# Validity of photo-oxidative stress markers and stress-related phytohormones as predictive proxies of mortality risk in the perennial herb *Plantago lanceolata*

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## ARTICLE INFO

## ABSTRACT

Oxidative stress and hormonal regulation are hallmarks of a/biotic stress responses in plants. However, little is known about their linkage with whole-organismal mortality in long-lived species. Here, we examined the validity of photo-oxidative stress markers and stress-related phytohormones as predictive proxies of mortality risk in the perennial herb *Plantago lanceolata*. Capitalizing on its broad ecological niche, we examined photo-oxidative stress markers (Fv/Fm ratio, contents of chlorophylls, carotenoids, and tocochromanols, and the extent of lipid peroxidation) and stress-related phytohormones (ABA, salicylic acid and jasmonates contents) as proxies of mortality in three populations of sub-tropical and Mediterranean habitats: Virginia (VA, U.S.A.), Catalonia (CAT, Spain), and Queensland (QLD, Australia). Stress markers were measured together with the vital rates of survival, growth, and reproduction on a total of 279 individuals. Stress marker data were collected during the summer and death/survival was monitored after two and four months. Whole-organism mortality was similarly high in both sub-tropical non-native populations (*ca.* 30 % after a drought in VA and QLD), but lower in the native population (*ca.* 10 % in CAT). The contents of antioxidants (lutein, zeaxanthin, β-carotene) and the de-epoxidation state of the xanthophyll cycle (DPS) were good proxies of mortality risk in VA and QLD. DPS and all carotenoid contents per unit of chlorophyll were lower four months in advance in dead than in alive plants in VA and QLD, thus suggesting reduced photoprotective capacity increased the mortality risk in non-native populations. We show that whole-organismal mortality in *P. lanceolata* is associated with a reduced capacity to enhance photoprotection under abiotic stress conditions. The validity of various stress markers as predictive proxies of mortality risk is discussed.

## 1. Introduction

During their lifecycles, organisms must invest energy not only in growth and reproduction, but also in maintenance and defence mechanisms that will allow them to survive under biotic and abiotic stress (Rejeb et al., 2014). Limited resources and physiological constraints may cause trade-offs in investment patterns and one major mechanism that might drive trade-offs is oxidative stress, a topic that has recently received much interest. The role of oxidative stress as a mechanism underlying life-history trade-offs in plants remains poorly understood

(Morales and Munné-Bosch, 2016), particularly when compared to animals (Stamps, 2007; Christensen et al., 2016; Smith et al., 2016). As aerobic organisms, plants use oxygen in several crucial metabolic processes such as photosynthesis, photorespiration, and respiration, among many others. All these vital biochemical processes produce reactive oxygen species (ROS), such as the superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, most particularly in chloroplasts (Asada, 2006; Triantaphylidès and Havaux, 2009). When produced transiently, ROS produced as a result of excess excitation energy in chloroplasts activate cell-signalling pathways (both inside and outside the chloroplasts) to promote biological processes through antioxidant and redox (reduction/oxidation) systems and/or plant hormones regulation (Foyer and Noctor, 2005; Muñoz and Munné-Bosch, 2020). Contrary to this optimal redox balance regulation, high and sustained increases in ROS levels cause damage to macromolecules (*e.g.* lipids, proteins and DNA; Asada, 2006) and irreversibly alter chloroplasts and overall cellular function. Therefore, a sustained disruption of the redox balance can cause intracellular oxidative damage that may lead chloroplasts, cells and, last, the whole organism to death (Morales and Munné-Bosch, 2016).

Photo-oxidative stress is a hallmark of organismal responses to abiotic and biotic stress (Baxter et al., 2014) and it is tightly linked to whole-plant senescence in monocarpic plants (Sedigheh et al., 2011). In these plants, their only reproductive event triggers a full cascade of biochemical and physiological degradation (Zimmermann and Zentgraf, 2005). However, the occurrence of photo-oxidative stress linked to plant senescence at the organismal level (*i.e.* the physiological decline after maturity that results in a cascade of mechanisms ultimately increasing the hazard of mortality and diminishing reproduction) appears to be more complex for polycarpic plants, where exploring trade-offs between reproduction and survival is complicated because reproduction does not necessarily trigger immediate death (Morales and Munné-Bosch, 2016). In addition to intrinsic (plant age) factors, extrinsic factors (adverse environmental conditions and/or biotic stressors) may cause mortality in perennial plants (García et al., 2011; Oñate et al., 2012; Morales et al., 2013, 2015, Roach and Gampe, 2004, Quarles and Roach 2018). Indeed, photo-oxidative stress markers and photoprotective molecules have been commonly used to better understand the causes and underlying mechanisms preventing photoinhibitory damage in plants at the organelle, cellular and organ levels in both monocarpic and polycarpic plants, and even at the whole-organism level in monocarpic plants, (Zimmermann and Zentgraf, 2005; Pintó-Marijuan and Munné-Bosch, 2014), but the validity of these markers as predictors of mortality at the organism level in polycarpic plants has not been examined thus far. The maximum efficiency of the photosystem II (PSII) ($F_v/F_m$), as an indicator of photoinhibition, the contents of photosynthetic pigments (chlorophylls and carotenoids), photoprotection capacity (xanthophyll cycle components, carotenoids and tocochromanols), as well as the extent of lipid peroxidation (estimated by malondialdehyde and lipid hydroperoxide contents) may be considered, in particular when measured simultaneously, good proxies of photo-oxidative stress and show therefore a strong potential as markers of mortality risk in perennial plants. Furthermore, stress-related phytohormones may be an excellent complement to these measurements. Indeed, changes in endogenous phytohormone contents may reflect variation in the activation of the plant defence systems regulating abiotic and biotic responses. Specifically, as a first line of defence, abscisic acid (ABA) regulates the stomatal closure under water availability limitations to prevent dehydration, while jasmonates (JAs) and salicylic acid (SA) regulate defences against herbivore and pathogen attack, respectively, being all three hormonal groups additionally related to redox processes occurring in chloroplasts (Davies, 2010; Muñoz and Munné-Bosch, 2020).

The aim of this study was to examine the validity of photo-oxidative stress markers ($F_v/F_m$ ratio, contents of chlorophylls, carotenoids, and tocochromanols, and the extent of lipid peroxidation) and stress-related phytohormones (ABA, SA and JAs), as markers of mortality in the perennial herb ribwort (*Plantago lanceolata* L.) a few months prior to death. We hypothesised that (i) a limited set of photo-oxidative stress markers and photoprotective molecules, together with the Fv/Fm ratio, can be used to evaluate photo-oxidative stress and photoinhibitory damage in chloroplasts and, by extension, (ii) can indicate mortality at the whole-organism level before death occurs in this cosmopolitan perennial herb. In addition, we also hypothesised that (iii) the contents of stress-related phytohormones, and most particularly those of ABA, might be associated with the degree of drought stress and hence mortality risk in *P. lanceolata* plants.

## 2. Materials and methods

### 2.1. Studied species, field sites description, climate data and habitats

Ribwort or English plantain (*P. lanceolata* L., Plantaginaceae) is a short-lived perennial herb that forms a rosette and is green year-round, making following an individual's vital rates (*i.e.*, mortality, growth, and reproduction) through time possible. Leaves are lanceolate, scarcely toothed, with 3–5 strong parallel veins narrowed to short petiole. Leaf stems are grouped deeply furrowed on a basal rosette; where it grows several erect, leafless, silky, and hairy flowering stems, that are taller than the leaves and end on an ovoid inflorescence of many small flowers. The flowering period generally starts in early spring and goes until the end of summer, depending on weather conditions. *P. lanceolata* is native to Eurasia but has been introduced by humans to North America and many other parts of the world with suitable habitats. This species is considered an invasive weed in North America, while it is present and widespread in the Americas and Australia as an introduced species (Global Biodiversity Information Facility, 2019). Across its distribution, populations of *P. lanceolata* are found in rather different habitats: from very dry meadows to rain forests, to open vegetation, to dense hayfields, and roadsides (Mook et al., 1989).

