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ORIGINAL ARTICLE

Photochemistry differs between male and female *Juniperus communis* L. independently of nutritional availability

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Abstract

Key message ***Juniperus communis males are better adapted than females to changing, seasonal environmental conditions due to their higher photosynthetic capacity and the higher concentration of photosynthetic pigments in their needles. Males cope with ROS more efficiently than females having greater carotenoids concentration in needles.***

Abstract In dioecious woody plants, females often exhibit greater reproductive effort than male plants and as a result, they can be more vulnerable to different stressors. We hypothesized that female plants of *J. communis* L. could have a lower photochemical capacity and a higher level of antioxidant enzyme activity and that these differences between males and females would be more pronounced under conditions where nutrient availability is limited. We also assume that additional stressors connected with different seasons would increase those differences. Male and female plants of *J. communis* growing in fertilized or non-fertilized soils were used to test this hypothesis. The effect of fertilization and sex on photochemical parameters derived from chlorophyll *a* fluorescence light curves, and on the concentrations of photosynthetic pigments in needles, was determined in different seasons within 2 years. To assess the tolerance of male and female plants to the nutrient deficit, antioxidant enzyme activity, and the level of reactive oxygen species (ROS) were determined. Results revealed sex-related differences in photochemical parameters, level of antioxidant enzyme activity, H_2O_2 levels, the concentration of photosynthetic pigments, and in the leaf mass-to-area ratio. This indicates that *J. communis* males could be better adapted than females to changing, seasonal environmental conditions due to their higher photosynthetic capacity, as reflected by their higher ETR_{max}, and a higher concentration of photosynthetic pigments in their needles. The sex-related differences concerning photosynthetic capacity and stress response found in our study are constitutive traits of each sex and are genetically based as they occurred independently of fertilization.

Keywords Dioecious plants · Chlorophyll *a* fluorescence · Nutrient stress · Photosynthetic pigments · Antioxidants

Abbreviations

ETR	Apparent electron transport rate (μmol m^{-2} s^{-1})
ETR_{max}	Maximum value of ETR
F_v/F_m	Maximum quantum yield of photosystem II photochemistry
F_m	Maximum fluorescence yield
F'_m	Maximum fluorescence in the light
F_0	Minimum fluorescence yield
F_s	Steady-state fluorescence
NPQ	Non-photochemical quenching of fluorescence
PPF	Photosynthetic photon flux (μmol m^{-2} s^{-1})
PPF_{sat}	Saturating level of photosynthetic photon flux
PSII	Photosystem II
Φ_{PSII}	Quantum yield of PSII photochemistry
$\Phi_{PPF_{sat}}$	Quantum yield of PSII photochemistry at the saturation level of PPF
SOD	Superoxide dismutase
POX	Guaiacol peroxidase
CAT	Catalase
APX	Ascorbate peroxidase

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Introduction

Dioecious species of plants form male and female flowers on separate individuals. Due to their different reproductive roles, sexes vary not only in the structural units they produce but also have differences in their morphology, physiology, and in their response to environmental conditions. The differences between sexes can be reflected by variations in growth (Lloyd and Webb 1977; Allen and Antos 1993; Obeso et al. 1998; Iszkuło and Boratyński 2011; Huang et al. 2018), the morphology of individual structures (Leigh and Nicotra 2003; Delph et al. 2011), physiology (Dawson and Bliss 1989; Obeso et al. 1998; Robakowski et al. 2018) as well as gene expression in flowers tissue (Sanderson et al. 2019).

Female plants of dioecious woody species often exhibit a greater reproductive effort than male plants (Obeso 2002; Montesinos et al. 2006) and require more favourable habitats to survive (Chen et al. 2015; Song et al. 2018). However, assessing reproductive investments in different genders is not an obvious task and recent studies revealed that measurements based on biomass may be less informative than those based on costs of nutrient and construction of the reproductive tissue (Lei et al. 2017b). Female plants have the potential to compensate costs associated with greater reproductive effort through increasing stomatal density, leaf area and gas exchange per unit leaf area as well as different strategies for N storage (Wallace and Rundel 1979; Kohorn 1994; Meagher 1999; Obeso 2002; Iszkuło et al. 2009; Montesinos et al. 2012; Iszkuło et al. 2013; Nowak-Dyjeta et al. 2017).

Dioecy is often associated with spatial segregation of male and female individuals, in particular a sex-related preference to inhabit specific types of microhabitats (Freeman et al. 1976; Hultine et al. 2016) and can result in a biased sex ratio (Field et al. 2013), especially within nutrient stress conditions (Song et al. 2018). Such a separation helps to limit intraspecific and cross-sex competition, which can influence the fitness of at least one sex (Chen et al. 2014).

Minerals greatly influence plant growth and different levels of species-specific tolerance to nutrient levels do exist (Laliberté et al. 2012), which can be observed by different nutrient requirements and tolerance to different soil N:P ratios (Güsewell and Koerselman 2002; Güsewell and Bollens 2003). The concentration of nutrients in plant tissues differ depending on environmental conditions and species (Kutbay and Ok 2003; Schmidt et al. 2010; Sardans et al. 2012) as well as leaf age (Miller et al. 1990) and can have a significant impact on many different biochemical processes (Pozo et al. 1999; Hermans et al. 2006; Ren et al. 2010). Plants have evolved mechanisms that allow them to adapt to and survive limited nutritional environments by altering their metabolism and allocation of biomass (Hermans et al. 2006; Rouached et al. 2010); however, fertilized plants can generally cope more readily with various stresses than plants with limited access to resources (Starck et al. 2000). Notably, when several stresses are exerted together, negative impacts on plant physiology increase, and plant survival is more threatened (Holopainen and Gershenzon 2010).

Stress related with nutrient availability can alter the process of the photosynthesis as well as the production of the reactive oxygen species (ROS). Photosynthetic capacity and efficiency can be indirectly determined by measuring chlorophyll *a* fluorescence (Retuerto et al. 2006; Simancas et al. 2016). A decline in photosynthesis, defined as photoinhibition, is observed when plant is exposed to stressors (Adams et al. 2004) and can be assessed by measuring maximum quantum yield of photosystem II (PSII) photochemistry (F_v/F_m). F_v/F_m values lower than 0.8 are considered as a good indicator of photoinhibition (Björkman and Demmig 1987; Lüttge et al. 2003). Plants protect themselves from photoinhibition increasing losses of excessive energy as heat by non-photochemical quenching of fluorescence (NPQ) (Maxwell and Johnson 2000; Szabó et al. 2005; Kromdijk et al. 2016). We assume that NPQ can be higher in female plants, which often have greater costs of reproduction and are more sensitive to stress injury (He et al. 2016). Numerous studies, however, have reported no sex differences in NPQ values (de la Bandera et al. 2008; Ait Said et al. 2013).

Reactive oxygen species (ROS), which regulate the redox state and function as regulators of energy and metabolic fluxes, are generated during photosynthesis (Foyer 2018). Maintaining antioxidant systems that regulate ROS levels in cells is vitally important for maintaining high photosynthetic rates as ROS are capable of damaging electron transport chain structures. ROS, however, can also function as signalling molecules in plants (Foyer and Shigeoka 2011). Plants produce antioxidant enzymes, such as catalase (CAT) and ascorbate peroxidases (APX) that scavenge hydrogen peroxide, as well as superoxide dismutase (SOD) which scavenges superoxide. In response to stress conditions, the activity of these enzymes tends to increase more sharply in female than in male individuals (Gupta et al. 2012; He et al. 2016), but not always (Lu et al. 2014). An increase in SOD and APX activity can be induced by insufficient nitrogen, phosphorus, and potassium (Tewari et al. 2007) and nutrient limitation can impact the internal structures of cells and the concentration of photosynthetic pigments (Tewari et al. 2007). Other stresses can also induce changes in antioxidant enzyme activity (Alguacil et al. 2006) and the degree of alteration can differ between male and female (Zhang et al. 2010).

