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From canopy to single flowers: a downscale approach to flowering of the invasive species *Acacia longifolia*

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Summary

Flowering of the invasive species *Acacia longifolia* was assessed in three locations of Portugal with variable environmental conditions to characterize its reproductive strategy. From North to South of Portugal, and from a within-plant perspective, we observed similar flowering performances in plants along the coast. Flowers and pollen are short-lived, possibly counterbalancing the massive quantities produced every flowering season.

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Running title: Downscale approach to flowering of *Acacia longifolia*

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Abstract

Context: *Acacia longifolia* is a native legume of Southeast Australia and Tasmania and is invasive in many parts of the world. A key feature to its success is the production of a high quantity of flowers every season, resulting in a massive seed bank that remains dormant in the soil for decades. Many studies have been performed on this species' reproductive biology, but none have focused on flowering in detail.

Aims: Our main objective was to understand this species' resource allocation strategy that ensures its successful reproduction in the invasive range.

Methods: We developed an integrative approach, assessing flowering at different levels: canopy and branch flowering (macro scale), downscaling to individual flower functional stages and their duration, pollen longevity and stigma receptivity (micro scale). We performed this study in three different locations in sand dunes along the Portuguese coast with different environmental conditions.

Key results: Canopy flowering shows no difference among sites. Pollen and stigma assessment revealed that this species is protogynous, with the stigma being highly receptive long before pollen is released. Once released, pollen lasts roughly 72h. Individual flowers are relatively short-lived, with a rapid progression from closed flower buds to fully open flowers.

Implications: Our results indicate that *A. longifolia* has a resource trade-off strategy of investing in flowers and pollen that are relatively short-lived, which are counterbalanced by their massive quantities.

Keywords: *Acacia longifolia*, invasive species, flowering trade-offs, environmental conditions, pollen longevity, stigma receptivity.

Introduction

Australian acacias (genus *Acacia*, formerly classified as *Acacia* subgenus *Phyllodineae*) are considered one of the most invasive plants at a global scale, displaying many characteristics of a model invasive species (Richardson *et al.* 2011). *Acacia longifolia* (Andrews) Willd. is a leguminous species native of Southeast

mainland Australia and Tasmania and is particularly invasive in areas with a Mediterranean climate, such as the Iberian Peninsula, South Africa, southern Brazil, Uruguay, and Western Australia (see, for example, Richardson *et al.* 2011; Harris *et al.* 2012). In Portugal, this species was introduced for dune fixation and as an ornamental plant in the late 19th/early 20th century and has since then invaded most of the coastal regions (Marchante 2011). Previous studies have shown that the invasive capacity of *A. longifolia* is associated with its ability for symbioses with nitrogen-fixing rhizobia (e.g., Rodríguez-Echeverría *et al.* 2009) enabling its spread in sandy dunes with very nutrient-poor soils (Ulm *et al.* 2017a), impacting these ecosystems from an early stage of invasion (Hellmann *et al.* 2011; Rascher *et al.* 2012; Ulm *et al.* 2017a). *Acacia longifolia* promotes its own success and that of its offspring by increasing soil nutrients (Le Maitre *et al.* 2011; Badalamenti *et al.* 2014; Ulm *et al.* 2017a, 2017b) and could control the available sunlight to other plants, eventually outcompeting them and occupying their space (Badalamenti *et al.* 2014). This species is capable of quick regeneration after forest fires since high temperatures promote seed germination (Marchante *et al.* 2003; Gibson *et al.* 2011). Moreover, forest fires and human disturbances (e.g., vegetation removal and sometimes even management interventions) were shown to promote this species' invasion (Carvalho *et al.* 2010; César de Sá *et al.* 2017). A key feature for *A. longifolia's* invasive success is its massive seedbank, which is developed in a very short time (Marchante *et al.* 2010). This may be due to the large number of flowers and seeds it produces every flowering season (Gibson *et al.* 2011; Giovanetti *et al.* 2018).

Flowering is a key event in the life cycle of a plant, and it needs to occur when environmental conditions are optimal for flower development and pollen dispersal in order to ensure successful reproduction (Rathcke and Lacey 1985). Abiotic factors such

as rainfall, temperature and wind have a strong influence on flowering time, flower longevity and pollen performance. It is well described that rain delays the opening of flowers (Stone *et al.* 2003) and may hinder pollen dispersal. Furthermore, wind and temperature affect the activity of pollinators and, therefore, are also determinants of pollen dispersal. There have been several studies of the phenology of *A. longifolia* in Portugal. Fernandes *et al.* (2015) examined phenology in both the North (mesic habitat – lower temperature, higher humidity) and South (xeric habitat – higher temperature, lower humidity) of Portugal and found that phenological events, in particular the reproductive phenophases occurred earlier at the South site, where plants had a shorter flowering period. Also, flowering was advanced two weeks at the South site, and flowering peak occurred earlier when compared to the North site. The authors found that these differences were mainly due to higher temperatures in the South site, and that temperature is the main driver of the flowering peak date. Morais and Freitas (2015) examined phenology in four populations of acacias in Portugal: North *vs.* South and Inland *vs.* Coastal populations. The authors corroborated the findings of Fernandes *et al.* (2015) with respect to latitude, and showed that coastal populations flowered earlier when compared to inland populations. Therefore, *A. longifolia*'s phenological events, in particular flowering, depend strongly on environmental conditions in invaded ranges. *Acacia longifolia* was initially considered an entomophilous species (Stone *et al.* 2003), depending on insects for pollination, and indeed it exhibits scented bright yellow flowers and produces extrafloral nectar, both very attractive to insects (Giovanetti *et al.* 2018). However, this species flowering occurs in winter when insect abundance is low, which results in pollinator scarcity (Godoy *et al.* 2009; Gibson *et al.* 2011). The many small flowers, and the large amount of pollen released, are characteristics often displayed by anemophilous species (i.e., pollinated by wind; Ackerman 2000). Recent

studies have shown that *A. longifolia* is an ambophilous species, pollinated by both insects and wind (Giovanetti *et al.* 2018).

Flower longevity is an important trait for reproduction as it determines for how long flowers are functional and receptive to pollination, and it is influenced by both abiotic factors and the abundance of pollinators (Trunschke and Stöcklin 2017). There is a balance between the number of flowers exhibited per plant and the resource investement of keeping them open (Ashman and Schoen 1994; Spigler and Woodard 2019). Longer flower longevity means longer exposure to pollinators and pollen dispersal, but it also means higher resourse costs (Primack 1985; Ashman and Schoen 1996; Harder and Johnson 2005). Therefore, flower longevity determines the size and duration of flower display (i.e. number of flowers open at any given time; Primack 1985; Ashman and Schoen 1996), and the determination of reproductive assurance (Rathcke 2003). A previous study of the reproductive biology of several invasive acacias in Portugal found that *A. longifolia* invests in high numbers of inflorescences with mostly hermaphrodite flowers, resulting in high seed crop (Correia *et al.* 2014). Self-pollination is reduced due to protogyny (the female reproductive organs mature before the male ones), a feature shared by most Australian acacias (Stone *et al.* 2003, Gibson *et al.* 2011). *Acacia longifolia* in an invaded range (Portugal) maintains the same level of self-incompatibility as in the native range, despite exhibiting more and larger seeds (Correia *et al.* 2015).

Pollen longevity is also an important factor to consider in combination with stigma receptivity. Stigma receptivity is a determinant of effective pollen-pistil interactions, making its timing and duration important for successful reproduction. There is usually a negative correlation between pollen size and number (Aguilar et al. 2002) due to plants' resource division and allocation (Vonhof and Harder 1995; Gillet

and Gregorius 2020). Pollen longevity is usually balanced with pollinator availability, with higher pollen longevity when pollinators are scarse (Dafni and Firmage 2000). Conversely, pollen longevity tends to be shorter when the level of self-incompatibility of the plant is higher, thus lowering self-pollination events (Dafni and Firmage 2000). However, the longevity of *A. longifolia* pollen is still unknown.

The mentioned reproductive characteristics of *A. longifolia* are generally found in successful invasive plants (e.g., see Pyšek and Richardson 2008), and in other species of acacias regardless of their invasive status (Gibson *et al.* 2011). However, varying strategies of resource investment in the reproductive units have been identified for different acacias in the invaded ranges (Correia *et al.* 2014; Giuliani *et al.* 2016). For example, *A. dealbata* was shown to invest heavily in high inflorescence density, while *A. melanoxylon*, *A. saligna*, *A. longifolia* (Correia *et al.* 2014) and *A. pycnantha* (Giuliani *et al.* 2016) invest in higher number of flowers per inflorescence, but lower inflorescence density. Thus, a characterization of the specific reproductive strategy of *A. longifolia* is of great interest to better understand its invasiveness.

All the above-mentioned studies address *A. longifolia* reproduction from different perspectives, however detailed studies of individual flowers are still lacking. Considering this species' invasive success, in this study, we examined differences in flower development in three locations in Portugal, under different environmental conditions. At these locations, we addressed flowering of *A. longifolia* in a within-plant perspective, focusing on individual flowers, to understand this species' resource allocation strategy for reproduction. Taking into consideration the studies mentioned above, we hypothesize that shot-lived individual flowers and pollen counter the massive production of flowers observed at canopy level.

Materials and Methods

Study sites

This study took place in the flowering season of 2019 in Portugal (January to March). According to different edaphoclimatic conditions, three locations were selected: one in the North, Figueira da Foz (FF; latitude 40.0646, longitude -8.8642); and two in the South, Pinheiro da Cruz (PC; latitude 38.2561, longitude -8.7730) and Vila Nova de Milfontes (VNM; latitude 37.6880, longitude -8.7927) (Supplementary Material, Figure S1). Figueira da Foz has a meso-Mediterranean climate with an average yearly temperature of 12-15 °C and average yearly precipitation of 700-900 mm, while Pinheiro da Cruz and Vila Nova de Milfontes have a thermo-Mediterranean climate with an average yearly temperature of 15-18 °C and average yearly precipitation of 400-600 mm (data from Portal do Clima, https://portaldoclima.pt/en/). In all sites, acacias are scattered, with the distance to the ocean being 1900 meters in FF, 250 meters in PC, and 150 meters in VNM. Molecular (see Vicente *et al.* 2018) and phenology studies (see Fernandes *et al.* 2015) have been previously done in these locations, except for FF which is a substitute for a site from Fernandes *et al*. (2015) and Vicente *et al.* (2018) where plants burned in a forest fire in 2017.

