



# Growth, leaf freezing resistance and reproductive fitness differ between sexual diploid and apomictic tetraploid *Ranunculus kuepferi* plants along a climatic elevation transect and at natural growing sites

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## Abstract

Apomictic plant taxa often show latitudinally and elevationally larger distribution ranges than their sexual relatives and tend to colonize previously glaciated areas more frequently. Despite numerous studies, the impact of the environment on this phenomenon is not fully understood. The present common-garden experiment on the alpine model plant *Ranunculus kuepferi*, with diploid sexual populations restricted to the south-western European Alps and tetraploid apomicts widespread throughout the Alpine Arc, should reveal how the cytotypes respond to the increasingly colder climate along a 1000 m elevation transect from the subalpine to the subnival zone in the Austrian Alps. Individuals originating from different populations in the European Alps were transplanted to plots at 4 elevations, and growth, leaf freezing resistance and reproductive fitness were recorded during the following 3 years. Additional measurements were carried out in natural populations. Diploids and tetraploids differed in their response to the respective site conditions along the elevation transect. Sexuals performed equal or even better than apomicts also at the highest site. Otherwise, tetraploids showed features, which could be advantageous in a cold climate: leaf mass per leaf area and rhizome mass increased with elevation, higher leaf freezing resistance, and larger diaspores prone to persist in seed banks. These traits, in combination with other factors, may have facilitated the postglacial establishment of tetraploids in higher regions of the Alps. The results suggest that both different responses to site conditions and different modes of reproduction may have led to the cytotype-specific geographical distribution patterns.

**Keywords** Apomixis · Flowering · Geographical Parthenogenesis · Freezing resistance · Polyploidy · Reproductive fitness · Seed germination · Vegetative growth

## Introduction

A number of apomictic taxa show latitudinally and elevationally larger distribution ranges than their sexual congeners and tend to colonize previously glaciated areas more frequently— a phenomenon which had been termed

Geographical Parthenogenesis (GP) by Vandel (1928) for animals, and later for plants (Stebbins 1950). Various models tried to explain the phenomenon of GP, whereby polyploidy and possible niche differentiation (Hörandl 2006; Lundmark 2006; Schinkel et al. 2016; Karbstein et al. 2021), uniparental reproduction (Johnson et al. 2010; Kirchheimer et al. 2018; Alonso-Marcos 2019; Hörandl 2023), and reproductive barriers caused by interactions between sexuals and apomicts (Levin 1975; Hörandl and Tensch 2009; Dobeš et al. 2018) are possible explanatory approaches. However, there is no general GP pattern because of the manifold combinational effects between taxonomic predispositions and the abiotic and biotic environmental conditions in a species' geographical range (Hörandl 2006, 2023; Tilquin and Kokko 2016). This means that the causality of GP has to be explored separately for each species.

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Apomicts are mainly polyploids (Carman 1997), therefore polyploidy has generally been regarded to be an essential driver of GP (Bierzychudek 1985; Kearney 2005; Lowry and Lester 2006; Lundmark 2006). The combination of genetic information from different ancestors by interspecific hybridization (allopolyploidy) and/or the multiplication of alleles by intraspecific crossing (autopolyploidy) might provide a higher developmental plasticity compared with the diploid parents and might give apomicts an advantage over the diploid parents in stressful habitats (Schinkel et al. 2016; Van de Peer et al. 2021). But this interpretation is non-exclusive since sexual polyploids do not necessarily have wider distribution ranges than diploid relatives (Stebbins and Dawe 1987; Brochmann et al. 2004; Glennon et al. 2014). Similarly, a recent meta-analysis on GP in alpine and arctic plants did not indicate a significant association of polyploid apomicts with GP features as larger distributions, more polar distributions, or previously glaciated areas (Hörandl 2023). However, there is a strong correlation that diploid sexuals and polyploid apomicts have separated distributions which points at a possible indirect role of polyploidy for GP. It fits into the concept that polyploids of several genera have been found to be more resistant to cold than diploid progenitors (Levin 1983; Tsarouhas et al. 2004; Tossi et al. 2022) and there is a strong general tendency of polyploids to be associated with colder climates (Brochmann et al. 2004; Rice et al. 2019).

Apomicts reproduce uniparentally, which means that seeds develop either completely independent of pollen (autonomous apomixis) or - in most taxa - they use self-pollen to fertilize the central cell of the embryo sac for endosperm development (pseudogamous apomixis); (for review: Hörandl 2010; Hojsgaard and Hörandl 2019). In both cases, plants are largely independent from mating partners and pollen vectors which may be beneficial when colonizing new habitats and founding new populations (Baker 1967), and may provide an advantage in cold climates where pollinator activity is irregular (Garcia-Camacho and Totland 2009; Wagner et al. 2016).

