average minimum temperature during the coldest months) and water quality (orthophosphate, nitrate concentrations and electrical conductivity), with blooms of the invasive fern *Azolla filiculoides* Lam. in the Doñana marshland (SW Spain), one of the most extensive wetlands in Europe. *Azolla* was first detected in Doñana in 2001. Its annual cover has been quantified since then through remote sensing. In the last decade there has been a considerable increase of orthophosphate and yearly fluctuations of nitrate. The first orthophosphate increase coincided with the presence of *Azolla* in Doñana. Since then, there has been a positive relationship between inter-annual variation in *Azolla* cover and minimum temperature during the coldest months. Our study shows that the Doñana marshland is facing rapid eutrophication and invasion by *Azolla*, most notably in years with high temperatures during the winter.

Key words: Alien fern, global warming, eutrophication, invasion, macrophyte, phosphorus, remote sensing

Introduction

Eutrophication of wetlands and coastal ecosystems as a result of anthropogenic nutrient enrichment is a world-wide process affecting different levels of ecological complexity and ecosystem services (Caraco 1993; Verhoeven et al. 2006; Howarth et al. 2011). A major consequence of increased nutrient loading in water bodies is the displacement of functional groups responsible for primary production from submerged to floating macrophytes, some of which may be invasive (Morris et al. 2003; Meerhoff et al. 2007; Netten et al. 2010; Szabo et al. 2010). These shifts can affect sediment biogeochemistry and water quality, and consequently alter ecosystem services. Nevertheless, some studies have suggested that nutrients alone do not promote such changes and other environmental factors drive these transitions (Balls et al. 1989; Irvine et al. 1989; Feuchtmayr et al. 2009; Moran et al. 2010). For example, recent mesocosm studies suggest that in temperate ecosystems the combined effect of nutrient loading and climate warming might have a synergistic effect promoting the invasion of free floating species (Feuchtmayr et al. 2009; Moran et al. 2010; Netten et al. 2010). However, empirical information regarding how several global change factors influence invasions is scarce.

Azolla filiculoides Lam. (hereafter *Azolla*) is a mat-forming, free floating fern (Salviniaceae) native to the Americas (http://www.nobanis.org/files/factsheets/Azolla_filiculoides.pdf). *Azolla* has been reported as an invasive alien species in several European countries with its main occurrence in Atlantic-Mediterranean regions (Hussner 2012). Since 2012, it has been included in the EPPO Observation List (http://www.eppo.int/INVASIVE_PLANTS/ias_lists.htm#ObservList) as it can produce physical-chemical changes to the water environment and eliminate submerged plants and algae (Janes et al. 1996). *Azolla* has the ability to obtain atmospheric N due to its symbiotic association with the N-fixing blue-green alga *Anabaena azollae* Strasburger; thus, the major limiting factor for *Azolla* growth is not N but the availability of dissolved P in water (Chakrabortty and Kushari

1986). *Azolla* can grow in water with a wide range of P concentrations (from 0.01 to 20 mg P/l); however, its fitness increases strongly when the availability of P increases (Cary and Weerts 1992). Although *Azolla* can survive temperatures that are close to, and even below, freezing (0º C), biomass production (Janes 1998), N fixation (Vu et al. 1986) and relative growth rate (Van der Heide et al. 2006) are greatly reduced below 5ºC. Although several experimental studies conducted under controlled conditions have found that P addition and high temperatures increase *Azolla* biomass (Cheng et al. 2010), the interaction between P availability and temperature has not been investigated under field conditions over the long term. Moreover, *Azolla* can grow in brackish water with low levels of salinity (>8.4 g/l, Fernández-Zamudio 2011); however its tolerance to salinity in relation to other changes in water quality has not been explored in real field settings.

The primary aim of our study was to explore the association among the invasion of the free floating fern *Azolla*, water quality (i.e. orthophosphate, nitrate and electrical conductivity) and climatic conditions (i.e. temperature and rainfall) in the Doñana Protected Area (SW Spain), one of the largest wetlands in Europe. To this end, we analyzed an 11-year time series for *Azolla* abundance since its initial detection in the marshland, as well as parallel time series for nutrient content and climatic data.

