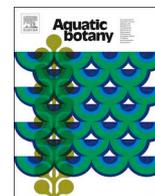




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Mineral nutrients, photosynthetic pigments and storage carbohydrates in turions of 21 aquatic plant species

Lubomír Adamec^{1,*}, Andrea Kučerová¹, Štěpán Janeček²¹ Institute of Botany of the Czech Academy of Sciences, Department of Experimental and Functional Morphology, Dukelská 135, CZ-379 82 Třeboň, Czech Republic² Faculty of Natural Science, Charles University, Department of Ecology, Viničná 7, CZ-128 43 Praha 2, Czech Republic

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ABSTRACT

Turions are vegetative, dormant overwintering storage organs produced by perennial aquatic plants in autumn. They are storage organs for non-structural carbohydrates and also mineral substances (N, P). Contents of photosynthetic pigments (chlorophyll *a* and *b*, carotenoids), storage carbohydrates (starch and five free sugars) and of mineral nutrients (N, P) were estimated in mature autumnal turions of 21 aquatic species from three different ecological groups and eight families; carbohydrates were also measured in the turions of eight species after an overwintering outdoors or in a refrigerator for 4-5 months. The mean turion N and P content was slightly higher than that reported in leaves or shoots of submerged aquatic plants. Assuming effective reutilization, > 54% of the total N and > 70% of the total P amount in mature turions could be theoretically allocated to newly sprouting shoots. The chlorophyll *a* + *b* contents in turions were the same to 2-6 times lower than those in adult leaves/shoots of submerged plants, but they ensure very high photosynthetic rates in sprouting turions. Starch and free sugars (total non-structural carbohydrates, TNC) created the main reserve substances in mature turions with variable ratios in different species. In mature turions, only TNC was significantly different between the rooted/unrooted species after application of a phylogenetic correction; the variability of other biochemical parameters has rather a taxonomic background. About 20% of the autumnal TNC content in turions declined over winter but the rest is available to support turion germination and sprouting. However, what is the minimal TNC pool remains unknown.

1. Introduction

The formation of specific overwintering organs – turions or winter buds – in aquatic plants is very common and represents the most advanced type of overwintering adaptation (Sculthorpe, 1967; Adamec, 2018). Turions are vegetative, dormant storage organs produced by perennial aquatic plants in response to unfavourable ecological conditions, usually at the beginning of autumn in subtropical to polar zones. They are formed at least in 14 genera of aquatic vascular plants, both in basal angiosperms, monocots and eudicots (APG, 2016), especially in submerged (or amphibious) and free-floating species (Adamec, 2018). Turions are formed by extreme condensation of apical shoot segments with modified shortened leaves or scales. Turions of all aquatic plants usually overwinter at the bottom of an aquatic habitat in darkness and under hypoxic or anoxic conditions but they can also overwinter at the water surface or on wet substrate (Sculthorpe, 1967; Adamec and Kučerová, 2013; Adamec, 2018). Two different ecological strategies of turion formation, germination and sprouting can be distinguished

(Sculthorpe, 1967; Adamec, 2008): turions of bottom-rooted species and the rootless *Ceratophyllum demersum* form and ripen at depth and also germinate and sprout at the bottom, in shade and colder water. Turions of submerged rootless and free-floating species, however, form, ripen and also germinate and sprout in light and warmer water at the water surface. In addition, two distinct ecophysiological strategies of autumnal turion sinking and spring rising (floating up, buoyancy) have been distinguished in the plants forming turions at the surface (Adamec, 2008, 2010, 2018). In *Aldrovanda*, *Hydrocharis* and *Spirodela*, an active mechanism of turion sinking and rising has developed. Mature autumnal turions usually break from the dying mother shoots and sink gradually to the bottom. In contrast, turions of various *Utricularia* species are usually less dense than water, but are firmly connected to the mother shoots and dragged to the bottom. By early spring, the turions usually separate and rise to the surface.

Turions as typical overwintering storage organs exhibit low rates of metabolism. Their aerobic respiration rate at 20 °C is 1.5-4 times lower per unit fresh weight (FW) or even 4-7 times lower per unit dry weight

* Corresponding author.

E-mail address: lubomir.adamec@ibot.cas.cz (L. Adamec).<https://doi.org/10.1016/j.aquabot.2020.103238>

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(DW) than that of growing summer shoots or leaves of the same or similar aquatic species (Adamec, 2008, 2011, 2018). The discrepancy between FW- and DW-based values is due to a very high DW:FW proportion (dry matter content, DMC) in turions, which is usually between 18-39% in mature turions and is thus 2.5-4 times higher than that in summer shoots or newly sprouting turions of the same species (Adamec, 2010, 2011, 2018). Mature turions accumulate starch, free sugars, amino acids, reserve proteins and lipids (Janauer, 1981; Villanueva et al., 1985; Ley et al., 1997; Płachno et al., 2014) but the knowledge of contents of these organic storage substances in turions is still fragmentary (cf. Adamec, 2018). The content of starch as the main storage substance in mature autumnal turions can usually range from 16-70% of DW and positively correlates with DMC (for the review, see Adamec, 2018) but Janauer (1981) reported only 0.73% DW of starch with a very low content of free sugars and organic acids in the dormant *Elodea canadensis* turions. The starch content gradually decreases in turions over winter and can reach only ca. 16-52% of its initial autumnal content (i.e., 4.1-37% of DW; Adamec, 2018). Relatively little is known about the total free sugar content in turions: available data indicate great variability (0.4-14% DW) among different species (cf. Janauer, 1981; Adamec, 2003; Heuschele and Gleason, 2014). Moreover, it can be expected from limited data that mainly raffinose family oligosaccharides (RFO) play an important role in the metabolism of storage carbohydrates in some aquatic plants. This can be true for stachyose in *Utricularia* species, as indicated by Janauer and Englmaier (1986): the Lentibulariaceae family belongs to the order Lamiales, where the RFO metabolism is common (Janeček et al., 2011).