Canopy flowering assessment

In each location, 10 adult plants were marked separated by at least 5 meters to avoid sampling closely related individuals. Periodic assessments were made every 15-18 days for a total of 4 assessments in each site, except for PC where the time between

the first and the second assessment was 24 days. Assessments in VNM and FF (the Southern and the Northern sites) were separated by approximately one week, as it is known that flowering occurs earlier in the South when compared to the North of Portugal (according to Fernandes *et al.* 2015), while assessments in PC were made in between those in VNM and FF. At every assessment, the percentage of closed, open, and senescent inflorescences was classified as 0-25%, 25-50%, 50-75% or 75-100% by direct observation of the canopy as a whole, since the flowers strikingly change colors as they progress throughout the flowering period and are easily identified: closed flowers are mostly green, while open flowers are bright yellow and senescent flowers are orange/brown. These data were then transformed into abundance levels from 1 to 4 as follows: level 1, when more than 8 plants were in the 0-25% class; level 2, when 2 or more plants were in the 25-50% class; level 3, when 2 or more plants were in the 50-75% class; and level 4, when 2 or more plants were in the 75-100% class. Two branches were also marked in each plant, one facing east and another facing west. At every assessment, the total number of inflorescences and of inflorescences with open flowers were counted also by direct observation.

Single flower characterization

The determination of flowering stages was performed on branches with flowers that were brought to the laboratory and observed under a dissecting microscope. Preliminary observations showed that flowers open in a non-synchronized way within an inflorescence, without any order (acropetal, from base to tip of the inflorescence, or basipetal), in accordance with Stone *et al.* (2003) for native range acacias formerly classified in the subgenus *Phyllodineae.* Taking this into account, instead of

characterizing inflorescence development as previously described for other acacias (Stone *et al.* 2003 and Gilpin *et al.* 2014), we characterized single flower development. Five flowering stages from the closed to the open flower were defined and described according to the position of petals, stamen, and style (Table 1).

The first stage (S0) is the fully closed flower bud. The stage S1 is the opening of the flower bud (with the petals still curved) followed by stage S2, where only the style is emerging from the flower bud with petals in a cup-like formation, and stage S3, where both style and stamen have emerged from the bud, but petals are in a vertical position. The last stage – S4 – is the fully open flower with horizontal petals, and both stamen and style are in a vertical position. This stage is when anther dehiscence occurs, and pollen is released. The three functional stages S1-S3 usually occur in rapid succession. Once flowers are fully open and pollen is released anthesis is complete, and flowers last only a few days until reaching senescence. On the other hand, since all flower buds are already formed, closed flowers can last from days to months depending on whether that bud opened at the beginning, middle or end of the flowering period.

An estimation of the duration of the functional flowering stages S1, S2 and S3 was performed by marking single inflorescences in the field and monitoring them for 8h in each location, at their respective flowering peaks (determined by the canopy flowering assessment). Five inflorescences from 5 individuals (n = 25 inflorescences) were marked in the morning and photographed at 9:00h, then photographed again at 13:00h (4h after the beginning of the assay) and at 17:00h (8h after the beginning of the assay), taking care to keep approximately the same angle for all photo records. The VNM location was excluded from this analysis as flowering progressed quickly at this site, and unfortunately it was not possible to take good quality photo records. The pictures were then examined in a computer where single, individual flowers were

marked (n = 100 flowers for each flowering time), and flowering stages were followed through the different photographic timings. Flowers were selected only if they were clearly visible in all photo records and if their flowering stage was easily identifiable at each photographic timing, regardless of their initial flowering stage. For each individual flower, the flowering stage at each time period was registered (see Supplementary Material, Figure S2, for an example), originating a heat map of the changing flowering stages through time. Through the heat map, for each flower, the duration of each stage was classified as less than 4h, 4h to 8h or equal or greater than 8h.

Pollen longevity assays

Pollen longevity studies were performed by assessing germinability in the appropriate medium through time. Assays were performed in all locations at their respective flowering peak. Firstly, three media were tested for this assay: *Arabidopsis thaliana* (At) pollen germination medium (Boavida and McCormick 2007), *Nicotiana tabacum* (Nt) pollen germination medium (Read *et al.* 1993), and Brewbaker and Kwack (BK) medium (Brewbaker and Kwack 1963), all with 5% sucrose. Pollen was collected and immediately placed in 20-25 μL of each medium on a microscope slide or in a microtube in a dark, humid chamber at room temperature. After 2h, the polyads were observed under the microscope. The Nt medium proved to be the most efficient and was thus selected for the assays, described below.

As a routine assay, pollen was collected from opened anthers into a microtube in the field in the morning. Five individuals were considered and pooled in a total of 6 samples (replicates) per site (i.e., 5 individuals per each of the 6 samples), and pollen was germinated in Nt medium at 0h, 6h, 24h, 48h and 72h after collection. At each time

point, pollen was placed in 20-25 μL of medium on a microscope slide in a dark, humid chamber at room temperature. Counts of germinated and non-germinated polyads were performed after 2h under a microscope. A polyad was considered germinated when at least one pollen tube was visible, and its length was approximately equal to the polyad's diameter or longer. To obtain the germinability at timepoint 0h as accurately as possible, germination was performed directly on the field by suspension of pollen in 20-25 μL of germination medium in an microtube with no cap, and then transported to the laboratory in a dark, humid chamber at room temperature. Once there, the media with the pollen was transferred to a microscope slide for counts as described above.

Assay with closed anthers were also performed only once with flowers from VNM to check the maturation of pollen throughout the flowering stages. One flower per each flowering stage S1-S3 was collected from 6 trees in VNM. Anthers were forced to release the pollen by squashing them on a microscope slide containing 20-25 μL of Nt medium and germination was checked as previously described.

Stigma receptivity assays

The stigma receptivity assay was performed at each location in its respective flowering peak, based on the hydrogen peroxide test as previously reported (e.g., Souza *et al.* 2016; Thimmaiah *et al.* 2018). Thirty flowers per site (from 3-5 inflorescences of 3 different trees) in each stage S2, S3 and S4 were detached from the inflorescences under a dissecting microscope and submerged in 3% hydrogen peroxide. The presence of air bubbles visible under the microscope confirms catalase activity, an enzyme present in receptive stigmas that degrades hydrogen peroxide into water and oxygen.

Statistical analysis

For the branch flowering assessment, the mean ratio of the number of inflorescences with open flowers and the total of number of flowers was computed for each of the marked branches at every location, and differences between East- and West-facing branches were tested by applying the Wilcoxon Signed Rank test. Since no pattern was found, we considered that branch orientation had no influence on flowering, and branches were pooled. Differences in the ratio among locations were tested using the Kruskal-Wallis test at each location's flowering peak date, as the data was not normally distributed (Shapiro-Wilk test $p < 0.05$). All the above-mentioned tests were performed using IBM SPSS Statistics v26.

In the single flower characterization assays, as the data is categorical and matched, Cochran-Mantel-Haenszel Test for 3-dimentional tables and a post-hoc Fisher exact test were performed in R (R Core Team 2016) using R/psych, R/vcd and R/rcompanion packages according to Mangiafico (2016) to check for significant differences in the duration of the stages at the flowering peak among locations and to check for significant differences in duration among stages at each location, using the duration data extracted from the heat maps.

Results

Canopy flowering

All locations presented an abundance of closed inflorescences at the end of January and of senescent inflorescences in mid-March, while the peak of open

inflorescences appears in mid-February. The maximum abundance of open flowers (peak of flowering) was observed on the 9th of February in VNM, on the 16th of February in FF and on the 20th of February in PC (Figure 1). It should be noted that the observation of the flowering peak in PC corresponded to a 24-day interval from the previous observation, instead of the 15/18-day interval that occurred for the other locations. This is the reason why the reported flowering peak occurs later in PC than in FF (the northern site). In the period this study was conducted, the high abundance of closed inflorescences was visibly shorter on the dunes in FF in the north as well as at PC in the south, when compared to VNM (Figure 1). The peak of flowering was fairly synchronized at all locations. In PC, open flower abundance was the shortest and, conversely, abundance of senescent flowers was the longest. **Figure 2: Ratio of the number of inflorescences with open flowers and the number of total inflorescences in branches during the flowering peak.** The Kruskal-Wallis test was applied with pairwise comparisons. VNM – Vila Nova de Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz. The peak of flowering was observed on the 9th of February in VNM (both sites), on the 16th of February in FF and on the 20th of February in PCWe also checked for differences in the ratio of the number of open over the number of total inflorescences among locations on their corresponding flowering peaks (Figure 2) and found no differences among locations (Kruskal-Wallis test chi-square = 0.163, p = 0.922, df = 2). Proximity to the ocean had no influence on canopy flowering.

Single flower development

The duration of the functional stages S1, S2 and S3 (Table 1) was estimated in each location at its corresponding flowering peak (Figure 3A and B). The duration

frequency plots obtained from the heat maps show that flowering stages S1-S3 have significantly different durations (Cochran-Mantel-Haenszel Test chi-square = 60.0720, df = 4, $p < 0.05$, and post-hoc Fisher exact test $p < 0.05$ for all stages), with stage S2 being the shortest and stage S3 being the longest. However, the duration of each stage is similar among locations (Cochran-Mantel-Haenszel Test chi-square = 3.5402, df = 2, $p > 0.05$). Even if not significant, the heat maps highlight some trends in the different locations (Figure 3). FF was the site with the shortest overall flowering, quickly moving to the senescent phase, while stage S3 lasted longer in PC, thus extending flowering.

