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Gender- and season-related variability in the content of proteins, amino acids, and carbohydrates in *Taxus baccata* needles of different age

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Abstract

The present study aimed to determine if the needles of male and female European yew (*Taxus baccata* L.) trees differ in their content of basic compounds (proteins, amino acids, and carbohydrates), and whether the observed differences result only from the gender factor or if they are also associated with the needles' age and season. The study was conducted on male and female European yew specimens, collected from the Botanical Garden of the Jagiellonian University in Krakow during three seasons. Male specimens had significantly higher content of insoluble carbohydrates compared to the females ones. In the first year of the needles life, the dry mass and content of soluble carbohydrates increased significantly. In the third year of the needles' life, the content of amino acids increased significantly and the content of soluble proteins decreased. The highest differences between the genders in the individual months were observed in the soluble carbohydrates and amino acids amounts and the least in the soluble proteins. The growth of the new needles seems to be at least partly sustained by carbohydrates remobilization from the older needles. In conclusion, male and female yews differ in their metabolism. These gender differences may vary with the needles' age. For this reason, the needle samples of different age should not be mixed, and the sampling time should be chosen carefully. For one-year-old needles, a potentially interesting gender marker may be an increased content of free amino acids in June and July, and high content of soluble carbohydrates in January, while for two-year-old needles, an increased content of free amino acids in male individuals from September. March seems to be an interesting month, as it shows significant differences between the genders in terms of all biochemical features studied in this research.

Keywords: European yew, gender differences, needles age, reproductive effort, sex differences.

Introduction

European yew (*Taxus baccata* L.) is a shade-tolerant species, found mainly in the lower canopy layer of the stands (Garcia *et al*. 2000, Thomas and Polwart 2003). Due to the habitat degradation, human activities, and anthropopressure, it grows in dispersed and isolated populations within its natural range. Therefore, European yew is considered as an endangered species (Hilfiker *et al.* 2004, Iszkuło *et al*. 2009). The increasing climate changes are also not conducive to the growth of yew. In addition, the gradual rise in temperature and water shortages tend to change the natural range of the species. Because of its habitat requirements and breeding biology (dioecy, limited seed spread, slow growth, longevity) yew cannot react quickly to climate changes and therefore its population continues to decline (Iszkuło *et al*. 2012). An additional threat to this species results from its increased popularity after the discovery of anticancer substances in its tissues (Iszkuło *et al*. 2013).

Yew is a dioecious species - the male and female reproductive structures are found on separate plants. Similar to other tree species, after reaching sexual maturity, yew uses the available resources for both vegetative development and generative reproduction (Koenig and Knops 1998). Gender differences, observed in different

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Abbreviations: *ANOVA* - analysis of variance; d.m. - dry mass; RuBisCo - ribulose bisphosphate carboxylase.
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species, are often attributed to unequal reproductive efforts. Male specimens invest their resources in the growth, which enables them to better spread their pollen, and in the production of as much pollen as possible. On the other hand, female specimens incur considerable expenditure on the production of seeds and fruits (Obeso 2002, Montesinos *et al.* 2006, Cedro and Iszkuło 2011). Gender differences can be observed at different levels and may vary, according to the species (Obeso 2002). Phenotypically, the individuals of different genders may differ in, for example, growth rate (Iszkuło *et al.* 2011) and survival under stress conditions, which may lead to a gender imbalance (Letts *et al.* 2008, Garbarino *et al.* 2015, Litkowiec *et al.* 2015). Physiologically, gender differences may be expressed by responses to water stress (Rowland 2001, Leigh and Nicotra 2003, Dudley and Galen 2007) or gas exchange characteristics (Leigh and Nicotra 2003, Letts *et al.* 2008). Physiological differences are also reflected by the changes in the content of various organic compounds such as photosynthetic pigments (Kumar *et al.* 2006, Yang *et al.* 2011, Zarek 2016), proteins (Ajibade *et al.* 2006, Khryanin 2007), amino acids (Chen *et al.* 2014), carbohydrates (Liu *et al.* 2018, Cheng *et al.* 2019), and low molecular mass compounds (Golubkina *et al.* 2017), as well as in the enzymatic activities (Robakowski *et al.* 2018, Ruuhola *et al.* 2018).

Currently, because of the lack of molecular markers, gender differences of yew are also analyzed at biochemical and physiological levels. Although they have not been studied extensively so far, in recent years there has been a growing interest in this topic. Pers-Kamczyc *et al.* (2019) stated that the availability of the nutrients affects the reproductive strategy of male yew trees. The photosynthetic and antioxidant properties (Robakowski *et al.* 2018) of yew and its content of the photosynthetic pigments (Zarek 2016, Robakowski *et al.* 2018) were also studied in the context of the gender. Previous studies indicated a higher number and density of stomata and the presence of longer needles in female specimens, compared to the males (Iszkuło *et al.* 2009), which could be associated with a higher gas exchange (Iszkuło *et al.* 2013). However, subsequent studies (Stefanović *et al.* 2017) did not confirm sexual dimorphism in terms of the needles' size in all studied populations. Another study showed higher content of nitrogen in male needles and a comparable content of carbon in both genders (Nowak-Dyjeta *et al.* 2017), whereas female needles were found to contain higher amounts of taxanes (Iszkuło *et al.* 2013). According to Iszkuło *et al.* (2009), male specimens are taller and, after reaching maturity, also show a higher gain in the thickness than the female ones (Cedro and Iszkuło 2011).

The existence of biochemical differences in the trees growing in a temperate climate zone is also associated with the changes of seasons and the periods of growth and dormancy (Jach and Ceulemans 2000, Bowling *et al*. 2018). An additional factor in coniferous species is the existence of the needles belonging to the different annual increments on the shoots (Silkina and Vinokurova 2009). Carbohydrates have an extremely important effect on the relationship between photosynthesis and biomass production and are connected to carbon content (Mandre *et al*. 2002). The number and variety of carbohydrates, detected in the plants, vary in plant organs and conditions throughout the growing season. As a result, the plants exhibit various strategies connected to development and growth, productivity and quality, and resistance to cold (Deligöz *et al.* 2018). Seasonal pattern of foliar sugars and starch have been observed for many evergreen conifer trees *i.e.* in *Abies alba* (Yang *et al*. 2021), *Abies balsamea* (Little 1970), in potted (Alscher *et al*. 1989, Schaberg *et al.* 2000) and mature (Amundson *et al*. 1992) *Picea rubens*, in mature *Pinus cembra* (Gruber *et al*. 2011), *Pinus elliottii* (Gholz and Cropper 1991), *Pinus nigra* (Deligöz *et al.* 2018), *Pinus pinaster* (Desalme *et al*. 2017), *Pinus sylvestris* (Oleksyn *et al*. 2000), *Pseudotsuga menziesii* (Webb and Kilpatrick 1993, Billow *et al*. 1994), *Taxus baccata* (Meyer and Splittstoesser 1971, Nowak *et al*. 2021), and *Tsuga heterophylla* (Billow *et al*. 1994).