Material and methods

Study site description

We conducted the study in the Doñana Protected Area marshland (Doñana marshland, hereafter). The Doñana marshland (360 Km^2) is a seasonal brackish water marsh located in Andalucía, at the lower part of the Guadalquivir River basin on the Atlantic coast of SW Spain (36°58'43.1"N, 6°21' 35.9"W). The Doñana marshland is a major waterfowl refuge in Europe and has been designated as a Special Protection Area for birds by the European Union, a Ramsar Site, a Biosphere Reserve, and a World Heritage Site (García and Marín 2006).

Currently, the Doñana marshland consists of shallow, temporal and brackish water bodies (20–80 cm depth) separated by geomorphological units that can be connected during high flood events and isolated in the dry season or during dry years. The marshland has a strong salinity gradient from North to South and from West to East (Bodelón et al. 1994), with a maximum value of 23.9 g/l. In this Mediterranean marshland, the level of isolation of the water bodies determines the flooding period, salinity, and turbidity. The climate is Mediterranean with Atlantic influence. Average annual precipitation (mean values ca. 550 mm.yr^{-1}) has a high inter-annual variation, and a marked seasonality that determines both a dry period (May–October) and a wet period (November–April).

Flooding of the marshland is mainly due to water discharge from northern streams and direct rainfall. All streams present high seasonal variation in water flow associated with annual rainfall variation, which produces flash flooding events during heavy rains. Main water losses occur by evapotranspiration and water discharges to the estuary through lock-gates. Most of the P in the sediment is dominated by the inorganic P fraction, reaching the highest content in the fraction bound to calcium carbonates ($CaCO_3$), followed by the fraction bound to Fe oxides (Reina et al. 2006). The Doñana marshland has been considered a nutrient poor water body (Serrano et al. 2006; Espinar and Serrano 2009) covered by dense meadows of submerged native macrophytes such as *Chara galioides* and *C. canescens* (Espinar et al. 2002). However, during 2001 the free floating alien fern *Azolla* was first detected (Espinar 2006).

Data sets

Azolla cover data set

Azolla annual cover was mapped in the Doñana marshland from 2001 to 2011 using medium resolution remote sensing imagery captured by different sensors onboard Landsat satellites (TM and ETM+ sensors IFOV = 30 m). Annual *Azolla* cover was measured during maximum coverage (April–May). Due to the extreme seasonality of the Doñana marshland, *Azolla* cover completely dries up in summer when the marshland dries, and then spreads out during early spring when the marshland is flooded. *Azolla* has a characteristic spectral signature across the optical spectrum with a steep red edge (680 to 730 nm) signature (Díaz-Delgado et al. 2010). These characteristics were critical factors in selecting the most representative image acquisition date (largest area covered by the fern during the year) and to enhance *Azolla* discrimination from the rest of the aquatic plants present in the study area. Since 2003 we have carried out several field sampling campaigns to determine the presence and abundance of *Azolla* with information gathered in the satellite images (ground truth). Field sampling consisted of linear transects registering presence and abundance of *Azolla* together with other aquatic plants and homogeneous *Azolla* patch delineation with differential GPS. Sampling always occurred in conjunction with Landsat TM or ETM+ overpasses (30 m pixel size).

Among the different available mapping methods, we selected the Spectral Angle Mapper algorithm (SAM, Yuhas et al. 1992), which is based on image spectral signatures and provides sub-pixel percent cover maps as output. SAM has been shown to be the best method for mapping dense patches of *Azolla* among standard per-pixel classification procedures (Díaz-Delgado et al. 2011).

To correct bias in the multi-temporal images, the time series of Landsat images was semi-automatically processed for metadata retrieval and co-registration of every scene into a single geometrically corrected ETM+ panchromatic scene (independent RMS test value < 15m). Every single scene was radiometrically corrected to surface reflectance image using a Lambertian simple dark object model (Pons and Solé-Sugrañes 1994). Finally, radiometric normalization was applied to the whole time series by means of pseudo-invariant areas and cloud cover masking, thus providing consistency to any temporal analysis (Bustamante et al. 2009).

The predictive ability of each model was assessed by the Area Under the Curve (AUC) of Receiver Operating Characteristic (ROC) plots (Pearce and Ferrier 2000) comparing predicted *Azolla* presence with field data. The AUC is a threshold-independent measure of discrimination ability (Zweig and Campbell 1993), and as a result, it is more appropriate than overall agreement or simple classification accuracy. AUC values for predicted yearly maps were greater than 0.6, except for 2006 (AUC=0.45). Therefore, data from this year was not considered for analysis. Data for 2005 was also excluded because a severe drought prevented flooding of the marshland (170 mm annual rainfall) and therefore *Azolla* failed to establish in Doñana that year. Floating *Azolla* fronds usually carpet open water bodies, but they may also appear sparsely in the shadow of dominant helophytes. The utilized method only detects *Azolla* present in open water. Thus, its actual marshland cover might have been underestimated by our method. Spring *Azolla* cover was estimated as the percentage of cover with respect to the total flooded area (see below).