Pollen longevity and stigma receptivity

Pollen longevity was determined by germination of polyads collected from closed anthers from stages S1, S2 and S3 in VNM, and from anthers from open flowers (S4) in all locations at their corresponding flowering peak (Figure 4). Since pollen longevity was similar across locations (see Supplementary Material, Figure S3), the mean percentage of germinated polyads was considered. Despite high variability, there was an increase of polyad germination from stage S1 to the stage S3. Once the flower is fully open (S4), pollen is released, and germination is at its maximum. After release, polyads last for roughly 72h, steadily losing germinability through time.

Stigma receptivity was assessed in all locations from stage S2 to S4, which is when the style has emerged from the flower bud. However, receptivity was similar at all locations (data not shown), therefore the mean receptivity was considered and shown in Figure 4. The stigma is receptive for several hours before the pollen is released (stage S4) and receptivity is very high (above 90% receptive stigmas) in all evaluated stages.

Discussion

Acacia longifolia is an invasive species still lacking understanding of its flowering developmental stages. Elucidating flower development will highlight resource allocation strategies and trade-offs in this species reproduction ability, with a view to the context of its invasive behavior. We showed that while the flowering period of *A. longifolia* is long, individual flowers have short functional periods lasting only a few hours. Furthermore, we also showed that *A. longifolia* pollen is short-lived, with germinability lasting about 72h, and stigmas are highly receptive before pollen release and maintain their receptivity throughout the flower functional period. These results support our original hypothesis that the huge quantities of flower and pollen produced are counterbalanced by their short longevity. However, our study took place during a single flowering season, bringing some limitations to our results. It will be necessary to verify how changes in plant phenology and reproductive ability in different years influence current observations. It will also be very interesting to replicate this study in the native range of the species to elucidate fixed and flexible flowering traits.

In our downscale approach, we checked for differences in flowering at various levels, starting from the (macro) canopy-inflorescences level through a visual assessment (Figure 1). Our results show that, at any given time of the flowering period, closed, open and senescent inflorescences are present simultaneously, meaning that flowers do not open all at the same time, making this species' flowering period long-lasting. In general, our findings at canopy level are in accordance with Fernandes *et al.* (2015) and Morais and Freitas (2015). At all locations there was a peak of flowering around mid-February quickly followed by flower senescence. The constant presence of open flowers enhances their availability to pollinators and wind (Giovanetti *et al*. 2018),

375 increasing the chance of cross-pollination and reproductive success, as previously
376 shown by Fernandes *et al.* (2015).

377 From the macro-level of the canopy, we downscaled our attention to flowers.
378 Flower longevity is an adaptive trait that can be altered by biotic (e.g., pollinators) and
379 abiotic (e.g., temperature, rainfall) factors to optimize reproductive success (Trunschke
380 and Stöcklin 2017), similarly to the timing and duration of the flowering period
381 (Fernandes *et al.* 2015; Morais and Freitas 2015). Traits related to fertility, such as
382 flower longevity and flowering duration, are crucial for invasive species, as they need to
383 quickly colonize new environments. For example, Pyšek *et al.* (2003) studied several
384 alien species from the Czech Republic and suggested that invasive species tend to have
385 longer blooming phases compared to native species, possibly to outcompete the native
386 species for pollinators. Indeed, *A. longifolia* has a long-lasting flowering period of
387 approximately 4-5 months. However, within this species, flowering duration does not
388 seem to change from the native to the invaded ranges, where it usually lasts 4 months
389 (Milton and Moll 1982; Fernandes *et al.* 2015; Morais and Freitas 2015). This is in
390 accordance with Noble's (1989) hypothesis, who stated that the reproductive characters,
391 such as phenology, should remain unchanged between similar native and invasive
392 ranges, while rates of establishment and reproductive losses may differ (see also Roy
393 1990). Indeed, Weiss and Milton (1984) found decreased reproductive losses in South
394 African (invasive) populations of *A. longifolia* when compared to their Australian
395 counterparts. Similarly, Correia *et al.* (2015) found a significantly higher number of
396 seeds per pod and a significantly lower percentage of aborted seeds per pod in
397 Portuguese *A. longifolia* populations when compared to Australian ones. Moreover, in
398 the invaded range, seed and seedling sizes are larger than their native counterparts,
399 showing a positive correlation between seed size and offspring growth (Correia *et al.*

2015, 2016) that can be expected to turn into higher rates of establishment. Thus, despite similar flowering duration among native and invasive ranges, fertility in the invaded range was proved to be higher.

Maybe the key to understand this difference relies in individual flowers. Our observations underlined that the long-lasting flowering period is achieved by a strategic investment in flowers. The flowers of *A. longifolia* are functional only for a few hours (flowering stages S1-S3), but their short life is counterbalanced by a massive flower production and by a non-synchronized way of opening (Gibson *et al.* 2011; Correia *et al.* 2014; Giovanetti *et al.* 2018). The resource allocation trade-off between quantity, longevity and blooming of flowers result in higher fertility even in the face of expected disrupted pollination due to winter flowering and the low activity of insect pollinators, in contrast with the expectations of Noble (1989).

The intra-flower level reinforces the above. We assessed pollen longevity and stigma receptivity (Figure 4 and Supplementary Material, Figure S3). Regarding pollen germinability, there was an increase in the germination rate from stage S1 to stage S3, as pollen was maturing and preparing for release until the flower reached S3. Once pollen is released, it retains germinability for roughly 72 hours. Stigmas, on the other hand, are receptive from stage S2 through the fully open flower, and no differences were found among locations, similarly to pollen longevity. Protogyny is confirmed for this species, in accordance with Stone *et al.* (2003). Our results sustain findings of Sedgley and Harbard (1993) and Kenrick (2003) on short-lived pollen in this species. Pollen germinability was similar among all locations, showing no effect of environmental variability on its longevity. Gibson *et al.* (2011) and Correia *et al.* (2014) inferred that *A. longifolia* guarantees pollination by investing in the production of mass amounts of pollen. This occurs also in other species, as depicted by Dafni and Firmage

(2000). Mass-production of pollen sustains pollination in several ways, by counterbalancing pollinator scarcity in winter-flowering species (Godoy *et al.* 2009; Gibson *et al.* 2011; Giovanetti *et al.* 2018) and self-incompatibility (Correia *et al.* 2015). Moreover, *A. longifolia* pollen grains are packed as groups of 16 into polyads. This might provide protection from damage or dehydration during travel via wind or pollinators and reduce the need for several pollination events, as one might be enough to fertilize all the ovules in the ovary (Kenrick and Knox 1982). To summarize, in this species we observe a long-lasting flowering period that may intercept contrasting conditions: active pollinators *vs.* windy climate. The maintenance of a long-lasting flowering period is obtained by controlling the flower amount and opening, and by ensuring that individual flowers may accomplish mass pollen release (enhanced by the polyads) and highest receptivity at any time.

The last point we would like to raise regards methodology. Previously, Stone *et al.* (2003) and Gilpin *et al.* (2014), characterized stages of inflorescence development in other *Acacia* species by the presence or absence of elongated stigma and/or stamen in the flowers and their abundance on the flower head. Here, we defined five flowering stages (Table 1) and estimated the duration of each functional flowering stages S1, S2 and S3 using a novel method of sequential photographic records (see Supplementary Material, Figure S2) easy to apply in the field without excessive flower manipulation. Indeed, photo records can be analyzed at a later date without interference with the collection of other data on the focal plants. In further studies with *A. longifolia*, shortening the photographic intervals to 1h instead of 4h is recommended, since progression through stages S1-S3 is fast. Stone *et al.* (2003) described the subgenus *Phyllodineae* (former classification) as having long-lived flowers by observation of flower heads. The methodology applied in the present study is based on detailed

observation of individual flowers instead of inflorescences, rendering it impossible to make a straightforward comparison, but highlighting the importance of the flower level.

It is important to understand *A. longifolia*'s reproductive biology as it will have a tremendous impact on its invasive behavior, as this species rapidly produces massive seedbanks that are "ticking time-bombs" for further invasion. In this study, we performed an assessment of *A. longifolia*'s flowering on different environmental conditions in an invaded area. Our results indicate the species to be outstanding in its allocation of resources, independently from the environmental conditions. Its intrinsic ability in resource optimization could indicate the species to be acknowledged by itself in its invading ability. Studies comparing other species in the genus or other species with similar abilities should be fostered, in a view to better understand invading processes.

Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of Funding

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Data Availability Statement

The data originated in this study are available from the authors upon request.

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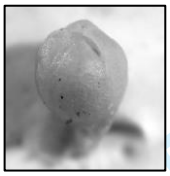
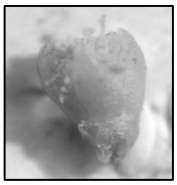
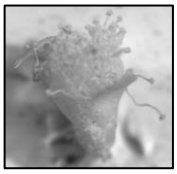
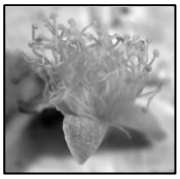
661 **Table 1: Detailed description of each flowering stage S0-S5 based on the position of**

662 **petals, stamen, and style.**

Stage	Photo	Petal position	Stamen position	Anthers state	Style position
S0		Curved, closing the bud.	Enclosed, not visible.	Closed.	Enclosed, not visible.
S1		Curved but separating at the top of the bud allowing inner view.	Visible, but not emerging from the cup-like curved petals.	Closed.	Enclosed, not visible.
S2		Curved, forming a cup-like structure.	Starting to emerge from the cup-like structure of petals.	Closed.	Elongating out of the cup-like structure of petals.
S3		Straight, vertically distended.	Elongated and straight.	Mostly closed, some open with pollen on the surface.	Elongated and straight.
S4		Horizontally distended and curving down.	Some straight, some curving.	Open, with or without pollen on the surface.	Some straight, but mostly curving.

663

664 **List of Figures**

665

666 **Figure 1: Overall canopy flower phenology circular plots.** Inflorescences were
667 classified as closed, open and senescent in 10 trees at each location. The yy axis
668 represents the abundance of each type of inflorescence, with the inner circles
669 representing lower abundance, and the outer circles representing higher abundance.
670 Abundance was determined by direct observation on the field. VNM – Vila Nova de
671 Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz.