Evergreen conifers accumulate nitrogen (N) in their foliage primarily in the form of photosynthetic proteins such as ribulose bisphosphate carboxylase (RuBisCo), which has also a storage function in addition to its enzymatic functions (Millard *et al*. 2007). These resources are then mobilized from the start of the next growing season (Dickson 1989). During the season remobilized nitrogen has been estimated to yield from 30 to 60 % of the N incorporated into the current growth (Nambiar and Fife 1987). Free amino acids presented in the leaves can also serve as a storage form of N for protein synthesis and future growth (Pietilä *et al.* 1991, Nordin *et al*. 2001). Seasonal content changes of soluble proteins and/or amino acids were determined, *i.e.* in *Abies alba* (Escher *et al.* 2004, Yang *et al.* 2021), in the seedlings of a mixture of *Picea glauca* and *P. englemannii*, and *Pseudostuga menziesii* (Roberts *et al*. 1991), *Pinus massoniana* (Xu and Xiao 2017), *Pinus pinaster* (Desalme *et al.* 2017), *Pinus sylvestris* (Näsholm and Ericsson 1990), and *Taxus baccata* (Meyer and Splittstoesser 1971).

The aim of this study was to verify the hypothesis that male and female specimens of European yew differ in their content of basic biochemical compounds (*i.e.*, proteins, amino acids, and carbohydrates) in their needles, as well as in the whole dry mass. An additional aim was to determine whether these gender differences are dependent on the age of the needles and the month at which the sample material is collected.

Materials and methods

Plants and sampling: The study was conducted on *Taxus baccata* L. specimens obtained from the Botanical Garden of the Jagiellonian University in Krakow. Gender of the specimens was determined from the generative reproduction organs. Shoots with the needles were collected once a month from 10 individuals (including five males and five females) during 36 months of the needles' life. Material collection was carried out from May 2013 to April 2016, because May is the first month of the year, when the species starts forming new needles. This period was divided into three seasons as follows: the first season - May 2013 to April 2014, the second season - May 2014 to April 2015, and the third season - May 2015 to April 2016. In the first season, the needles belonging to the current growth, (*i.e.*, those aged from 1 to 12 months; denoted as current-year needles), as well as one-year and two-year-old needles, were collected. The aim of the simultaneous collection of all three needle age classes in the first season was to determine the differences between the needle age classes under the influence of the same meteorological conditions. In the second season, only one-year-old needles (*i.e.*, those aged from 13 to 24 months of age) and in the third season, two-year-old needles (*i.e.*, those aged from 25 to 36 months of age) were collected. The purpose of collecting the needles belonging to the increasing older age classes for three seasons was to observe the changes occurring during 36 months of their life and gradual ageing. All the collected data, both in the first and in the remaining two seasons, were used to calculate the long-term mean values of all determined parameters, taking into account the age of the needles and the gender of the individuals. Five male and five female individuals of related age (about 90 years), of comparable size and growing under even light conditions, were chosen for the study. The shoots with the needles were harvested from the northern side of the crown to reduce the impact of direct solar radiation, from a height of 1.5 - 2 m. In the laboratory, the shoots were divided into fragments, based on their age, and the needles were obtained. Whole needles from the middle part of the annual increment were used for the analysis, and the needles from the upper and lower parts of the increment, varying considerably in their length from the average value, were rejected. Then, the needles were ground in liquid nitrogen, and the resulting powder was lyophilized and used for biochemical analyses. All analyses were performed in three repetitions.

Meteorological data: Meteorological data were obtained from the Scientific Station of the Department of Climatology of the Jagiellonian University IGiGP (latitude 50°04′N, longitude 19°58′E, altitude 201 - 208 m a.s.l.), which is located in the area of the Botanical Garden of the Jagiellonian University in Krakow. To evaluate the course of the changes in the basic climatic factors and their influence on the studied factors, the mean daily temperature as well as the minimum and maximum temperature on 7 d preceding the day of collection of plant material was calculated for each date of sample collection. In addition, the total precipitation that occurred during the 7 d preceding the sampling date was determined.

Dry mass: Before lyophilization, a part of the obtained powder was used for estimating the content of the dry mass. For this purpose, the powder samples were carefully weighed and dried at 70 °C for 72 h. After drying, the samples were weighed again and the percentage of dry mass (% d.m.) was calculated on the basis of weight loss. The remaining part of the powder intended for further analysis was lyophilized for 120 h using *FreeZone* 2.5 liter freeze dry system (*Labconco*, Kansas City, MO, USA).

Soluble and insoluble proteins: The soluble proteins were extracted in a solution containing 100 mM potassium phosphate buffer (pH 7.8) and 2.0 mM ethylenediaminetetraacetic acid with 0.5 % (m/v) *Triton X* and 1 mM dithiothreitol. Then, 0.5 cm^3 of the extraction buffer was added to 10 mg of lyophilized plant powder and shaken on ice for 1 h. The extract obtained after centrifugation was used to determine the content of soluble proteins, while the pellets were reused to extract and determine the content of insoluble proteins. The insoluble proteins were extracted in 0.5 cm^3 buffer of the same composition as that applied for the extraction of soluble proteins but with the addition of 0.05 % (m/v) sodium dodecyl sulfate. The content of both soluble and insoluble proteins was determined using the Bradford method (1976), with the modification proposed by Ernst and Zorr (2010). Absorbance was read at the wavelengths of 590 and 450 nm. The protein content was calculated from the calibration curve using bovine serum albumin as a standard and expressed in mg g^{-1}(d.m.). Analyses were performed using *Synergy 2* microplate reader (*Biotek*, Winooski, VT, USA).

Free amino acids: The content of free amino acids was determined as described by Kraj (Kraj 2014), using the method developed by Yokoyama and Hiramatsu (2003). For this purpose, approximately 10 mg of the lyophilized and powdered plant material was extracted using 0.5 cm^3 of 3 % (m/v) sulfosalicylic acid on a shaker table at room temperature for 1 h. After centrifuging the pellets, 400 mm^3 of 0.2 M citrate buffer (pH 5.0), and 400 mm^3 of ninhydrin reagent were added to 200 mm^3 of the filtrate. The resulting reaction mixture was heated in a water bath at 97 °C for 10 min and then cooled in an ice bath to inhibit the course of the reaction. Then, 200 mm^3 of 60 % (v/v) ethanol was added to stabilize the reaction mixture, and the absorbance was read at a wavelength of 570 nm. Glycine solution in HCl (100 mM) was used as a standard. Analyses were performed using a *Synergy 2* microplate reader. The content of free amino acids was expressed in mg g^{-1}(d.m.).