Most studies up to date tried to reconstruct important steps in the evolution of GP on the basis of molecular, cytological and morphological data collected across the distribution range of model species representing different taxons (for an overview of published studies see Hörandl 2023). One of these well-researched sexual/apomictic species is *Ranunculus kuepferi* Greut. et Burdet (Ranunculaceae). The species shows a distinct GP pattern within the European Alps, with diploid sexuals restricted to their glacial refugia in the South-Western Alps, and tetraploid apomicts colonizing nearly the whole Alpine Arc, the Northern Apennines and the Corsica mountains (Burnier et al. 2009; Cosendai and Hörandl 2010; Cosendai et al. 2011). Other cytotypes

such as triploids and hexaploids occur only in the geographical contact zone of diploids and tetraploids. In the Alps, apomicts colonize higher elevations (1700–2800 m a.s.l.) than sexual diploids (1300–2500 m a.s.l.); (Cosendai and Hörandl 2010; Schinkel et al. 2016). Diploid sexuals exhibit stylar self-incompatibility (SI) whereas tetraploid apomicts are aposporous, pseudogamous (with rare autonomous apomixis) and self-compatible (SC); (Huber 1988; Cosendai et al. 2013; Schinkel et al. 2016). Thus, sexuals - in contrast to apomicts - depend on pollinators and mating partners to set seeds. Nevertheless, sexual *R. kuepferi* plants generally have a higher seed set than apomicts which due to developmental disturbances show reduced pollen fertility and often a high percentage of sterile ovules and aborted seeds (Huber 1988; Cosendai and Hörandl 2010; Cosendai et al. 2013; Schinkel et al. 2016). A number of studies sought to reconstruct the Holocene range expansion of the sexual and apomictic cytotypes of *R. kuepferi* across the European Alps using different methodical approaches. Based on the cytogeographical studies (Cosendai and Hörandl 2010; Cosendai et al. 2011, 2013), Schinkel et al. (2016) measured in situ growth parameters, and analyzed seed set and the reproductive mode in 81 populations from the whole distribution range in relation to location, elevation, and climate parameters. The analyses do not suggest a long-distance dispersal for founding apomictic populations in the Alps after the retreat of the Pleistocene glaciers, but rather a stepwise dispersal aided by self-fertility and apomixis. Furthermore, Schinkel et al. (2016) and Kirchheimer et al. (2016) found a clear correlation of ploidy and apomixis with elevation and the respective climate and soil conditions, whereby it was not possible to disentangle the effect of each single factor. In the similar direction point the spread models of Kirchheimer et al. (2018) who simulated the Holocene range expansion for *R. kuepferi* and concluded that a niche shift of the apomicts towards colder conditions in combination with competitive advantages of asexuality were probably crucial determinants to overcome topographical barriers. This assumption is supported by a distinct methylation profile of obligate apomictic tetraploids which suggests that epigenetic changes during postglacial recolonization of the Alps have taken place, together with the shift to polyploidy and apomixis (Schinkel et al. 2020).

So far, the research results on GP in *R. kuepferi* provide plausible explanations for a possible evolution and spatial separation of the cytotypes. Experimental work in climate growth chambers and analysis of growth, gene expression and methylation patterns suggested a better cold acclimation of tetraploids than for diploids (Syngelaki et al. 2020a, b, 2021). Cold shocks with freezing temperatures further affected reproductive development and triggered apomictic seed formation in diploid sexual plants (Klatt et al. 2018). However, there is a lack of direct experimental evidence

how cytotypes perform at the same mountain sites under the same climatic conditions, and over longer time periods. We therefore pursued an experimental ecological approach and tested several functional traits of *R. kuepferi* in multiple common gardens along a 1000 m elevation transect. Experimental plots with apomictic and sexual individuals of different geographical origin were established at four elevation levels between 1800 m a.s.l. (subalpine zone) and 2800 m a.s.l. (subnival zone) in the Austrian Central Alps. In order to test temperature effects in particular and to exclude other environmental factors such as soil and competition from the surrounding vegetation, the test plants were grown uniformly in containers with standard alpine soil. The study aimed to capture as much response parameters as possible concerning growth performance, reproduction, ontogenesis, developmental dynamics and functionality of floral structures. The present report concentrates on vegetative growth, survival, leaf freezing resistance, flowering frequency and reproductive success which was investigated during three consecutive years following transplantation. Single examinations were also carried out in a natural sexual and an apomictic population. Ontogenesis, developmental dynamics and functionality of floral structures have been published in a separate paper (Ladinig et al. 2024).

Preconditions to colonize high-mountain areas are to grow and propagate at generally low temperatures in a short growing season (Wagner et al. 2012), and to survive unpredictable cold spells during the growing season whose frequency and severity increases with elevation (Ladinig et al. 2013). Because of the - in several cases evidenced - association between polyploidy and a more flexible stress response (Van de Peer et al. 2021), we expected tetraploid apomicts to cope better with the increasingly colder climate in the elevation transect than the diploid parents. Since diploids are depending on mating partners and thus on pollinators, the reproductive success could be reduced due to unreliable pollinator activity at the uppermost transect sites. If so, the advantage of a higher reproductive fitness in sexuals compared with apomicts should be weakened. Overall, the common garden experiment in the climatic elevation transect was designed to show whether apomictic polyploid and sexual diploid *R. kuepferi* individuals from different source populations differ in functional traits relevant for growth, survival and reproductive fitness, to gain answers whether such traits may have contributed to the different elevational ranges of the cytotypes.

## Materials and methods

### Morphological characteristics of the study plant

*Ranunculus kuepferi* Greuter & Burdet (Ranunculaceae) is a perennial clonally growing herbaceous plant. The shoot system is a belowground sympodial rhizome, consisting of a cluster of individually rooted ramets. Each ramet forms a variable number of grass-like leaves forming a basal rosette (Fig. ESM\_1, S1a). After a vegetative phase, the apical meristem of a ramet becomes floral giving rise to a uni- or multiflorous inflorescence. Soon after seed dispersal, all aboveground structures senesce. As in *Ranunculus glacialis* (Mauracher and Wagner 2021) belowground renewal shoots develop from axillary buds and after rooting disintegrate into daughter ramets.