672

673 **Figure 2: Ratio of the number of inflorescences with open flowers and the number**
674 **of total inflorescences in branches during the flowering peak.** The Kruskal-Wallis
675 test was applied with pairwise comparisons. VNM – Vila Nova de Milfontes. PC –
676 Pinheiro da Cruz. FF – Figueira da Foz. The peak of flowering was observed on the 9th
677 of February in VNM (both sites), on the 16th of February in FF and on the 20th of
678 February in PC. n = 20 at each location.

679

680 **Figure 3: Frequencies of the duration intervals of flowering stages S1, S2 and S3 at**
681 **each location (A) obtained through the heat maps (B).** Each vertical column
682 represents one individual flower, and each square within the column represents the
683 flowering stage (S0-S4) at a different photographic timepoint. PC – Pinheiro da Cruz.
684 FF – Figueira da Foz.

685

686 **Figure 4: Mean percentage of germinated polyads and mean percentage of stigma**
687 **receptivity.** Pollen germinability and stigma receptivity were assessed through the three

functional stages S1, S2 and S3. Once the flower is fully open (S4), pollen is released and remains viable for roughly 72h. Bars represent standard deviation.

From canopy to single flowers: a downscale approach to flowering of the invasive species *Acacia longifolia*

Running title: Downscale approach to flowering of *Acacia longifolia*

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Abstract

Context: *Acacia longifolia* is a native legume of Southeast Australia and Tasmania and is invasive in many parts of the world. A key feature to its success is the production of a high quantity of flowers every season, resulting in a massive seed bank that remains dormant in the soil for decades. Many studies have been performed on this species' reproductive biology, but none have focused on flowering in detail.

Aims: Our main objective was to understand this species' resource allocation strategy that ensures its successful reproduction in the invasive range.

Methods: ~~In this study, w~~We developed an integrative approach, assessing flowering at different levels: canopy and branch flowering (macro scale), downscaling to individual flower functional stages and their duration, pollen longevity and stigma receptivity (micro scale). We performed this study in three different locations in sand dunes along the Portuguese coast with different environmental conditions.

Key results: ~~According to our results, c~~Canopy flowering shows no difference among sites. Pollen and stigma assessment revealed that this species is protogynous, with the stigma being highly receptive long before pollen is released. Once released, pollen lasts roughly 72h. Individual flowers are relatively short-lived, with a rapid progression from closed flower buds to fully open flowers.

Implications: Our results indicate that *A. longifolia* has a resource trade-off strategy of investing~~s~~ in flowers and pollen that are relatively short-lived, which are counterbalanced by their massive quantities.

Keywords: *Acacia longifolia,* invasive species, flowering trade-offs, environmental conditions, pollen longevity, stigma receptivity.

Introduction

Australian acacias (genus *Acacia*, formerly classified as *Acacia* subgenus *Phyllodineae*) are considered one of the most invasive plants at a global scale, displaying many characteristics of a model invasive species (Richardson *et al.* 2011). *Acacia longifolia* (Andrews) Willd. is a leguminous species native of Southeast

mainland Australia and Tasmania and is particularly invasive in areas with a Mediterranean climate, such as the Iberian Peninsula, South Africa, southern Brazil, Uruguay, and Western Australia (see, for example, Richardson *et al.* 2011; Harris *et al.* 2012). In Portugal, this species was introduced for dune fixation and as an ornamental plant in the late 19th/early 20th century and has since then invaded most of the coastal regions (Marchante 2011). Previous studies have shown that the invasive capacity of *A. longifolia* is associated with its ability for symbioses with nitrogen-fixing rhizobia (e.g., Rodríguez-Echeverría *et al.* 2009) enabling its spread in sandy dunes with very nutrient-poor soils (Ulm *et al.* 2017a), impacting these ecosystems from an early stage of invasion (Hellmann *et al.* 2011; Rascher *et al.* 2012; Ulm *et al.* 2017a). *Acacia longifolia* promotes its own success and that of its offspring by increasing soil nutrients (Le Maitre *et al.* 2011; Badalamenti *et al.* 2014; Ulm *et al.* 2017a, 2017b) and ~~has the ability to~~could control the available sunlight to other plants, eventually outcompeting them and occupying their space (Badalamenti *et al.* 2014). This species is capable of quick regeneration after forest fires since high temperatures promote seed germination (Marchante *et al.* 2003; Gibson *et al.* 2011). Moreover, forest fires and human disturbances (e.g., vegetation removal and sometimes even management interventions) were shown to promote this species' invasion (Carvalho *et al.* 2010; César de Sá *et al.* 2017). A key feature for *A. longifolia's* invasive success is its massive seedbank, which is developed in a very short time (Marchante *et al.* 2010). This may be due to the large number of flowers and seeds it produces every flowering season (Gibson *et al.* 2011; Giovanetti *et al.* 2018).

Flowering is a key event in the life cycle of a plant, and it needs to occur when environmental conditions are optimal for flower development and pollen dispersal in order to ensure successful reproduction (Rathcke and Lacey 1985). Abiotic factors such

as rainfall, temperature and wind have a strong influence on flowering time, flower longevity and pollen performance. It is well described that rain delays the opening of flowers (Stone *et al.* 2003) and may hinder pollen dispersal. Furthermore, wind and temperature affect the activity of pollinators and, therefore, are also determinants of pollen dispersal. There have been several studies of the phenology of *A. longifolia* in Portugal. Fernandes *et al.* (2015) examined phenology in both the North (mesic habitat – lower temperature, higher humidity) and South (xeric habitat – higher temperature, lower humidity) of Portugal and found that phenological events, in particular the reproductive phenophases occurred earlier at the South site, where plants had a shorter flowering period. Also, flowering was advanced two weeks at the South site, and flowering peak occurred earlier when compared to the North site. The authors found that these differences were mainly due to higher temperatures in the South site, and that temperature is the main driver of the flowering peak date. Morais and Freitas (2015) examined phenology in four populations of acacias in Portugal: North *vs.* South and Inland *vs.* Coastal populations. The authors corroborated the findings of Fernandes *et al.* (2015) with respect to latitude, and_ ~~also~~ showed that coastal populations flowered earlier when compared to inland populations. Therefore, *A. longifolia*'s phenological events, in particular flowering, depend strongly on environmental conditions in invaded ranges. *Acacia longifolia* was initially considered an entomophilous species (Stone *et al.* 2003), depending on insects for pollination, and indeed it exhibits scented bright yellow flowers and produces extrafloral nectar, both very attractive to insects (Giovanetti *et al.* 2018). However, this species flowering occurs in winter when insect abundance is low, which results in pollinator scarcity (Godoy *et al.* 2009; Gibson *et al.* 2011). The many small flowers, and the large amount of pollen released, are characteristics often displayed by anemophilous species (i.e., pollinated by wind;

Ackerman 2000). Recent studies have shown that *A. longifolia* is an ambophilous species, pollinated by both insects and wind (Giovanetti *et al.* 2018).

Flower longevity is an important trait for reproduction as it determines for how long flowers are functional and receptive to pollination, and it is influenced by both abiotic factors and the abundance of pollinators (Trunschke and Stöcklin 2017). There is a balance between the number of flowers exhibited per plant and the resource investement of keeping them open (Ashman and Schoen 1994; Spigler and Woodard 2019). Longer flower longevity means longer exposure to pollinators and pollen dispersal, but it also means higher resourse costs (Primack 1985; Ashman and Schoen 1996; Harder and Johnson 2005). Therefore, flower longevity determines the size and duration of flower display (i.e. number of flowers open at any given time; Primack 1985; Ashman and Schoen 1996), and the determination of reproductive assurance (Rathcke 2003). A previous study of the reproductive biology of several invasive acacias in Portugal found that *A. longifolia* invests in high numbers of inflorescences with mostly hermaphrodite flowers, resulting in high seed crop (Correia *et al.* 2014). Self-pollination is reduced due to protogyny (the female reproductive organs mature before the male ones), a feature shared by most Australian acacias (Stone *et al.* 2003, Gibson *et al.* 2011). *Acacia longifolia* in an invaded range (Portugal) maintains the same level of self-incompatibility as in the native range, despite exhibiting more and larger seeds (Correia *et al.* 2015).

Pollen longevity is also an important factor to consider in combination with stigma receptivity. Stigma receptivity is a determinant of effective pollen-pistil interactions, making its timing and duration important for successful reproduction. There is usually a negative correlation between pollen size and number (Aguilar et al. 2002) due to plants' resource division and allocation (Vonhof and Harder 1995; Gillet

and Gregorius 2020). Pollen longevity is usually balanced with pollinator availability, with higher pollen longevity when pollinators are scarse (Dafni and Firmage 2000). Conversely, pollen longevity tends to be shorter when the level of self-incompatibility of the plant is higher, thus lowering self-pollination events (Dafni and Firmage 2000). However, the longevity of *A. longifolia* pollen is still unknown.

The mentioned reproductive characteristics of *A. longifolia* are generally found in successful invasive plants (e.g., see Pyšek and Richardson 2008), and in other species of acacias regardless of their invasive status (Gibson *et al.* 2011). However, varying strategies of resource investment in the reproductive units have been identified for different acacias in the invaded ranges (Correia *et al.* 2014; Giuliani *et al.* 2016). For example, *A. dealbata* was shown to invest heavily in high inflorescence density, while *A. melanoxylon*, *A. saligna*, *A. longifolia* (Correia *et al.* 2014) and *A. pycnantha* (Giuliani *et al.* 2016) invest in higher number of flowers per inflorescence, but lower inflorescence density. Thus, a characterization of the specific reproductive strategy of *A. longifolia* is of great interest to better understand its invasiveness.

All the above-mentioned studies address *A. longifolia* reproduction from different perspectives, however detailed studies of individual flowers are still lacking. Considering this species' invasive success, in this study, we examined differences in flower development in three locations in Portugal, under different environmental conditions. At these locations, we addressed flowering of *A. longifolia* in a within-plant perspective, focusing on individual flowers, to understand this species' resource allocation strategy for reproduction. Taking into consideration the studies mentioned above, we hypothesize that shot-lived individual flowers and pollen counter the massive production of flowers observed at canopy level.