Soluble and insoluble carbohydrates: The content of soluble carbohydrates was determined using the method of Dubois *et al.* (1951), which was adapted to work on microplates by Marcińska *et al.* (2013), with minor modifications. Briefly, about 5 mg of lyophilized and homogenized plant tissue was extracted with 0.5 cm^3 of 95 % ethanol in a water bath at 50 °C for 30 min. The obtained extract was diluted 50 times with the same solvent. Then, the reaction mixture consisting of 150 mm^3 of diluted extract, 450 mm^3 of concentrated H_2SO_4, and 90 mm^3 of 5 % (m/v) phenol in water was prepared. The mixture was heated in a water bath for 15 min at 90 °C, and the absorbance was then read using *Synergy2* microplate reader at a wavelength of 490 nm. Glucose was used as a standard. The content of soluble carbohydrates was expressed in mg g^{-1}(d.m.) For determining the content of insoluble carbohydrates, they were first hydrolyzed according to the modified method of Kraj and Grad (2013). Briefly, 0.5 cm^3 of 75 % (v/v) H_2SO_4 was added to the dried pellets obtained from the previous experiment and the resulting solution was extracted at room temperature for 1 h. The extract was prediluted with distilled water and centrifuged, then the sample was finally diluted 100 times and analyzed according to the method described earlier for soluble carbohydrates. Glucose was used as a standard for the analysis. The content of insoluble carbohydrates was expressed in mg g^{-1}(d.m.).

Statistical analysis: All collected data were used to determine the average values of the investigated factors ± standard errors of the mean (SEs), taking into account both gender and age of needles. To test statistically significant differences between the groups, a two-way *ANOVA* was applied. Homogeneity of variance was tested using Bartlett's test (Sokal and Rohlf 1995), and the normality of data was determined by Shapiro-Wilk's test. For the data that did not meet the assumptions of *ANOVA*, Kruskal-Wallis *ANOVA* nonparametric test was performed. The material that was collected only in the first season but belonged to three different age classes was used to present the pattern of the changes in the content of individual biochemical components during the season, taking into account the age of the needles and assuming the same weather conditions for all age classes. To determine the interactions between the gender and the age class of the needles, and between the gender and the sampling date in the first season (May to February), *Repeated Measures ANOVA* was used. The research material covering 36 months of the needles' life over three seasons was used to present the pattern of the changes in the content of particular biochemical compounds. The significance of the differences between the genders in every month in the all seasons, and between the three age classes of the needles in every month in the first season were determined using Kruskal-Wallis *ANOVA* nonparametric test as a *posthoc* test. Correlation between the biochemical parameters and the climatic factors was also investigated. All statistical analyses were conducted using *Statistica* software, *v. 12.0* (*StatSoft*, Tulsa, OK, USA). Average values are presented as means ± SEs. Statistical significance was established at $P \leq 0.05$.

Results

The mean temperatures (maximum and minimum) and the precipitation in each season did not differ significantly ($P < 0.05$) (Table 1 Suppl.). The climatic data covering the period of 36 months of plant material collection (Fig. 1) indicated a regular course of climate changes during the seasons 2013/14 and 2014/15, whereas they showed irregular breaks in the trend in the season 2015/16. In particular, the local increase in the precipitation recorded in May and July of 2014 was noteworthy. Correlations of the tested compounds with the weather factors (7 d temperature: mean, maximum, and minimum and the sum of 7 d precipitation) are included in Table 2 Suppl.

In females, the percentage of dry mass increased significantly with the age in the one-year-old needles. A significant decrease in the content of soluble proteins in the needles was observed with the age, especially between the second and the third age classes. Without dividing individuals by their gender, the content of amino acids increased with the age between the one- and two-year-old needles. The content of soluble carbohydrates increased with the age from the current- to one-year-old needles of both sexes. The mean content of insoluble carbohydrates was significantly higher in the male needles (Table 3 Suppl.).

Comparing different age classes of the needles in the

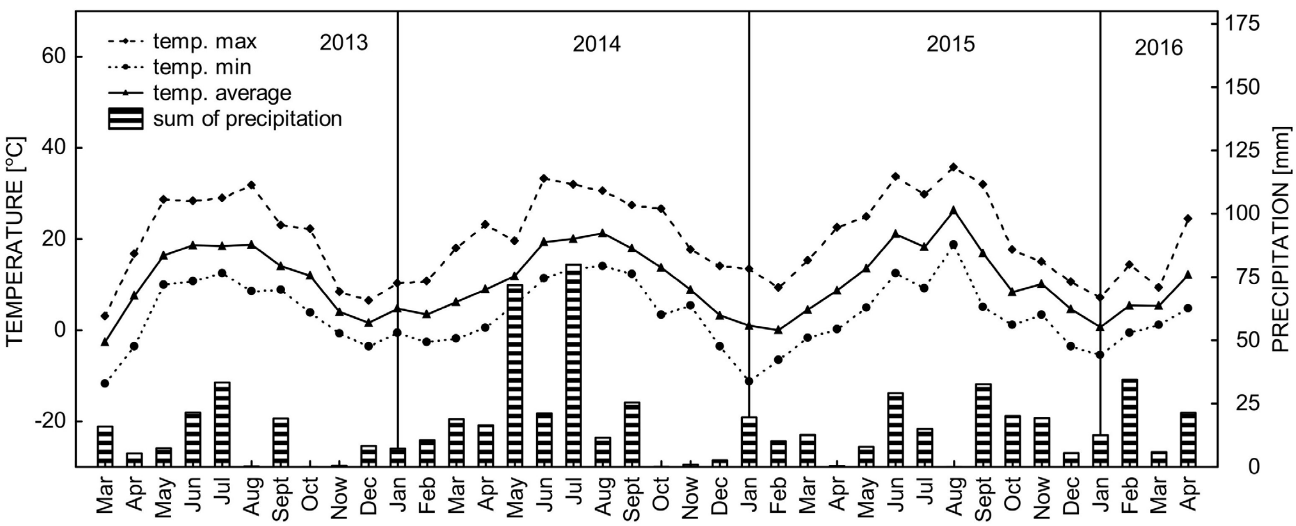

Fig. 1. Weather conditions during sampling periods from March 2013 to April 2016. The *bars* represent the sum of precipitation [mm] and the *lines* correspond to the average (*solid line*), maximum (*dashed line*), and minimum (*dotted line*) temperature [°C] during the seven days preceding sampling time.