This study was carried out in three sites with contrasting environments located in Virginia (VA; Southeastern of the United States of America), Catalonia (CA; Northeastern of Spain) and Queensland (QLD; Northeastern of Australia) (Fig. 1). In accordance with the climate classification by the World Map of Köppen-Geiger (last update published by Kottek et al., 2006), VA and QLD vegetation are classified as humid-subtropical climate, whereas CAT is described as Mediterranean climate. Both humid sub-tropical populations are dense mown grassland vegetation, whereas the Mediterranean population was established in arid-soil and open-vegetation habitat. For all field sites, climatological conditions were recorded six months prior and after the field sampling points (Fig. 2a).

The VA population was located at the Morven Farm near the University of Virginia (37° 57′ 30.24″ N; 78° 28′ 25.94″ W; 155 m.a.s.l.). Climatological conditions during the experiment (from March 2016 to March 2017) were monitored at the "Charlottesville 2 SSE VA US" meteorological station (Station USW00003759, data obtained from NOAA.gov website, Fig. S4a). Mean monthly precipitation during the sampling for stress markers (September 2016) was 74.9 mm. Most rain accumulated during previous months (May-August, 514.9 mm), and a relatively dry season continued after sampling (October-December, 136.7 mm). Summer season had the highest mean maximum and minimum monthly temperatures (above 25 °C and 17 °C during June-September, respectively). In September, mean maximum and minimum monthly temperatures were 25.9 °C and 18.2 °C (Fig. 2a).

The CAT population was located at the Garraf Natural Park, near Barcelona (41° 19′ 4.67″ N, 1° 57′ 00.75″ E; 466 m.a.s.l.). Climatological conditions during the experiment (from January 2015 to March 2016) were registered in the "Sant Pere de Ribes" meteorological station (data provided by the Catalonia's Meteorological Service; www.meteocat.com), at the same Natural Park and 4,6 km from field sampling site (41° 15.0′ N, 01° 46.0″ E; 161 ma.s.l.) (Fig. 2b). Mean monthly precipitation during the sampling for stress markers analysis (July 2015)
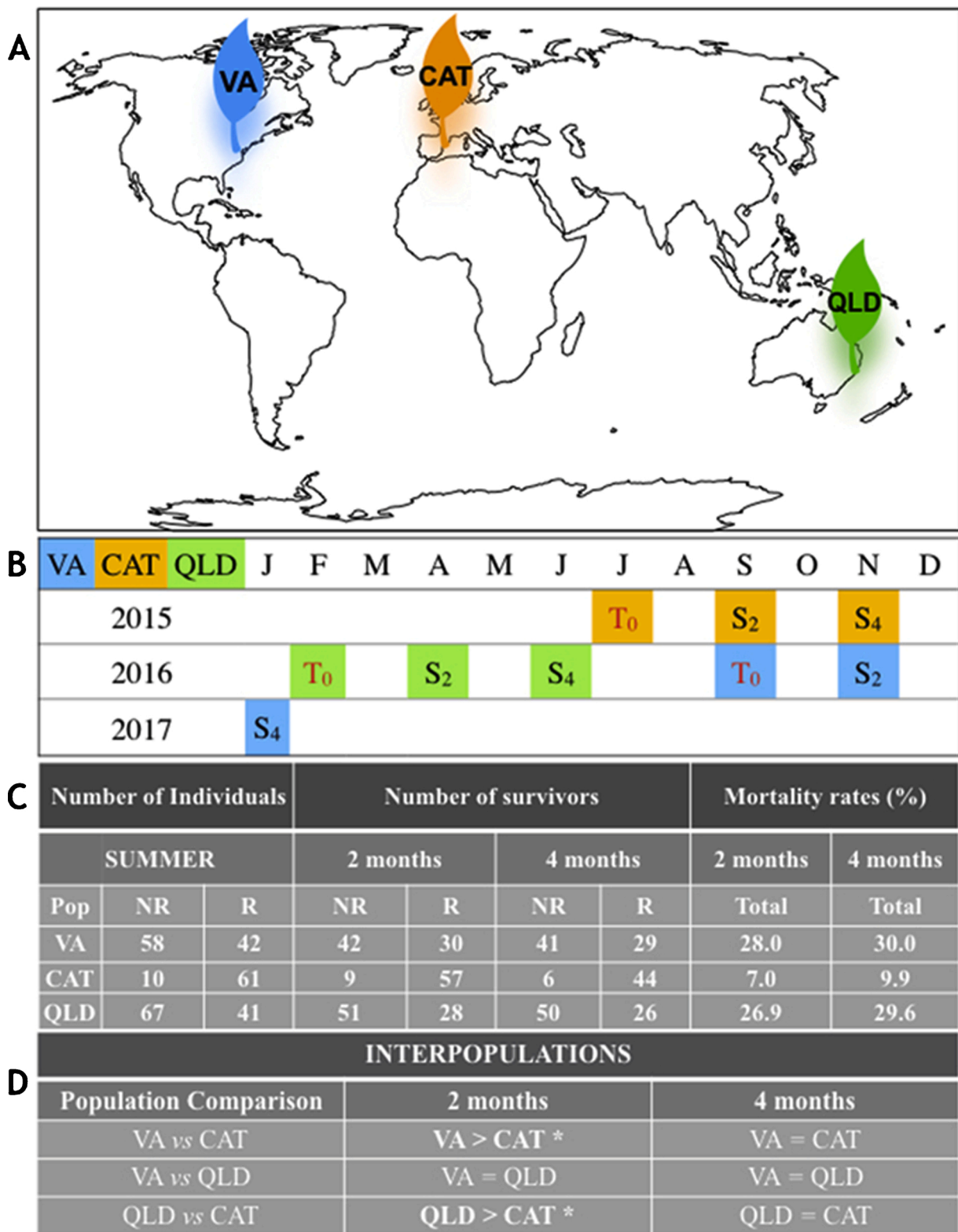
**Fig. 1.** Location, sampling time points, plant survival *censuses* and mortality comparisons across three populations of *P. lanceolata*. (a) Field sampling locations for the three studied *P. lanceolata* populations. VA, CAT and QLD respectively correspond to Virginia (southeastern United States of America), Catalonia (northeastern Spain) and Queensland (eastern Australia). (b) Data collection times for demographic, physiological and survival *censuses*. Demographic and physiological data were collected simultaneously on consecutive days (T0), and survival census (S) were checked after two (S2) and four (S4) months for each field site. (c) Monitoring of survival was carried out two and four months after physiological data collection for all plants. Some of the plants of CAT population, which were initially marked but later lost (both above- and belowground) between the second and fourth month of the study for unknown reasons, were not considered for the estimation of mortality rates at four months. NR, non-reproductive plants. R, reproductive plants.
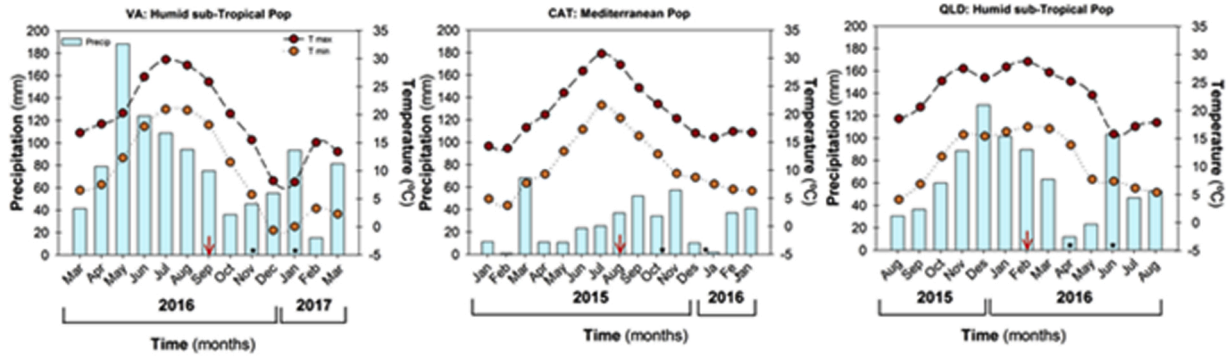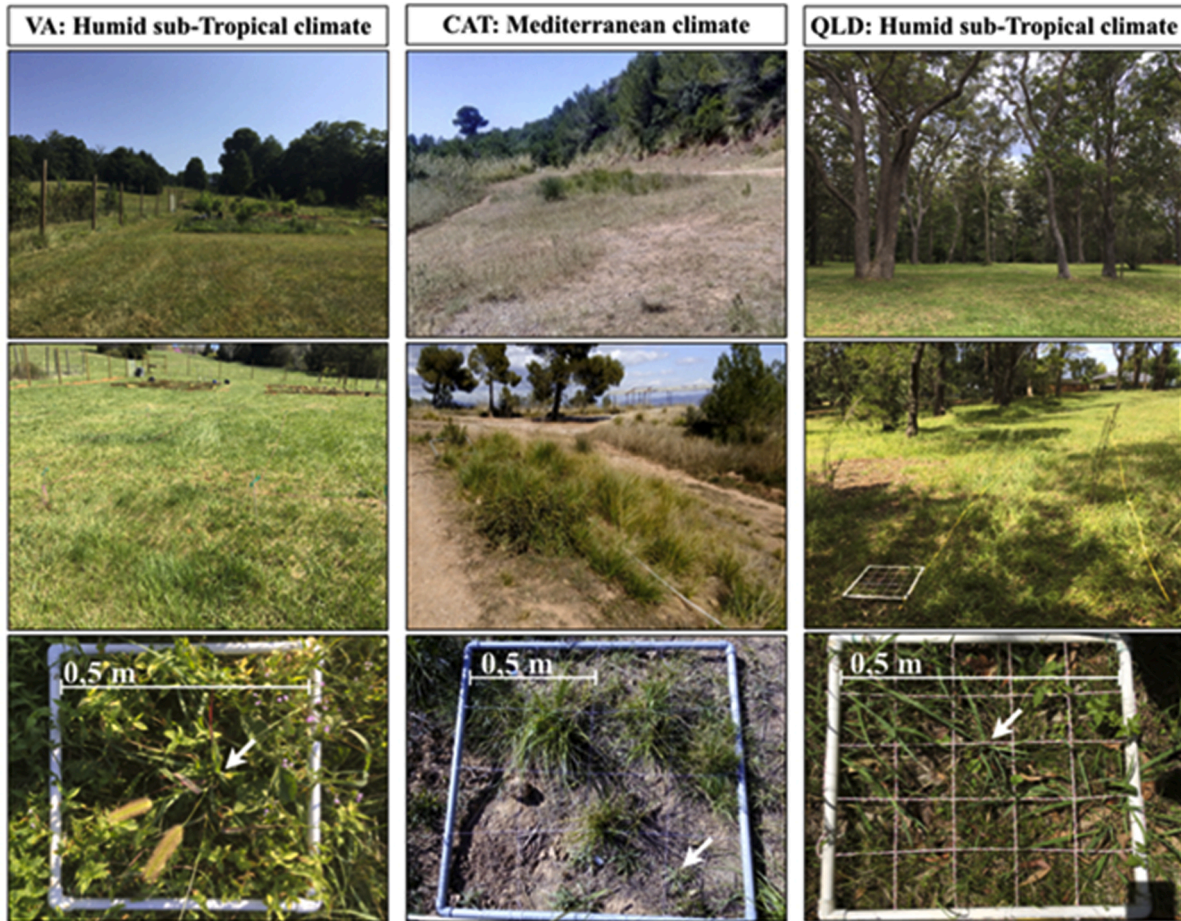
**Fig. 2.** Weather conditions experienced at each field site before and during the study. (a) Monthly mean precipitation, maximum and minimum temperatures during the period 6 months before and after data collection. These periods were March 2016 to March 2017 for VA, January 2015 to January 2016 for CAT and August 2015 to August 2016 for QLD. The red arrows indicate initial sampling times and asterisks indicate the survival censuses that followed. (b) Photos of the sampling sites in each of the three P. lanceolata populations showing the broader sampling area (at the top), the establishment of transects (in the middle) and plots (at the bottom). Field sites correspond to invasive, native and introduced habitats for the VA, CAT and QLD populations, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