Turions are also storage organs for mineral nutrients (at least N, P, S, Mg, Fe) but their storage capacity is considered less marked than that for organic substances (Adamec, 2010, 2011, 2018). In mature turions of 12 aquatic plants, the mean turion N and P content (in % DW) and the ranges were as follows (Adamec, 2010, 2018): N, 1.87 (range 0.56-3.05) and P, 0.26 (0.15-0.41). Generally, these values are comparable to or slightly lower than those compiled from the literature (Dykyjová, 1979) on the leaf/shoot nutrient content in growing plants of the same species. Nevertheless, due to the high DMC of turions of about 30%, the growth of new organs from turions could be supported by about 30% of the total turion's N and 50% of P (Adamec, 2010). All turions contain chlorophyll (Adamec, 2018) but the net photosynthetic rate of innately dormant turions of eight species was slightly negative or very low even under optimum conditions (Adamec, 2003, 2011, 2018). In contrast, sprouting turions can efficiently photosynthesise, which gives them an ecological (phenological) benefit for rapid population growth during the spring season (Adamec, 2011). However, the data on chlorophyll content in turions are very scarce (Heuschele and Gleason, 2014; Oláh et al., 2017).

To fill the knowledge gap in the content of photosynthetic pigments (chlorophyll *a* and *b*, carotenoids), storage (i.e., non-structural) carbohydrates (starch and five free sugars) and mineral nutrients (N, P) in turions, the contents of all these substances were estimated in mature autumnal, innately dormant turions of 21 aquatic plant species (from 22 populations). Carbohydrates were also estimated in the turions of eight species in the state of imposed dormancy after overwintering outdoors or in a refrigerator for 4-5 months, to find out which part of the storage carbohydrates was consumed over winter. The studied 21 species from eight families belong to different ecological groups of aquatic plants. Thus, the biochemical parameters estimated were correlated with functional ecological traits and phylogeny of the species. Stoichiometric relationships were analysed between biochemical parameters.

2. Materials and Methods

2.1. Plant material and sampling

2.1.1. Mature autumnal turions

In total, 21 species of aquatic plants from the temperate zones of

Europe and North America and one tropical population of *Aldrovanda vesiculosa* were used for the study on autumnal, innately dormant turions (for the list of species and their taxonomic and ecological traits and origin, see Table S1 in Suppl.). Out of the 21 species used, 12 are rootless, nine form roots, from which seven root at the bottom and two are free-floating; 13 sprout at the surface; 11 are carnivorous and 10 are non-carnivorous; 13 species are eudicots and eight of them are monocots (after APG, 2016). Mature turions of five species were collected from field sites in the Czech Republic between September and October 2018 (*Potamogeton crispus* turions were collected on 1 July) and those of the remaining species were collected from various outdoor cultivations in 1-2.5 m² plastic containers from the collection of aquatic and wetland plants in the Institute of Botany CAS at Třeboň, Czech Republic (see Adamec, 2008, 2010) between September to November 2018. *Spirodela polyrhiza* and *Myriophyllum verticillatum* grew in a large container densely inhabited by other submerged aquatic plants and, therefore, a strong depletion of mineral N in the water could happen during the turion formation at the end of the growing season. A tropical *A. vesiculosa* population from Katherine, N.T., N Australia, which can form dormant turions, was grown outdoors in a 3.5 l aquarium (Elansary et al., 2010) and formed mature turions in November.

Freshly collected mature turions were thoroughly cleaned of algae and mother shoots, washed by tap water, rinsed by distilled water and blotted dry using a soft paper tissue. In turions of *Caldesia parnassifolia*, *Hydrocharis morsus-ranae*, *Potamogeton acutifolius* and *P. obtusifolius*, the outermost scale-shaped, pale membranous leaflets were removed before analysis. Around 15-60 mg of turion FW was weighed and stored frozen at ca. -25 °C until the chlorophyll *a*, *b* and carotenoids analyses. Around 25-100 mg of turion FW (usually as mixed samples) was weighed and dried at 80 °C for DW and DMC estimation; the dry matter was then used for estimation of turion N and P content. Another ca. 80-200 mg of turion FW was frozen, lyophilised and used for estimation of starch and free sugar content. Where possible, all chemical analyses were performed four times on parallel material which usually consisted of several turions within each sample.

2.1.2. Overwintered turions

Another experiment was established to find the loss of turion reserve carbohydrates and DMC during an experimental overwintering outdoors. At the time of turion sampling (October-November 2018), parallel turions of 10 species (see Table S1) were put into three porous (mesh size 1.5 mm) polyester bags (10 × 10 cm), which were allowed to overwinter at the depth of ca. 55 cm at the bottom of an outdoor 2.5 m² plastic container embedded in the ground in the collection of aquatic plants. This setup mimicked natural overwintering in the field. A submersible temperature datalogger (Minikin T, EMS Brno, Czech Republic) was placed close to the turions to monitor the water temperature every 1 h over winter. On 29 March 2019, the measurement of redox potential using a Pt electrode close to the turions revealed a mean redox potential of 100.5 mV (SD 58.8 mV, *n* = 6). On 29 March 2019, the overwintering turion exposure was terminated. Overall, between 17 November to 29 March, the mean water temperature close to the turions was 3.7 °C and the minimum 1.8 °C. Over the last week of the overwintering (22-29 March), the water temperatures were still rather low between 5.4 to 6.4 °C, but surprisingly, turions of *Utricularia australis*, *Elodea canadensis* and *Myriophyllum verticillatum* distinctly sprouted and could not be used for analyses; turions of the other species showed no signs of germination or sprouting. The same turions of *U. australis* and *M. verticillatum*, overwintered in a refrigerator (at 4.3 ± 0.8 °C in darkness) for the same time, were used instead. Turions of *A. vesiculosa* (from E Poland) overwintered in a refrigerator were also used for comparison with those overwintered outdoors in the container. Moreover, the carbohydrate content was also estimated in turions of *U. australis* and *U. vulgaris*, collected from outdoor collection containers in November 2017 and kept in a refrigerator for 12 months, after a long-term storage on the boundary of their life-span (Adamec, 2018).

2.2. Chemical analyses

Chlorophyll *a*, *b* and carotenoids in mature turions were estimated spectrophotometrically after Lichtenthaler (1987). In summary, frozen turions were ground with 80% acetone, the homogenate was filtered and centrifuged at 13,400 rpm for 2 min and the supernatant was measured for absorbance at 663.2 nm (chl *a*), 646.8 nm (chl *b*) and 470.0 nm (carotenoids). The results were expressed per unit DW. The DMC was estimated in a parallel sample. The sum of chl *a* + chl *b* and the chl *a* to chl *b* ratio are shown.

Dried turions (1-3) were homogenized by a pair of forceps. The homogenised material was digested and mineralised by concentrated acids, diluted and analysed for N and P content colorimetrically by an automatic FIAstar 5010 Analyzer (Tecator, Sweden; for all analytical details, see Adamec, 2002). The molar N to P content ratios are also shown.