In the present study, the effect of long-term nutrition limitation on the photosynthetic apparatus and antioxidant

system in male and female plants of common juniper (*Juniperus communis* L.) was investigated. *Juniperus communis* is a dioecious, evergreen shrub or small tree and is described as a pioneer species that plays an important role in natural forest succession (Thomas et al. 2007). It naturally occurs in cool, temperate areas of the Northern Hemisphere; including Asia, Europe, and North America (Adams et al. 2003). Despite its wide range, decreases in the population of this species have been occurring in Europe (Oostermeijer and De Knegt 2004; Verheyen et al. 2009). A male-biased sex ratio is also observed in many populations of this species, which may be related to stress conditions as more male than female individuals occur in sites with limited water availability (Ortiz et al. 2002). After reaching maturity, male plants have a greater radial growth rate (Iszkuło and Boratyński 2011) and smaller needle area (Nowak-Dyjeta et al. 2017) than female plants.

We hypothesized that male and female *J. communis* plants would exhibit constitutive differences in several physiological parameters and those differences would be more pronounced in nutrient limited environment.

Materials and methods

Plant material

The study was conducted at the Institute of Dendrology, Polish Academy of Sciences in Kórnik, Poland. Shoots of *J. communis* L. were rooted in 2012. Specifically, fifty shoots were collected from each of the ten male (♂) and ten female (♀) mature plants growing in the Rokita forest district, Western Pomerania, Poland. Cuttings of a similar size of about 10 cm were taken from the middle part of each crown, growing in similar light conditions. In total, 1000 shoots were rooted and kept in a greenhouse in 10 L pots in which they were grown till the end of the experiment. They were placed under 2-m-high scaffolding that was covered with a shading net (reducing full sunlight by 50%). The relative photosynthetic photon flux density inside the shaded canopy was measured using a line quantum sensor (Apogee Inc.) according to the method of Messier and Puttonen (1995). The soil substrate used in the experiment was taken from a mixed broadleaved forest and 10% of the total soil volume originated from the location in which the maternal plants grew to introduce mycorrhizae.

Experimental design and sampling

In March 2013, all seedlings were randomly divided into two blocks containing both genders, and then within each block, two fertilization groups were established. Half of the seedlings were fertilized and plants were described as fertilized plants (F), whereas the remaining plants were grown without any fertilizer and were described as non-fertilized plants (NF). Plants from the same paternal or maternal origin were present in both treatment groups with the same number of individuals in each group. Since 2013, F plants were fertilized every year after flowering in May. The fertilizer consisted of 6 grams of slow-release fertilizer (15.0% N, 9.0% P, 12.0% K, 2.5% MgO and microelements, Osmocote Exact, ICL, Israel) per liter. Plants in the NF group were grown without any fertilizer. Pots were placed at the same distance of 30 cm to avoid density effects. Each plant was irrigated separately with an automatic drop irrigation system. As fertilization increases the growth of plants and it is related to water availability, plants from both groups were irrigated with different water doses. Fertilized plants were irrigated twice more than nonfertilized plants to keep the medium soil moisture during the whole vegetation season.

Measurements were conducted during two following years and 1 year needles were sampled four times per year (May, July, October, January) during 2015 and 2016. Sampling dates were linked to plant phenology with the flowering beginning in May, the most intensive plant growth occurring in July, biomass allocation primarily to roots in September/October, and a period of dormancy in January. Needles were collected from three similarly shaded branches, located one-third of the way below the top of plants. At each sampling point, 1 year needles from 32 seedlings were used for all analysis (4 seedlings × 2 fertilization treatments × 2 sexes × 2 blocks). Seedlings were randomly chosen; however, seedlings from the same genotype were analyzed from both treatment groups to reduce the impact of genotype on the investigated physiological traits.

Microclimate

Temperature and humidity were monitored by four EL-USB-2 + data loggers (EasyLog, Inc.) placed near the top of plants. Meteorological parameters were measured every hour over the 2 year duration of the study. Monthly mean, minimal, and maximal temperatures, as well as monthly mean relative humidity values, occurring over the sampling period are presented in Table 1. Monthly mean temperatures varied between 20.3 °C ± 7.9 °C (mean ± SE) in July, 2016 and −0.7 °C ± 4.6 °C in January, 2017. The highest temperature occurred in July, 2015 and the lowest in January, 2016. The difference between the highest and lowest temperature over the course of the entire study was 60.5 °C. The lowest relative humidity occurred at the beginning of the experiment in May, 2015 ($RH_{min} = 65.0 \pm 23.6$) and the highest was observed at the end of the experiment, in January, 2017 ($RH_{max} = 100.0 \pm 7.5$). The difference between

Table 1 Air temperature and relative humidity monitored over the course of the 2-year study

Dates		Monthly temperature (°C)			Relative air humidity (%)
		Mean	Minimum	Maximum	
I Year	May 2015	12.8 ± 8.7	−2.5	35.5	65.0 ± 23.6
	July 2015	18.9 ± 8.4	4.0	45.0	71.4 ± 22.9
	October 2015	13.0 ± 7.8	−2.0	38.0	73.8 ± 19.2
	January 2016	1.0 ± 7.0	−15.5	14.5	92.4 ± 8.2
II Year	May 2016	11.4 ± 8.3	−3.5	36.0	69.6 ± 23.8
	July 2016	20.3 ± 7.9	4.5	44.5	72.7 ± 23.4
	October 2016	14.3 ± 7.9	0.0	40.0	78.8 ± 21.3
	January 2017	−0.7 ± 4.6	−15.0	10.5	100.0 ± 7.5

Data were registered hourly near plants of *J. communis*. Mean monthly, minimum and maximum temperatures and mean monthly values of relative humidity are presented (means with standard errors, $n = 744$)

the lowest and the highest mean relative humidity over the course of the study was 35.6%.

Determination of enzyme activity and the level of reactive oxygen species (ROS)

The activity of ascorbate peroxidase (APX, EC.1.11.1.11), superoxide dismutase (SOD, EC. 1.15.11), guaiacol peroxidase (GP, EC. 1.11.1.7), and catalase (CAT, EC 1.11.1.6) in the cytosolic fraction of needle samples was determined. ROS [superoxide (O_2^-) and hydrogen peroxide (H_2O_2)] levels were also measured.

All enzyme assays were performed on samples of whole, 1 year needles and the same crude extract was used to determine the activity for all of the measured enzymes. All extraction procedures were carried out at 4 °C. Samples were ground in liquid nitrogen and extracted for 1 h in 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mM EDTA and 20% polyvinylpolypyrrolidone. Homogenates were filtered through two layers of cheesecloth and were subsequently centrifuged at 4 °C at 20,000*g* for 20 min. The supernatants were used to determine enzyme activity assays after they were desalted on a Sephadex G25 (Sigma-Aldrich) standard column as described by Helmerhorst and Stockes (1980).

APX activity was measured according to Nakano and Asada (1981) and was based on the decrease in absorbance at 290 nm due to ascorbic acid (ASA) oxidation for 5–10 min. The first step of the reaction included the addition of H_2O_2 with low oxidation of ASA by H_2O_2 as a control. The reaction was conducted in a mixture containing 50 µl of the enzyme extract, 1 ml of 0.68 mM ASA, 1 ml of 4 mM H_2O_2, and 0.1 mM EDTA in 0.1 M phosphate buffer at pH 7.0. APX activity was expressed as nmol ASA min^{-1} mg $protein^{-1}$.

Catalase activity was assessed as the ability to catalyze the decomposition of H_2O_2 according to the procedure described by Chance and Maehly (1955). The reaction was initiated by the addition of H_2O_2 to the crude extract. Two controls were used: one without H_2O_2 and one without enzyme extract. The reaction mixture contained 50 µl enzyme extract, 1 ml of 30 mM H_2O_2, and 1 ml of 0.1 M phosphate buffer, pH 7.0. The decrease in absorbance was measured at 240 nm. CAT activity was expressed as mmol H_2O_2 min^{-1} mg $protein^{-1}$.