was 25.3 mm, and previous months (April-June, 44.7 mm) were also dry. Mean maximum and minimum monthly temperature during July were 30.8 °C and 21.6 °C, respectively (Fig. 2a).

The QLD population was located in a parkland area in the city of Toowoomba, 125 km West of Brisbane (27° 34′ 51.96″ S, 151° 59′ 16.224″ E), at 709 m.a.s.l. Climatological conditions during the experiment (from August 2015 to August 2016) were registered in the

Toowoomba meteorological station. Mean monthly precipitation during the sampling for stress markers (February 2016) was 89.6 mm, with a previous rainy spring (November 2015-January 2016; above 80 mm monthly). The hottest month was February with a mean maximum monthly temperature of 28.7 °C (Fig. 2a).

The three populations were thus established in different sites: a farm, a National Park and an urban park in VA, CAT and QLD, respectively.

There was no disturbance in the VA and CAT sites, although both areas had been mowed prior to the experiment. In contrast, QLD site was an informal parkland area that was mown approximately 5 times a year (when the grass got quite tall) and was sporadically grazed by macropods (eastern grey kangaroos).

### 2.2. Establishment of demography transects and plots

Within each field site, we established 0.5 m wide belt transects comprising enough contiguous $0.5 \times 0.5$ m$^2$ plots to capture at least 100 individual plants. The number of plots differed between the sites because of differences in plant density. The maximum transect length was 10 m (20 contiguous plots; Fig. 2b). When this first transect did not capture 100 individuals, then a second parallel transect was established 2 m from the first to minimise effects of trampling plots. Each transect and plot were geo-referenced (record latitude and longitude with a GPS device) and run from left to right along the contour of any slope. Within a field site, plants were marked, and their $x$ and $y$ co-ordinates were recorded within the plot, where the bottom left corner of the plot was (0,0). These positions were used to relocate individuals and census survival two and four months after marking. During the initial measurements, the number of rosettes per plant, and on each rosette the number of leaves and the length and width of the largest leaf were recorded as proxies for plant size. If reproductive stems were present, the number of stems and the length of the longest stem and its inflorescence were measured.

### 2.3. Field sampling design and data collection

Leaf collection for stress markers were carried out during the summer season, at each field site during 2015 and 2016 (Fig. S4). Fully expanded, mature leaves with no visual damage were collected at midday (at maximum daily incident solar radiation) on sunny-clear days. For each individual, one leaf was used to measure relative water content (RWC), maximum efficiency of photosystem II and the leaf mass per area ratio (LMA), and another leaf was frozen *in situ* in liquid nitrogen and immediately transported to the laboratory, where all samples were stored at –80 °C until they were used for biochemical analyses. Mortality censuses were done two and four months after sampling at each field site.