For the determination of non-structural carbohydrates, 20-70 mg of lyophilised turions were homogenised with a small steel ball in a 2 ml Eppendorf vial in a ball mill (MM301, Retsch, Haan, Germany) for 30 s. The starch content was determined using a commercially available total starch assay kit (K-TSTA 01/05, AOAC Method 996.11, AACC Method 76.13) developed by Megazyme International (Wicklow, Ireland; www.megazyme.com; Janeček et al., 2015). In summary, 15-60 mg of the milled homogenate was extracted three times by 5 mL of 80% (v/v) aqueous boiling ethanol for 10 min. After centrifugation at 1800 g for 10 min, the filtered supernatant was then evaporated and the ethanol-soluble sugars transferred to distilled water. Starch remaining in the undissolved pellet after extraction was enzymatically reduced to glucose by thermostable α -amylase and amyloglucosidase and the glucose was subsequently determined colorimetrically with the GOPOD reagent containing glucose oxidase, peroxidase and 4-aminoantipyrine. Glucose, fructose and sucrose concentrations in the aqueous solution were estimated using a high-performance anion exchange chromatography with a pulsed amperometric detector (HPAE-PAD) using a Dionex ISC-3000 system (Dionex, Prague) and a CarboPac PA1 analytical column (Dionex). For other analytical details, see Janeček et al. (2015). Similarly, raffinose-family oligosaccharide concentrations were calculated as the difference between ethanol-soluble carbohydrates (galactose, glucose, fructose and sucrose) before and after addition of α -galactosidase (*Aspergillus niger*, Megazyme) to the aqueous extract of ethanol-soluble carbohydrates. The turion contents of five free sugars (glucose, fructose, sucrose, galactose and raffinose) are expressed per unit DW and their sum is denoted as "free sugars". Total non-structural carbohydrates (TNC) represent the sum of free sugars and starch.

2.3. Statistical treatment

Where possible, all analytical data are stated as means \pm 1 SE, $n = 4$. Only chl *a* to chl *b* and starch to TNC ratios were calculated from individual values. As the N and P analyses were conducted on different turions, only the means could be used for the N to P content ratio and correlated with other parameters. One-way ANOVA was used to test the difference in all parameters between the turions of the Polish and Australian *A. vesiculosa* populations, turions of *A. vesiculosa* overwintered outdoors and in a refrigerator, and between *U. australis* and *U. vulgaris* turions overwintered as normal and those overwintered for one year.

The similarity in the biochemical compositions of turions of individual plant species was analysed by Principal Component Analysis (PCA) using the Canoco 5 program (Ter Braak and Šmilauer, 2012). Data were log ($x+1$) transformed to decrease the effect of extreme values. Both the ordination considering true and standardized contents (data for individual species were centered and standardized) was conducted. The relationship between turion N and P contents was analysed by the standardized major axis approach using the Smart package running under the R software environment (Warton et al., 2012) and

was plotted on a graph showing a possible N or P limitation.

The effect of turion overwintering and species identity on carbohydrate content was tested by mixed-effect models, where the factor "Species" was treated as a random factor and "Period" (i.e., autumn vs. spring) as a fixed factor. In analyses including a phylogenetic correction, we included significant phylogenetic eigenvectors (see below) as covariates.

For all parameters estimated in autumnal turions for all 21 species, the differences between functional plant groups (Rooted vs. Unrooted; Bottom vs. Surface sprouting; Carnivory vs. Non-carnivory) and between eudicots and monocots, were tested by one-way ANOVA. The parallel tests with the phylogenetic correction were performed by ANCOVA with significant phylogenetic eigenvectors used as covariates. The similar analyses of the effect of overwintering on carbohydrate content (free sugars, starch, TNC) were performed for eight species. All these analyses of the effect of overwintering and between the functional groups were performed using Statistica (StatSoft, 2011).

Phylogenetic corrections were done using the phylogenetic eigenvector approach (Bauman et al., 2018). Firstly, we calculated phylogenetic distances among all target species using the phylogenetic tree for Spermatophyta, which includes estimated divergency times (Smith and Brown, 2018). The matrix was constructed using the ape package in R (Paradis and Schliep, 2019). For *U. ochroleuca*, which is missing in the phylogenetic tree and which is considered a hybrid species between *U. minor* and *U. intermedia* (Astuti et al., 2019), we calculated the average phylogenetic distances for these two parental species. This distance matrix was used to extract phylogenetic eigenvectors using Principal Coordinate Analysis (PCoA). Significant eigenvectors, which were later used as covariates in the analyses, were selected by forward stepwise selection in General Linear Models using Statistica (StatSoft, 2011).

Linear regression models were used to look for statistically significant relationships between means of different parameters. Eight and three linear regression models are presented for autumnal and spring turions, respectively, including statistically significant or expected and physiologically important relationships. Bonferroni correction was used and, therefore, $P = 0.0063$ or $P = 0.017$ are used as the critical P level in the regression models.

3. Results

3.1. Mature autumnal turions

3.1.1. DMC, mineral nutrients and photosynthetic pigments

The mean DMC of mature autumnal turions of 21 species of aquatic plants was 28.0% FW but the range was very wide from 9.1% (*C. demersum*) to 37.9% (*P. berchtoldii*; Table S2). Turion N content ranged over 10 times, with the extreme minimum in *S. polyrhiza* (0.37% DW) and maximum in the Hydrocharitaceae (3.7-4.0%). Turion P content ranged only 4.1 times among species and the highest values (0.73%) were also found in both Hydrocharitaceae species. The mean molar N to P content ratio in all species was 13.1 ± 1.0 (and/or 5.93 ± 0.43 as a mass ratio; see Fig. 1, Table S2) but ranged 15 times: the highest values were found in all *Utricularia* and *Potamogeton* species. As shown in Fig. 1, the majority of species (except for *S. polyrhiza*, *M. verticillatum* and *C. demersum*) occur between the lines demarcating the N to P mass ratio between 5 and 10, indicating a weak N limitation of the adult plants or higher efficiency of P reutilization from aged shoots. The turion chlorophyll *a* content was very variable across the species and ranged almost 25 times from 0.15 mg g⁻¹ DW in *U. vulgaris* to 3.68 mg g⁻¹ in *P. acutifolius* (Table S2). Whereas the mean chlorophyll *b* content was 0.66 mg g⁻¹ and ranged only 8.8 times. The mean sum of chlorophyll *a*+*b* content was 1.96 mg g⁻¹ and ranged 10.6 times with the minimum in *U. tenuicaulis* and *U. vulgaris* and maximum in *P. acutifolius* turions. The mean chl *a* to chl *b* content ratio was 2.28 ± 0.28 with the minimum in *U. australis* and *U. vulgaris*, while the maximum occurred in *U. intermedia* and *E. canadensis*. The carotenoids content in turions

Table 1

DMC and content of free sugars, starch and total non-structural carbohydrates (TNC) in autumnal turions at the stage of innate dormancy (upper part) and in spring turions at the stage of imposed dormancy after overwintering outdoors or in a refrigerator (lower part). Means ± 1 SE are shown; n = 4. For species abbreviations, see Fig. 1.