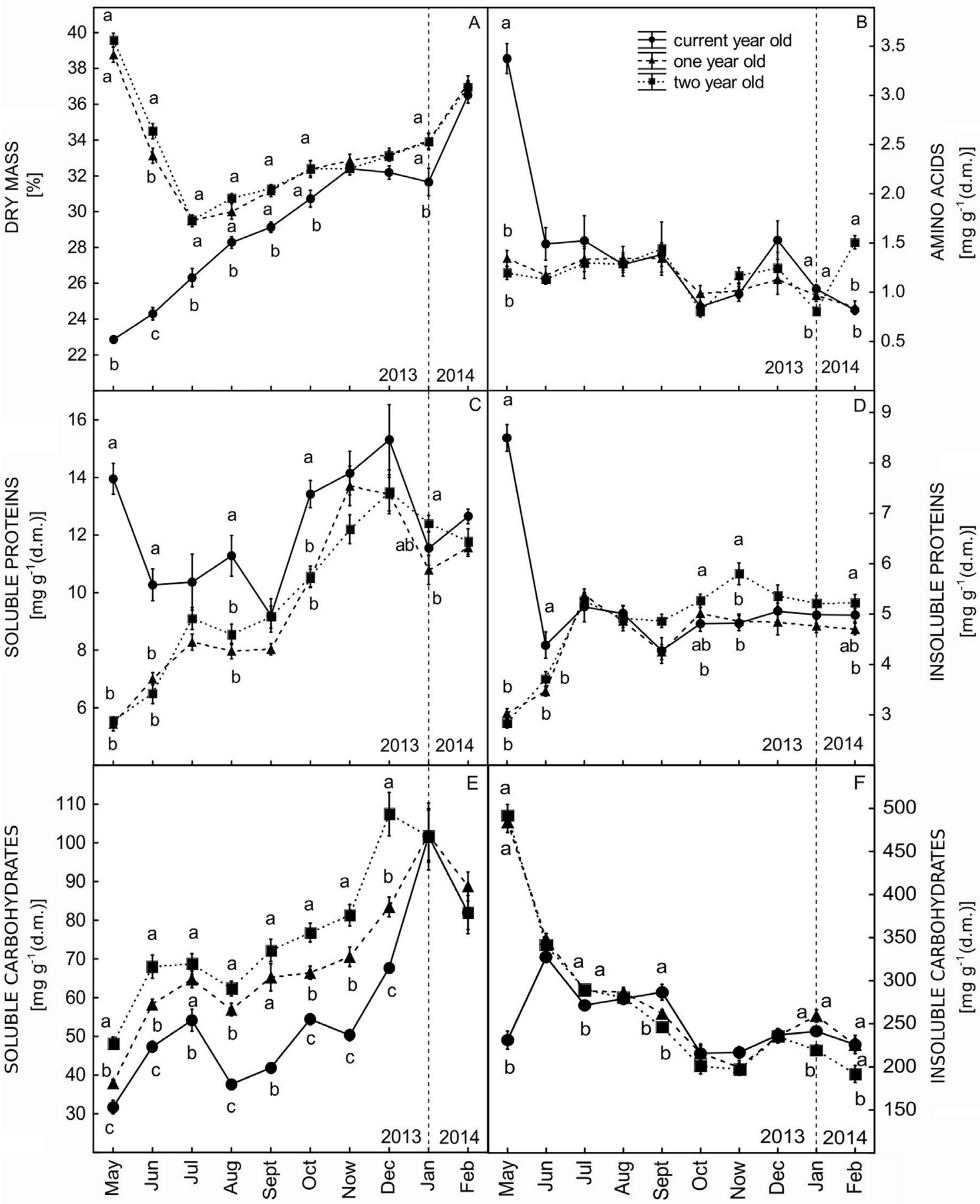

Fig. 2. Changes in the dry mass (*A*), and content of total free amino acid (*B*), soluble proteins (*C*), insoluble proteins (*D*), water soluble carbohydrates (*E*), and water insoluble carbohydrates (*F*) in *T. baccata* needles in different age classes: current-year-old (*circle mark*, *solid line*), one-year-old (*triangle mark*, *dashed line*), and two-year-old needles (*square mark*, *dotted line*) during one season (from May 2013 to February 2014). Means of 10 measurements ± SEs. Means in the particular months denoted by different letters are significantly different at $P \leq 0.05$.

first season (Fig. 2*A*), the lowest content of the dry mass was found in the current-year needles for most of the months of sample collection. The content of the dry mass in the current-year needles gradually increased during the season, whereas in one- and two-year-old needles the content abruptly decreased during the first three months (May, June, and July). No significant interaction was found between the gender and the needles' age nor between the gender and the month of the sample collection ($P > 0.05$) (Table 1).

The trend of the seasonal variations in the content of the dry mass and the differences between the genders are presented in Fig. 3*A*. During the first season, the content of the dry mass gradually increased in the needles of both

Table 1. Repeated measures *ANOVA* analysis of dry mass, soluble and insoluble proteins, amino acids, and soluble and insoluble carbohydrates in *T. baccata* needles during one season (from May to February). * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, F - value of Snedecor function, d.m. - dry mass.

Factor	Dry mass [%]	Soluble proteins [mg g^{-1}(d.m.)] F-value	Insoluble proteins [mg g^{-1}(d.m.)]
Gender	0.00	0.21	1.11
Needle age class	29.47 ***	5.14 *	5.61 *
Sampling month	45.38 ***	26.74 ***	8.91 ***
Gender × needle age class	0.57	0.76	0.68
Gender × sampling month	1.22	2.19 *	4.54 ***

Factor	Total free amino acids [mg g^{-1}(d.m.)]	Soluble carbohydrates [mg g^{-1}(d.m.)] F-value	Insoluble carbohydrates [mg g^{-1}(d.m.)]
Gender	0.45	0.31	3.99
Needle age class	0.91	17.38 ***	14.67 ***
Sampling month	11.09 ***	28.34 ***	67.54 ***
Gender × needle age class	0.15	0.27	0.08
Gender × sampling month	3.94 ***	3.19 **	0.98

genders. Male needles showed higher content for 8 months of sample collection (distributed mainly in the first season), whereas two-year-old female needles showed higher dry mass for 3 months (July, August, and January in the third season). In the first season, the males showed a faster increase in the content of the dry mass, which resulted in significant differences in June, September, March, and April. In the second season, such a phenomenon was observed in February and March.