### 2.4. RWC, LMA and $F_v/F_m$ ratio

RWC is an accurate indicator of plant water status as well as a useful physiological indicator of cellular water deficit (Tanentzap et al., 2015). To measure RWC, leaves were collected, transported to the laboratory in thermal bags at about 4 °C in darkness, and weighed to estimate fresh weight (FW). Leaves were then immersed in distilled water for 24 h at 4 °C and weighed for turgid weight (TW). To determine dry weight (DW) the leaves were then dried at 80 °C until they reached a constant weight. Relative leaf water content (RWC) was calculated as $100 \times$ (FW – DW) / (TW – DW) and, in the same leaves, LMA was calculated by measuring its dry weight and leaf area (g DW· m$^{-2}$). The maximum efficiency of photosystem II, or $F_v/F_m$ ratio, was estimated following Van Kooten and Snel (1990) by using chlorophyll fluorescence that was measured with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany) in the field on leaves maintained for at least 1 h in darkness.

### 2.5. Pigments and tocochromanols

The analyses of chlorophylls, carotenoids, and tocochromanols were performed by high performance liquid chromatography (HPLC), whereas total anthocyanin levels (as cyanidin-3-glucoside equivalents) were determined spectrophotometrically, all from the same extract. Specifically, 100 mg of tissue was ground in liquid nitrogen with the mixer mill MM400 (Retsch GmbH, Germany) and extracted with cold methanol containing 0.01 % butylated hydroxyltoluene, using repeated vortexing and ultrasonication (Branson 2510 ultrasonic cleaner, Bransonic, USA). After centrifuging at 12,000 rpm for 10 min at 4 °C, the supernatant was collected, and the pellet was re-extracted with the same solvent until it was colourless. The collected supernatants were merged, filtered and separated in three homogeneous aliquots to carry out the three independent analytical methods. Two aliquots were put in respective vials for each corresponding HPLC method (photosynthetic pigment and tocochromanol analyses), whereas the other aliquot was acidified with 30 % HCl and used for anthocyanin quantification spectrophotometrically (Cecil Aquaris CE7400, Cecil Instruments, Cambridge, UK) as described by Gitelson et al. (2001).

Chlorophylls and carotenoids were separated on a binary-solvent gradient using reverse-phase HPLC system and quantified with a diode array detector as described by Munné-Bosch and Alegre (2000). Shortly, pigments were separated on a non-endcapped Zorbax ODS-5 mm column (250 mm long, 4.6 mm i.d., 20 % Carbon, Teknokroma, St.Cugat, Spain) at 30 °C for 38 min at a flow rate of 1 mL min$^{-1}$ and the injection volume of 80 μL. The solvent mixture for the gradient consisted on (A) acetonitrile:methanol (85:15, v/v) and (B) methanol:ethylacetate (68:32, v/v). The gradient used was: 0–14 min 100 % A, 0% B; 14–16 min decreasing to 0% A, 100 % B; 16–28 min 0 % A, 100 % B; 28–30 min increasing to 100 % A, 0% B; and 30–38 min 100 % A, 0% B. Detection was carried out at 445 nm and compounds were identified and quantified as described previously (Munné-Bosch and Alegre, 2000).

Tocochromanols (vitamin E and plastochromanol-8 [PC-8]) were separated isocratically on a normal-phase HPLC system and quantified with a fluorescent detector as described by Amaral et al. (2005). The HPLC equipment consisted on an integrated system with a Jasco PU-2089 Plus pump, a Jasco AS-2055 Plus auto-sampler and a FP-1520 fluorescence detector (Jasco, Tokyo, Japan). Tocochromanols were separated on an Inertsil 100 A (5 μm, 30 × 250 mm, GL, Sciences Inc, Tokyo, Japan) normal-phase column, operating at room temperature. The flow rate was 0.7 mL min$^{-1}$ and the injection volume was 10 μL. The mobile phase was a mixture of *n*-hexane and *p*-dioxane (95.5:4.5, v/v). Detection was carried out at an excitation of 295 nm and emission at 330 nm. Quantification was based on the results obtained from the fluorescence signal and compared to that of a calibration curve made with authentic standards.

### 2.6. Lipid peroxidation

The extent of lipid peroxidation was estimated from the amount of lipid hydroperoxides and MDA in leaves. For lipid hydroperoxides, frozen tissue was ground with the mixer mill MM400 and extracted with cold methanol containing 0.01 % (w/v) butylated hydroxytoluene (to avoid further peroxidation) by repeated vortexing and ultrasonication. Lipid hydroperoxides contents were determined by the FOX-2 method (Bou et al., 2008), with the extract treated with triphenylphosphine as a negative control for each sample and expressed as hydrogen peroxide equivalents, after a concurrently assayed standard curve. MDA was measured by HPLC following the method described by Iturbe-Ormaetxe et al. (1998) with some modifications. The cold methanol 100 % (v:v) containing 1 ppm butylated hydroxytoluene was selected as the optimal extracting medium for MDA in comparison with ethanol 100 %, 80:20 (v:v) ethanol:water and 80:20 (v:v) methanol:water (data not shown). The HPLC equipment (Agilent) included a photodiode-array detector controlled by Millennium software. The (TBA)$_2$-MDA adduct was resolved on an Hypersyl ODS-5 μm column (250 × 4.6 mm, Teknokroma, St. Cugat, Spain) and eluted in the mobile phase with 5 mm potassium phosphate buffer (pH 7.0) containing 14 % acetonitrile and 0.6 % tetrahydrofuran (Draper et al., 1993). The flow rate was 1 mL/min and the injection volume 20 μL. The (TBA)$_2$-MDA adduct was quantified by its absorbance at 532 nm (Diode array detector 1000S, Applied Biosystems), and was identified by its characteristic spectra and by coelution with an authentic standard of 1,1,3,3-tetraethoxypropane

(Sigma, Steinheim, Germany), which is stoichiometrically converted into MDA during the acid-heating step of the assay.

## 2.7. Stress-related phytohormones

Extraction and analyses of stress-related phytohormones, including ABA, SA and JAs, were performed using ultra-HPLC coupled to electrospray ionisation tandem mass spectrometry (UHPLC/ESI-MS/MS) as described by Müller and Munné-Bosch (2011). JAs profiling included the measurement of jasmonic acid (JA), its precursor OPDA (12-*oxo*-phytodienoic acid) and the JA amino acid conjugate jasmonoyl-isoleucine (JA-Ile). Deuterium-labelled compounds for all phytohormones were used as internal standards to estimate recovery rates for each sample.

## 2.8. Statistical analyses

A Chi-square test was used to determine differences in plant mortality between populations. Logistic regression between the binary individual mortality response and either plant size (number of leaves) or reproductive stage (number of inflorescences) was performed for each population. To evaluate whether any of the functional traits predict individual survival and longevity, both Multiple Logistic Regression (MLR) and COX Proportional Hazard Regression models were run. The effect of each stress marker on probability of mortality was tested

comparing statistical differences between dead and alive individuals for each of the functional traits using a one-way analysis of variance (ANOVA, Bonferroni adjusted). For these analyses, the values of stress markers were log-transformed, as required, to achieve homogeneity and normal distribution of variances. Bivariate Pearson parametric correlations between all stress markers were performed. In all cases, differences between dead and alive individuals were considered significant at a probability level of Bonferroni adjusted $P < 0.0024$. Statistical analysis was performed using the SPSS 20.0 statistical package (SPSS, Inc., Chicago, IL, USA/Release Version 20; IBM, USA), and graphs were performed using SigmaPlot 10.0 (Systat Software, Inc., San Jose, CA, USA).