Flooding area data set

Time series images of the Doñana marshland, captured by different sensors onboard Landsat satellites from 1975 to the present, provide relevant information on Doñana marshland flooding dynamics (Díaz-Delgado et al. 2011). Flooded areas were mapped using single threshold slicing of Landsat band TM5 (Short Wave Infrared, 1550 –1750 nm, a water-sensitive spectral region) once every 16 days (whenever images were cloud free) and every 7 days from 2003 onwards (both Landsat 7 ETM+ and Landsat 5 TM operating).

Water quality data set

We used nitrate concentration, reactive soluble phosphorus concentration (hereafter orthophosphate) and electrical conductivity as water quality indicators. Water quality data were collected and analysed using standard methods in five permanent areas of the marshland, namely Resolimán, Cancela Millán, Honduras del Burro, Vetalengua and lucio del Rey, every two months during the rainy season from 1996 to 2010. Due to annual variation in rainfall and the difficulties in reaching several remote sampling points, sampling data were not consistent across years. Data for 1999 and 2011 were not available.

Marshland sampling points included several water bodies spread across the whole Doñana marshland. Most of them are fixed sampling stations integrated into an extensive monitoring network for water quality coordinated by the Doñana Biological Reserve ICTS (http://icts-rbd.ebd.csic.es). Water samples were analyzed by C+E Laboratory® according to standard procedures (APHA 1985).

Climatic data set

In Andalucía, during the last 60 years, there has been a clear climatic warming tendency (Appendix I). With the aim of elucidating to what degree climate variables explain *Azolla* blooms, we used climatic data from El Palacio de Doñana, the closest weather station to the study area, for the years 1996 to 2011. Because *Azolla* growth is limited by low temperatures (Janes 1998), aside from annual rainfall, we selected average minimum air temperatures of the coldest months (temperature hereafter) as the predictor variable. In the last 60 years, Andalucía has experienced a significant increase in minimum temperatures (Appendix I).

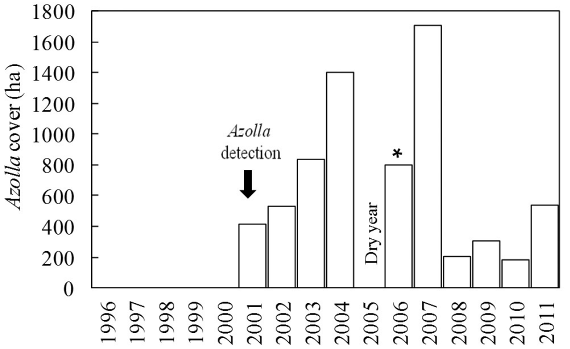

Figure 1. Annual variation in *Azolla* cover. Asterisk (*) in 2006 indicates AUC values for predicted yearly maps smaller than 0.6 (AUC=0.45).