Comparing different age classes of the needles in the first season (Fig. 2*C*), the highest content of soluble proteins was found in the current-year needles, especially at the beginning of the season. Then, the content decreased rapidly during the first month in the current-year needles, while in one- and two-year-old needles it increased gradually until December. Since September, the course of the changes in all age classes of the needles showed a similar pattern. A significant interaction was found between the gender and the month of the sample collection ($P < 0.05$) (Table 1).

The trend of seasonal variations in the content of soluble proteins and the differences between the genders are presented in Fig. 3*B*. In the first season, a higher content of soluble proteins was observed in males in May and October, whereas in females the content was higher in June and January. From May to June, the males showed a sharp decrease in the content of soluble proteins, whereas in the period from September to October the content increased rapidly. Similarly, in the female individuals, the content of soluble proteins increased gradually from September to December. In the second season, the course of changes in both genders was similar; however, the highest content was recorded in the females during the beginning of the winter in December, while in the case of the males the local highest content was noted in November. In the females, the content of soluble proteins gradually decreased from December to May, while in the males the content showed a large increase in March and then a rapid decrease until May. The course of the changes was also similar in the third season in both genders, but the female individuals showed higher content of soluble proteins than the males (June, August, November, and March).

Comparing different age classes of the needles in the first season (Fig. 2*D*), in the beginning (May and June) the highest content of insoluble proteins was found in the current-year needles, while in the second half (October, November, and February) the highest content was observed in two-year-old needles. Since July, the course of the changes in all age classes of the needles was similar. A significant interaction was found between the gender and the month of the sample collection ($P < 0.0001$) (Table 1).

The trend of seasonal variations in the content of insoluble proteins and the differences between the genders are presented in Fig. 3*C*. In the first season, the males showed a higher content of insoluble proteins in May which further decreased rapidly, while from June to August, the content was higher in female individuals. The higher content of insoluble proteins was again observed in the males at the end of the first season (April) and at the beginning of the second season (May), whereas in the second half of the second season, the content of insoluble proteins was higher in the females (December, March, and April). In the third season, the course of changes in the content of insoluble proteins was found to be similar in both genders; however, the content was higher in females (August and March), while in male individuals the content was higher only in the last investigated month (April).

Comparing different age classes of the needles in

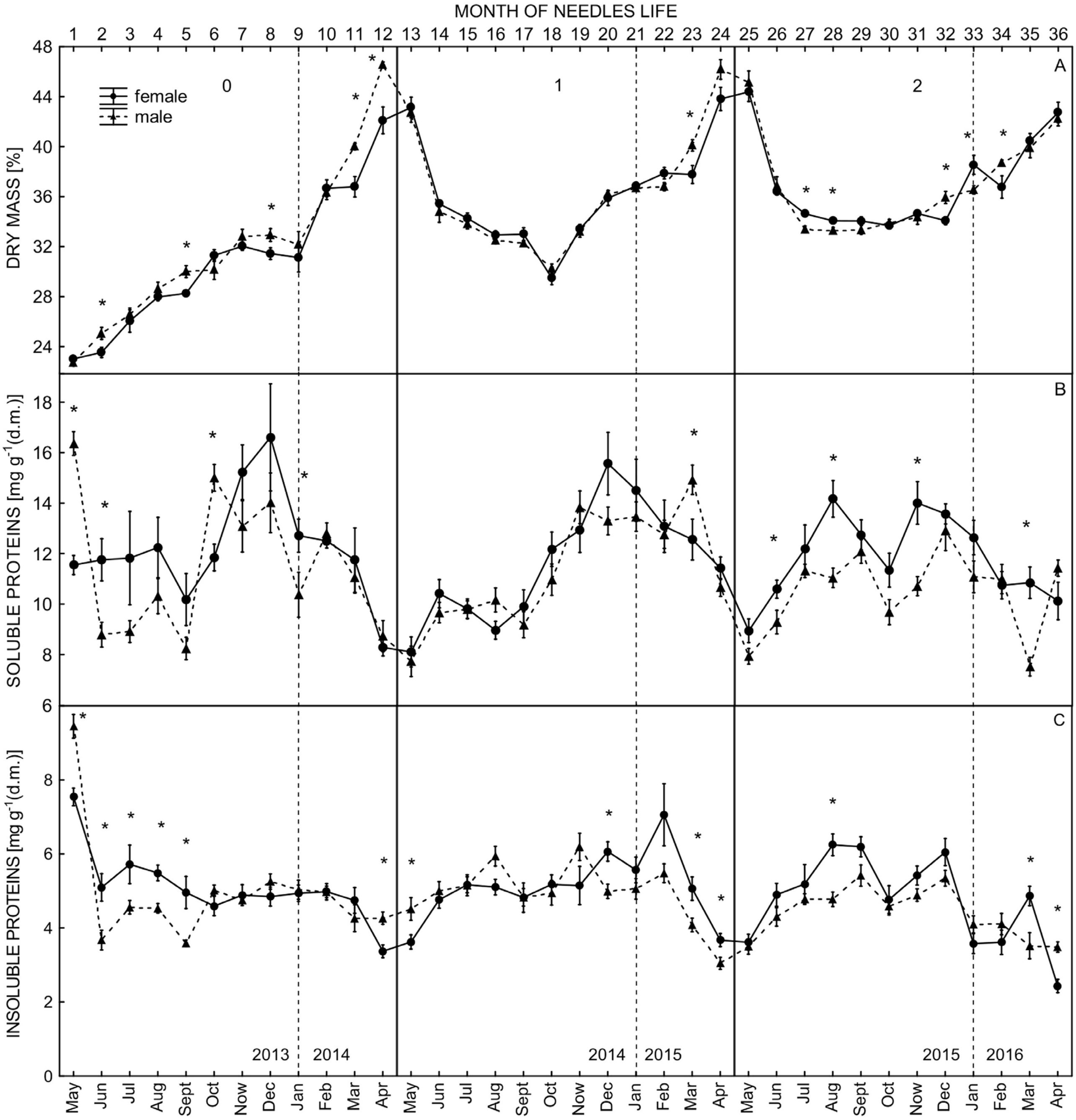

Fig. 3. Changes in the dry mass (*A*), soluble proteins (*B*), and insoluble proteins (*C*) in male (*triangle mark, dashed line*) and female (*circle mark, solid line*) *T. baccata* needles during 36 months of needles life (from May 2013 to April 2016). Mean of 5 measurements ± SEs. Means denoted by * are significantly different at $P \leq 0.05$. The *vertical dashed line* indicates the beginning of the year and the *vertical solid line* indicates the beginning of the new season (0, 1, or 2).

the first season, in the first month (May) the current-year needles showed the highest content of amino acids; however, the content decreased rapidly, and since June the needles of all three age classes showed a similar course of changes (Fig. 2*B*). In addition, there was an increase in the content of amino acids in two-year-old needles at the turn of January and February. A significant interaction was found between the gender and the month of the sample collection ($P < 0.0001$) (Table 1).