POX activity was measured accordingly as described by Chance and Maehly (1955). The reaction was initiated by the addition of H_2O_2 to the crude extract. Two controls were used: without H_2O_2 and without guaiacol. The reaction was conducted in a mixture containing 50 µl of enzyme extract, 1 ml of 1% guaiacol, 1 ml of 0.2 mol/l H_2O_2, and 1 ml of 0.1 mol/l phosphate buffer, pH 7.0. POX activity was measured as the oxidation of guaiacol at 470 nm ($\varepsilon = 26.6$ mM^{-1} cm^{-1}) and was expressed as mmol H_2O_2 min^{-1} mg $protein^{-1}$.

SOD activity was determined as described by Giannopolitis and Ries (1977). The ability of SOD to inhibit the photochemical reduction of 4-nitroblue tetrazolium chloride (NBT) was measured as described in Pukacka and Pukacki (2000). A non-irradiated reaction mixture was used as a control. The reaction mixture contained 50 µl enzyme extract, 63 mM NBT, 1.3 mM riboflavin, 0.1 mM dithiothreitol, and 50 µM phosphate buffer. The reaction mixture from test samples was placed in glass test tubes and illuminated. The amount of enzyme required to cause 50% inhibition of the rate of NBT reduction was defined as one unit of SOD activity (U mg^{-1} protein).

Bovine serum albumin was used as a standard to estimate protein concentration of crude enzyme extracts (Bradford 1976).

ROS levels were determined on the same day, on freshly sampled needles that had been placed on ice immediately after collection. The level of superoxide (O_2^-) was determined according to the method described by Doke (1983). The capacity to reduce nitroblue tetrazolium (NBT) in the dark at room temperature was determined. Samples were incubated in 3 ml of 0.05 M K-phosphate buffer (pH 7.8) containing 0.05% NBT and 10 mM sodium azide, at room temperature in the darkness, for 30 min. Then, 2 ml of sample were heated to 85 °C for 15 min and subsequently cooled on ice. The final product absorbance was measured at 530 nm and O_2^- content was expressed as ΔA530 g fresh $weight^{-1}$. Finely powdered fresh needles were homogenized

in 5% trichloroacetic acid containing 10 mM EDTA to measure hydrogen peroxide (H_2O_2). The homogenate was centrifuged at 26,000*g* for 20 min and 4 °C. The ferrithiocyanate method was used to analyze the total amount of hydrogen peroxide in the supernatant (Sagisaka 1976).

Chlorophyll and carotenoids concentration

Chlorophyll and carotenoid concentration were determined according to the methods described by Barnes et al. (1992). Approximately, 25 mg of fresh needles were cut into small pieces (about 1 mm^2) and incubated for 4 h in 4 ml of 100% dimethyl sulfoxide (DMSO) saturated with $CaCO_3$ at 60 °C. The incubation continued until needles lost all color. The absorbance of the extract was measured at 665, 648, and 470 nm in a Cary 60 UV–Vis spectrophotometer (Agilent Technologies). A solution of DMSO was used as a blank.

Measurements of chlorophyll *a* fluorescence

Fluorescence measurements were conducted using a Fluorescence Monitoring System (FMS 2, Hansatech, Norfolk, UK) operating in an online mode according to the procedure described by Robakowski et al. (2018). On each occasion, needles were harvested from four plants per fertilization treatment per sex per block ($n = 8$), wrapped in moist paper, placed in an Eppendorf tube, and left in darkness for 30 min at room temperature (21–22 °C, min–max) before measuring fluorescence. Then, 4–5 needles were placed close to each other, abaxial surface facing down, on a piece of transparent, self-adhesive tape, and introduced into a leaf clip. Needles among those collected for the fluorescence measurements were also used to determine a leaf mass-to-area ratio (LMA).

A minimum (F_0) and a maximum fluorescence values (F_m) as well as steady state fluorescence (F_s) and maximum light-adapted fluorescence (F'_m) were measured accordingly to Robakowski et al. (2018). Maximum quantum yield of PSII photochemistry was calculated according to the following formula: $F_v/F_m = (F_m - F_0)/F_m$.

A quantum yield of PSII photochemistry (Φ_{PSII}) was calculated for each light level according to the formula: $\Phi_{PSII} = (F'_m - F_s)/F'_m$ (Genty et al. 1989); non-photochemical quenching of fluorescence (NPQ) was calculated with $NPQ = (F_m - F'_m)/F'_m$ (Maxwell and Johnson 2000); an apparent electron transport rate (ETR) with $ETR = \alpha \times \Phi_{PSII} \times 0.5 \times PPF$, where α—needle absorptance and 0.5 factor means the assumption that both photosystems obtained an equal amount of excitation energy (Maxwell and Johnson 2000).

Since leaf absorptance may be species specific and undergo adaptation to microclimate conditions, the leaf absorptance value was calculated from total chlorophyll concentration according to the empirical model provided by Evans (1993).

Determination of cardinal points of light response curves

To determine the cardinal points of light response curves, the maximum apparent rate of photosynthetic electron transport of PS II (ETR_{max}) and the saturation level of photosynthetic photon flux density (PPF_{sat}), were fitted to the functions developed by Ye et al. (2013): $ETR_{max} = \alpha\left(\frac{\sqrt{\beta+\gamma}-\sqrt{\beta}}{\gamma}\right)^2$ and $PPF_{sat} = \frac{\sqrt{(\beta+\gamma)/\beta}-1}{\gamma}$, where α—initial slope of light response curve of ETR, β—range of light response curve of dynamic down-regulation of PSII and γ—a saturation term of light response curve for photosynthetic electron transport rate (ETR-PPF; where PPF is photosynthetic photon flux). ETR values increase with increasing PPF until it reaches saturation irradiance (PPF_{sat} at which $ETR = ETR_{max}$), but then decreases because of down-regulation of PSII in high irradiance.

To determine the maximum value of quantum yield of PSII photochemistry at the saturation value of PPF ($\Phi_{PPF_{sat}}$), the following formula was used (Rascher et al. 2000; Robakowski 2005):

$\Phi_{PPF_{sat}} = m + ae^{-bPPF_{sat}} + ce^{-dPPF_{sat}}$, where m, a, e, b, c, d are independent parameters.

PPF versus NPQ was fitted with the exponential function: $NPQ = m + a(1 - e(-bPPF))$.

NPQ increases to infinity with actinic light (Maxwell and Johnson 2000). Therefore, values of NPQ_{530} at the arbitrary chosen PPF value = 530 µmol m^{-2} s^{-1} were used in statistical analysis.

Statistical analysis

All data were tested for normality with the Shapiro–Wilk's test and the homogeneity of variance was evaluated using the Levene's test. Data were subsequently analyzed using a two-way ANOVA with sex and fertilization treatment as the sources of fixed effects and with blocks as the source of random effects. Analyses were run at each sampling date. When the interaction between sex and fertilization treatment occurred, the Tukey's post hoc test was used to find differences between means. Means were considered to differ statistically at $P < 0.05$. Data were presented as means with standard errors (SE). Statistical analysis was conducted using JMP 13 software (SAS Institute Inc., Cary, NC, USA 1989–2007). Figures were prepared in R package (R Core Team 2018).