Flowers of both, sexual diploid (2x) and apomictic tetraploid (4x) plants are hermaphroditic (ESM\_1, Fig. S1b, c). At the study sites in natural populations (2x: Colle della Lombarda, 4x: Kaun Valley, EMS\_2), sexual flowers consisted of 5 sepals, 5 petals, 32–85 stamens and 3–30 free uniovular carpels in spiral arrangement (ESM\_1, Fig. S1b). In apomictic flowers, sepal and petal number (0–5) were irregular; the number of stamens was lower (11–37), and the number of carpels was significantly higher (up to 100) than in sexuals (ESM\_1, Fig. S1c). Each flower gives rise to an aggregate fruit consisting of a variable number of fruitlets (=achenes) which correspond to diaspores. Since apomixis is facultative, both sexual and apomictic seeds can be produced by the same plant in variable proportions (Schinkel et al. 2016; Klatt et al. 2018; Syngelaki et al. 2020a).

### Origin of the plant material and experimental design

*Container plots along the elevation transect.* To test, whether there are differences in the growth performance and reproductive fitness between diploid sexuals and tetraploid apomicts depending on the site climate, individuals of both cytotypes of different origins in the European Alps were transplanted along an elevation transect of about 1000 m in the Stubai Alps (North Tyrol, Austrian Central Alps, 46° 59' N, 11° 06' E). In this region, *R. kuepferi* does not naturally occur, which is important to exclude uncontrolled cross-breeding between experimental and autochthone individuals and thus an influence on the reproductive development. There exist geographically close tetraploids populations in North Tyrol in the Tux Alps and in the Ötztal Alps, which experience very similar climatic conditions as the transplanted individuals in the selected mountain area. Since the present study focused on the influence of abiotic environmental conditions, in particular the effect of temperature

and length of the vegetation period, the selected transect was ideal for carrying out the transplantation experiment. It offered a climatic gradient from the sub-alpine to the sub-nival elevation belt and had access via cable car (important for the transportation of soil and equipment), allowing good accessibility and rapid transportation of samples to the laboratory in Innsbruck within 1–2 h.

To investigate reproductive traits the test plants had to be adult, i.e. capable of flowering. Plant individuals should ideally have been grown from seeds under controlled conditions to exclude the effect of plant age and the impact of the natant environment. To our experience from earlier studies, raising a sufficient number of flowering individuals of the same age is extremely challenging if not impossible in the case of the perennial species *R. kuepferi*. The seedlings do not survive for a long time under greenhouse conditions or in an experimental garden in the valley. Furthermore, apomictic seeds germinate poorly and show different levels of dormancy (see results in the present study). Generally, in perennials, the biological age does not always correspond to the chronological age, which means that the year of transition from the juvenile to the adult stage can vary among individuals. For these reasons, container plots were set up with adult individuals collected in different populations across the Alps (for details on the plant material see below).

In the second half of August 2014, experimental plots were installed at a subalpine site (1770 m a.s.l., hereinafter referred to as P1800), a lower alpine site (2290 m a.s.l., P2300), a higher alpine site (2600 m a.s.l., P2600) and a subnival site (2830 m a.s.l., P2800); (ESM\_1, Fig. S2a–d). The subalpine and lower alpine plots were situated within dense grasslands, which are extensively grazed by cows (subalpine site) and sheep (alpine site). The higher alpine plots were set up on a flat moraine ridge, and the subnival plots on a flat, rocky plateau in the immediate vicinity of the Stubai glacier area. The vascular plant cover was sparse at both upper sites. To eliminate the influence of competition with the natural vegetation and different soils at the different sites, and to focus on the effect of the different site climates, container plots were established. Plastic grid baskets (26 × 28 cm) were lined with garden fleece, filled with lime-free standard alpine soil and sunk into the ground (ESM\_1, Fig. S5a). Two directly adjacent containers formed one plot in which 18 single ramets from different individuals of different provenances per cytotype were planted in a random arrangement. To follow the development of each single individual, the root zone was delimited with a plastic mesh (ESM\_1, Fig. S5b). Per site and ploidy, 3 plots with a total of 54 individuals were arranged in groups (ESM\_1, Fig. S5c). For the plot structure regarding the different provenances of the study plants see Supplements ESM\_2. To avoid uncontrolled pollen transfer between cytotypes - which might

have affected seed development and reproductive success - diploid and tetraploid plot groups had a distance of at least 50 m. Plots were protected from damage caused by grazing animals and birds with wire baskets (ESM\_1, Fig. S5d).

The plant material was collected in July/August 2014 in pure diploid and tetraploid *R. kuepferi* populations along the European Alps, documented by Cosendai and Hörandl (2010). The diploid individuals originated from two populations in the Western Alps (for details on the collection sites see Supplements ESM\_2). Within the tetraploid plots about half each of the individuals originated from populations in the Western Alps and the Eastern Alps. Adult plant individuals (recognizable by fruit stalks) - growing at least 5 m apart - were excavated with the root balls at the end of the fruiting period, when the aboveground organs started to senesce, transported in cooler bags to the Institute of Botany in Innsbruck and kept in a cooling chamber at 5–10 °C in dim light during the daytime until further processing. The belowground budlike renewal shoots for overwintering of each individual were separated from each other; the largest bud from each individual (further referred to as main shoot) was planted into the relevant container according to the planting plan (see Supplements ESM\_2, plot structure with individual number and lists with corresponding provenances). The containers were then transferred to the sites along the transect. Main investigations in the plots were carried out in the growing seasons 2015–2017, single additional measurements in 2018 (see Supplements ESM\_3, test parameters).