The trend of seasonal variations in the content of free amino acids and the differences between the genders are presented in Fig. 4*A*. In the first months of the first season (May, June, and September) and the second season (June and July), higher content of amino acids was observed in the females. In the second half of the first season (October, January, March, and April) and the second season (September, March, and April), and also throughout the third season, higher content of amino acids were observed

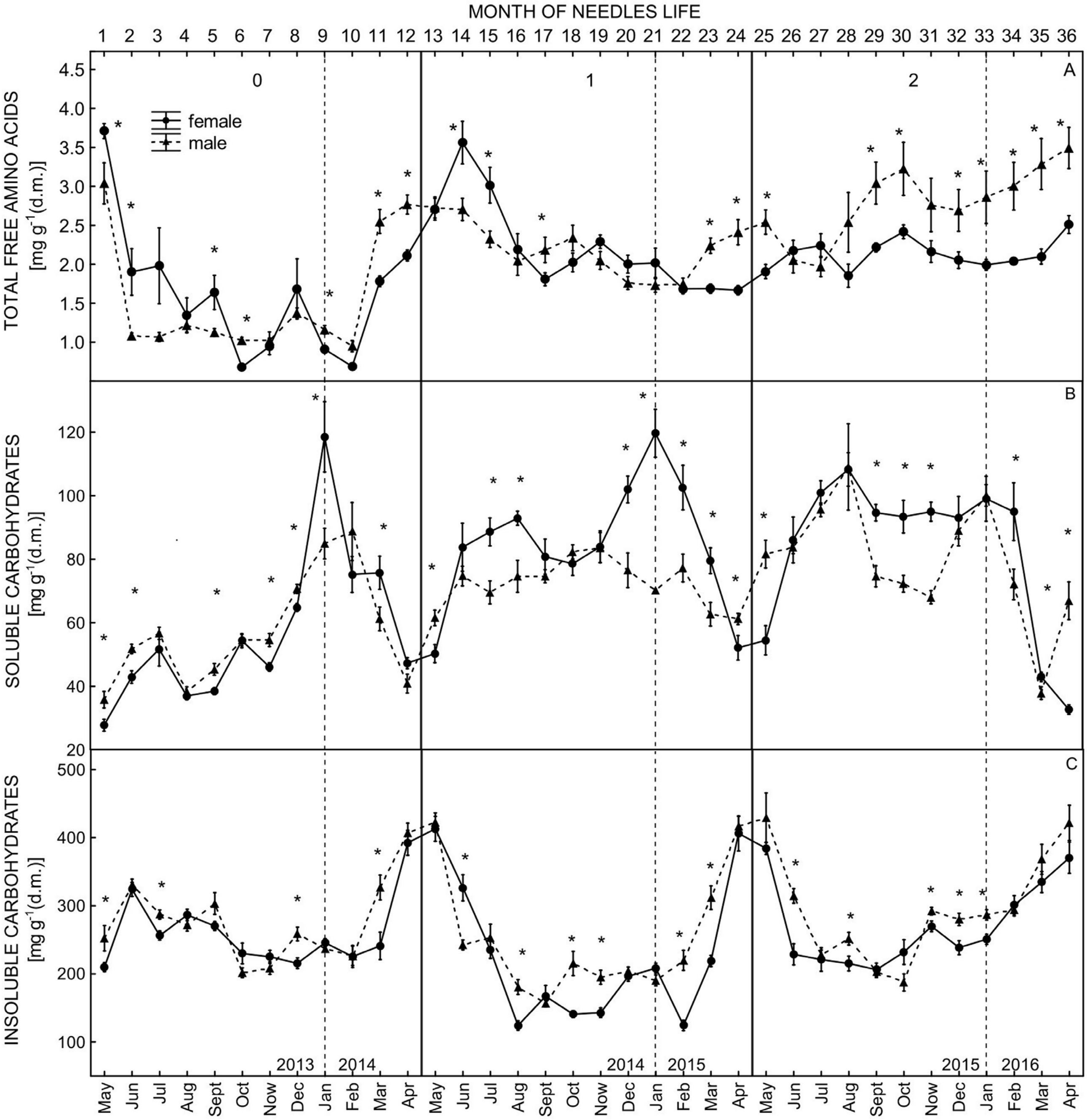

Fig. 4. Changes in the total free amino acid (*A*), water soluble carbohydrates (*B*), and water insoluble carbohydrates (*C*) in male (*triangle mark, dashed line*) and female (*circle mark, solid line*) *T. baccata* needles during 36 months of needles life (from May 2013 to April 2016). Means of 5 measurements ± SEs. Means denoted by * are significantly different at $P \leq 0.05$. The *vertical dashed line* indicates the beginning of the year and the *vertical solid line* indicates the beginning of the new season (0, 1, or 2).

in the males. It is worth noting that at the turn of the first and second season, the males showed maximum content of amino acids earlier (March and April) than the females (June and July). At the turn of the next season, the pattern was similar, although the content of amino acids in the females was much lower than in the second season.

Comparing different age classes of the needles in the first season (Fig. 2*E*), a similar course of the changes was observed in all age classes, but each month, until January, the amount of soluble carbohydrates was significantly higher in the older needles. A significant interaction was found between the gender and the month of sample collection ($P < 0.001$) (Table 1).

The trend of seasonal variations in the content of soluble carbohydrates and the differences between the genders are presented in Fig. 4*B*. For most of the first season, until December, higher content of soluble carbohydrates was found in the males, while the females showed higher content only in January and March. In the second and the third season, higher content was more often observed in the females, while only in the first and last month of each of these seasons (May and April) higher content was observed in the males. It should be mentioned that the content of soluble carbohydrates was very high in January of the first and second season; however, in the first season, this high content was observed in the males and in the second season in the females.

Comparing different age classes of the needles in the first season (Fig. 2*F*), the highest content of insoluble carbohydrates was found in one- and two-year needles in the first month (May). Between May and June, the content decreased rapidly in the older needles, while in the current-year needles it increased sharply. In the rest of the season, a similar course of changes was observed in all the age classes. Another difference was observed at the end of the season (from December) when the two-year-old needles started to show an earlier significant decrease in carbohydrates content compared to needles of age classes 0 and 1. No significant interaction was found between the gender and the needles' age nor between the gender and the month of the sample collection ($P > 0.05$) (Table 1).