## 3. Results

### 3.1. Photo-oxidative stress markers as a proxy of mortality risk

Both VA and QLD, the two non-native populations, showed higher mortality than the native CAT population two and four months after the photo-oxidative stress markers were collected (Fig. 1). After four months, both non-native populations showed similar mortality rates around 30 %, while the native population experienced a lower mortality rate of around 10 % (Fig. 1). Stress indicators, including photosynthetic pigments and photo-oxidative stress markers revealed some physiological performance differences between survivor and non-survivor plants
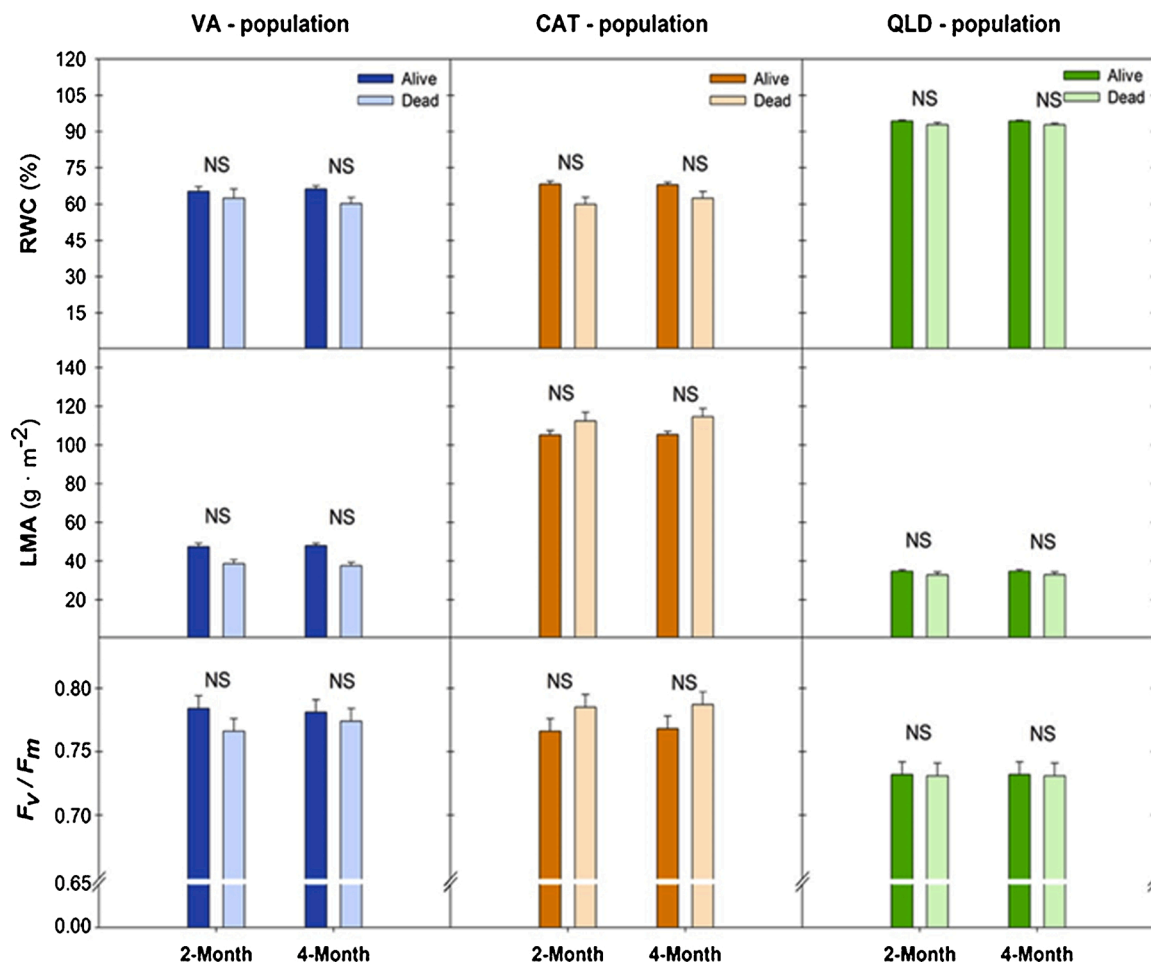


**Fig. 3.** Relative leaf water content (RWC), leaf mass per area ratio (LMA; g·m$^{-2}$) and maximum efficiency of PSII photochemistry (*Fv/Fm* ratio) as proxies of mortality risk in three populations of *P. lanceolata*. Data for alive and dead individuals were collected during the summer, that is two and four months before death/survival was monitored (see Fig. 1 for details of the experimental design). Data represent the mean SE of n = 20/69, 5/66 and 29/79 individuals (two months) and 24/65, 21/50 and 32/75 individuals (four months) for dead/alive individuals in Virginia (VA), Catalonia (CAT) and Queensland (QLD), respectively. With a $P < 0.0024$ (Bonferroni adjusted) these proxies were not significant.

in VA and QLD but not in CAT. More specifically, while the RWC, LMA and $F_v/F_m$ ratio did not differ between survivors and non-survivor plants either two or four months in any of the studied populations (Fig. 3), carotenoids resulted to be a strong proxy of mortality, in particular in VA and QLD (Figs. 4 and 5).

Carotenoids, as photoprotective molecules, including xanthophylls (lutein, zeaxanthin and VZA) and β-carotene, as well as the de-epoxidation state of xanthophyll cycle (DPS), were significantly lower (Bonferroni corrected *P*-values) in dead compared to living individuals when measurements were performed four months in advance in both non-native habitats (VA and QLD) (Fig. 5). The contents of antioxidants (lutein, zeaxanthin, β-carotene) and the de-epoxidation state of the xanthophyll cycle (DPS) were good proxies of mortality risk in VA and QLD. DPS and all carotenoid contents per unit of chlorophyll were lower four months in advance in dead than in alive plants. Furthermore, among all stress markers evaluated in this study, the contents of total carotenoids and zeaxanthin were significant predictors of survival, although only in the native population and following COX Proportional Hazard Regression (not when using Multiple Logistic Regression, Table S1). In contrast, the contents of tocochromanols (α-tocopherol and plastochromanol-8) were not significant predictors of mortality neither

when results were tested by ANOVA (Fig. S1) nor following COX Proportional Hazard or Multiple Logistic Regressions (Table S1).