Results

Light response curves

Curves for Φ_{PSII} and ETR vs. PPF were both higher in fertilized individuals than in non-fertilized individuals and the mean values of Φ_{PSII} and ETR were significantly different between the fertilization treatments from the low PPF values (Fig. 1a, b). The fertilization treatment also affected the loss of absorbed energy from needles as heat, expressed as NPQ, which was significantly higher in non-fertilized plants in the range of 45 µmol m^{-2} s^{-1} to 530 µmol m^{-2} s^{-1} actinic PPF (Fig. 1c). Similarly to fertilization, the sex also had a significant effect on the shape of curves of Φ_{PSII}, ETR, and NPQ vs. PPF. Female (♀) plants had significantly lower values of Φ_{PSII} than male (♂) plants (e.g., ♀: 0.12 ± 0.01 vs. ♂: 0.14 ± 0.01 at 783 µmol m^{-2} s^{-1} of fluorescence induction light, Fig. 1d) and ETR (e.g., ♀: 59.78 ± 4.03 vs. ♂: 71.62 ± 4.81 at 1075 µmol m^{-2} s^{-1} of fluorescence induction light, Fig. 1e), but significantly higher values of NPQ (e.g., ♀: 2.40 ± 0.08 vs. ♂: 2.28 ± 0.08 at 345 µmol m^{-2} s^{-1} of fluorescence induction light, Fig. 1f).

Time-related changes in chlorophyll *a* fluorescence-based parameters

All of the chlorophyll *a* fluorescence-based parameter (PPF_{sat}, ETR_{max}, F_v/F_m, $\Phi_{PPF_{sat}}$ and NPQ_{530}) differed significantly between the F and NF (Fig. 2a–e, S-Table 1). ETR_{max} had significantly higher values in fertilized plants within all but one sampling time points (e.g., July, 2016 F: 74.86 ± 3.74 vs. NF: 54.58 ± 3.74, $P = 0.001$, Fig. 2a). Lower $\Phi_{PPF_{sat}}$ values were observed in general in fertilized than non-fertilized plants (e.g., July, 2016 F: 0.12 ± 0.004 vs. NF: 0.14 ± 0.004, $P = 0.038$, Fig. 2b). However, fertilized plants had higher values of F_v/F_m (e.g., July, 2016: 0.92 ± 0.002, Fig. 2c) and PPF_{sat} (e.g., July, 2016: 1226.43 ± 56.44, Fig. 2d) when compared to non-fertilized plants (F_v/F_m: 0.91 ± 0.002, $P = 0.018$; PPF_{sat}: 803.82 ± 56.44, $P < 0.001$). Values of NPQ_{530} were significantly different between fertilized and non-fertilized plants only within two sampling time points (in July 2015 and October 2016, Fig. 2e).

In May 2015 and in July 2016, ETR_{max} values were higher in male when compared to female plants (e.g., July 2016 ♂: 71.51 ± 3.74 vs. ♀: 57.93 ± 3.74, $P = 0.016$, Fig. 3a). Values of F_v/F_m were significantly different between the sexes; however, it was true within two sampling points (May 2015 and January 2016, Fig. 3b). These values were on one date higher in females (May

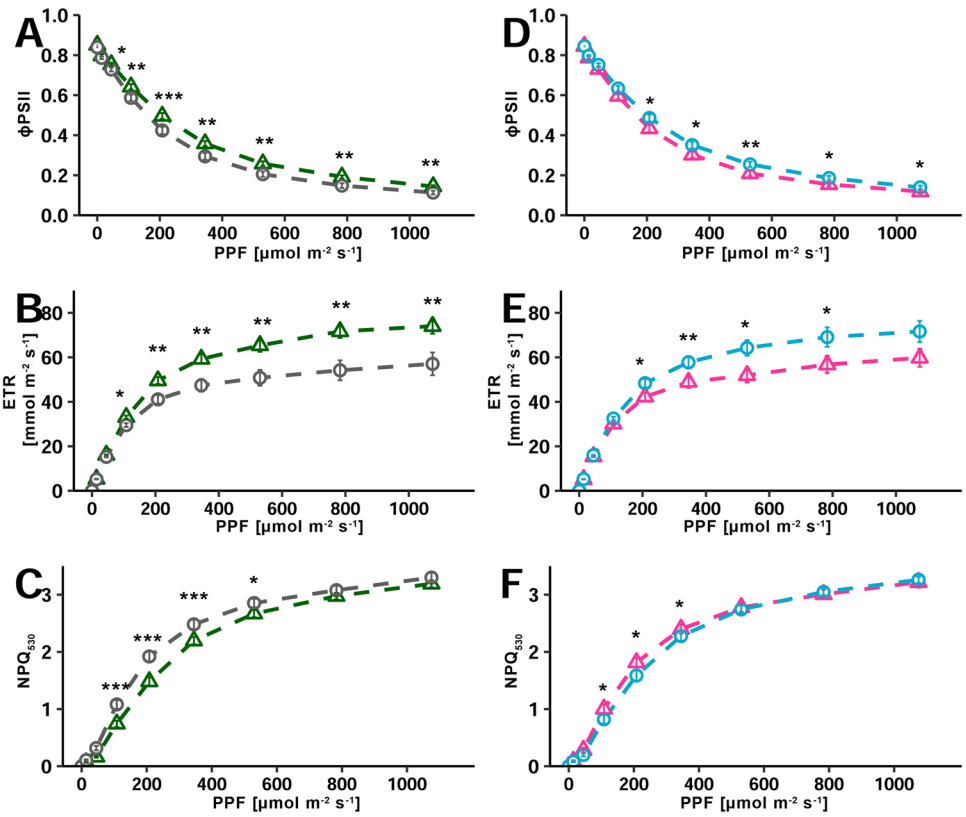

Fig. 1 Photosystem II (PSII) quantum yield (Φ_{PSII}, **a**, **d**), apparent electron transfer rate (ETR, **b**, **e**) and non-photochemical quenching of fluorescence (NPQ, **c**, **f**) versus photosynthetic photon flux (PPF) in needles from fertilized (green) vs. non-fertilized (grey) (**a**, **b**, **c**) plants of *Juniperus communis* and male (blue) vs. female (pink) plants (**d**, **e**, **f**). Measurements of chlorophyll *a* fluorescence were conducted in July, 2016. Data are means with standard errors (SE, $n = 16$). Asterisks indicate significant differences between fertilization treatments or sexes at each value of PPF ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$)