Materials and Methods

Study sites

This study took place in the flowering season of 2019 in Portugal (January to March). According to different edaphoclimatic conditions, three locations were selected: one in the North, Figueira da Foz (FF; latitude 40.0646, longitude -8.8642); and two in the South, Pinheiro da Cruz (PC; latitude 38.2561, longitude -8.7730) and Vila Nova de Milfontes (VNM; latitude 37.6880, longitude -8.7927) (Supplementary Material, Figure S1). Figueira da Foz has a meso-Mediterranean climate with an average yearly temperature of 12-15 °C and average yearly precipitation of 700-900 mm, while Pinheiro da Cruz and Vila Nova de Milfontes have a thermo-Mediterranean climate with an average yearly temperature of 15-18 °C and average yearly precipitation of 400-600 mm (data from Portal do Clima, https://portaldoclima.pt/en/). In all sites, acacias are scattered, with the distance to the ocean being 1900 meters in FF, 250 meters in PC, and 150 meters in VNM. Molecular (see Vicente *et al.* 2018) and phenology studies (see Fernandes *et al.* 2015) have been previously done in these locations, except for FF which is a substitute for a site from Fernandes *et al*. (2015) and Vicente *et al.* (2018) where plants burned in a forest fire in 2017.

Canopy flowering assessment

In each location, 10 adult plants were marked separated by at least 5 meters to avoid sampling closely related individuals. Periodic assessments were made every 15-18 days for a total of 4 assessments in each site, except for PC where the time between

the first and the second assessment was 24 days. Assessments in VNM and FF (the Southern and the Northern sites) were separated by approximately one week, as it is known that flowering occurs earlier in the South when compared to the North of Portugal (according to Fernandes *et al.* 2015), while assessments in PC were made in between those in VNM and FF. At every assessment, the percentage of closed, open, and senescent inflorescences was classified as 0-25%, 25-50%, 50-75% or 75-100% by direct observation of the canopy as a whole, since the flowers strikingly change colors as they progress throughout the flowering period and are easily identified: closed flowers are mostly green, while open flowers are bright yellow and senescent flowers are orange/brown. These data were then transformed into abundance levels from 1 to 4 as follows: level 1, when more than 8 plants were in the 0-25% class; level 2, when 2 or more plants were in the 25-50% class; level 3, when 2 or more plants were in the 50-75% class; and level 4, when 2 or more plants were in the 75-100% class. Two branches were also marked in each plant, one facing east and another facing west. At every assessment, the total number of inflorescences and of inflorescences with open flowers were counted also by direct observation.

Single flower characterization

The determination of flowering stages was performed on branches with flowers that were brought to the laboratory and observed under a dissecting microscope. Preliminary observations showed that flowers open in a non-synchronized way within an inflorescence, without any order (acropetal, from base to tip of the inflorescence, or basipetal), in accordance with Stone *et al.* (2003) for native range acacias formerly classified in the subgenus *Phyllodineae.* Taking this into account, instead of

characterizing inflorescence development as previously described for other acacias (Stone *et al.* 2003 and Gilpin *et al.* 2014), we characterized single flower development. Five flowering stages from the closed to the open flower were defined and described according to the position of petals, stamen, and style (Table 1).

The first stage (S0) is the fully closed flower bud. The stage S1 is the opening of the flower bud (with the petals still curved) followed by stage S2, where only the style is emerging from the flower bud with petals in a cup-like formation, and stage S3, where both style and stamen have emerged from the bud, but petals are in a vertical position. The last stage – S4 – is the fully open flower with horizontal petals, and both stamen and style are in a vertical position. This stage is when anther dehiscence occurs, and pollen is released. The three functional stages S1-S3 usually occur in rapid succession. Once flowers are fully open and pollen is released anthesis is complete, and flowers last only a few days until reaching senescence. On the other hand, since all flower buds are already formed, closed flowers can last from days to months depending on whether that ~~particular~~ bud opened at the beginning, middle or end of the flowering period.

An estimation of the duration of the functional flowering stages S1, S2 and S3 was performed by marking single inflorescences in the field and monitoring them for 8h in each location, at their respective flowering peaks (determined by the canopy flowering assessment). Five inflorescences from 5 individuals (n = 25 inflorescences) were marked in the morning and photographed at 9:00h, then photographed again at 13:00h (4h after the beginning of the assay) and at 17:00h (8h after the beginning of the assay), taking care to keep approximately the same angle for all photo records. The VNM location was excluded from this analysis as flowering progressed quickly at this site, and unfortunately it was not possible to take good quality photo records. The

pictures were then examined in a computer where single, individual flowers were marked (n = 100 flowers for each flowering time), and flowering stages were followed through the different photographic timings. Flowers were selected only if they were clearly visible in all photo records and if their flowering stage was easily identifiable at each photographic timing, regardless of their initial flowering stage. For each individual flower, the flowering stage at each time period was registered (see Supplementary Material, Figure S2, for an example), originating a heat map of the changing flowering stages through time. Through the heat map, for each flower, the duration of each stage was classified as less than 4h, 4h to 8h or equal or greater than 8h.

Pollen longevity assays

Pollen longevity studies were performed by assessing germinability in the appropriate medium through time. Assays were performed in all locations at their respective flowering peak. Firstly, three media were tested for this assay: *Arabidopsis thaliana* (At) pollen germination medium (Boavida and McCormick 2007), *Nicotiana tabacum* (Nt) pollen germination medium (Read *et al.* 1993), and Brewbaker and Kwack (BK) medium (Brewbaker and Kwack 1963), all with 5% sucrose. Pollen was collected and immediately placed in 20-25 μL of each medium on a microscope slide or in a microtube in a dark, humid chamber at room temperature. After 2h, the polyads were observed under the microscope. The Nt medium proved to be the most efficient and was thus selected for the assays, described below.

As a routine assay, pollen was collected from opened anthers into a microtube in the field in the morning. Five individuals were considered and pooled in a total of 6 samples (replicates) per site (i.e., 5 individuals per each of the 6 samples), and pollen

was germinated in Nt medium at 0h, 6h, 24h, 48h and 72h after collection. At each time point, pollen was placed in 20-25 µL of medium on a microscope slide in a dark, humid chamber at room temperature. Counts of germinated and non-germinated polyads were performed after 2h under a microscope. A polyad was considered germinated when at least one pollen tube was visible, and its length was approximately equal to the polyad's diameter or longer. To obtain the germinability at timepoint 0h as accurately as possible, germination was performed directly on the field by suspension of pollen in 20-25 µL of germination medium in an microtube with no cap, and then transported to the laboratory in a dark, humid chamber at room temperature. Once there, the media with the pollen was transferred to a microscope slide for counts as described above.

Assay with closed anthers were also performed only once with flowers from VNM to check the maturation of pollen throughout the flowering stages. One flower per each flowering stage S1-S3 was collected from 6 trees in VNM. Anthers were forced to release the pollen by squashing them on a microscope slide containing 20-25 µL of Nt medium and germination was checked as previously described.

Stigma receptivity assays

The stigma receptivity assay was performed at each location in its respective flowering peak, based on the hydrogen peroxide test as previously reported (e.g., Souza *et al.* 2016; Thimmaiah *et al.* 2018). Thirty flowers per site (from 3-5 inflorescences of 3 different trees) in each stage S2, S3 and S4 were detached from the inflorescences under a dissecting microscope and submerged in 3% hydrogen peroxide. The presence of air bubbles visible under the microscope confirms catalase activity, an enzyme present in receptive stigmas that degrades hydrogen peroxide into water and oxygen.

Statistical analysis

For the branch flowering assessment, the mean ratio of the number of inflorescences with open flowers and the total of number of flowers was computed for each of the marked branches at every location, and differences between East- and West-facing branches were tested by applying the Wilcoxon Signed Rank test. Since no pattern was found, we considered that branch orientation had no influence on flowering, and branches were pooled. Differences in the ratio among locations were tested using the Kruskal-Wallis test at each location's flowering peak date, as the data was not normally distributed (Shapiro-Wilk test $p < 0.05$). All the above-mentioned tests were performed using IBM SPSS Statistics v26.

In the single flower characterization assays, as the data is categorical and matched, Cochran-Mantel-Haenszel Test for 3-dimentional tables and a post-hoc Fisher exact test were performed in R (R Core Team 2016) using R/psych, R/vcd and R/rcompanion packages according to Mangiafico (2016) to check for significant differences in the duration of the stages at the flowering peak among locations and to check for significant differences in duration among stages at each location, using the duration data extracted from the heat maps.

Results

Canopy flowering

All locations presented an abundance of closed inflorescences at the end of January and of senescent inflorescences in mid-March, while the peak of open inflorescences appears in mid-February. The maximum abundance of open flowers (peak of flowering) was observed on the 9th of February in VNM, on the 16th of February in FF and on the 20th of February in PC (Figure 1). It should be noted that the observation of the flowering peak in PC corresponded to a 24-day interval from the previous observation, instead of the 15/18-day interval that occurred for the other locations. This is the reason why the reported flowering peak occurs later in PC than in FF (the northern site). In the period this study was conducted, the high abundance of closed inflorescences was visibly shorter on the dunes in FF in the north as well as at PC in the south, when compared to VNM (Figure 1). The peak of flowering was fairly synchronized at all locations. In PC, open flower abundance was the shortest and, conversely, abundance of senescent flowers was the longest. **Figure 2: Ratio of the number of inflorescences with open flowers and the number of total inflorescences in branches during the flowering peak.** The Kruskal-Wallis test was applied with pairwise comparisons. VNM – Vila Nova de Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz. The peak of flowering was observed on the 9th of February in VNM (both sites), on the 16th of February in FF and on the 20th of February in PCWe also checked for differences in the ratio of the number of open over the number of total inflorescences among locations on their corresponding flowering peaks (Figure 2) and found no differences among locations (Kruskal-Wallis test chi-square = 0.163, p = 0.922, df = 2). Proximity to the ocean had no influence on canopy flowering.