Lipid hydroperoxide content (a marker of primary lipid peroxidation) was a significant mortality risk proxy in the non-native QLD population following both Multiple Logistic Regression and COX Proportional Hazard Regression (Table S1). However, there were no significant differences between survivors and non-survivors for lipid peroxidation markers used as proxies of mortality, neither for MDA nor lipid hydroperoxide contents in none of the three studied populations (Fig. S2). Furthermore, jasmonoyl-isoleucine (JA-Ile), an oxylipin produced by lipid peroxidation and mainly involved in biotic defence, was a significant time-dependent covariate in the QLD population using COX Proportional Hazard Regression (Table S1b), additionally to SA content, also involved in biotic stress responses. However, no significant differences were detected between living and dead plants in the endogenous contents of SA and JAs as proxies of mortality (Figs. S2 and S3). On the other hand, ABA was a significant time-dependent covariate with mortality in the native population using Multiple Logistic Regression, but not with COX Proportional Hazard Regression (Table S1a), without endogenous ABA contents differing significantly between living and dead plants as a proxy of mortality (Fig. S3).
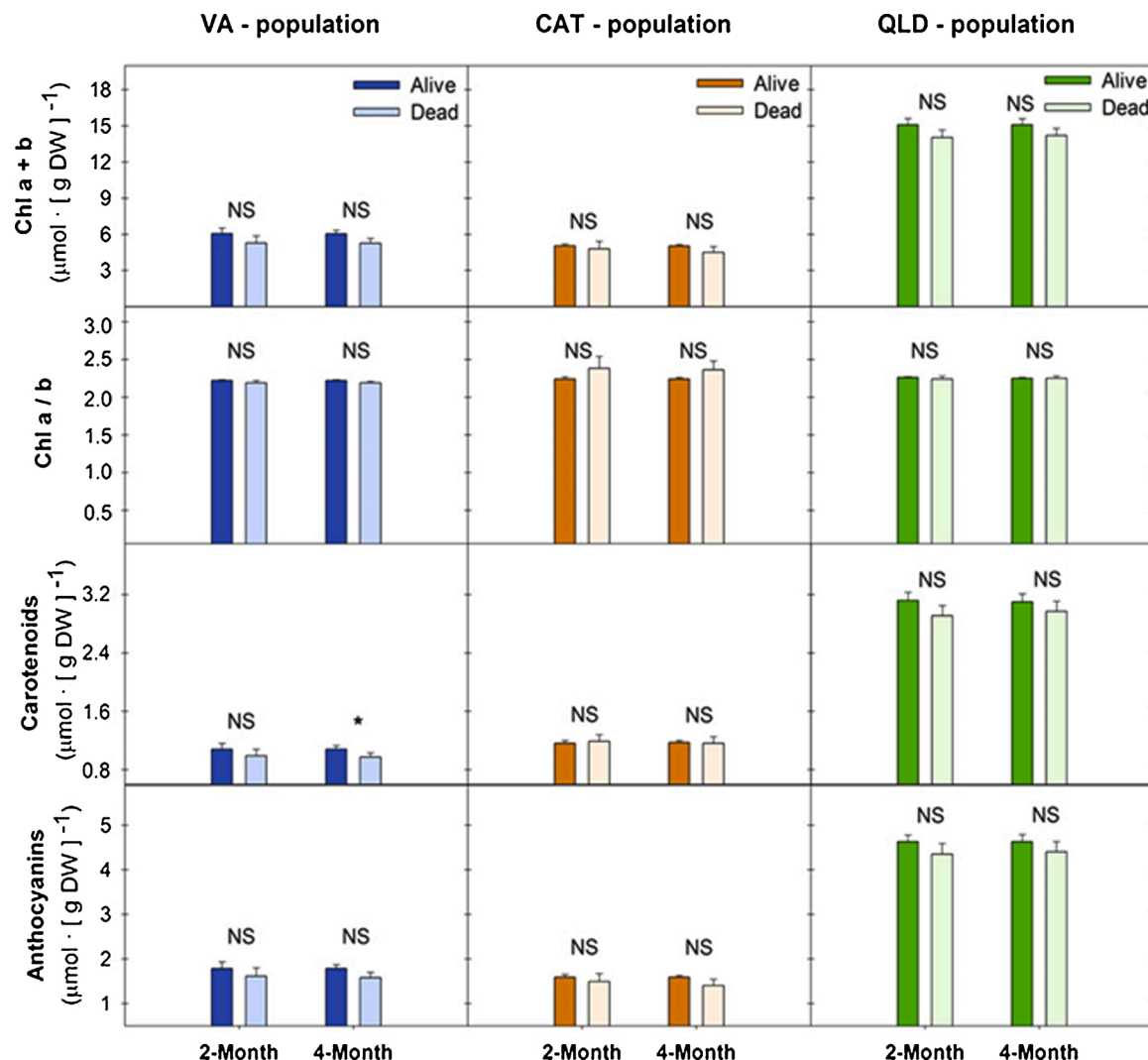


**Fig. 4.** Plant pigments as proxies of mortality risk in three populations of *P. lanceolata*. Total chlorophyll (Chl) contents, Chl a/b ratio, total carotenoids and total anthocyanin contents of leaves are shown. Data for alive and dead individuals were collected during the summer, that is two and four months before death/survival was monitored (see Fig. 1 for details of the experimental design). Data represent the mean and SE of n = 16/76, 5/66 and 29/78 individuals (two months) and 29/78, 21/50 and 32/75 individuals (four months) for dead/alive individuals in Virginia (VA), Catalonia (CAT) and Queensland (QLD), respectively. *P* < 0.0024 (Bonferroni adjusted) is shown by an asterisk (*).
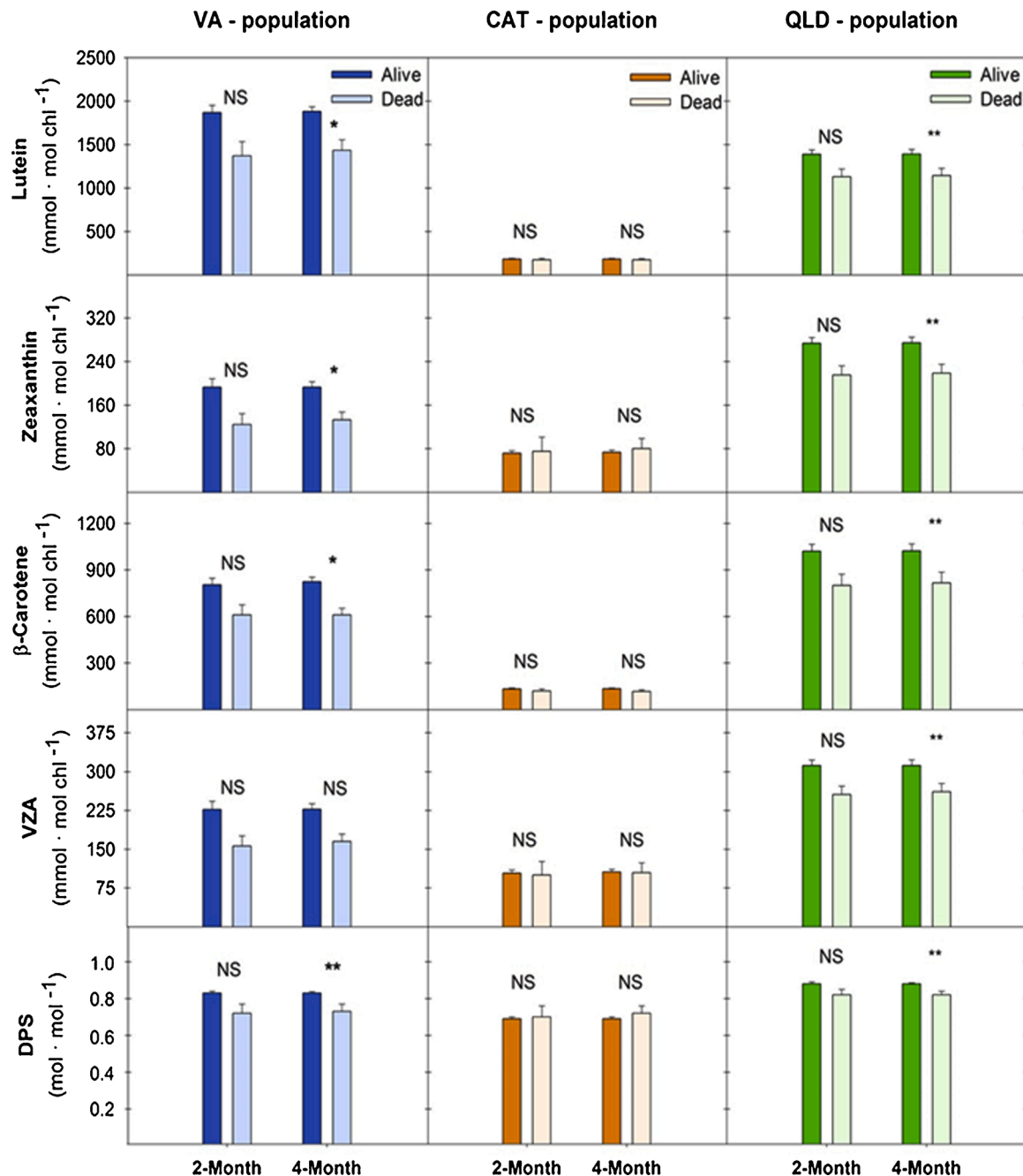
**Fig. 5.** Lutein, zeaxanthin, β-carotene and xanthophyll cycle pool (VZA) contents, as well as the de-epoxidation state of the xanthophyll cycle (DPS) as proxies of mortality risk in three populations of *P. lanceolata*. All carotenoid contents are given per chlorophyll (Chl) unit. Data for alive and dead individuals were collected during the summer, that is two and four months before death/survival was monitored (see Fig. 1 for details of the experimental design). Data represent the mean±SE of n = 16/67, 5/66 and 29/78 individuals (two months) and 19/64, 21/50 and 32/75 individuals (four months) for dead/alive individuals in Virginia (VA), Catalonia (CAT) and Queensland (QLD), respectively. $P < 0.0024$ (Bonferroni adjusted) and $P < 0.001$ are shown by an asterisk (*) and double asterisk (**), respectively.