The trend of seasonal variations in the content of insoluble carbohydrates and the differences between the genders are presented in Fig. 4*C*. Gender differences were observed in 15 collection dates, of which a higher content was detected in the males in 14 dates. Only in the second season, in January, a higher content of insoluble carbohydrates was recorded in the female needles. The course of the changes in the content of insoluble carbohydrates was similar in both genders. An interesting observation was the earlier but slower increase in the content in the males (from February to April) and the later but much more rapid increase in the content in the females (from May to April).

Considering all 6 examined parameters, the highest differences between the males and the females were observed in March (Table 4 Suppl.). The differences in 4 out of 6 parameters were observed for the needles from the current year and two-year-old needles, while for one-year-old needles, the differences for all 6 parameters studied were noted. Five parameters differentiated the gender also in May and June, and 4 parameters in September for the needles from the current year increment, while for two-year-old needles, 4 parameters differentiated the gender in August.

Discussion

According to the hypothesis of the "uneven reproductive effort" (Obeso 2002), the male and the female specimens differ in certain characteristics due to their different reproductive efforts. Reproduction cost divergences can vary in different plant species, but for many species it is generally accepted that reproductive effort is higher for the females (Krischik and Denno 1990, Cipollini and Stiles 1991, Cipollini and Whigham 1994, Massei *et al*. 2006, Montesinos *et al*. 2006, Zunzunegui *et al*. 2006, Iszkuło *et al*. 2009). As a general rule, growing fruits requires large quantities of the energy to mature, whereas pollen production is much less expensive (Lovett Doust and Lovett Doust 1988, Delph 1990). Of 41 dioecious species reviewed by Leigh *et al*. (2006), female reproductive effort was higher in 39 cases. In *Taxus baccata*, the female specimens produce the seeds and the arils every year after reaching sexual maturity and thus have a higher "cost of living" than the male specimens which produce only pollen. The previous observations such as the higher growth rates in males (Cedro and Iszkuło 2011), the greater requirements for the water in females (Iszkuło *et al.* 2009, Vessella *et al.* 2015), the greater needle area of female specimens, the greater nitrogen content of males (Nowak-Dyjeta *et al.* 2017), or the greater sensitivity of females to irradiance (Robakowski *et al*. 2018) indicate higher living costs in female yews. This is also overlapped by phenological differences. The author's observations made during the fieldwork (Zarek unpublished) showed that the females begin the vegetative growth of the shoots in spring with a delay of about two weeks in relation to the males. Male yew flowers start to build up in the summer during the year preceding pollination. In autumn or winter, pollen production is performed, which is wind-propagated in February and March. Then the pollination starts. Fertilization takes place between May and June and then the growth and the development of the embryo occurs. Production of the arils and the seeds ends in September. Simultaneously from May to June, the development of female "flowers" is started, which will be pollinated the next year (Szajnawski 1978, Thomas and Polwart 2003, Zarek 2016).

One of the objectives of this study was to examine whether these gender differences can be observed at the biochemical level in the content of basic biogenic compounds. In long-term studies, these differences are often blurred due to the seasonal changes in the content of individual compounds. In the present study male specimens only had a significantly higher content of insoluble carbohydrates, among the metabolites tested. Our observation of an increased content of insoluble carbohydrates in male specimens with similar resources available for both genders supports the hypothesis of the uneven reproductive effort. The most likely reason for the higher content of insoluble carbohydrates in the males is the high cost and the duration of the energy needed for seeds production in female trees compared to pollen production in male trees, and accordingly, females may deposit fewer reserve compounds (Krischik and Denno 1990, Cipollini and Whigham 1994, Obeso 2002, Massei *et al*. 2006, Iszkuło *et al*. 2009). This is also in line with our previous study of the content of photosynthetic pigments in European yew (Zarek 2016), which showed higher content of chlorophyll *a* and *b* in female specimens. A higher content of insoluble carbohydrates with a lower content of photosynthetic pigments may indicate a lower consumption of resources for daily needs in male individuals. This gives them the opportunity for allocating the resources in the form of reserve materials or using them to increase their size. Also, according to Iszkuło *et al.*

(2009), for the studied yew populations, male specimens had greater height and breast height diameter. On the other hand, according to a study by Nowak *et al*. (2021), female individuals had a higher starch content than male individuals, but this discrepancy may result from a different method of determining insoluble carbohydrates and less frequent sampling (only four times a year). The differences observed between the needles of different age classes may reflect their different developmental and physiological state in a given period of the season (Silkina and Vinokurova 2009). In our research the largest differences were always seen in the youngest needles, regardless of the gender. In the first period of life, the youngest needles undergo an intensive vegetative growth involving most of the resources produced mainly by the neighboring older needles and also those locally produced later. This is suggested by the high content of amino acids and proteins (which are the main nitrogen depots) and insoluble carbohydrates (which are the source of carbon) in the youngest needles, and low content in the older needles especially at the beginning of the new season. The highest content of N was observed also in current year needles for other conifers (Millard and Grelet 2010, Schaeffer *et al.* 2017), highlighting how the bulk of a host's N is stored in the youngest age class of the needles. The vegetative growth gradually decreases during the season. In evergreen conifers, the carbohydrates from the previous year's needles support the shoot growth until the current-year needles develop (Hansen and Beck 1994). This is also confirmed by our study, which showed a decrease in the dry mass in older needles in the period from May to July, when the youngest needles are intensively growing. Then the available resources are used, among others, to produce generative structures, and the part of the produced compounds are accumulated in the form of reserve compounds. After pollination in March, one-year-old male needles can focus on vegetative function, whereas one-year-old female needles are forced to supply not only vegetative growth of the youngest shoots but also developing embryos and produce seeds and arils as showed, *inter alia*, by their lower content of insoluble carbohydrates and higher content of soluble carbohydrates in the present study. Female individuals are associated with a greater demand for carbohydrates used in the production of seeds (Wallace and Rundel 1979, Blake-Mahmud and Struwe 2020). According to Robakowski *et al.* (2018), although the green parts of the arils are capable of performing photosynthesis, they can cover only a part of the resources used to produce and maintain seeds and red arils.

Comparing the average content of the tested compounds, we can obtain only a simplified picture of the study subject. The metabolism of dioecious plants varies significantly in different months of the year, and depends not only on the gender but also on the age of the needles and the weather conditions in a given year (Deligöz *et al.* 2018). Studies limited to plant materials collected once a year were often unable to show differences between the genders (Ågren *et al.* 1999, Jiang *et al.* 2016). Therefore, it is recommended that long-term studies should be conducted to investigate the pattern of gender differences (Suzuki *et al.* 2014, Juvany and Munné-Bosch 2015, Retuerto *et al.* 2018, Robakowski *et al.* 2018).