Single flower development

The duration of the functional stages S1, S2 and S3 (Table 1) was estimated in each location at its corresponding flowering peak (Figure 3A and B). The duration frequency plots obtained from the heat maps show that flowering stages S1-S3 have significantly different durations (Cochran-Mantel-Haenszel Test chi-square = 60.0720, df = 4, $p < 0.05$, and post-hoc Fisher exact test $p < 0.05$ for all stages), with stage S2 being the shortest and stage S3 being the longest. However, the duration of each stage is similar among locations (Cochran-Mantel-Haenszel Test chi-square = 3.5402, df = 2, $p > 0.05$). Even if not significant, the heat maps highlight some trends in the different locations (Figure 3). FF was the site with the shortest overall flowering, quickly moving to the senescent phase, while stage S3 lasted longer in PC, thus extending flowering.

Pollen longevity and stigma receptivity

Pollen longevity was determined by germination of polyads collected from closed anthers from stages S1, S2 and S3 in VNM, and from anthers from open flowers (S4) in all locations at their corresponding flowering peak (Figure 4). Since pollen longevity was similar across locations (see Supplementary Material, Figure S3), the mean percentage of germinated polyads was considered. Despite high variability, there was an increase of polyad germination from stage S1 to the stage S3. Once the flower is fully open (S4), pollen is released, and germination is at its maximum. After release, polyads last for roughly 72h, steadily losing germinability through time.

Stigma receptivity was assessed in all locations from stage S2 to S4, which is when the style has emerged from the flower bud. However, receptivity was similar at all locations (data not shown), therefore the mean receptivity was considered and shown in

Figure 4. The stigma is receptive for several hours before the pollen is released (stage S4) and receptivity is very high (above 90% receptive stigmas) in all evaluated stages.

Discussion

Acacia longifolia is an invasive species still lacking understanding of its flowering developmental stages. Elucidating flower development ~~and pollen longevity~~ will highlight resource allocation strategies and trade-offs in this species reproducti~~ve strategy~~on ability, with a view to ~~in~~ the context of its invasive behavior. We showed that while the flowering period of *A. longifolia* is long, individual flowers have short functional periods lasting only a few hours. Furthermore, we also showed that *A. longifolia* pollen is short-lived, with germinability lasting about 72h, and stigmas are highly receptive before pollen release and maintain their receptivity throughout the flower functional period. ~~Our~~ These results support our original hypothesis that the huge quantities of flower and pollen produced ~~(Gibson *et al.* 2011; Correia *et al.* 2014)~~ are counterbalanced by their short longevity. However, our study took place during a single flowering season, bringing some limitations to our results. It will be necessary to verify how changes in plant phenology and ~~their~~ reproductive ability in different years influence current ~~change our~~ observations. It will also be very interesting to replicate this study in the native range of the species to elucidate fixed and flexible flowering traits.

~~Firstly~~In our downscale approach, we checked for differences in flowering at various levels, starting from the (macro) canopy-inflorescences level ~~by~~ through a visual assessment (Figure 1). Our results show that, at any given time of the flowering period, closed, open and senescent inflorescences are present simultaneously, meaning

that flowers do not open all at the same time, making this species' flowering period long-lasting. In general, our findings at canopy level are in accordance with Fernandes *et al.* (2015) and Morais and Freitas (2015). At all locations there was a peak of flowering around mid-February quickly followed by flower senescence. The constant presence of open flowers ~~that are open for longer periods of time~~ enhances their availability to pollinators and wind (Giovanetti *et al.* 2018), increasing the chance of cross-pollination and reproductive success, as previously shown by Fernandes *et al.* (2015).

From the macro-level of the canopy, we downscaled our attention to flowers. Flower longevity is an adaptive trait that can be altered by biotic (e.g., pollinators) and abiotic (e.g., temperature, rainfall) factors to optimize reproductive success (Trunschke and Stöcklin 2017), similarly to the timing and duration of the flowering period (Fernandes *et al.* 2015; Morais and Freitas 2015). ~~This is especially important~~Traits related to fertility, such as flower longevity and flowering duration, are crucial for invasive species, as they need to quickly colonize new environments ~~with different conditions than in their native ranges~~. For example, Pyšek *et al.* (2003) studied several alien species from the Czech Republic and suggested that invasive species tend to have longer blooming phases compared to native species, ~~since they~~possibly to outcompete ~~with~~ the native species for pollinators. Indeed, *A. longifolia* has a long-lasting ~~winter~~ flowering period of approximately 4-5 months. However, within this species, flowering~~,~~ ~~but its~~ duration does not seem to change from the native to the invaded ranges, where it usually lasts 4 months (Milton and Moll 1982; Fernandes *et al.* 2015; Morais and Freitas 2015). This is in accordance with Noble's (1989) hypothesis, who stated that the reproductive characters, such as phenology, should remain unchanged between similar native and invasive ranges, while rates of establishment and reproductive losses may

differ (see also Roy 1990). Indeed, Weiss and Milton (1984) found decreased reproductive losses in South African (invasive) populations of *A. longifolia* when compared to their Australian counterparts. Similarly, Correia *et al.* (2015) found a significantly higher number of seeds per pod and a significantly lower percentage of aborted seeds per pod in Portuguese *A. longifolia* populations when compared to Australian ones. Moreover, in the invaded range, seed and seedling sizes are larger than their native counterparts, showing a positive correlation between seed size and offspring growth (Correia *et al.* 2015, 2016) that can be expected to turn into higher rates of establishment. Thus, despite similar flowering duration among native and invasive ranges, fertility in the invaded range was proved to be higher.

Maybe the key to understand this difference relies in individual flowers. Our observations underlined that the long-lasting flowering period is achieved by a strategic investment in flowers. The flowers of *A. longifolia* are functional only for a few hours (flowering stages S1-S3), but their short life is counterbalanced by a~~. This indicates that that the~~ massive ~~quantity of flowers produced~~flower production and by a non-synchronized way of opening (Gibson *et al.* 2011; Correia *et al.* 2014; Giovanetti *et al.* 2018). The ~~is counterbalanced by their relatively short-lived functional period, thus highlighting a~~ resource allocation trade-off between quantity~~, and~~ longevity and blooming of flowers result in higher fertility even in the face of expected disrupted pollination due to winter flowering and the low activity of insect pollinators, in contrast with the expectations of Noble (1989).~~.~~

The intra-flower level reinforces the above. We assessed pollen longevity and stigma receptivity (Figure 4 and Supplementary Material, Figure S3). Regarding pollen germinability, there was an increase in the germination rate from stage S1 to stage S3, as pollen was maturing and preparing for release until the flower reached S3. Once

pollen is released, it retains germinability for roughly 72 hours. Stigmas, on the other hand, are receptive from stage S2 through the fully open flower, and no differences were found among locations, similarly to pollen longevity. Protogyny is confirmed for this species, in accordance with Stone *et al.* (2003). Our results sustain findings of Sedgley and Harbard (1993) and Kenrick (2003) on short-lived pollen in this species. Pollen germinability was similar among all locations, showing no effect of environmental variability on its longevity. Gibson *et al.* (2011) and Correia *et al.* (2014) inferred that *A. longifolia* guarantees pollination by investing in the production of mass amounts of pollen. This occurs also in other species, as depicted by Dafni and Firmage (2000). Mass-production of pollen sustains pollination in several ways, by counterbalancing pollinator scarcity in winter-flowering species (Godoy *et al.* 2009; Gibson *et al.* 2011; Giovanetti *et al.* 2018) and self-incompatibility (Correia *et al.* 2015). Moreover, *A. longifolia* pollen grains are packed as groups of 16 into polyads. This might provide protection from damage or dehydration during travel via wind or pollinators and reduce the need for several pollination events, as one might be enough to fertilize all the ovules in the ovary (Kenrick and Knox 1982). To summarize, in this species we observe a long-lasting flowering period that may intercept contrasting conditions: active pollinators *vs.* windy climate. The maintenance of a long-lasting flowering period is obtained by controlling the flower amount and opening, and by ensuring that individual flowers may accomplish mass pollen release (enhanced by the polyads) and highest receptivity at any time. ~~Second, we assessed pollen longevity and stigma receptivity (Figure 4 and Supplementary Material, Figure S3). Regarding pollen germinability, there was an increase in the germination rate from stage S1 to stage S3, as pollen was maturing and preparing for release when the flower is fully open, as was observed when characterizing the flowering stages described above. Once pollen is~~

~~released, it retains germinability for roughly 72 hours. This suggests that *A. longifolia* pollen is relatively short-lived, in accordance with Sedgley and Harbard (1993) and Kenrick (2003). Pollen germinability was similar among all locations, showing no effect of environmental conditions on its longevity. Therefore, and taking into consideration our results, *A. longifolia* guarantees pollination by investing in the production of mass amounts of pollen (Gibson *et al.* 2011; Correia *et al.* 2014) which is relatively short-lived (see Dafni and Firmage 2000 for examples of pollen longevity in several species). The production of high amounts of pollen, able to travel longer distances, could be important for an invasive species to enhance cross-pollination, especially if pollen is short-lived. For *A. longifolia*, this is particularly important since it has a level of self-incompatibility (Correia *et al.* 2015) and faces pollinator scarcity, as flowering occurs during winter (Godoy *et al.* 2009; Gibson *et al.* 2011; Giovanetti *et al.* 2018). Pollen grains of *A. longifolia* are packed as groups of 16 into polyads (Kenrick and Knox 1982), and this might confer an advantage as packing might provide protection from damage or dehydration during travel via wind or pollinators (Kenrick and Knox 1982). Also, polyads reduce the need for several pollination events, as one might be enough to fertilize all the ovules in the ovary (Kenrick and Knox 1982).~~

~~Stigmas, on the other hand, are receptive from stage S2 through the fully open flower, and no differences were found among locations, similarly to pollen longevity. Protogyny has been confirmed for this species, in accordance with Stone *et al.* (2003) observations, and the level of self-incompatibility is not lowered during invasion (Correia *et al.* 2015). Stigma receptivity throughout the flower functionality duration might be a strategy to ensure pollination.~~