Species	DMC (%)	Glucose (% DW)	Fructose	Sucrose	Raffinose	Galactose	Sum of free sugars	Starch	TNC	Starch/TNC (% TNC)
Cer_dem	9.13	0.70 ± 0.19	2.73 ± 0.63	0.20 ± 0.03	1.95 ± 0.29	0.18 ± 0.01	5.77 ± 0.64	20.7 ± 1.3	26.5 ± 1.93	78.3 ± 1.0
Ald_ves_P	23.4	5.81 ± 0.19	3.13 ± 1.05	2.80 ± 0.46	5.13 ± 0.38	0.14 ± 0.004	17.0 ± 0.64	11.6 ± 0.97	28.6 ± 0.70	40.5 ± 2.6
Ald_ves_A	24.6	5.70 ± 0.03	3.11 ± 0.37	2.34 ± 0.14	8.05 ± 0.41	0.32 ± 0.01	19.5 ± 0.40	18.1 ± 0.19	37.6 ± 0.27	48.1 ± 0.7
Utr_aus	33.6	0.93 ± 0.10	0.64 ± 0.07	1.29 ± 0.06	5.04 ± 0.21	0.16 ± 0.01	8.06 ± 0.24	11.5 ± 0.20	19.5 ± 0.25	58.7 ± 1.0
Utr_bre	26.2	0.49 ± 0.05	0.74 ± 0.17	0.23 ± 0.01	5.57 ± 0.09	0.36 ± 0.02	7.38 ± 0.18	9.10 ± 0.51	16.5 ± 0.63	55.1 ± 1.1
Utr_int	25.3	2.35 ± 0.16	2.69 ± 0.24	0.71 ± 0.06	5.11 ± 0.26	0.17 ± 0.02	11.0 ± 0.15	13.9 ± 0.16	24.9 ± 0.31	55.8 ± 0.1
Utr_mac	26.3	1.21 ± 0.14	1.56 ± 0.24	0.62 ± 0.10	6.63 ± 0.30	0.41 ± 0.11	10.4 ± 0.50	3.75 ± 0.57	14.2 ± 1.2	26.4 ± 3.9
Utr_och	31.1	2.27 ± 0.03	1.71 ± 0.05	0.34 ± 0.03	6.65 ± 0.18	0.37 ± 0.06	11.3 ± 0.14	14.0 ± 0.35	25.3 ± 0.36	55.1 ± 0.7
Utr_sty	27.0	2.57 ± 0.12	0.82 ± 0.19	0.22 ± 0.07	7.13 ± 0.12	0.29 ± 0.01	11.0 ± 0.05	15.2 ± 0.88	26.2 ± 0.84	57.7 ± 1.5
Utr_ten	37.4	0.73 ± 0.01	0.46 ± 0.03	0.62 ± 0.05	4.87 ± 0.22	0.12 ± 0.01	6.80 ± 0.29	20.2 ± 1.9	27.0 ± 2.18	74.5 ± 1.2
Utr_vul	23.4	1.11 ± 0.42	1.75 ± 0.33	0.72 ± 0.13	7.52 ± 0.44	1.00 ± 0.42	12.1 ± 0.57	2.97 ± 0.45	15.1 ± 0.89	19.5 ± 2.0
Myr_ver	26.8	1.43 ± 0.25	16.1 ± 1.34	2.86 ± 0.65	0.00	0.04 ± 0.01	20.5 ± 1.1	32.7 ± 0.96	53.1 ± 1.08	61.5 ± 1.8
Pot_acu	31.3	0.06 ± 0.02	0.49 ± 0.06	2.01 ± 0.34	3.85 ± 0.35	0.04 ± 0.01	6.44 ± 0.32	29.0 ± 0.82	35.5 ± 1.12	81.9 ± 0.4
Pot_ber	37.9	0.08 ± 0.02	0.21 ± 0.09	0.77 ± 0.14	1.86 ± 0.34	0.04 ± 0.004	2.96 ± 0.22	41.5 ± 1.1	44.5 ± 0.87	93.3 ± 0.6
Pot_cri	37.0	0.37 ± 0.04	0.88 ± 0.37	0.34 ± 0.13	0.71 ± 0.18	0.00	2.31 ± 0.27	59.4 ± 4.9	61.7 ± 4.71	96.1 ± 0.8
Pot_obt	34.5	0.29 ± 0.02	0.36 ± 0.03	0.73 ± 0.14	1.07 ± 0.69	0.02 ± 0.01	2.47 ± 0.72	46.9 ± 1.7	49.4 ± 1.05	94.9 ± 1.6
Elo_can	13.6	0.09 ± 0.002	1.91 ± 0.09	1.18 ± 0.03	0.08 ± 0.08	0.02 ± 0.002	3.29 ± 0.08	23.6 ± 0.40	26.9 ± 0.44	87.8 ± 0.3
Hyd_mor	25.2	0.22 ± 0.07	1.07 ± 0.19	0.43 ± 0.14	0.78 ± 0.22	0.01 ± 0.01	2.51 ± 0.34	42.5 ± 0.63	45.0 ± 0.77	94.4 ± 0.7
Cal_par	35.8	0.37 ± 0.07	1.02 ± 0.23	0.90 ± 0.15	2.13 ± 0.29	0.24 ± 0.08	4.65 ± 0.11	51.6 ± 0.66	56.2 ± 0.63	91.7 ± 0.2
Spi_pol	32.4	0.41 ± 0.04	0.98 ± 0.09	0.12 ± 0.03	0.61 ± 0.20	0.01 ± 0.001	2.13 ± 0.28	60.5 ± 1.2	62.7 ± 1.33	96.6 ± 0.4
Cer_dem	10.4	0.30 ± 0.06	1.29 ± 0.15	0.053 ± 0.004	3.40 ± 0.15	0.057 ± 0.004	5.10 ± 0.33	27.6 ± 3.6	32.7 ± 3.7	83.9 ± 1.8
Ald_ves_P	20.5	6.94 ± 0.23	2.91 ± 0.27	5.17 ± 0.39	4.42 ± 0.93	0.11 ± 0.02	19.6 ± 1.0	3.15 ± 0.30	22.7 ± 1.2	13.9 ± 1.2
Ald_ves_P*	23.9	6.13 ± 0.16	2.92 ± 0.11	5.39 ± 0.13	5.34 ± 0.38	0.12 ± 0.01	19.9 ± 0.32	2.81 ± 0.28	22.7 ± 0.32	12.4 ± 1.2
Utr_aus*	35.0	1.58 ± 0.14	0.56 ± 0.05	3.76 ± 0.16	6.29 ± 0.39	0.25 ± 0.02	12.4 ± 0.36	6.53 ± 0.32	19.0 ± 0.32	34.4 ± 1.6
Utr_sty	19.1	3.28 ± 0.12	0.75 ± 0.26	1.01 ± 0.41	8.76 ± 0.20	0.18 ± 0.02	14.0 ± 0.43	7.79 ± 0.91	21.8 ± 0.64	35.5 ± 3.3
Utr_vul	21.2	1.42 ± 0.34	1.34 ± 0.18	3.26 ± 0.23	6.08 ± 0.99	0.19 ± 0.03	12.3 ± 1.2	2.04 ± 0.18	14.3 ± 1.2	14.4 ± 1.1
Myr_ver*	25.4	2.73 ± 0.15	12.6 ± 1.55	8.73 ± 0.62	0.66 ± 0.26	0.16 ± 0.02	24.8 ± 1.5	5.89 ± 0.33	30.7 ± 1.8	19.2 ± 0.9
Pot_acu	26.2	0.14 ± 0.01	2.08 ± 1.44	0.96 ± 0.24	0.87 ± 0.45	0.023 ± 0.012	4.07 ± 0.77	22.3 ± 0.22	26.4 ± 0.59	84.7 ± 2.5
Hyd_mor	23.5	0.24 ± 0.04	1.04 ± 0.17	2.15 ± 0.52	4.05 ± 0.51	0.011 ± 0.004	7.49 ± 0.45	25.5 ± 1.4	33.0 ± 1.3	77.2 ± 1.5
Utr_aus_1Y	20.2	1.41 ± 0.33	1.13 ± 0.55	2.73 ± 0.46	7.65 ± 0.52	0.57 ± 0.16	13.5 ± 0.46	9.95 ± 0.46	23.4 ± 0.54	42.4 ± 1.5
Utr_vul_1Y	33.5	3.31 ± 0.28	2.24 ± 0.44	2.94 ± 0.65	5.34 ± 0.54	0.81 ± 0.45	14.6 ± 1.1	1.65 ± 0.35	16.3 ± 1.0	10.2 ± 2.2