*Study sites in natural populations.* Diploids were studied in the extended stands around the Colle della Lombarda in the Western Alps (ESM\_2, population 1) at three different sites between 2260 and 2350 m a.s.l. in summer 2014 (ESM\_1, Fig. S3). Tetraploids were investigated in the upper Kaun Valley in the Eastern Alps (ESM\_2, population 6) at three different sites between 2470 and 2580 m a.s.l. in the growing season 2014 (ESM\_1, Fig. S4).

## Climate measurements

Microenvironmental temperatures were recorded at all sites at hourly intervals throughout the investigation period, using small data loggers (Tidbit, Onset, Bourne, MA, USA). Temperature loggers were placed at the soil surface within each plot and covered with a white plastic boot to avoid solar radiation errors. Periods with snow cover and the exact melting dates could be derived from the logger data.

## Survival

Plots were visited at short intervals between snow melt and fruit dispersal to monitor survival and to record the

numerous plant parameters in the experiment. Because of substantial losses in a part of the plots during the first and second winter, individuals had to be replaced to carry out the investigation program with a sufficient number of individuals. Substitutes came from reserve plots which had been established separately for each cytotype at each site (see ESM\_1, Fig. S5c for an example). Reserve plots had been established in the same year as the container plots from a surplus of adult individuals of different proveniences. As for the container culture, reserve plants were grown from the largest belowground overwintering bud of an individual in lime-free standard alpine soil. But unlike in container culture, the soil substrate was filled in a hand-dug planting bed and the root zones of the individuals were not delimited with plastic meshes. Replacement planting took place at the beginning of the growing season, when the plant losses in the experimental plots became visible. Whole substitute plants that had developed in parallel with the individuals in the plots were carefully dug out, provided with root barriers and planted in the gaps in the containers. As the root system of *R. kuepferi* is not extensive, and the transplanting occurred within minutes, the disturbance was probably only minor.

## Vegetative traits

**Number of ramets and leaves.** Starting with a single planted ramet per individual in the container plots, the formation of lateral ramets during the subsequent 3 growing seasons was recorded by counting the number of basal leaf rosettes above ground. Additionally, the number of leaves of each rosette - and by summing up - of each individual was determined. Because of clonal growth it was not possible to delimit neighboring individuals in natural populations. Thus, only the number of leaves in randomly selected ramets of similar size (2x:  $n=75$ , 4x:  $n=70$ ) was assessed.

**Leaf size, leaf mass and Specific Leaf Area (SLA).** From the individuals in the plots, 24–30 (2x) and 23–32 (4x) fully mature leaves per cytotype and elevation were sampled from the main shoots in the last year of investigation. In the natural populations,  $n=153$  (2x plants) and  $n=83$  (4x plants) fully mature leaves from different, randomly selected individuals were collected. Leaves were individually scanned and leaf areas were determined using an image analysis software (Optimas Corp., Seattle, Washington, USA). Leaves were dried at 80 °C and weighted to the nearest 1 mg. SLA was calculated as the ratio of leaf area (dm<sup>2</sup>) to leaf dry mass (g).

**Epidermis parameters.** Clear nail polish was painted on the lower epidermis of fully expanded leaves in the middle part of the lamina. The dried polish was pulled from the leaf surface by means of clear adhesive strips. The strips with

the epidermis imprint were stuck on microscope slides. Under a light microscope, the number of epidermis cells and stomata per mm<sup>2</sup> was counted and stoma length and width were measured by image analysis (Olympus, Cell<sup>^</sup>D). Per cytotype and elevation, 10 leaves of different individuals were investigated. Cell counts were made in 4 randomly chosen measuring squares (0.175 mm<sup>2</sup> each) per leaf, and on average the length of 23 stomata per leaf was measured.

**Rhizome mass and root number** were determined in 10 individuals each from the natural 2x and 4x populations and, along the transect, on average in 20 individuals per cytotype and elevation. Main ramets were dug out, washed and the number of thicker roots was determined. Rhizomes including preformed buds but without roots were dried for 72 h at 80 °C and weighted to the nearest 1 mg.

**Freezing resistance test.** Exposure to freezing temperatures took place in temperature-controlled chest freezers (Liebherr, Lienz, Austria). Temperature inside the freezing compartment was controlled using adequately programmed software (LabView 2012, NI, Austin, TX, USA) that realizes user-defined cooling profiles with a temperature accuracy of  $\pm 0.2$  K. The plant material was subjected to step cooling procedure consisting of a cooling down phase, a cooling phase at the target temperature and a warming-up phase. Target freezing temperatures were -3, -5, -7, -8, -9, -10, -11, and -12 °C. In a single freezing experiment, cooling started at +5 °C and temperatures were lowered at a rate of -2 K.h<sup>-1</sup> down to the target temperature, which is recommended for temperature-controlled freezing experiments (Arora 2018). Plants remained for 5 h at the target temperature. During the subsequent warming-up phase, temperatures rose at a rate of 2 K.h<sup>-1</sup> until +5 °C was reached again. Plant temperatures were continuously recorded using fine-wire copper-constantan thermocouples (welding spot diameter 0.15 mm) which were placed on the leaves. Treatment temperatures are presented in °C and temperature differences in Kelvin (K) as is the custom in bioclimatology (Leuzinger et al. 2010). To minimize the effect on growth, freezing resistance tests took place in the third year of investigation at the end of anthesis on larger, fully grown leaves of the main shoot. For each test temperature, cytotype and elevation up to 10 leaves were harvested randomly within the plots and kept at 5 °C until the test started on the same day or the day after. Leaves were placed side by side on moist filter paper and enclosed in plastic bags with a zip closure. On average  $n=5$  control leaves per cytotype and elevation were bagged in the same way and further kept at +5 °C. After the freezing treatments bags were slightly opened and, together with the control samples, exposed at room temperature and diffuse light for 4 days to develop the damage symptoms. Percent damage was visually rated for each single leaf. Because of

the small sample size per test temperature, it was not reasonable to specify subsets with regard to plots and origin.