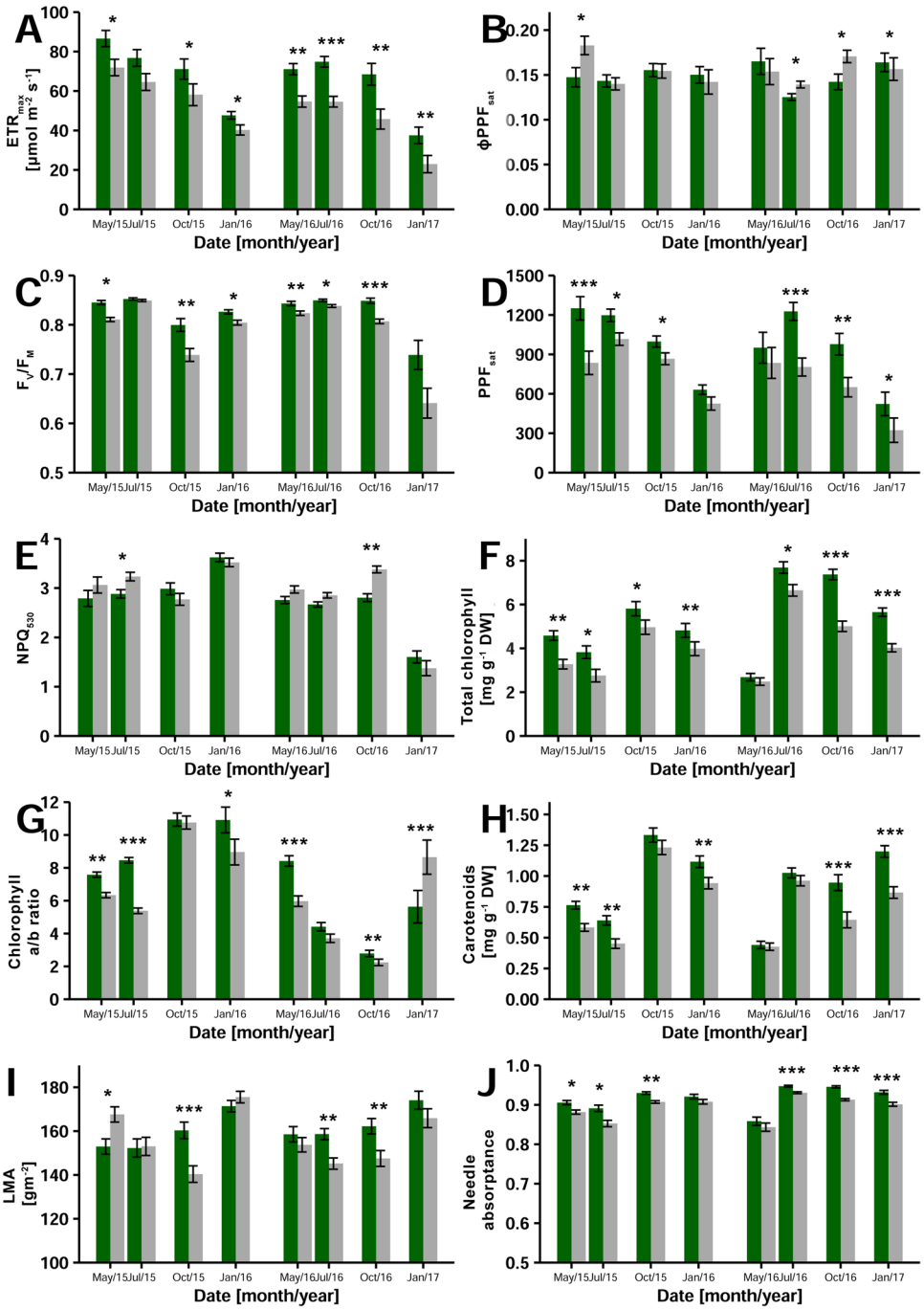

Fig. 2 Seasonal changes in photosynthetic parameters in needles of *Juniperus communis* L. individuals of plants grown with (green) or without fertilization (grey). **a** Apparent maximum electron transport rate (ETR_{max}) (µmol m^{-2} s^{-1}); **b** quantum yield of PSII photochemistry at the saturation value of photosynthetic photon flux ($\Phi_{PPF_{sat}}$); **c** maximum quantum yield of PSII photochemistry (F_v/F_m); **d** saturation photosynthetic photon flux corresponding to maximum electron transport rate (PPF_{sat}) (µmol m^{-2} s^{-1}); **e** non-photochemical quenching of fluorescence at PPF = 530 µmol m^{-2} s^{-1} (NPQ_{530}); **f** total chlorophyll (mg g^{-1} DW); **g** chlorophyll *a/b* ratio; **h** carotenoids (mg g^{-1} DW); **i** leaf mass-to-area ratio (LMA, gm^{-2}); **j** needle absorptance**.** Data are means with standard errors (SE, $n = 16$). Asterisks indicate statistical differences between means at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

2015 ♀: 0.912 ± 0.001 vs. ♂: 0.907 ± 0.001, $P = 0.050$), otherwise in males (January 2016 ♀: 0.897 ± 0.003 vs. ♂: 0.909 ± 0.003, $P = 0.012$). Moreover, male plants had higher values of $\Phi_{PPF_{sat}}$ (in January 2017 ♂: 0.17 ± 0.01, Fig. 3c) and NPQ_{530} (in July 2015 ♂: 3.24 ± 0.10, Fig. 3d) than female plants ($\Phi_{PPF_{sat}}$ ♀: 0.15 ± 0.01, $P = 0.05$; NPQ_{530} ♀: 2.88 ± 0.10, $P = 0.020$). No interaction between fertilization treatment and sex was noticed for values of chlorophyll *a* fluorescence-based parameters.

Time course changes in total chlorophyll and carotenoids concentration, absorptance and leaf-mass-to-area ratio

Total chlorophyll, chlorophyll *a/b* ratio, carotenoid concentration as well as LMA values were affected by lack of fertilization regardless to sampling date (Fig. 2f-j; S-Table 2). Non-fertilized plants had significantly lower concentration of total chlorophyll than fertilized plants, especially in autumn (e.g., October 2016 NF: 5.01 ± 0.32 vs. F: 7.37 ± 0.32,

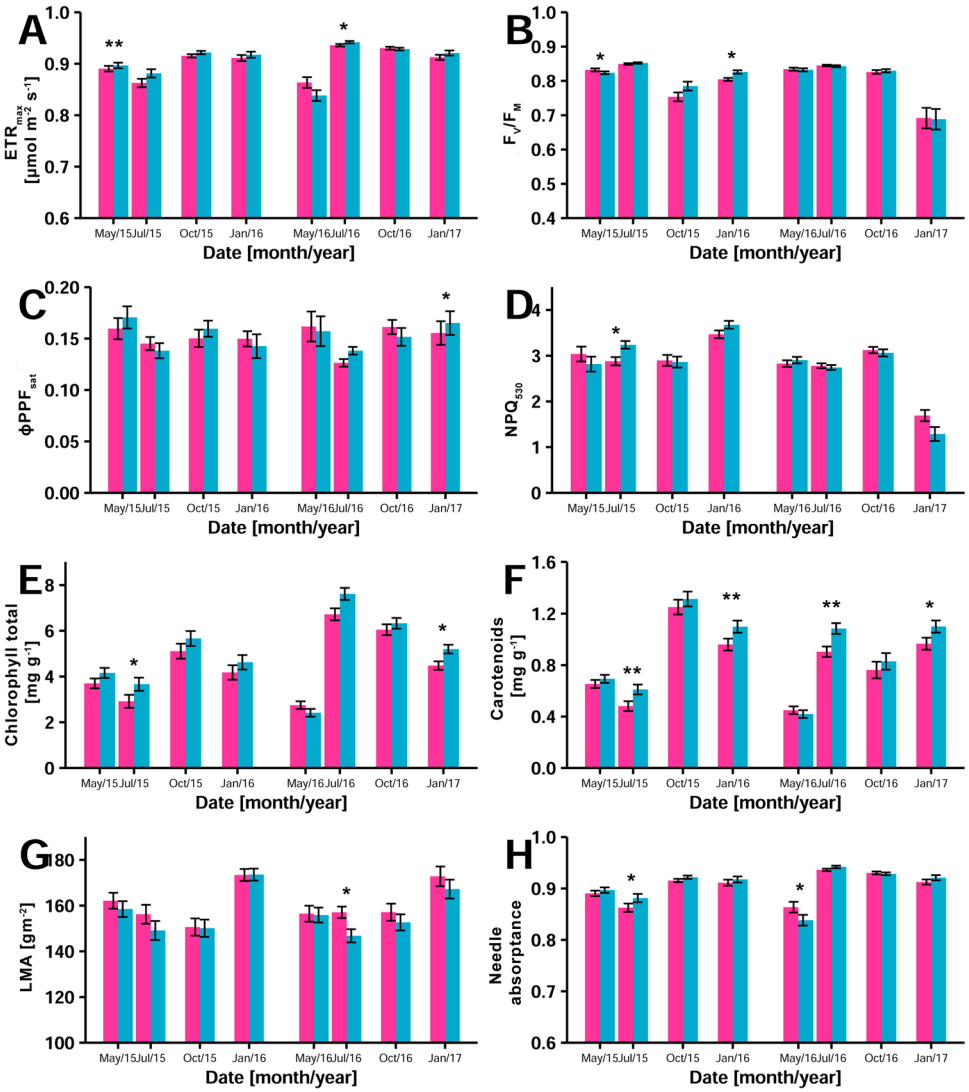

Fig. 3 Seasonal changes in leaf parameters in needles of male (blue) or female (pink) *Juniperus communis* L. plants: **a** apparent maximum electron transport rate (ETR_{max}) (µmol m^{-2} s^{-1}); **b** maximum quantum yield of PSII photochemistry (F_v/F_m); **c** quantum yield of PSII photochemistry at the saturation value of photosynthetic photon flux ($\Phi_{PPF_{sat}}$), **d** non-photochemical quenching of fluorescence at PPF = 530 µmol m^{-2} s^{-1} (NPQ_{530}); **e** total chlorophyll (mg g^{-1} DW); **f** carotenoids (mg g^{-1} DW); **g** leaf mass-to-area ratio (LMA, gm^{-2}); **h** needle absorptance. Data are means with standard errors (SE, $n = 16$). Asterisks indicate statistical differences between means at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