Overwintering in a refrigerator for the same time is labelled by an asterisk. 1Y, turions overwintered in a refrigerator for one year.

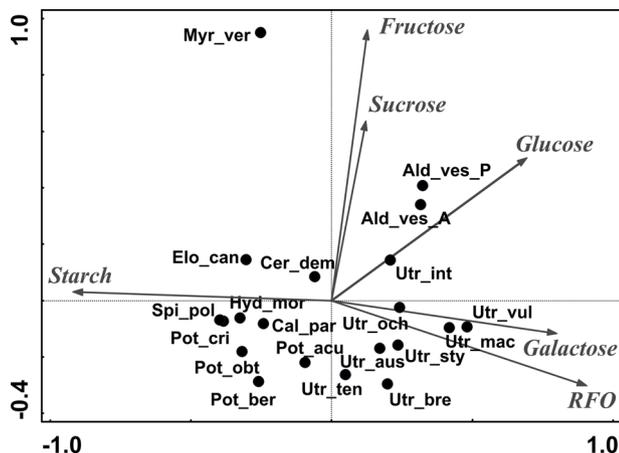


Fig. 3. Patterns of non-standardized carbohydrate contents in autumnal turions of 20 plant species as shown by the PCA. Explained variation: 1st axis 60.1%; 1st + 2nd axes 82.4 %. For species abbreviations, see Fig. 1; RFO, raffinose.

found for TNC content (data not shown). Free sugar content weakly ($P = 0.0078$) and negatively correlated with starch content, indicating a distinct complementarity of both components of the TNC.

No statistically significant difference in turion DMC, N and P contents or pigment contents was estimated between functional (Rooted vs. Unrooted; Bottom vs. Surface sprouting; Carnivory vs. Non-carnivory) or taxonomic groups (Eudicots vs. Monocots) in 21 plant species regardless of the application of the phylogenetic correction, only chlorophyll *a* content was significantly different between bottom- and

Table 2

All meaningful linear regression models between mean contents of various metabolites or parameters in dormant autumnal turions of 21 aquatic plant species (A) or in spring turions of eight aquatic plant species (B). Statistically significant regressions labelled by bold letter. Due to Bonferroni correction, only $P < 0.0063$ are considered significant for A and only $P < 0.017$ for B; *r*, correlation coefficient.

No.	Linear regression	n	r	P
A. Autumnal turions				
1	$N = 1.56 + 1.83 P$	22	0.325	0.14
2	$N = 3.55 - 0.0442 DMC$	22	-0.369	0.091
3	$P = 0.716 - 0.0109 DMC$	22	-0.516	0.014
4	$chl\ a + b = 1.66 + 0.210\ carotenoids$	22	0.251	0.26
5	$carotenoids = 4.39 - 0.106 DMC$	22	-0.598	0.0033
6	$N = 3.27 - 0.0273 TNC$	20	-0.479	0.033
7	$free\ sugars = 13.2 - 0.181\ starch$	20	-0.576	0.0078
8	$starch = -3.94 + 1.08 DMC$	20	0.451	0.046
B. Spring turions				
1	$free\ sugars = 23.1 - 0.848\ starch$	9	-0.800	0.0097
2	$starch = 24.1 - 0.553 DMC_{spring}$	9	-0.346	0.362
3	$TNC = 32.3 - 0.327 DMC_{spring}$	9	-0.334	0.380

Abbreviations used: N, P: turion nitrogen or phosphorus content (% DW); DMC: dry matter content (% FW); chl *a* + *b*: chl *a* + chl *b* ($mg\ g^{-1}\ DW$); TNC: non-structural carbohydrates (% DW); free sugars (% DW); DMC_{spring} , spring value of DMC.

surface-sprouting species without the correction (Table S3). Thus, these parameters are attributable to neither functional-ecological nor taxonomic traits of the species. The starch and TNC contents in autumnal turions were always significantly different ($P < 0.024$) within all functional groups without the correction and also between the taxonomic groups. However, out of all groups, only the TNC content remained significantly different ($P = 0.015$) between the rooted/unrooted species when the correction was applied (Table S3); the higher TNC content was in the rooted species. Thus, the autumnal starch and TNC contents in turions are prevalently phylogenetically based.