Additionally, chlorophyll fluorescence was measured before and 4 d after the exposure to freezing temperatures. The maximum quantum yield of photosystem II—reflected by the ratio of variable fluorescence  $F_v$  to maximum fluorescence  $F_m$  ( $F_v/F_m$ )—is a good indicator for stress (Buchner et al. 2014) and temperature causing tissue damage (Krause et al. 2010).  $F_v/F_m$  was determined in dark adapted leaves (30 min) using a portable chlorophyll fluorometer (PEA MK2, Hansatech Instruments Ltd., Norfolk, UK). The percentage of reduction of  $F_v/F_m$  compared to baseline before the freezing treatment is a measure for tissue damage. To calculate the temperature that causes 50% tissue damage, a sigmoidal function was fitted to the data within the temperature range between full (lethal temperature  $LT_{100}$ ) and no ( $LT_0$ ) visual damage. The inflection point of the curve indicates  $LT_{50}$ . Dummy values were used to improve curve fitting, i.e. 10 values for zero leaf injury at +4 °C for all groups and 10 values for 100% leaf injury at -11 °C for leaves from P1800 and P2300 sites and -15 °C for leaves from P2600 and P2800 sites, which corresponds to the maximum freezing resistance capacity in leaves of perennial herbs during the growth period (Neuner et al. 2020). According to a modified protocol in Stegner et al. (2020), three  $F_v/F_m$  values were randomly selected by bootstrapping (250 replicates) for each target temperature. The  $LT_{50}$  data set created by the bootstrap runs was used for statistical analyses and to show the differences between 2x and 4x plants in a box plot.

## Flowering traits

In the transect plots, percent flowering individuals per cytotype and elevation was assessed during three growing seasons after transplantation. Additionally, the number of inflorescences per flowering individual, the number of flowers per inflorescence, and the number of flowers per individual were recorded. The number of flowering individuals per year and elevation strongly varied between 7 and 51 in 2x plants and 9–44 in 4x plants. In total, 74–142 inflorescences in 2x plants and 42–138 inflorescences in 4x plants per elevation were analysed during 3 study years; for more details on the test parameters see ESM\_3. In natural populations, where neighboring individuals are not clearly definable, only the numbers of flowers in  $n=75$  (2x plants) and  $n=70$  (4x plants) inflorescences were counted.

## Reproductive success

About one week before seed maturity, all available aggregate fruits in the plots and 100 in each of the natural stands were individually bagged with transparent fine-mesh organza

to prevent a loss of fruitlets (ESM\_1, Fig. S5e). Mature aggregate fruits were harvested and individually stored in Eppendorf tubes at 5 °C. Under a dissecting microscope, the number of intact and undeveloped (ovules not fertilized or aborted) seeds per aggregate fruit was counted. At each elevation in the transect, the total number of analyseable aggregate fruits during 3 study years was 29–109 in 2x plants and 42–71 in 4x plants. At the natural sites, 70 (2x) and 95 (4x) aggregate fruits from different individuals were analysed. To determine the Fruit/Flower (Fr/FI) ratio, the number of flowers maturing at least one mature fruitlet was divided by the total number of flowers under observation.

Mature seeds could not be scooped out from the thin, dry pericarp without damage. To determine the mass of a single achene, up to 5 intact appearing fruitlets of a single aggregate fruit were weighted together to the nearest 0.01 mg and the value divided by the number of weighted fruitlets. Depending on the number of flowers that matured fruits, 43–87 (2x) and 22–48 (4x) aggregate fruits per elevation were analysed.

Seed germination was tested in 66 fruitlets per cytotype and elevation. The seeding material originated from random samplings in the natural stands Colle della Lombarda (2x), and Kaun Valley (4x). Intact appearing fruitlets were sawn in plastic grid cylinders (hair curlers) which were filled with lime-free standard alpine soil and buried next to a plot at each elevation (ESM\_1, Fig. S5f). To prevent washing out, garden fleece was used to close the bottom of each cylinder and to cover the seedbed.

## Statistical analysis

For all traits measured in the transect experiment, two-factorial models were computed with elevation of the transplantation site (i.e. P1800, P2300, P2600, P2800), cytotype (i.e. 2x, 4x) and their interaction as predictors. Additionally, we compared traits between cytotypes separately at each transplantation site. Traits measured at the natural sites NAT (and therefore at only one elevation) were analysed separately using cytotype as predictor.

Metric data (rhizome mass, leaf dry weight, leaf area, specific leaf area (SLA), stoma length, achene mass, fruit/flower ratio) were analysed by linear mixed effect models (LMEs) or by ANOVAs. Model assumptions were checked by diagnostic plots and Levene's test; in the case the model assumptions were not met, traits were transformed by natural logarithm (leaf area, leaf dry weight) or square root (SLA). For count data generalized linear (mixed) models (GLMs, GLMMs) were applied assuming a Poisson distribution (i.e. number of ramets, number of roots per main ramet, leaves per individual, epidermis cells per mm<sup>2</sup>, stomata per mm<sup>2</sup>, inflorescences per flowering individual,