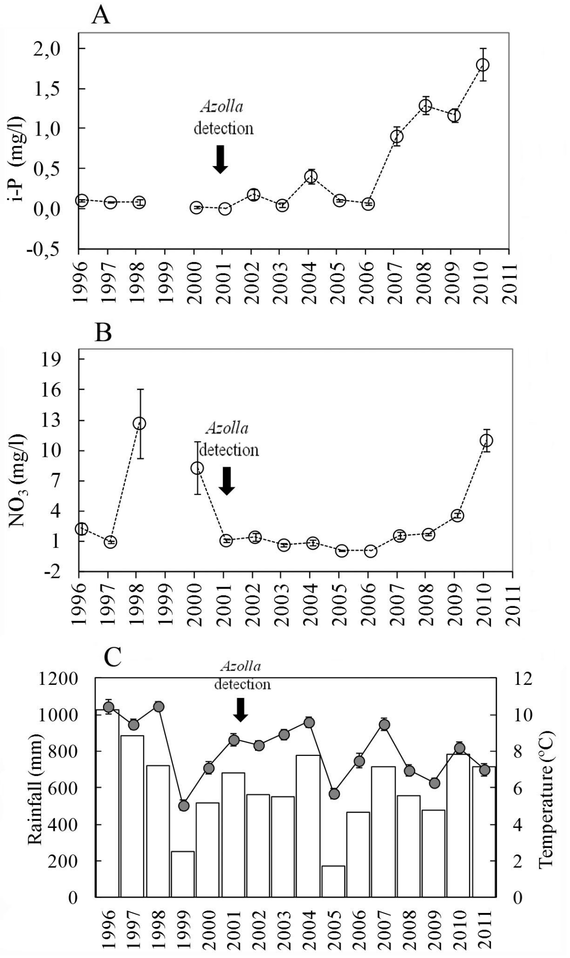

Figure 2. Orthophosphate and nitrate concentrations, annual rainfall and minimum temperature of the coldest months (October to March) in the Doñana marshland, from 1996 to 2011. Mean values (± SE) are indicated.

Data analysis

To adapt the data to the flooding seasonality in the Doñana marshland, throughout the paper, we assign "year" to the hydrological year and not to the annual year. For example, the year 2004 is the 12-month period from September 2003 to August 2004. Notice also that the time series for both water quality and climate are larger than for *Azolla* cover. By using water quality and climatic data from 1996 to 2011 we were able to explore the temporal context of these variables before *Azolla* was detected in Doñana (2001). A linear regression analysis was conducted to test whether there was a temporal trend in water quality and climate variables.

General Lineal Models (GLM) were then used to model annual variation in the percentage of flooded area covered by *Azolla* and the predictors mentioned above for the period 2001–2010 (STATISTICA version 6, StatSoft Inc., Tulsa, OK, USA). Akaike's information criterion corrected for small sample size (Burnham and Anderson 2002) were used to select a suitable model among a set of potential models that best explains *Azolla* blooms in the Doñana marshland.

Results

Nutrient concentration changes and climatic trends

Average annual orthophosphate concentration in the Doñana marshland increased significantly with time (r^2=0.603, p=0.001). It increased more than one order of magnitude (from 0.1 to > 2 mg/l) from 2006 to 2010. *Azolla* spread coincided with a smooth early increase in orthophosphate concentration. During 2004 there was a peak in orthophosphate concentration, followed by a period with low orthophosphate coinciding with a very dry period (170 mm in 2005) and a subsequent strong increase from 2007 to 2010 (Figure 2a).

Although nitrate concentration did not increase significantly during the time series (p=0.94), there was a tendency of increased nitrate from 2007 to 2010 (Figure 2b).

From 2006 to 2011 there was no significant trend in annual rainfall or temperature (p=0.94, p=0.18, respectively). However, there was high variation particularly in annual rainfall, with the wettest period (1027 mm) in 1996 and the driest period (170 mm) in 2005 (Figure 2c). Within the Andalucía warming context of the last 60 years (Appendix I), temperatures during the coldest months were warm, particularly in 2004 (mean±SE,

Table 1. *Azolla* cover model in the Doñana marshland using Akaike´s Information Criterion corrected for small sample size (AICc). ΔAICc indicates differences in AICc related to minimum AICc and K indicates number of parameters. Values of model fit are also indicated.

Model	L.Ratio χ^2	*P*	*K*	ΔAICc
Temperature	5.91	0.014	1	0.000
Rainfall, Temperature, Orthophosphate	13.4	0.004	3	1.84
Rainfall, Temperature	7.3	0.025	2	2.34
Nitrate, Temperature	7.04	0.029	2	2.6
Nitrate	3.18	0.07	1	2.73
Temperature, Orthophosphate	6.17	0.045	2	3.47
Temperature, Electrical conductivity	6.11	0.047	3	3.53
Temperature, Orthophosphate, Nitrate	11.06	0.011	3	4.18
Rainfall, Nitrate	4.88	0.086	2	4.76
Nitrate, Electrical conductivity	4.55	0.1	2	5.09
Nitrate, Orthophosphate	4.31	0.11	3	5.33
Temperature, Rainfall, Orthophosphate, Nitrate	17.3	0.001	4	7.27

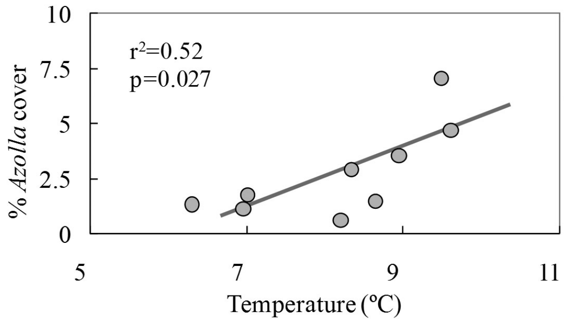

Figure 3. Bivariate relationships between annual *Azolla* cover measured as a percentage of flooded area and average minimum temperature. Values for 2005 were excluded because it was a dry year and for 2006 because AUC values for predicted *Azolla* cover was smaller than 0.6 (see results).