The last point we would like to raise regards methodology. Previously, Stone *et al.* (2003) and Gilpin *et al.* (2014), characterized stages of inflorescence development in

other *Acacia* species by the presence or absence of elongated stigma and/or stamen in the flowers and their abundance on the flower head~~, but for *A. longifolia* this kind of study has never been performed~~. Here, we defined five flowering stages (Table 1) and estimated the duration of ~~the~~ each functional flowering stages S1, S2 and S3 using a novel method of sequential photographic records (see Supplementary Material, Figure S2) easy to apply in the field without excessive flower manipulation. Indeed, photo records can be analyzed at a later date without interference with the collection of other data on the focal plants. ~~However, in~~In further studies with *A. longifolia*, shortening the photographic intervals to 1h instead of 4h is recommended, since progression through stages S1-S3 is ~~fairly~~ fast. ~~Previously,~~ Stone *et al.* (2003) described the subgenus *Phyllodineae* (former classification) as having long-lived flowers by observation of flower heads. The methodology applied in the present study is based on detailed observation of individual flowers instead of inflorescences, rendering it impossible to make a straightforward comparison, but highlighting the importance of the flower level.~~ Still, it is important to note that the methodology described in the present paper has the advantage of providing a more accurate approach on individual flower development. However, in further studies with *A. longifolia*, shortening the photographic intervals to 1h instead of 4h is recommended, since progression through stages S1-S3 is fairly fast.~~

~~In conclusion it~~It is important to understand *A. longifolia*'s reproductive biology as it will have a tremendous impact on its invasive behavior, as this species rapidly produces massive seedbanks that are "ticking time-bombs" for further invasion. In this study, we performed an assessment of *A. longifolia*'s flowering on different environmental conditions in an invaded area. Our results indicate the species to be

outstanding in its allocation of resources, independently from the environmental conditions. Its intrinsic ability in resource optimization could indicate the species to be acknowledged by itself in its invading ability. Studies comparing other species in the genus or other species with similar abilities should be fostered, in a view to better understand invading processes.~~Our results suggest an explanation for the high investment *A. longifolia* makes on production of massive quantities of flowers and pollen every flowering season: the massive number of flowers produced is counterbalanced by their relatively short functional period, while the mass amount of pollen is counterbalanced by its short longevity and extended receptivity of the stigma, ensuring this species successful reproduction.~~

Conflicts of Interest

The authors declare no conflicts of interest.

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Data Availability Statement

The data originated in this study are available from the authors upon request.

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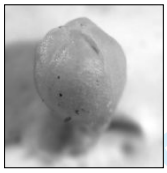
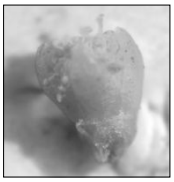
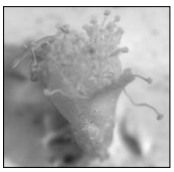
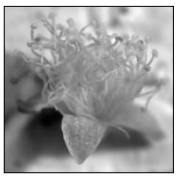
Table 1: Detailed description of each flowering stage S0-S5 based on the position of petals, stamen, and style.

Stage	Photo	Petal position	Stamen position	Anthers state	Style position
S0		Curved, closing the bud.	Enclosed, not visible.	Closed.	Enclosed, not visible.
S1		Curved but separating at the top of the bud allowing inner view.	Visible, but not emerging from the cup-like curved petals.	Closed.	Enclosed, not visible.
S2		Curved, forming a cup-like structure.	Starting to emerge from the cup-like structure of petals.	Closed.	Elongating out of the cup-like structure of petals.
S3		Straight, vertically distended.	Elongated and straight.	Mostly closed, some open with pollen on the surface.	Elongated and straight.
S4		Horizontally distended and curving down.	Some straight, some curving.	Open, with or without pollen on the surface.	Some straight, but mostly curving.

List of Figures

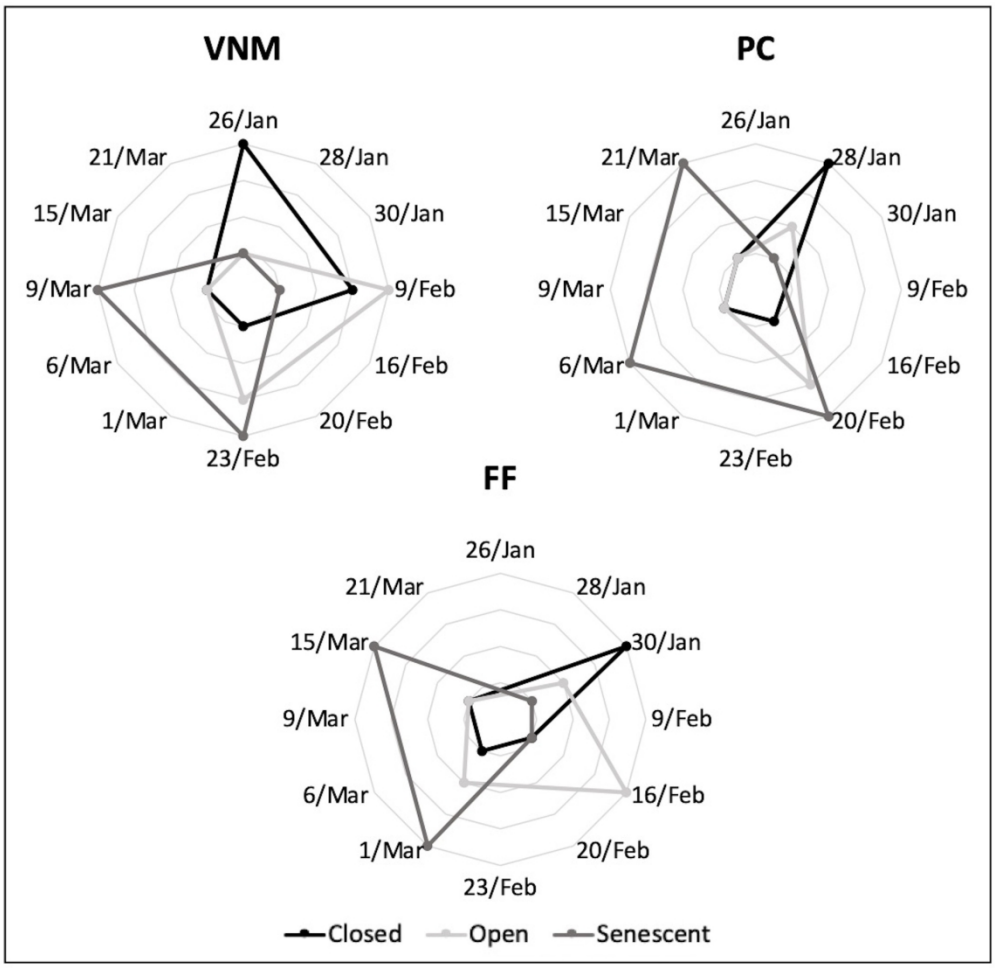
Figure 1: Overall canopy flower phenology circular plots. Inflorescences were classified as closed, open and senescent in 10 trees at each location. The yy axis represents the abundance of each type of inflorescence, with the inner circles representing lower abundance, and the outer circles representing higher abundance. Abundance was determined by direct observation on the field. VNM – Vila Nova de Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz.

Figure 2: Ratio of the number of inflorescences with open flowers and the number of total inflorescences in branches during the flowering peak. The Kruskal-Wallis test was applied with pairwise comparisons. VNM – Vila Nova de Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz. The peak of flowering was observed on the 9th of February in VNM (both sites), on the 16th of February in FF and on the 20th of February in PC. n = 20 at each location.

Figure 3: Frequencies of the duration intervals of flowering stages S1, S2 and S3 at each location (A) obtained through the heat maps (B). Each vertical column represents one individual flower, and each square within the column represents the flowering stage (S0-S4) at a different photographic timepoint. PC – Pinheiro da Cruz. FF – Figueira da Foz.

Figure 4: Mean percentage of germinated polyads and mean percentage of stigma receptivity. Pollen germinability and stigma receptivity were assessed through the three

734 functional stages S1, S2 and S3. Once the flower is fully open (S4), pollen is released
735 and remains viable for roughly 72h. Bars represent standard deviation.



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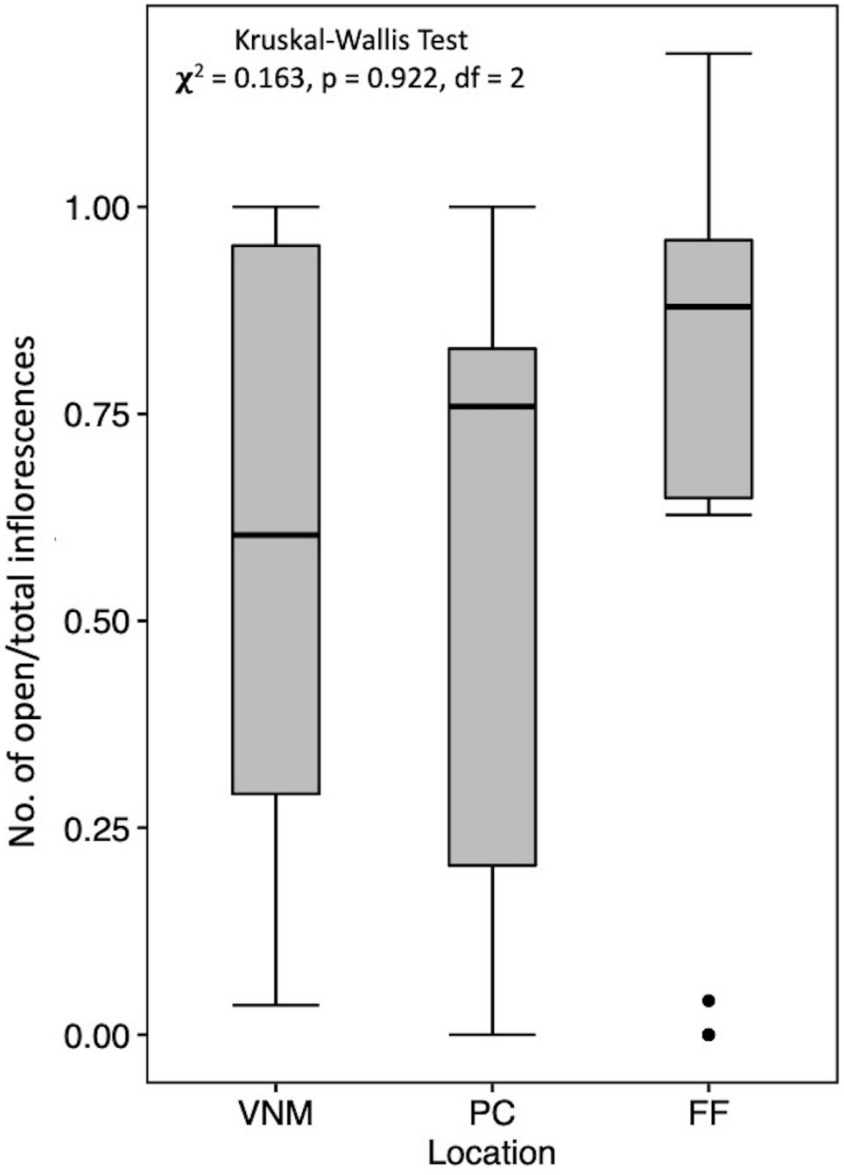