$P < 0.0001$) and winter (e.g., January 2017 NF: 3.58 ± 0.13 vs. F: 4.7 6 ± 0.14, $P < 0.0001$, Fig. 2f) as well as by lower chlorophyll *a*/*b* ratio (Fig. 2g). Moreover, non-fertilized plants were characterized by lower carotenoid concentration (e.g., October 2016 NF: 0.64 ± 0.04 vs. F: 0.95 ± 0.04, $P < 0.0001$; January 2017: 0.87 ± 0.04 vs. F: 1.20 ± 0.04, $P < 0.0001$, Fig. 2h). This was accompanied by lower LMA in autumn (e.g., October 2016 NF: 147.52 ± 3.95 vs. F: 162.30 ± 3.83, $P = 0.0127$), but not in winter (e.g., January 2017 NF: 165.38 ± 4.37 vs. F: 174.10 ± 4.23, $P = 0.189$, Fig. 2i) and by lower needle absorptance observed in non-fertilized plants at both sampling dates (e.g., October 2016 NF: 0.91 ± 0.003 vs. F: 0.95 ± 0.003, $P < 0.0001$; January 2017 NF: 0.90 ± 0.01 vs. F: 0.93 ± 0.01, $P < 0.0001$, Fig. 2j).

Female plants had significantly lower total chlorophyll concentration in July 2015 (♀: 2.91 ± 0.20 vs. ♂: 3.66 ± 0.20, $P = 0.015$) and January 2017 (♀: 4.48 ± 0.18 vs. ♂: 5.20 ± 0.19, $P = 0.010$, Fig. 3e). In both years, female had lower carotenoid concentration than male plants, and significant differences between sexes occurred in July (e.g., in 2016 ♀: 0.90 ± 0.04 vs. ♂: 1.08 ± 0.04, $P = 0.004$) and January (e.g., in 2016 ♀: 0.96 ± 0.03 vs. ♂: 1.10 ± 0.03, $P = 0.007$; Fig. 3f, Table S1). Moreover, the significant interaction between the sex and fertilization treatments on carotenoids concentration were detected in January 2015 ($P = 0.004$) and in June 2016 ($P = 0.029$). In January 2015, fertilized male plants (F ♂: 1.26 ± 0.05 a) had the higher carotenoid concentration than other groups (F ♀: 0.97 ± 0.05 b; NF ♀: 0.95 ± 0.05 b and NF ♂: 0.94 ± 0.05 b, $P = 0.004$). In July 2016, carotenoid concentration had the highest value in males regardless to fertilization (NF ♂: 1.12 ± 0.06 a and F ♂: 1.05 ± 0.06 a), whereas non-fertilized females had the lowest value (♀: 0.81 ± 0.06 b) and fertilized female plants were similar to all of above (♀: 1.00 ± 0.06 ab, $P = 0.0285$). Female plants had also higher LMA, but significant differences were observed only in July 2016 (♀: 156.78 ± 3.13

vs. ♂: 147.35 ± 3.23, $P = 0.0465$, Fig. 3g). Moreover, the significant interaction between sex and fertilization treatment on LMA was observed in June 2016 ($P = 0.048$), thus non-fertilized male plants (NF ♂: 135.8 ± 4.57 b) had the lowest LMA value when compared with other groups of plants [female non-fertilized (NF ♀: 154.58 ± 4.57 a), male fertilized (F ♂: 158.91 ± 4.57 a) and female fertilized (F ♀: 158.97 ± 4.27 a)]. Needle absorptance was significantly lower in female plants (♀: 0.86 ± 0.006) when compared with male plants (♂: 0.88 ± 0.006; $P = 0.05$) in July 2015; however, in May 2016, the opposite relationship was observed—female (♀: 0.864) had higher needle absorptance than male plants (♂: 0.840 ± 0.007, $P = 0.018$, Fig. 3h).

Antioxidant enzymes activity and reactive oxygen species

Needles for fertilized (F) plants exhibited higher enzyme activity and a lower concentration of free radicals (O_2^- and H_2O_2) than needles from non-fertilized (NF) plants, especially within the first season of sampling (Fig. 4, S-Table 3). The activity of most of the antioxidant enzymes was higher in fertilized plants in July, 2015 (APX 1.27 ± 0.04; POX 0.21 ± 0.01; SOD 37.77 ± 1.78) than in non-fertilized plants (APX 1.00 ± 0.04, $P = 0.002$; POX 0.14 ± 0.01, $P = 0.005$; SOD 29.29 ± 1.78, $P = 0.005$) (Fig. 4a, c, d). In contrast, only the activity of CAT was higher in fertilized plants (0.21 ± 0.02) than in non-fertilized plants (0.15 ± 0.01, $P = 0.005$, Fig. 4b) in July, 2016. Additionally, the level of free radicals on this sampling date was lower in needles of fertilized plants (O_2^- 1.53 ± 0.22; H_2O_2 24.33 ± 2.79) than in needles of non-fertilized plants (2.55 ± 0.27, $P = 0.007$ and 35.03 ± 2.79, $P = 0.011$, respectively, Fig. 4e, f).

Plant sex had a significant effect on antioxidant enzyme activity and on the level of H_2O_2 (Fig. 5a–d). Specifically, in October, 2015, higher level of H_2O_2 was observed in needles of female plants (♀: 27.92 ± 2.02 vs. ♂: 20.76 ± 1.95, $P = 0.017$, Fig. 5a) and also SOD activity was lower in needles of female plants (24.80 ± 1.90) than in needles of male plants (30.29 ± 1.90, $P = 0.046$, Fig. 5c). During October, 2016, the level of H_2O_2 was lower in needles of female plants (74.49 ± 2.84) than in needles of male plants (84.76 ± 2.84, $P = 0.036$), which was inversely related to the level of APX activity observed in needles of female (♀: 1.16 ± 0.10 vs. ♂: 0.85 ± 0.10, $P = 0.048$, Fig. 5b). Male plants had higher CAT activity (♂: 0.085 ± 0.007) than female plants (♀: 0.063 ± 0.007, $P = 0.033$, Fig. 5d) in January 2016. No interaction between fertilization treatment and sex was observed for enzymes activity and the concentration of free radicals.