### 3.2. Plant size may differentially influence mortality across diverse environments

Plant size was estimated as the number of leaves on each individual, and reproductive stage as the presence of flowers and its total number of inflorescences across populations from diverse environments. The three populations showed significant differences in plant size (ANOVA test; $P < 0.001$). CAT had the largest individuals (mean of $17 \pm 0.36$ leaves per plant), followed by VA (mean of $7.21 \pm 0.36$ leaves per plant) and the smallest was QLD (mean of $5.5 \pm 0.14$ leaves per plant). Populations in both humid sub-tropical habitats (VA and QLD) were represented by a higher number of non-reproductive plants than CAT (Fig. 1). In

accordance, two months after field sampling (late summer), mortality was higher in reproductive plants in both VA and QLD (28.6 and 31.7 %, respectively; Fig. 1C), whereas CAT showed higher mortality in non-reproductive plants (but at low rates, with only 10 %) (Fig.1). Logistic regressions between mortality and plant size for each population revealed a significant relationship for non-native VA population only, in which smaller plants had lower survival probabilities than larger plants (Table S2). Reproductive investment was not significantly related to future mortality in any of the *P. lanceolata* populations (Table S2).

A comparative analysis of stress indicators, including RWC, LMA, photosynthetic pigments, lipid peroxidation markers, photo- and antioxidant protection and stress-related phytohormones between the three

studied populations revealed some physiological performance differences between native (CAT) and non-native (VA and QLD) populations (Figs. 3–5; Fig. S1-S3). LMA was three-fold higher in CAT compared to both VA and QLD populations (Fig. 3), the contents of lutein and β-carotene per unit of chlorophyll were 80–90 % lower in CAT compared to both VA and QLD populations (Fig. 5), and MDA contents were between 3- and 4-fold higher in CAT than VA and QLD populations (Fig. S2). Therefore, the native population with the lowest intrinsic mortality was characterised by having the largest individuals and a higher fraction of mature ones, showing the population in average smaller but thicker leaves (as indicated by higher intrinsic LMA), chloroplasts with less carotenoids per unit of chlorophyll (as indicated

particularly by the amounts of lutein and β-carotene per unit of chlorophyll), and constitutively higher MDA contents, thus indicating intrinsically higher lipid peroxidation.

## 4. Discussion

Harsh environmental conditions can trigger photo-oxidative stress and whole-organism mortality. Here, we found that some photoprotective and photo-oxidative stress markers measured during the summer (characterised by a combination of water deficit and high radiation) may be associated with increases in plant mortality during and after this stress period in *P. lanceolata* plants. Photoprotective molecules
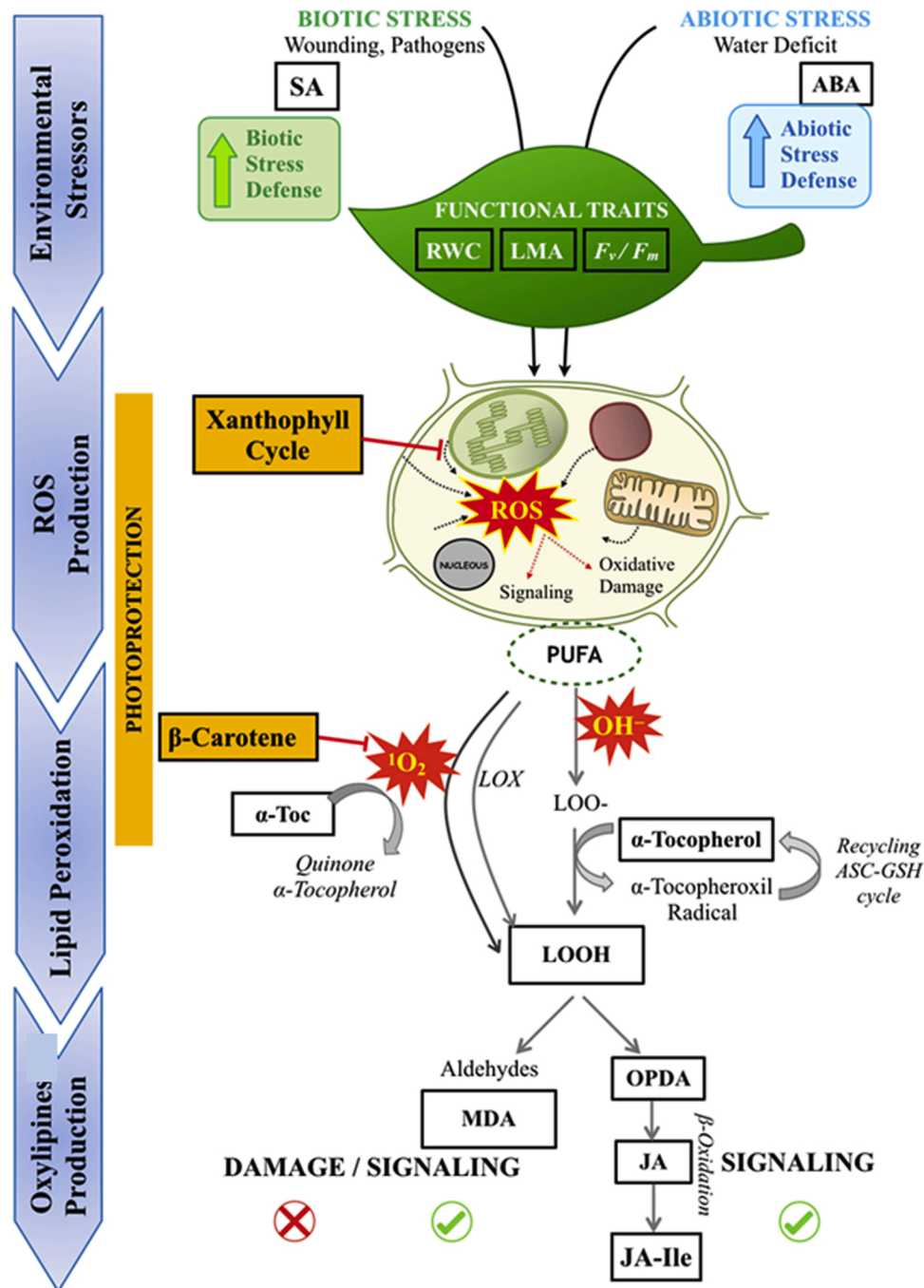