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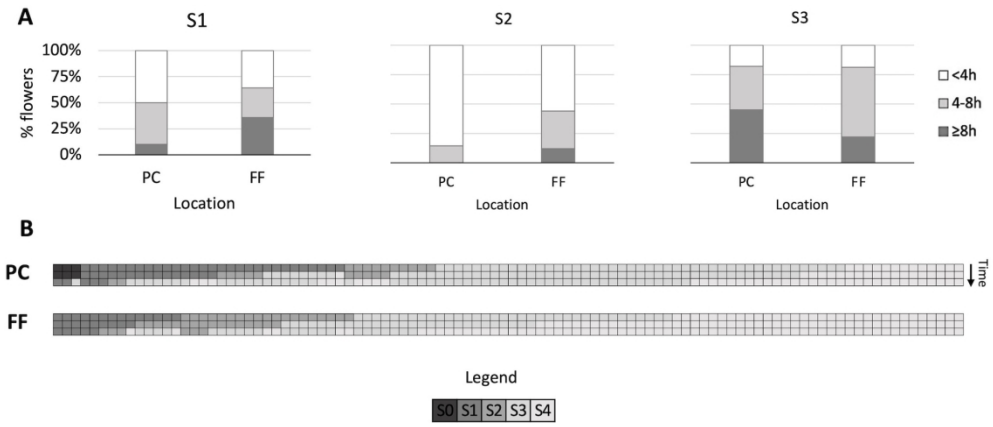

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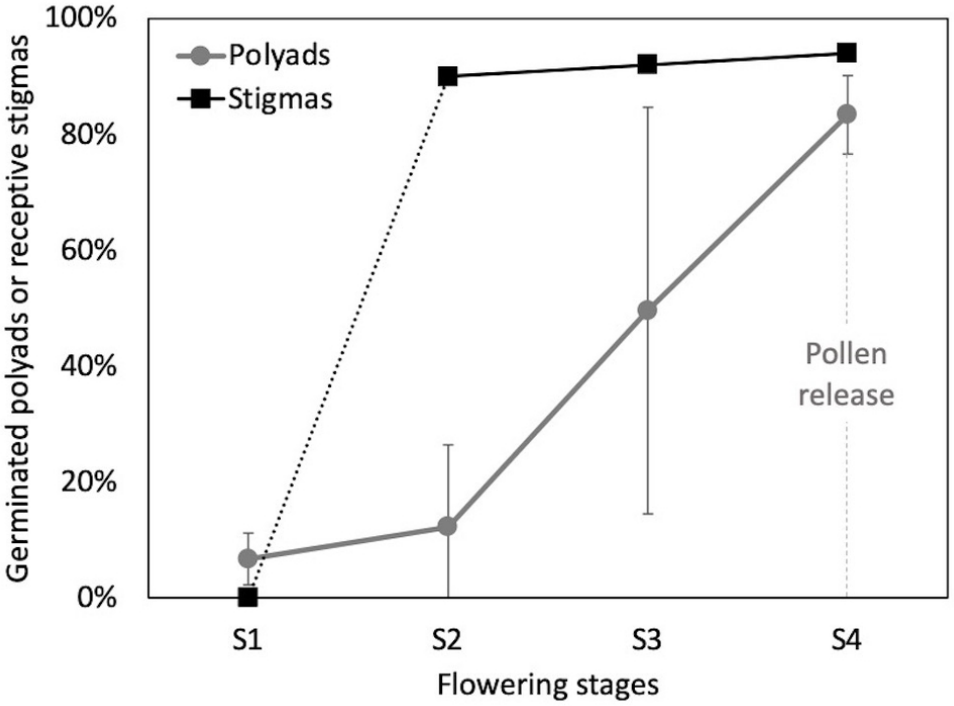

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Supplementary Material

From canopy to single flowers: a downscale approach to flowering of the invasive species *Acacia longifolia*

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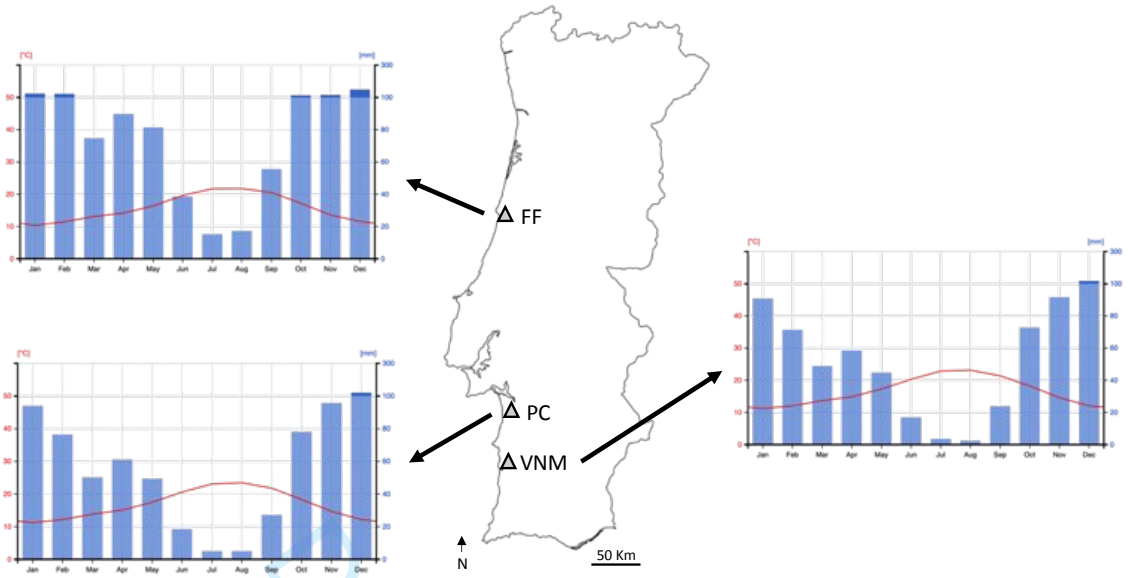

Figure S1. Map of the study locations in Portugal and their respective climate charts. FF – Figueira da Foz. PC – Pinheiro da Cruz. VNM – Vila Nova de Milfontes. Climate charts adapted from ClimateCharts.net.

Figure S2. Diagram of the method applied to estimate the duration of stages S1, S2 and S3. Firstly, inflorescences were followed on the field through sequential photography for an 8h period (pictures taken at 9:00h, 13:00h and 17:00h). Next, photos were analyzed on a computer, and the flowering stage of each clearly visible flower were registered at each photographic time, creating a heat map. This heat map was then used to estimate the stages' duration intervals.

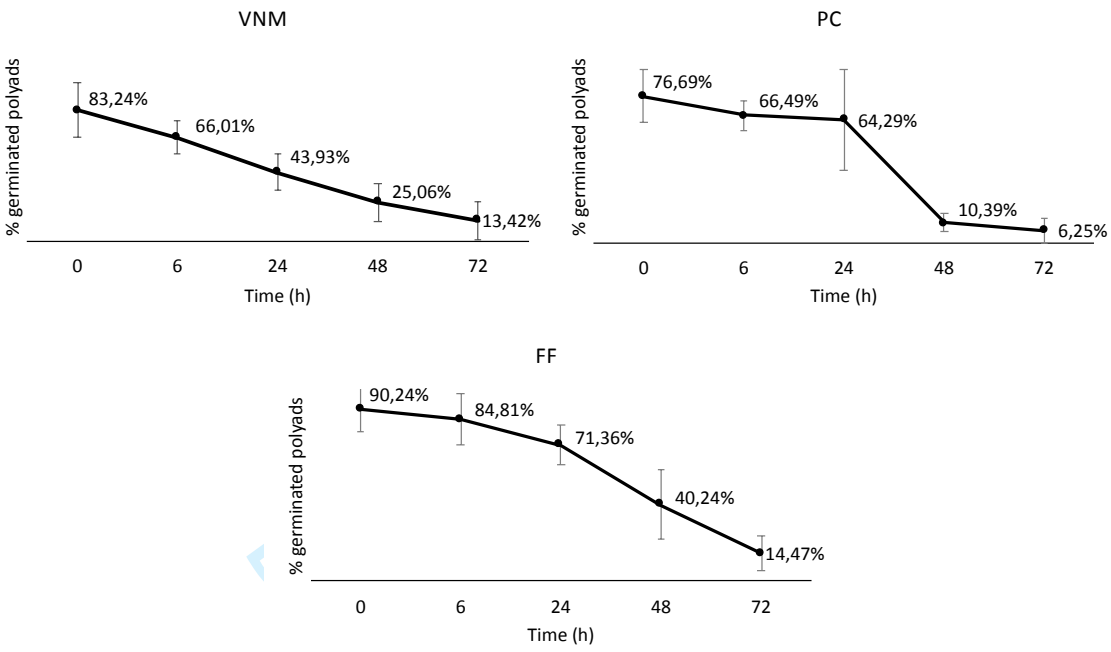

Figure S3. Percentage of germinated polyads in a 72h time-period for each location at its respective flowering peak. VNM – Vila Nova de Milfontes. PC – Pinheiro da Cruz. FF – Figueira da Foz.