flowers per inflorescence, flowers per flowering individual, ovules per flower, seeds per flower). Data that was recorded at a binomial scale (0/1) were analysed by GLMs/ GLMMs with binomial error distribution (i.e. survival in the 3rd year, individuals flowering y/n, seed germination 1st year, seed germination 2nd year). LMMs or GLMMs with random intercepts were applied (instead of simple ANOVAs and GLMs) if at least one of three potential sources of non-independence in the data should be considered: (i) nesting of individuals within plots; (ii) data from several years; data from several regions of origin. Other data were obtained by a random selection procedure: individuals were selected randomly within each cytotype at each elevation. For the variables temperature, leaf area, leaf mass and SLA statistical differences among sites within the same cytotype were tested with a one-factorial ANOVA and Games-Howell post hoc test. Statistical differences between cytotypes at the same site were tested by *t* tests, LMEs, GLMs or GLMMs depending on the data structure. LT50 was compared between cytotypes at each elevation using Wilcoxon rank sum test, due to inhomogeneity of error variances. Most statistical analyses were computed in R version 4.4.2.: The functions *glm*, *aov* and *wilcox.test* are included in base R. LMEs were computed using the function *lmer* implemented in the package *lmerTest* (Kuznetsova et al. 2017). To model GLMMs we used the function *glmmTMB* implemented in the *glmmTMB* package (Brooks et al. 2017). McFadden's  $R^2$  and Nakagawa's  $R^2$  were computed using the R package performance (Lüdtke et al. 2021), which provides various measures of model fit for generalized and mixed-effects models. One-factorial ANOVAs and *t* tests were performed with the statistical package SPSS ver. 29.0.2.0 (IBM, Armonk, NY, USA). All test were carried out at the critical level of significance  $\alpha=0.05$ .

## Results

### Climate and growth period

Daily mean canopy temperatures in the period between snowmelt and seed maturity differed significantly along the transect ( $p<0.001$ , one-way ANOVA), but not between 2x and 4x plots at the same elevation ( $P\geq 0.17$ , *t* test); (Fig. 1a). Because of different melting dates (Fig. 1b), plot temperatures did not linearly decrease with elevation but were about the same between the early melting site P1800 und late melting site P2600. Mean temperatures were only lower in the subnival plots P2800 where night temperatures regularly dropped close to zero. The significantly highest temperatures among all sites were measured in the natural (NAT) 2x population in the Western Alps. Temperatures in the 4x

NAT population in the Eastern Alps were significantly lower than in 2x NAT population ( $p<0.001$ , *t* test) and within the range measured at the highest plot site P2800 - despite similar snow melt dates as in the 2x NAT population (Fig. 1b). Depending on the study year, *R. kuepferi* plants experienced a varying number of snow days during the growth period (snowmelt until seed maturity; Fig. 1a, numbers below the boxes). Most snow days were recorded in P2300 plots around anthesis, fewer at the later thawing sites higher up, and none at the natural sites.

### Survival and vegetative growth

**Transect.** Cytotypes differed significantly with respect to elevation in most of the investigated vegetative traits reflected by a significant cytotype\*elevation interaction (Table 1; for the effect size  $R^2$  as overall indication of how well the fixed and, where applicable, random effects explain variance in the data see Supplementary Material ESM\_4, Table S1). The direction of the response differed between cytotypes and depended on trait and elevation (for details see statistics in the individual figures).

Survival rates of the initially planted individuals were rather inconsistent over the years along the transect (Fig. 2, closed symbols, full lines); (Table 1, cytotype\*elevation interaction  $P<0.001$ ). Individual losses were highest in 2x plots at P1800 and in 4x plots at P2600 and vice versa lowest in 4x and 2x plots at these sites. In two fully destroyed 4x plots at P2600, clear burrowing traces indicated snow vole feeding during winter; due to the loss of all 4x individuals at two out of three plots, no statistical test was applied for P2600. At P2300 and P2800, mortality rates seemed to reflect the transplantation effect without significant difference between the two cytotypes (2x-4x comparison for the 3rd year:  $P\geq 0.33$ ). In addition, replanting could largely stabilize the pool of individuals (Fig. 2, open symbols, broken lines).

After transplantation of a single ramet (=main shoot) per individual up to 56% of 2x individuals and up to 37% of 4x individuals formed new daughter ramets in the following 3 years without a statistically clear difference between cytotypes (Fig. 3a; Table 1). Mean leaf number per individual increased with elevation in 2x plants but not in 4x plants, resulting in significant cytotype-differences at the higher sites in the third study year ( $P<0.01$ ); (Fig. 3b).

Striking differences between diploids and tetraploids were recorded in the rhizome mass of the main shoot in dependence of elevation (Fig. 3c); (cytotype\*elevation interaction,  $P<0.001$ ; Table 1). The mean dry mass of a single 2x rhizome peaked at 22 mg at P2600. In comparison, mean dry mass of the 4x rhizomes increased with elevation from 24 mg at the lowest sites to 53 mg at the highest