**Fig. 6.** Schematic diagram to illustrate the formation of lipid peroxidation products measured in this study, including lipid hydroperoxides, 12-*oxo*-phytodienoic acid (OPDA), jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile) and malondialdehyde acid (MDA), as well as its relationship between reactive oxygen species (ROS), antioxidants, and the hormonal interactions between abiotic and biotic stresses.

resulted to be particularly interesting to predict mortality at the organismal level in non-native populations of the cosmopolitan perennial herb *P. lanceolata*. Ecotypic variation occurs broadly in plant populations (Bradshaw, 1984). However, how individual plants handle multiple stresses is poorly understood under natural environments (Nguyen et al., 2016). In this study, a native population grown in a Mediterranean habitat was compared to two non-native populations located in humid sub-tropical climates, in southern-eastern USA and eastern Australia (GBIF, 2017). This study focused on individual performance differences between populations to understand physiological processes related to mortality under different environmental conditions. Multiple abiotic stressors enhance ROS accumulation that can trigger oxidative damage as a result of the imbalance between ROS production and ROS scavenging (Asada, 2006; Xie et al., 2019). Lower photoprotective capacity in plants from both non-native populations may promote a sustained accumulation of high ROS levels causing irreversible damage (Mittler, 2002) eventually leading to whole-plant mortality (Petrov et al., 2015; Xie et al., 2019), which is consistent with the higher mortality observed in these non-native populations (*ca.* 30 % in VA and QLD).

Carotenoids (xanthophylls and carotenes) act as antioxidants to mitigate photo-oxidative stress (Demmig-Adams and Adams, 1996; Esteban et al., 2015), in which zeaxanthin and β-carotene can physically quench singlet oxygen and other ROS, as therefore help maintaining photosystem membrane stabilisation in combination with tocopherols (Havaux, 1998; Trebst, 2003, 2005; simplified diagram represented in Fig. 6). The contents of lutein and β-carotene, together with those of zeaxanthin and the overall pool of the xanthophyll cycle (VZA), were more strongly reduced in plants from non-native populations that died after periods of lower precipitation and higher temperature, suggesting that photoprotection mechanisms play a key role in plant survival. Regarding the importance of carotenoids in leaves to mitigate photo-oxidative damage (Dall'Osto et al., 2006; Demmig-Adams and Adams, 2006; Ramel et al., 2012) under environmental stressors (Havaux, 2014), this study revealed how carotenoids per unit of chlorophyll can be used as a proxy of mortality risk in an invasive plant species. Therefore, this study shows that photoprotective molecules can serve as proxies for mortality risk in plants. However, strong inter-population variability and the complexity of association between various markers also indicate that these proxies of mortality are only useful to predict mortality within a given population and when used in combination with various physiological stress markers that give a complete picture of the physiology of plants. Furthermore, it is interesting to note that the quick signalling response of stress-related phytohormones to multiple stresses may also limit the potential of stress-related phytohormones as proxies of mortality in plants. Finally, it should be noted that the Fv/Fm values were never below 0.70 in any of the populations studied, thus supporting the idea that Fv/Fm is quite insensitive to environmental stress factors such as drought (see *e.g.*, Bussotti et al., 2020).

Both intrinsic and extrinsic factors were considered as potential predictors of individual mortality in this study. Although plant size-differences between populations were observed, only VA showed size-dependent mortality, in which smaller plants had lower survival probabilities than larger plants. Individuals from the wetter non-native environments had leaves with greater area per unit biomass (low LMA), a characteristic that has been described in most herbaceous and woody species across different climate conditions to be in general more sensitive to drought stress (Símová et al., 2016). These results support the idea that morphological features such as LMA can determine plant survival (Símová et al., 2016) and nicely illustrate how plants adapted to low water availability (*e.g.* CAT population in the native range) are less sensitive to sudden rainfall decrease during the summer than those growing in more humid habitats (VA and QLD populations in the non-native, invasive range). The present study is also in agreement with previous work showing that functional traits can be good proxies of

mortality risk in plants (Guadagno et al., 2017), and extends this knowledge showing photo-oxidative stress markers, in particular those related to photoprotection (specifically, the ratio of carotenoids per unit of chlorophyll and DPS) may be particularly useful for the study of invasion biology.

Both Multiple Logistic Regression and COX Proportional Hazard Regression models were run to test whether any of the covariates influence how long individuals survive within each population. Although these modelling approaches differ in terms of methodology, in both cases RWC and LMA were significant modulators of mortality through time in non-native populations. However, RWC and LMA were not associated with mortality neither in the native nor in the introduced habitats, when differences between alive and dead individuals were compared by ANOVAs. Although these parameters (which are very cheap and easy to measure) could help to predict plant mortality in other herbaceous perennials (Guadagno et al., 2017), it is unclear if they are valid for *P. lanceolata*, since various statistical approaches provide different results. It is likely the limited sampling size used in the present study prevents obtaining more reliable results for these stress markers when using ANOVA tests. In any case, it should be noted that while *P. lanceolata* was sensitive to summer season (drought events), in particular in the non-native populations studied here, other aspects must be considered to evaluate its invasive potential allowing plants by-passing this limitation and giving them the capacity to colonise new habitats. These mechanisms include the continuous introduction of genotypic diversity by humans (Smith et al., 2020) and its capacity to develop effective biological strategies for invasion, such as inhibiting the germination of native species, altering soil nutrient stoichiometry, combining clonal with sexual reproduction or developing a huge genotypic and phenotypic plasticity, as it has been described in this (Kuiper and Bos, 1992; Dietz et al., 2012; Smith et al., 2020) and other invaders (Conser and Connor, 2009; Novoa and González, 2014; Fenollosa et al., 2017).

In the context of global change with more severe and frequent drought events, studies to predict plant mortality are increasing, but unfortunately most of them are still focused on trees and native species (McDowell et al., 2008; Guadagno et al., 2017; Choat et al., 2018; Anderegg et al., 2019; Blackmann et al., 2019; McAllister et al., 2019; Menezes-Silva et al., 2019; Stovall et al., 2019). Under prolonged stress events, hydraulic failure and carbon starvation occur at the whole-plant scale, and membrane disruption at a cellular scale has been reported as a proxy of tree mortality (Guadagno et al., 2017). Although ROS trigger lipid peroxidation and membrane disruption under environmental stressors (both a/biotic; Noctor et al., 2018), the ability of oxidative stress markers to predict plant mortality has not been investigated to date, except for one case in two gymnosperms (*Picea engelmannii* and *Pinus contorta*) that experienced lethal water stress in the field and in laboratory conditions (Guadagno et al., 2017). Therefore, our study may be particularly useful and pave the way for future studies investigating the validity of photo-oxidative stress markers as proxies of mortality in invasive plants, an aspect that has not been investigated thus far in other invasive plants.

In conclusion, higher mortality at the individual level in the non-native populations was associated with a lower photoprotection capacity in English plantain. Although it would be clearly a mistake to rely on a single proxy of mortality, overall, mortality in *P. lanceolata* can be reflected at the whole-plant level by a combination of stress markers prior to death. Inter-population variability, clonal growth and previous life histories can limit, however, the validity of these markers as proxies of mortality if we aim at evaluating mechanisms underlying differences in mortality between various populations.

**Author contributions**

M.M., D.R., R.S.-G., and S.M.-B. conceived and designed the study; M.M., A.C., D.R., B.Q., R.S.-G and J.D. performed field sampling in their

respective field sites; M.M. performed all biochemical analyses; M.M. analysed the data with the help of B.Q.; M.M. and S.M.-B. wrote the first draft of the paper, with subsequent input from all co-authors.

## Declaration of Competing Interest

All the authors declare that they have no conflict of interest

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2021.104598.

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