3.2. Overwintered turions

In spring, for turions of eight species after overwintering outdoors or in a refrigerator in the stage of imposed dormancy, the mean DMC was 22.8% FW with a wide range from 10.4% (*C. demersum*) to 35.0% (*U. australis*; Table 1). The mean TNC content in these turions was $24.8 \pm 2.1\%$ DW within a relatively narrow range (14.3–33.0%; Fig. 4). Its main component was usually free sugars (mean $13.3 \pm 2.4\%$ DW) ranging from 4.1% DW in *P. acutifolius* to 24.8% in *M. verticillatum*. Raffinose followed by sucrose, fructose and glucose reached the highest

mean content of free sugars (Table 1). The mean starch content was only $11.5 \pm 3.5\%$ DW (range 2.0–27.6%) and only in *P. acutifolius*, *C. demersum* and *H. morsus-ranae* exceeded 36% TNC. No significant difference (one-way ANOVA) in any parameter was found between the two *A. vesiculosa* variants overwintered either as standard outdoors or in a refrigerator for a similar period (data not shown). Turions of *U. australis* overwintered in a refrigerator for one year had significantly (at $P < 0.001$) higher starch and TNC content than those overwintered as standard, but no difference was found between analogical *U. vulgaris* turions (Table 1). Linear regression analysis revealed a significant negative correlation ($P = 0.0097$) between the starch and free sugar content in overwintered turions of eight species (Table 2), indicating a distinct complementarity of both storage resources. Neither starch nor TNC were correlated with the spring value of DMC. As shown in Table 1 and Fig. 4A, the content of free sugars in overwintered spring turions (except for *C. demersum*, *U. vulgaris* and *P. acutifolius*) was increased when compared with mature autumnal turions. In contrast, the starch content in spring turions was lower than in autumnal turions in all species but *C. demersum* and *U. vulgaris* (Fig. 4B) and the same differences also applied to the TNC content (Fig. 4C). A statistical evaluation of data on carbohydrate content between autumnal and spring turions

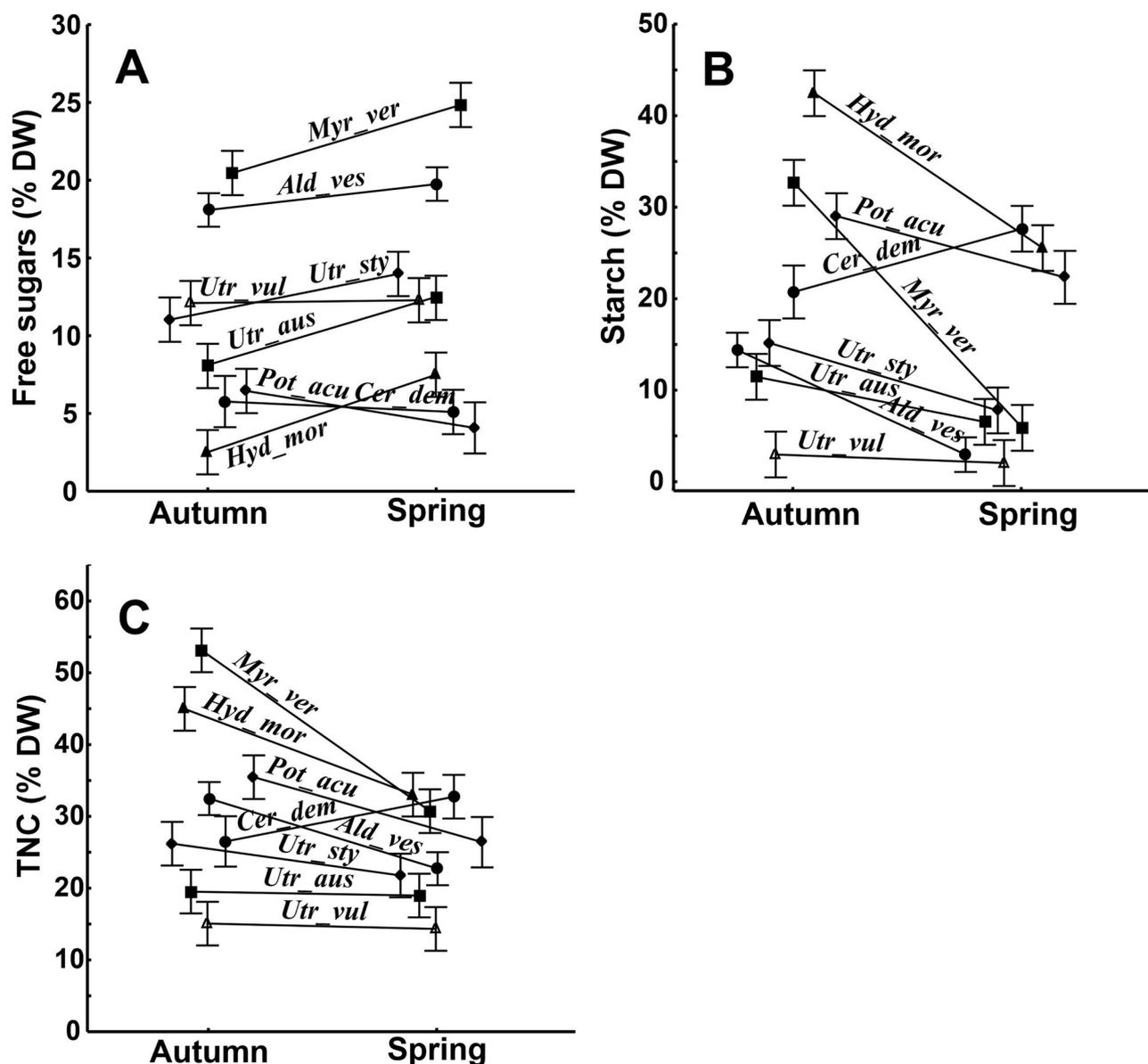


Fig. 4. Carbohydrate reserves in autumnal and spring turions of eight species. Means ± 1 SD are shown. For species abbreviations, see Fig. 1.

without the phylogenetic correction revealed a significant difference both in free sugars, starch and TNC content between species and for the overwintering \times species interaction (Table S4). With the correction, only the overwintering \times species interaction for free sugars, starch and TNC was significant; the overwintering effect was significant only for starch both with and without the correction.