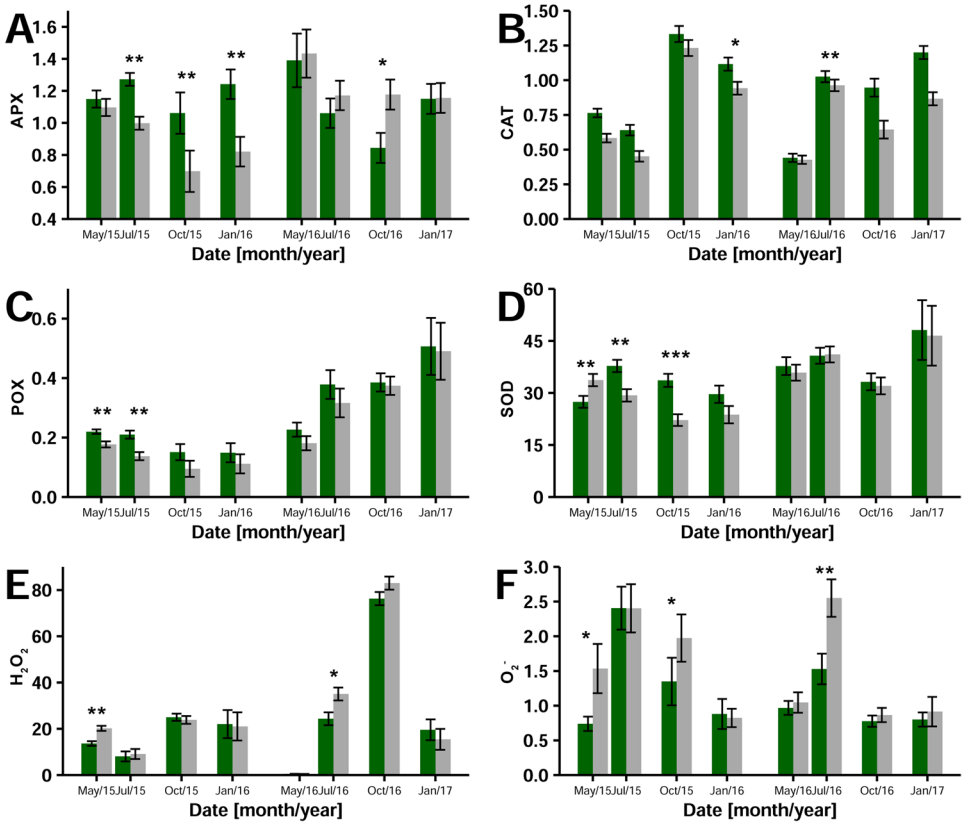

Fig. 4 Seasonal changes in photosynthetic parameters in needles of *Juniperus communis* L. individuals of plants grown with (green) or without fertilization (grey). **a** ascorbate peroxidase (1 nmol ASA min^{-1} mg $protein^{-1}$, APX); **b** catalase (mmol H_2O_2 min^{-1} mg $protein^{-1}$, CAT); **c** guaiacol peroxidase (nkat min^{-1} mg $protein^{-1}$, POX); **d** superoxide dismutase (U mg $protein^{-1}$, SOD); **e** hydrogen peroxide (μg g^{-1} DW, H_2O_2); **f** superoxide (μg g^{-1} DW, O_2^-) ascorbate peroxidase (1 nmol ASA min^{-1} mg $protein^{-1}$, APX), Data are means with standard errors (SE, $n = 16$). Asterisks indicate statistical differences between means at $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

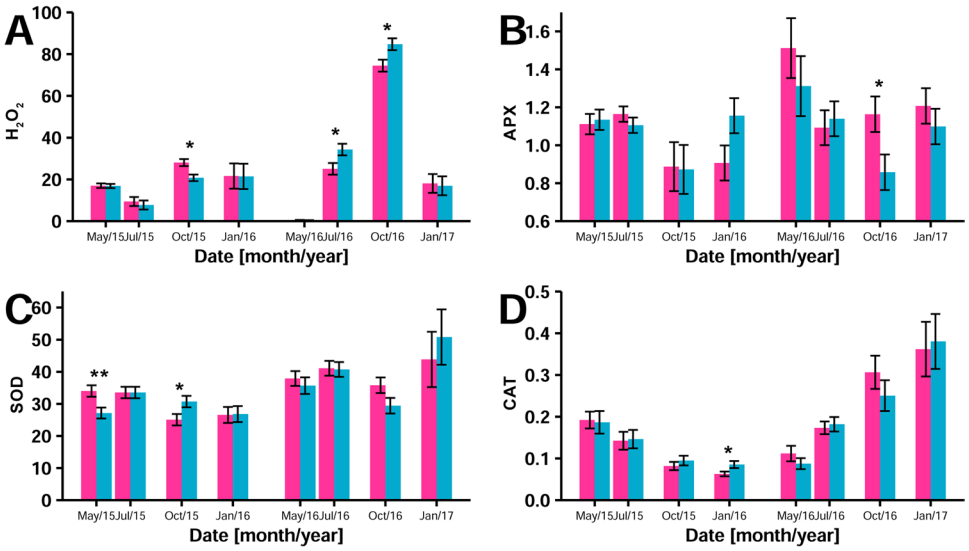

Fig. 5 Seasonal changes in leaf parameters in needles of male (blue) or female (pink) *Juniperus communis* L. plants: **a** hydrogen peroxide (μg g^{-1} DW, H_2O_2), **b** ascorbate peroxidase (1 nmol ASA min^{-1} mg $protein^{-1}$, APX), **c** superoxide dismutase (U mg $protein^{-1}$, SOD), **d** catalase (mmol H_2O_2 min^{-1} mg $protein^{-1}$, CAT). Data are means with standard errors (SE, $n = 16$). Asterisks indicate statistical differences between means at $^{*}P < 0.05$; $^{**}P < 0.01$

Discussion

In needles of *J. communis* plants, sex-related differences were found in photochemical parameters, the concentration of photosynthetic pigments, LMA, and H_2O_2 and in antioxidant enzyme activity. Results indicate that needles from male plants have a higher photosynthetic capacity than female plants, as determined by ETR_{max}, $\Phi_{PPF_{sat}}$, and a higher concentration of photosynthetic pigments, and female plants had higher LMA. In our experiment, due to higher needle carotenoid concentration even under limited nutritional conditions, abiotic stress could affect male plants less than female plants. We did not observe, however, the consistent relationship between sex and antioxidant enzymes activity and ROS formation. Our study showed the significant impact of nutritional availability on photochemical parameters, the concentration of photosynthetic pigments, antioxidant enzyme activity, and the formation of ROS in *J. communis*.

In accordance to our hypothesis, *J. communis* male plants exhibit a greater ability to maintain higher photosynthetic activity than female plants and they do this regardless of nutrient availability. On certain dates, needles of male plants had the higher values of ETR_{max}, $\Phi_{PPF_{sat}}$ and NPQ_{530} than needles of female plants, as well as the higher concentration of total chlorophyll and carotenoids, but the lower LMA. Recent studies on *Salix paraplesia* revealed an opposite situation when female plants had higher values of, inter alia, net CO_2 assimilation rates, and chlorophyll concentration. However, opposite to *J. communis*, this species shows female-biased sex ratio (Liao et al. 2019). The higher LMA in female *J. communis* plants suggests that their needles are thicker with more developed palisade parenchyma than male needles, which can be considered as a structural leaf adaptation to high light (Givnish 1988).

Resource availability can affect male and female individuals differently, with males being more tolerant to resource limitation than female plants (Montesinos et al. 2012; Zhang et al. 2014), which can result from different preferences in nitrogen sources (Zhao et al. 2018) or different strategies of growth *vs* defense trade-offs (Jiang et al. 2016). *Juniperus communis* females exhibit lower photosynthetic capacity than males, which can result from greater reproductive effort of females in this species (Iszkuło and Boratyński 2011). In dioecious species, however, females can compensate for a greater reproductive effort by increased gas exchange per leaf area unit, greater stomatal density, or greater leaf area relative to male plants (Wallace and Rundel 1979; Kohorn 1994; Meagher 1999; Obeso 2002; Montesinos et al. 2012; Iszkuło et al. 2013). Additionally, male and female plants can exhibit different levels of sensitivity to different stresses (Zhang et al. 2017; Qin et al. 2018). Importantly, stress tolerance can be constitutive and/or induced based on the genetics of the species (reviewed in Geber et al. 1999).

In the present study, ETR_{max} and $\Phi_{PPF_{sat}}$ values were higher in *J. communis* males than in females. F_v/F_m value was higher in females during flowering and it was opposite in winter.

Our results for photosynthetic parameters are in agreement with previous studies on *Pistacia lentiscus* (Correia and Diaz Barradas 2000) and *Populus cathayana* Xu et al. (2008), but they are not consistent with the results reported by Mitchell (1998) for *Taxus baccata* and *T. brevifolia*, who did not detect any differences between sexes in foliar physiology. However, similarly to our results, the value of F_v/F_m in *Pistacia lentiscus* was higher in female plants than male plants, especially during spring (Ait Said et al. 2013). Also *T. baccata* males had a lower F_v/F_m than females but no sex-based differences in ETR_{max} and PPF_{sat} were observed (Robakowski et al. 2018).