The present study also showed that the timing of plant material collection is an important factor. Barbasz *et al.* (2012) found that the biochemical composition of yew tissues is strongly influenced by the month of harvesting and thus the plant growing season. Apart from the biochemical differences seen in the individual months, the reversal of the trends in the individual periods, connected with local maxima and minima shifts in the content of the individual compounds, seems to be equally important. This indicates the differences in the behaviors of the individuals of different genders in certain months of the year, which is probably related to their role in the reproduction process and the associated periodic needs but also may result from different gender responses to weather conditions (Bochenek and Eriksen 2010). In our previous study, similar maxima shifts were also observed in yew in the content of photosynthetic pigments in the individual months of the season (Zarek 2016). A similar phenomenon occurs, *e.g.*, in the deciduous tree *Fraxinus excelsior* where pollen production entirely depends on the resources gained during the previous vegetative season, whereas growing fruits use current resources (Bochenek and Eriksen 2010). Developing seeds work as powerful sinks, and compete with growing shoots and leaves (Delph 1990, Miyazaki *et al*. 2002).

The present study conducted during 36 months of the needles' life showed that the most significant differences between the genders in the individual months were observed for the two of the studied parameters - the content of soluble carbohydrates and the content of free amino acids. Both parameters showed high dynamics, which is probably related to a number of metabolic functions performed by these compounds. One of them is that carbohydrates and proteins are the sources of energy and building blocks for organic compounds and storage components in both genders (Chapin and Kedrowski 1983, Mandre *et al*. 2002), but also are used in the production of the seeds and the fruit in the females (Delph 1990, Miyazaki *et al.* 2002) and amino acids and proteins as a source of nitrogen needed for the pollen production in the males. Producing large amounts of nitrogen-rich pollen in a short period of the growing season creates a high nitrogen demand (Obeso 2002, Nowak-Dyjeta *et al*. 2017). In our study, a higher content of soluble carbohydrates in the period from July to August and of amino acids in June and July was observed in the female one-year-old needles, which is the time when the seeds and the arils begin to form. In the youngest male needles, during the pollen formation period, we observed a significantly higher content of free amino acids, which are the source of nitrogen needed for pollen production. Another role of carbohydrates and amino acids is regulating the osmotic potential of cells, which is particularly important in the case of evergreen species during the period of the lowest temperatures in the season to protect tissues against damage. Total content of soluble carbohydrates in plants rise during the onset of cold acclimation (Sakai and Larcher 1987) and these carbohydrates may participate in cold tolerance (Wong *et al*. 2003). In yew, the highest osmotic potential was observed in January (Szaniawski 1978). This is consistent with the regular periodic increase in the content of soluble carbohydrates around January shown by the present study, which underlines their significant role in maintaining high osmotic potential. This effect is more visible in the youngest needles, probably because of the higher water content of young tissues and maintenance of large amounts of soluble carbohydrates may be necessary to protect delicate tissues from the damage. With proceeding needle age, they become harder and less hydrated, which slightly reduces their need for osmoprotectants.

The above-mentioned differences between amino acids content in the oldest needles may indicate differences in the rate of aging between the genders. Female needles seem to age slightly slower, which was indicated by the higher content of insoluble proteins and lower content of free amino acids noted in the two-year-old needles. Beis *et al.* (2002) found that the age of leaves significantly affects the content of free amino acids in spinach, a finding that is similar to pine, in which a significant decrease is observed in the content of free amino acids with age (Xu and Xiao 2017).

In our research high content of soluble carbohydrates in winter months was particularly noticeable in female needles of younger age classes, indicating their higher resistance to low temperatures or a higher need for metabolites to protect the tissues. Also Schaberg *et al.* (2000) found that carbohydrates content peaked in winter and it is lowest in spring in *Picea rubens*. Although an earlier study by Iszkuło *et al.* (2009) did not confirm the higher cold resistance of female yew specimens that leads to a disruption of the population gender structure due to increased elimination of the specimens of one gender, as it is seen in *Pistacia lentiscus* (Palacio *et al.* 2005) and *Hippophae rhamnoides* (Li *et al.* 2005). The present study revealed that maintaining a similar cold resistance could be more demanding for female specimens. The population gender disruption is more common in habitats with limited resources. This also confirms the previous observations that female specimens prefer more humid and fertile habitats (Iglesias and Bell 1989, Bertiller *et al.* 2002, Vessella *et al.* 2015). The present study also showed that the content of soluble proteins in female specimens was higher in the late autumn period, which may indicate the increased activity of the enzymatic apparatus before the coldest period or as a reaction to mild water deficit in August and around October and November. In the case of soluble proteins, this effect was also more visible in younger needles. Mild water deficit contributes to the changes in lipid fluidity, a signal that initiates a chain of events leading to the expression of specific genes responsible for the activation of adjustment mechanisms (Hoekstra *et al.* 2001) while triggering the synthesis of specific functional and regulatory proteins (Munns 2002). In contrast, the role of amino acids as osmoprotectants was much less visible according to the results of the present study. Although an increased content of free amino acids was found in winter in both the youngest female and male needles - with the content being slightly higher in the female ones - the main function (protein biosynthesis and building blocks for several other biosynthesis pathways) was much more visible through an increase in the content of amino acids in spring, which was associated with an increase in the content of dry mass and insoluble carbohydrates.

Our research shows that during the year, the most pronounced differences between males and females occur in March, regardless of the age of the needles, and in May and June in the youngest needles. It is a transition period from one season to another. In March, plants intensively prepare for the beginning of forthcoming vegetation season (Hansen and Beck 1994) and female specimens are pollinated, followed by fertilization and gradual development of the embryos of the next generation (Thomas and Polwart 2003, Zarek 2016). In May and June, dynamic processes related to the intensive vegetative development of the new generation of needles take place (Mandre *et al*. 2002). All these factors undoubtedly affect the metabolism of male and female individuals because of their reproductive functions.

Conclusions

Average content of amino acids, soluble and insoluble proteins, and carbohydrates cannot be considered as a good sex-related marker, although their variations can indicate the differences in their metabolism. Gender differences in yew depend on both the age of the needles and the time of the material collection. The needle samples of different ages should not be mixed and sampling time should be chosen precisely. For one-year-old needles, an increased content of free amino acids in June and July and a high content of soluble carbohydrates in January, while for two-year-old needles, an increased content of free amino acids in males from September may become a potentially interesting gender markers. The increase in the differences with the age of the needles may indicate the different pattern of needle aging in both genders, which gives hope for finding more markers in the older needles. However, this should be confirmed by further research with more individuals. The pattern of changes during the 36 months of the needles' life shows that even if they have a similar content of biochemical compounds in both genders, the shift of the peaks over time is often visible, which is caused by metabolic differences. Especially, March shows high variability between the genders in terms of all biochemical features studied in this research.

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