4. Discussion

4.1. DMC, nutrients and pigments in turions

In this study, the turions were collected from various field sites and outdoor cultures differing greatly in their mineral nutrient and CO₂ availability. This means that the discrete turion contents of all analysed substances were also considerably influenced by this variability, regardless of the species (*sensu e.g.* Adamec, 2010; Huang et al., 2017; Li et al., 2017). In this study, the mean DMC in autumnal turions was 28.0% (Table S2; cf. Adamec, 2010); the extremely low DMC in *C. demersum* (9.1%) is due to the high proportion of voluminous lacunae in the turion leaves filled by water. In contrast, the DMC of growing leaves or shoots of a variety of submerged species ranges only from 7-18% (mean ca. 12-15%; Hussner et al., 2015, 2016; Dülger and Hussner, 2017; Dülger et al., 2017). Due to the high content of storage substances, turions are thus around 1.9-2.3 to 4 times more condensed than adult leaves/shoots.

The mean turion contents of N ($2.31 \pm 0.18\%$) and P ($0.41 \pm 0.03\%$; Table S2) in 21 species are comparable with those estimated in mature turions of 12 species (Adamec, 2010) also included in this study. Similar N (0.8-1.3%) and P contents (0.2-0.5%) were found in mature *P. crispus* turions formed in a growth experiment (Xie and Yu, 2011) and Xie et al. (2014) reported 0.7-3.1% N (mean 1.7%) and 0.2-1.5% P (mean 0.6%) in *P. crispus* turions collected from 9 field sites of different trophy. The extremely low turion N content in *S. polyrhiza* ($0.37 \pm 0.01\%$; cf. Appenroth and Adamec, 2015) growing in a large container densely inhabited by submerged aquatic plants incl. *M. verticillatum* could be explained by a strong depletion of mineral N in the water at the end of the growing season and, thus, by a strong N limitation but an excessive P availability; this also partly applies for *M. verticillatum* cultivated in the same container. Both Hydrocharitaceae species, *H. morsus-ranae* and *E. canadensis*, had the highest turion N and P contents, confirming the high nutrient demands of both species. After exclusion of *S. polyrhiza* and *M. verticillatum* as outliers, the molar N to P content ratio in turions was 9.8–20.3 equal to the mass ratio of 4.4-9.2 (Table S2, Fig. 1) with a significant linear correlation between N and P content ($N = 0.347 + 5.13 P$). However, even despite the great differences in turion N and P contents and their ratios, no significant differences between any functional or taxonomic groups were confirmed (Table S3).

Moreover, the turion contents of N and P as well as their ratios found in this study are comparable with those in adult leaves or shoots of the same or similar aquatic species compiled from the literature: e.g., Dykjavová (1979) reported the mean shoot/leaf N content 2.74% (range 1.31-3.80%), P content 0.36% (0.05-0.62%) and the mean mass N to P content ratio 7.6 in 12 species included in this study, Hussner et al. (2016) found the foliar N content 1.0-3.1% (mean 2.0%) in 10 mostly rooted submerged species, and Chou et al. (2019) found the foliar N content of 1.8-3.4% (mean 2.5%) in 18 submerged or free-floating plants at field sites in China. If the N and P contents in turions and adult shoots in aquatic plants are quite comparable, do the N and P pools in turions represent storage substances to be allocated to newly sprouting organs? In a nutrient-deficient medium, turions of six species produced newly sprouting organs from N, P, S and Mg stored in the turions and the proportion of N and P allocated to the newly sprouting shoots (as based on the proportion of the new biomass) could be about 25-60% of the total turion's amount (Adamec, 2011). Considering that the "critical" (growth limiting) content of N is about 1.3% DW and P is 0.13%

DW in aquatic shoots (Gerloff and Krombholz, 1966), and taking into account the mean turion N and P contents (Table S2 and Adamec, 2010), about 30-44% of the total turion's N and about 50-68% of the total P amount can theoretically be used immediately to support the growth of new shoots without limitation. Moreover, assuming a later effective reutilization of N and P from senescent turion segments (like from senescent shoot segments reported for three submerged species, see Adamec, 2014, 2016), at least 54-61% of the total N and 70-85% of P in turions could be allocated to the new growth.

Very variable contents of both chlorophyll *a*, *b* and carotenoids were found in mature turions in all 21 aquatic species (Table S2, Fig. 2) confirming the definition that all turions are green (Adamec, 2018). The mean sum of chlorophyll *a*+*b* content was $1.96 \pm 0.28 \text{ mg g}^{-1}$, the mean chl *a* to chl *b* content ratio was 2.28 ± 0.28 and the mean chlorophyll *a*+*b* to carotenoids content ratio was 1.97 ± 0.23 . However, when the phylogenetic correction was applied to look for differences in pigment contents between the functional or taxonomic groups, no significant difference was found (Table S3). This suggests that the great variability of pigment content is only species-specific. Literature data on photosynthetic pigments in turions are very scarce. Heuschele and Gleason (2014) reported the chlorophyll *a* content of 0.64 ± 0.12 - $0.97 \pm 0.12 \text{ mg g}^{-1}$ DW in mature ("green") *P. crispus* turions collected from the field, while only $0.50 \pm 0.16 \text{ mg g}^{-1}$ in one-year-old ("overwintering") turions in the field (cf. $0.74 \pm 0.03 \text{ mg g}^{-1}$ in Table S2). For tissue-culture-raised *S. polyrhiza* turions, Oláh et al. (2017) found the contents of chlorophyll *a*, *b* and carotenoids about 2-3 times lower than shown for this species in Table S2, but the mean chl *a* to chl *b* content ratio and chlorophyll *a*+*b* to carotenoids content ratio were comparable with our data. However, many studies bring data on pigment contents in dozens of aquatic species. For adult leaves or shoots of dozens of mostly rooted submerged species in the field or growth experiments, several studies report a chlorophyll *a*+*b* content between 2-21.8 (mean ca. 5-14) mg g^{-1} , the chl *a* to chl *b* content ratio between 1.5-2.7 (mean ca. 2.0) and the mean chlorophyll *a*+*b* to carotenoids content ratio between 2.6-3.4 (Ronzhina et al., 2004; Adamec, 2013; Hussner et al., 2015, 2016; Dülger and Hussner, 2017; Dülger et al., 2017). The chlorophyll *a*+*b* contents found in mature turions (Table S2) are thus comparable with or about 2-6 times lower than those in adult leaves/shoots of submerged plants, while the turion chl *a* to chl *b* content ratios are similar to those in leaves/shoots. Obviously, the content of photosynthetic pigments in mature turions is high enough to ensure a very high net photosynthetic rate in sprouting turions (Adamec, 2011).