The results of the present study indicate that the greater reproductive effort in female plants can manifest itself in lower photosynthetic activity. This was shown already in *Populus purdomii*, where males had greater photosynthetic capacity than females as well as lower construction costs (Lei et al. 2017a). We hypothesize that although females of *J. communis* exhibit a greater reproductive effort than males (Iszkuło and Boratyński 2011), they compensate for it in a manner other than increasing photosynthetic efficiency, as occurs in *Silene latifolia* (Gehring and Monson 1994). The compensation in *J. communis* may be brought about by the greater leaf area in female plants (Nowak-Dyjeta et al. 2017). The higher LMA in *J. communis* females suggests that females had thicker leaves and fertilization did not affect leaf structure in females as remarkably as in males. Moreover, females can achieve the same or even higher production of carbohydrates than males at the whole plant level (Nicotra et al. 2003). Females can also differ from males in regards to resource allocation (Gehring and Monson 1994), as nitrogen content in *J. communis* was reported to be higher in female needles before flowering (Nowak-Dyjeta et al. 2017). What is significant, greater nitrogen storage in *J. thurifera* shoot increased photosynthesis of male but did not affect female photosynthesis (Montesinos et al. 2012).

Higher ETR_{max} values in needles of male plants suggest a greater tolerance of their photosynthetic apparatus to photoinhibition relative to needles in female plants. Significantly, higher ETR_{max} values in males occurred during flowering in the first year of our study and during a period of intensive growth in the second year. In both cases, differences between male and female plants appeared when the highest temperatures occurred. This may indicate that females cannot maintain their photosynthetic capacity relative to males during the periods of most intensive growth (reviewed in Song et al. 2018). In wind-pollinated species, males produce a great amount of pollen during flowering to increase their reproductive success and allocation in reproduction can be greater in males during that time (Rocheleau and Houle 2001). Because of that, males may increase photosynthetic efficiency to obtain the energy necessary for mating, and at the same time, they are more prone to loss of energy even after flowering is over.

In addition to greater photosynthetic efficiency, males in our study were characterized by higher total chlorophyll and carotenoid concentration, especially during intensive vegetative growth in July. Total chlorophyll concentration may be an indicator for estimating the potential of plants to adapt to an unfavorable environment (Croft et al. 2017; Li et al. 2018). The higher total chlorophyll concentration in male plants potentially suggests better performance (Barradas and Correia 1999) and is also related to plant maturity (Laporte and Delph 1996). Notably, relative to our results, chlorophyll content in *Silene latifolia* males was higher than in unpollinated females, but not pollinated females (Laporte and Delph 1996). Chlorophyll *a*/*b* ratio, an indicator of leaf senescence and light growth conditions, did not differ between males and females suggesting that similar conditions for leaf expansion exist in both sexes (Adams et al. 1990). Additionally, a higher carotenoid concentration in male plants may indicate a greater ability to cope with stress, thus males are potentially better able to regulate ROS levels than females. Carotenoids, inter alia, protect the photosynthetic apparatus from photooxidative damage by quenching the triplet state of chlorophyll; thereby preventing the formation of harmful oxidative species, and also by directly quenching of activated oxygen species (Gupta et al. 2018). They also participate in the dissipation of excess energy and help to stabilize photosynthetic structures. Additionally, they can also act as signaling molecules that have a positive impact on plant response to nutritional stress (Gupta et al. 2018).

We observed a gender-based effect on H_2O_2 content in *J. communis* needles. In *Populus cathayana* N- and P-deficient females had higher levels of H_2O_2 as they showed lower peroxidase activity (Zhang et al. 2014). Similarly, we observed a higher level of H_2O_2 in females in the first year, but during the second year, it was higher in males. A higher level of H_2O_2 was also connected with male's lower activity of APX. Similarly, in studies by Zhang et al. (2010), females showed lower ROS levels, which were additionally connected with stress conditions. On the other hand, we did not trace the differences between genders in O_2^-. As we observed, SOD and CAT activities were higher in males in the first year during the time of slower metabolic processes and stresses connected with cold and low light intensity. It might indicate on males' better performance during cold stress and stay in opposition to findings of Gupta et al. (2012) where females showed higher SOD activity as a response to cold stress. In our studies, SOD activity was higher in females during flowering, which can indicate males' reproductive effort connected with pollen production, which may be very resource consuming in wind-pollinated species (Teitel et al. 2016).

Differences between fertilized and non-fertilized individuals of *J. communis* depended on the season, which is in agreement with other reports demonstrating seasonal differences in plant response to environmental conditions (Troeng and Linder 1982; Lewis et al. 1996; Nowak-Dyjeta et al. 2017; Wang et al. 2018). In the current study, nutrient availability (fertilized vs. non-fertilized) affected a wide range of metabolic processes. Almost all of the measured parameters including photosynthetic capacity assessed as ETR_{max}, were lower in needles of non-fertilized plants than in needles of fertilized plants during at least one measuring term. A similar experiment conducted on *Taxus baccata* revealed that non-fertilized plants were more sensitive to photoinhibition than fertilized (Robakowski et al. 2018). A stronger

photoinhibitory stress response in non-fertilized *T. baccata* plants was evidenced as lower F_v/F_m, ETR_{max}, and $\Phi_{PPF_{sat}}$ values, as well as a lower concentration of photosynthetic pigments and higher NPQ values (Robakowski et al. 2018). These parameters exhibited similar differences in response to nutrient availability (fertilized vs. non-fertilized conditions) in *J. communis* as they did in *T. baccata*, except for $\Phi_{PPF_{sat}}$ which was higher in non-fertilized plants within most measuring terms, when differences were significant. These findings are in accordance with other reports of nutrition limitation as a significant factor impacting photosynthetic efficiency (Lima et al. 1999; Starck et al. 2000; Zhao et al. 2018). A negligible effect of nutrient availability, however, has also been reported (Klooster et al. 2010; Msanne et al. 2017). In response to mineral starvation, plants exhibit a down-regulation of genes connected to photosynthesis (Wu et al. 2003) and can be more vulnerable to photoinhibition (Simancas et al. 2016). In contrast to the results of Simancas et al. (2016) and Zhao et al. (2018), and similar to the study in *T. baccata* (Robakowski et al. 2018), results of the current *J. communis* study indicate that nutrient supplementation increases the capacity of this woody plant to cope with stresses, due to the constitutive expression of higher antioxidant enzyme (SOD, POX, and CAT) activity, higher levels of carotenoids, and a lower formation of free radicals.

Conclusions

Our results indicate that needles in male and female plants of *J. communis* differ in photosynthetic efficiency, level of ROS formation, and antioxidant enzymes' activity. The differences were observed regardless of nutrient availability. Females have lower photosynthetic capacity than males, lower concentration of chlorophyll per unit dry mass, and lower leaf absorbed energy loss. Differences between males and females in photosynthetic efficiency appeared during the most intensive growth and flowering indicating that males increase energy gain and losses when they invest more resources into reproduction. Males showed higher values of ROS, and differences between sexes in enzymes' activities were dependent on enzyme and season. Males showed a higher concentration of carotenoids per unit dry mass, which shows that genders of *J. communis* have different adaptations to cope with stressors. The results of our study indicate that most of the gender-based differences in *J. communis* are genetically based constitutive traits and are characteristic for each sex, as they appear independently of nutrient supplementation.

Author contribution statement

Study conception and design: EPK, GI, and MR; acquisition of data: MR, ER, and RŻ; analysis and interpretation of data MR, PR, and EPK; drafting of first version of the manuscript: MR and final version: MR, PR, EPK, ER, GI, and RŻ.

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Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any personal, professional or financial relationships that could potentially be construed as a conflict of interest.

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