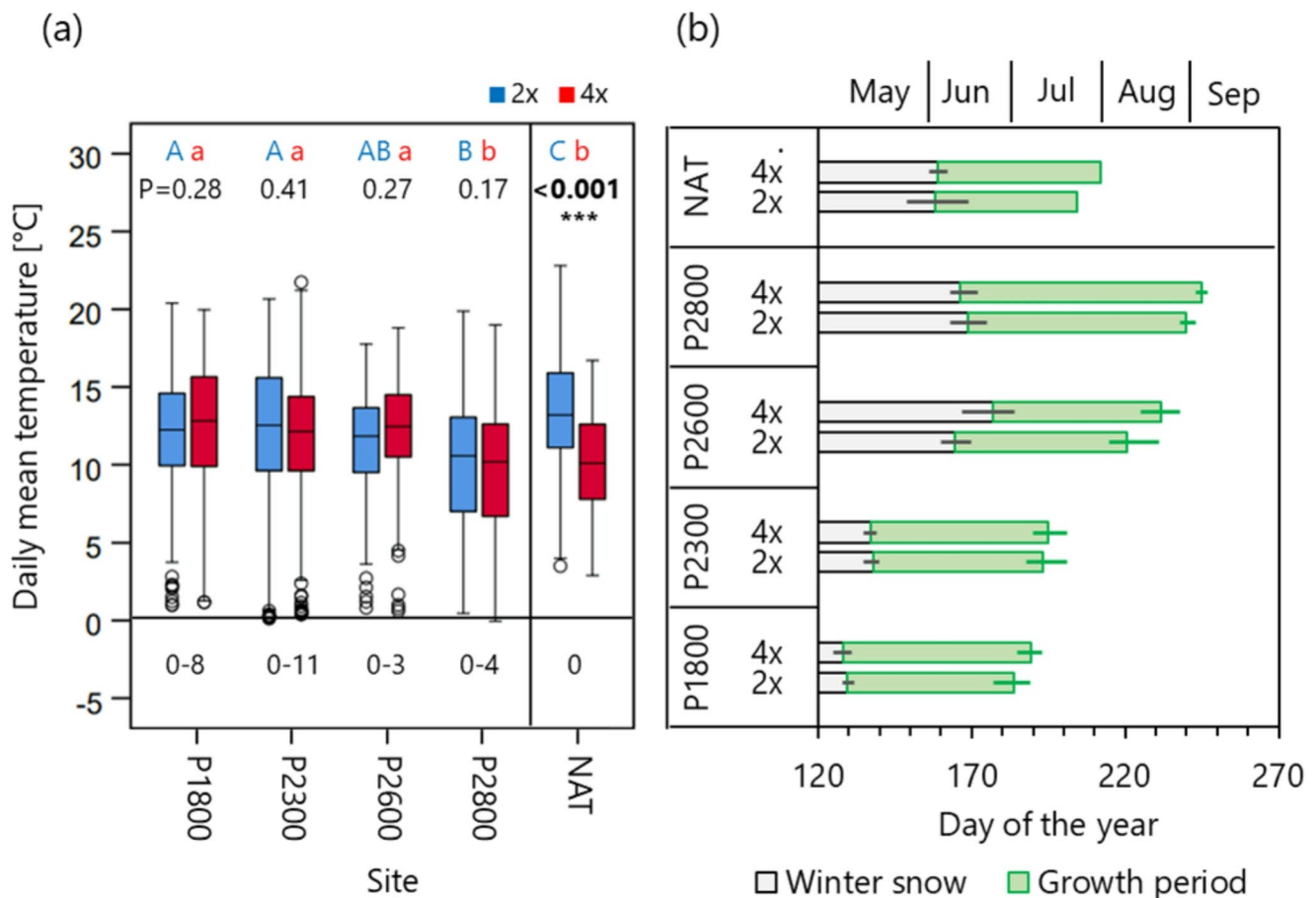


**Fig. 1** Site temperatures and growth period of *Ranunculus kuepferi*. **a** Range of daily mean temperatures in the plots along the transect (P1800– P2800), and at the natural sites (NAT) during the growth period (snowmelt until seed maturity) of 2x (blue) and 4x (red) plants. Temperatures were recorded at the ground level in two (4x NAT) and three study years (2x NAT and transect). Boxes show the median (line inside box), the 25th and 75th percentile (top and bottom of box), maximum and minimum values within the 1.5 times the interquartile range (whiskers), and outliers (circles). Numbers below the boxes indicate the number of snow days during the growth period

site where a part of the rhizomes grew bulb-like. Both cytotypes formed more roots at higher sites than at lower sites, whereby the increase was more pronounced in diploids than in tetraploids (Table 1; Fig. 3d).

Leaf dry weight and leaf area, determined on fully expanded leaves of the main shoot, decreased with elevation in both cytotypes. Values were partly higher in 4x plants than in 2x plants (Fig. 4a, b) which overall led to a significant cytotype\*elevation interaction ( $P < 0.001$ , Table 1). The resulting Specific Leaf Area (SLA, leaf area/dry weight) was about the same along the transect in 2x plants (Fig. 4c), but significantly lower in 4x plants at the lowest and at the highest site ( $P \leq 0.007$ ).

Unlike leaf size, leaf epidermis traits differed highly significantly between diploids and tetraploids at all sites

in the years of investigation. Significant differences among different sites are indicated by uppercase letters (2x) and lowercase letters (4x); (one-way ANOVA, Games-Howell post hoc comparison). Significant differences between the cytotypes at the natural sites and at the same elevation along the transect are indicated with asterisks ( $***P < 0.001$ , ns not significant,  $t$  test). **b** Duration of winter snow cover (grey bars) and growth period of *Ranunculus kuepferi* (green bars) at the different sites; error bars show the respective minimum and maximum duration in the years of investigation

(Fig. 4d–f). Cells of the lower leaf epidermis were smaller in 2x plants than in 4x plants, resulting in a significantly higher cell number (elevation means 1.4–1.8-fold) per unit area (Fig. 4d). Furthermore, diploids had a 1.2–1.7-fold higher stomatal density (Fig. 4e) and smaller stomata (Fig. 4f) than tetraploids. Apart from the intrinsic differences between the two cytotypes, site conditions had an additional impact on epidermal structures (cytotype\*elevation interaction,  $P \leq 0.001$ ; Table 1)