4.2. Carbohydrate content in turions

Our data from the 20 aquatic species show that the main organic storage substances (energy reserves) in mature autumnal turions are starch (range 3.0-60.5% DW) and free sugars (range 2.1-20.5%; Table 1, Figs. 3, 4). With the exception of three carnivorous species with prevailing free sugars content, starch content created the dominant proportion (> 50%) of the TNC content in the mature turions. The relative importance of the other four types of organic storage substances – proteins, amino acids, organic acids and lipids (Janauer, 1981; Villanueva et al., 1985; Płachno et al., 2014) – in turions remains unknown. It is generally accepted that starch is the main energy reserve substance in mature turions. We also demonstrate that mainly RFOs can be important in some phylogenetic groups of aquatic plants and should be considered in future studies. As RFOs do not only have only storage, but also membrane stabilization and stress tolerance functions (Van den Ende 2013), next exploration of their role in turion physiology is topical. As compiled by Adamec (2018) from the literature, the starch content in mature turions may range from about 16-70% DW and positively correlates with turion DMC (Winston and Gorham, 1979; Ley et al., 1997; Weber and Noodén, 2005). In our study, the highest starch content of around 60% was found in *S. polyrhiza* and *P. crispus* turions.

This value agrees with literature data (e.g., Ley et al., 1997) for the former species but strongly contrasts with the data by Heuschele and Gleason (2014) for turions of the latter species: with only ca. 3% in “overwintering” turions collected from the field and even ca. 1.5% in fresh “green” turions. As several studies conducted on field-collected or culture-raised, mature “green” *P. crispus* turions in China have also consistently confirmed high starch content between 7–70% (usually 30–50%; Xie and Yu, 2011; Wang et al., 2013; Xie et al., 2014; Zhu et al., 2015), the data by Heuschele and Gleason (2014) may not be correct.

In contrast with the high starch content, that of free (soluble) sugars (or the sum of sucrose, glucose and fructose) in mature turions of three aquatic species in the literature is very variable and usually one order of magnitude lower (range 0.42–14.4% DW), which could also be due to inconsistent methods. The mean starch content found in the mature turions of 20 species ($26.4 \pm 4.1\%$ DW; median 20.5%; Table 1) is evidently about 2–5 times higher than that reported in the adult leaves of several dozen submerged or free-floating plants (range 1–35% DW, mean about 5–10%; Ronzhina et al., 2010; Dülger and Hussner, 2017; Dülger et al., 2017; Chou et al., 2019). In general, the content of free sugars in mature turions found in this study or stated in the literature is comparable with or 2–3 times higher than that estimated in adult leaves of about 30 submerged or free-floating species (range 0.7–12.9%, mean about 3–5%; Janauer and Englmaier, 1986; Ronzhina et al., 2010; Chou et al., 2019). Therefore, the TNC content in mature turions is also ca. 2–5 times higher than that in submerged plant leaves.

In mature turions, the starch content in the present study weakly and negatively correlated with free sugars content (Table 2), which indicates a complementarity of both types of reserve substances in the most species. The variability of the TNC as the sum of both types of reserve substances was thus much lower. The difference in the contents of starch and TNC within three functional groups has remained statistically significant after the phylogenetic correction only for TNC between the rooted/unrooted species (Table S3). Highly significant differences for free sugars, starch and TNC contents were also confirmed between eudicots and monocots, with higher contents of free sugars but lower starch and TNC contents in eudicots. Therefore, these taxonomically-based patterns of carbohydrate content mask and underlie the differences within each functional group. Rooted species as such may have evolved higher TNC content than unrooted ones, as sprouting in deeper water is more energy demanding.

After overwintering outdoors or in a refrigerator, the content of free sugars usually increased but that of starch and TNC decreased (Tables 1, 2, Fig. 4): the mean TNC content decline was 6.4% DW, which was equal to only ca. 20.5% of the autumnal TNC content. Adamec (2018) compiled literature data which indicated that as much as 48–84% of the initial starch content could be lost in turions over winter. The exceptionally small TNC increase in *C. demersum* turions over winter could be interpreted as a conversion of other storage substances (e.g., organic acids) into starch over winter. With a few exceptions, raffinose was the dominant free sugar both in mature and overwintering turions. In the leaves of submerged plants, the contents of raffinose and sucrose were clearly complementary (Hussner et al., 2016). In the present study, the use of turions of two species stored for one year (Table 1) did not lead to clear results on a minimal turion carbohydrate threshold necessary for the subsequent turion germination and sprouting. Yet turions (except for *P. crispus*) are not adapted to overwintering for more than one winter season (see Adamec, 2018). Although neither free sugars nor starch nor TNC contents in the overwintered turions correlated with the spring values of the DMC (Table 2), the spring and autumnal values of the DMC significantly correlated with each other ($P = 0.001$, data not shown) so that the mean spring values formed about 86% of the autumnal ones. As opposed to significant negative linear correlations between starch content (and/or DMC) and N or total chlorophyll content found in leaves of many submerged species (Hussner et al., 2016; Dülger and Hussner, 2017; Dülger et al., 2017), such correlations were found only weakly significant in this study for mature turions

(Table 2). However, most of these weak correlations were negative, which indicates that N or P or pigments in turions are “diluted” by reserve carbohydrates (starch, TNC or DMC).

In conclusion, the mean turion N and P content is slightly higher than that usually found in leaves or shoots of submerged aquatic plants. Considering effective reutilization, > 54% of the total N and > 70% of the total P amount in mature autumnal turions could be theoretically reutilised and allocated to newly sprouting shoots. The chlorophyll *a + b* contents in mature turions are the same or several times lower than those in the adult leaves or shoots of submerged plants, but they are sufficient to ensure a very high net photosynthetic rate in sprouting turions. Starch and free sugars (TNC) create the main reserve substances in mature turions but their ratio is very variable among species. However, the significant differences in starch and TNC content between different functional groups of aquatic plants usually become non-significant when a phylogenetic correction is applied. This indicates that these differences are primarily taxonomically, but not ecologically or functionally based. About 20% of the autumnal TNC content in turions declined on average during overwintering but the rest is available to support turion germination and sprouting in the spring. Turions thus function as an important storage organ for carbohydrates but their proportion (pool) to be used for this function remains to be estimated.

5. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.aquabot.2020.103238>.

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