9.61±0.26 ºC) and 2007 (9.5±0.32 ºC). Temperatures for these two years were higher than the average for the period 2001–2011 (8.12±0.01 ºC). In addition, for these two years, there was a low occurrence of temperatures below 5 ºC (only 14 days in 2004 and 24 days in 2007), as well as the number of freezing days (only 2 in 2004 and 3 in 2007) (data not shown). Before the detection of *Azolla* in the study area, a warm period was also detected during 1996–1998; at that time, orthophosphate concentration in the marshland was low.

Relationship between Azolla *blooms and environmental data*

Since *Azolla* was first recorded in the Donaña marshland in 2001, the surface area covered by this species has increased, reaching a maximum in 2004 (Figure 1). During that year, *Azolla* was present in almost all flooded microhabitats in the Doñana marshland, i.e. open water, emergent helophyte perennial formations of woody Chenopodiaceae (J. L. Espinar, personal observation). A second major bloom occurred during 2007, a year with high minimum temperatures.

Based on corrected Akaike's model selection criterion, temperature was the most suitable model that best fit the data (ΔAICc = 0, k = 1, χ^2=5.918, *P*=0.014). A set of models with considerably lower support (i.e. ΔAICc between 2 and 4, Burnham and Anderson 2002) included the combined effect of orthophosphate concentration, temperature and rainfall (ΔAIC=1.84), temperature and rainfall (ΔAIC=2.34) and temperature and nitrate concentration (ΔAIC=2.6) (Table 1).

A bivariate positive relationship between *Azolla* cover and temperature was observed (r^2=0.52, p=0.027, Figure 3).

Discussion

We found an exponential increase in orthophosphate and nitrate concentrations in the Doñana marshland during the last 5 years. There is a strong positive correlation between orthophosphate and nitrate concentrations in the marshland and tributary streams indicating that eutrophication most likely occurs via external inputs, particularly in rainy years (Espinar et al., unpublished data). Some of these streams receive run-off water from agricultural areas irrigated by groundwater wells or small dams, waste-water effluent from urban areas, and sewage effluents from waste-water stations (Serrano et al. 2006).

The availability of P in water has been identified as a major limiting factor for *Azolla* growth (Chakrabortty and Kushari 1986; Kitoh et al. 1993).

However, we did not find a significant relationship between its cover and orthophosphate concentration in the water despite the fact that the establishment of *Azolla* runs parallel with marshland eutrophication in Doñana. This lack of a significant relationship might change in advanced stages of the invasion process, as has been observed in other free-floating plants in which abundance is related to a decrease in water quality. *Azolla* cover was not related to nitrate concentration.

Another major environmental change in the region has been an increase in average minimum temperatures (Appendix I). We found a significant positive relationship between *Azolla* annual cover and air temperature in winter. Our results are consistent with the hypothesis that temperature might be an important factor determining the fitness of floating macrophytes (Janes 1998; van der Heide et al. 2006; Netten et al. 2010; Szabo et al. 2010; Peeters et al. 2013). Warm temperatures during the winter might open “windows of opportunity” that promote the fast growth of *Azolla* mats before the spring establishment of submerged macrophytes. A positive increase in the fitness of floating species (*Azolla* among others) in response to local warming has also been described in temperate areas, such as in a thermal stream in Slovenia (Sajna et al. 2007), and in a portion of the River Erft (Germany) which has been abnormally warmed as a consequence of opencast mining water discharges (Hussner and Lösch 2005).

Furthermore, the early expansion of *Azolla* mats during late winter and early spring might enhance its own growth by warming up the top layer of water through the absorption of irradiance, and reducing water mixing through a reduction in wind action (Room and Kerr 1983; Netten et al. 2010). This process might be important in wind protected areas or in open waters where *Azolla* mats can be significantly large. Therefore, there might be a positive feedback between warming and *Azolla* growth.

In Doñana there has not been any attempt to manage and control *Azolla*. The *Azolla* feeding weevil, *Stenopelmus rufinasus*, has not been found in Doñana but has been observed in the vicinity (Dana and Vivas 2006). This weevil is a well-known biological control of *Azolla* (http://www.nobanis.org/files/factsheets/Azolla_filiculoides.pdf) that might be playing a significant role in the dynamics of the *Azolla* invasion. Future monitoring surveys in Doñana should quantify both the abundance and distribution of *Azolla* and that of *Stenopelmus rufinasus*.

Several studies have pointed out that a major consequence of increasing nutrient loading in water bodies is the displacement of functional groups responsible for primary production from submerged to floating macrophytes, some of which may be invasive and outcompete floating macrophytes (Morris et al. 2003; Meerhoff et al. 2007; Netten et al. 2010; Szabo et al. 2010). This shift from floating to submerged macrophytes has been proposed as alternative stable states defined as the result of asymmetric competition mediated by nutrient and light availability (Scheffer et al. 2003). In water ecosystems these shifts can affect species assemblages, sediment biogeochemistry and water quality.

Therefore, one major consequence of dense blooms of *Azolla* is the decrease of submerged macrophyte cover (Janes et al. 1996). Although we did not study its effect on submerged macrophytes, threshold irradiance for maintaining autotrophic communities dominated by submerged macrophytes have been identified in Doñana (Geertz-Hansen et al. 2011). In the Doñana marshland, species diversity and primary productivity have historically been dominated by submerged species, while free floating macrophytes have had a minor role (Duarte et al. 1990; Espinar et al. 2002; Espinar 2006). We only estimated the cover of *Azolla* in open water bodies, and therefore, we underestimated its abundance in the marshland. In Doñana, the reduction of submerged species as a result of its replacement by *Azolla* might represent a potential threat as well as promote a regime shift. Changes in macrophyte communities could have important consequences for wetland geochemistry given that submerged macrophytes play a central functional role in controlling nutrient budgets in shallow water bodies.

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Appendix 1. Regional temperature changes.

To have an overview of regional temperature patterns, we analysed 60 year climatic data (1952–2010) of four climatic stations in Andalucía located less than 45 km from the study area in Doñana. We chose two coastal stations, Huelva (37º16'48"N, 06º54'35"W and 19 m a.s.l) and Cádiz (36º30'04"N, 06º15'24"W and 1 m a.s.l.) and two inland stations, Sevilla (37º25'00"N, 05º52'45"W, and 34 m a.s.l.) and Jerez (36º45'02"N, 06º00'21"W and 27 m a.s.l.). Data was provided by the Spanish Meteorological Service (AEMET). The selected variable was average daily minimum temperature of the coldest month. Generalized Lineal Models were used to detect trends with "year" as the continuous predictor and climatic "station" as the categorical predictor.

We found that in the last 60 years there has been a significant increase in minimum temperatures (F-value = 27, p<0.001). There has also been significant differences between stations (F-value = 45.31, p<0.001) due to the influence of the Atlantic Ocean in smoothing temperatures in the coastal stations (Cádiz and Huelva) in comparison with inland stations (Sevilla and Jerez) (Figure S1).

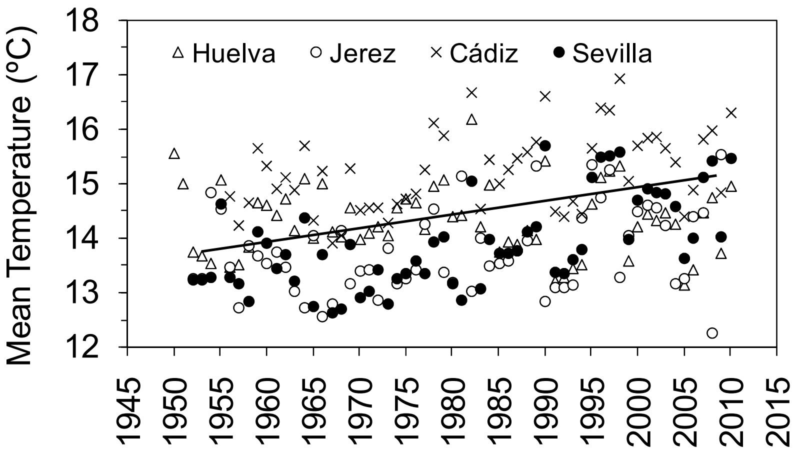

Figure S1. Time series data of mean minimum temperature during the cold months (October-March) in four stations of Andalucía located less than 45 km from the study area in Doñana.