### Leaf freezing resistance

Freezing resistance increased with elevation in both cytotypes, the increase was more pronounced in tetraploids than in diploids (Fig. 5). There was a good correspondence

**Table 1** Effect of cytotype and elevation and their interaction on vegetative traits of diploid sexual and tetraploid apomictic *Ranunculus kuepferi* plants in the transect experiment and at natural sites (NAT). Measures of explanatory power (i.e. Eta-square, McFadden's  $R^2$ , Nakagawa's  $R^2$ ) are summarized in supplementary material ESM 4, table S1)

| Vegetative traits                                              | Transect          |                         |                   |                         | NAT                          |                         |                                          |                                                                  |
|----------------------------------------------------------------|-------------------|-------------------------|-------------------|-------------------------|------------------------------|-------------------------|------------------------------------------|------------------------------------------------------------------|
|                                                                | Cytotype<br>df=1  |                         | Elevation<br>df=3 |                         | Cytotype * Elevation<br>df=3 |                         | Comparison<br>2x-4x<br>transect          | Comparison<br>2x-4x<br>NAT                                       |
|                                                                | Test<br>statistic | P                       | Test<br>statistic | P                       | Test<br>statistic            | P                       | P                                        | Test statistic                                                   |
| Survival y/n                                                   | $\chi^2$ 0.986    | 0.321                   | $\chi^2$ 3.152    | 0.369                   | $\chi^2$ 24.61               | <b>&lt;0.001</b><br>*** | <b>2x ≤ 4x</b>                           | -                                                                |
| Ramets per individual                                          | $\chi^2$ 3.649    | 0.056                   | $\chi^2$ 2.565    | 0.464                   | $\chi^2$ 2.73                | 0.436                   | 2x=4x                                    | -                                                                |
| Rhizome mass per<br>main ramet [mg]                            | F 36.58           | <b>1.02E-08</b><br>***  | F 6.24            | <b>0.0005</b><br>***    | F 9.32                       | <b>1.04E-05</b><br>***  | <b>2x ≤ 4x</b>                           | F 0.64 (1,18)<br>2x=4x<br>0.43                                   |
| Roots per main ramet                                           | $\chi^2$ 6.678    | <b>0.01</b><br>**       | $\chi^2$ 13.39    | <b>0.004</b><br>**      | $\chi^2$ 2.925               | 0.403                   | <b>2x ≥ 4x</b>                           | $\chi^2$ 2.09 (1,23)<br>2x=4x<br>0.15                            |
| Leaves per individual                                          | $\chi^2$ 9.25     | <b>0.002</b><br>**      | $\chi^2$ 3.90     | 0.273                   | $\chi^2$ 7.00                | 0.072                   | <b>2x ≥ 4x</b>                           | -                                                                |
| Leaf dry weight [g]                                            | F 22.05           | <b>&lt;0.001</b><br>*** | F 13.38           | <b>&lt;0.001</b><br>*** | F 7.8                        | <b>&lt;0.001</b><br>*** | <b>2x ≤ 4x</b>                           | F 58.57<br>(1,234)<br><b>2x &lt; 4x</b><br><b>5.12E-13***</b>    |
| Leaf area [dm <sup>2</sup> ]                                   | F 10.57           | <b>0.001</b><br>***     | F 23.03           | <b>&lt;0.001</b><br>*** | F 9.4                        | <b>&lt;0.001</b><br>*** | <b>2x ≤ 4x</b>                           | F 241.3<br>(1,234)<br><b>2x &lt; 4x</b><br><b>&lt;2.2E-16***</b> |
| Specific leaf area<br>(SLA, dm <sup>2</sup> .g <sup>-1</sup> ) | F 25.8            | <b>&lt;0.001</b><br>*** | F 8.8             | <b>&lt;0.001</b><br>*** | F 1.4                        | 0.249                   | <b>2x ≥ 4x</b>                           | F 69.55<br>(1,234)<br><b>2x &lt; 4x</b><br><b>6.4E-15***</b>     |
| Epidermis cells per<br>mm <sup>2</sup>                         | $\chi^2$ 6310.5   | <b>2.20E-16</b><br>***  | $\chi^2$ 294.9    | <b>2.20E-16</b><br>***  | $\chi^2$ 192.7               | <b>2.20E-16</b><br>***  | <b>2x &gt; 4x</b><br><b>&lt;0.001***</b> | -                                                                |
| Stomata per mm <sup>2</sup>                                    | $\chi^2$ 1104.1   | <b>2.20E-16</b><br>***  | $\chi^2$ 171.8    | <b>2.20E-16</b><br>***  | $\chi^2$ 124.8               | <b>2.20E-16</b><br>***  | <b>2x &gt; 4x</b><br><b>&lt;0.001***</b> | -                                                                |
| Stoma length [μm]                                              | F 681.4           | <b>&lt;0.001</b><br>*** | F 10.5            | <b>&lt;0.001</b><br>*** | F 59.4                       | <b>&lt;0.001</b><br>*** | <b>2x &lt; 4x</b><br><b>&lt;0.001***</b> | -                                                                |

Notes: Statistics for survival, ramets per individual, leaf traits and roots refer to the 3rd year, those for rhizomes to the 3rd and 4th year after transplantation

$\chi^2$  indicates Wald Chi-squared test statistics. Significance is indicated in bold, \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ . Bold letters in the column Comparison 2x-4x transect indicates significant difference between cytotypes at least at one site (details are shown in the figures); ≤ means that depending on the site, trait values were smaller, equal or larger in diploids than in tetraploids. NAT test statistic: df values in brackets, – not determined

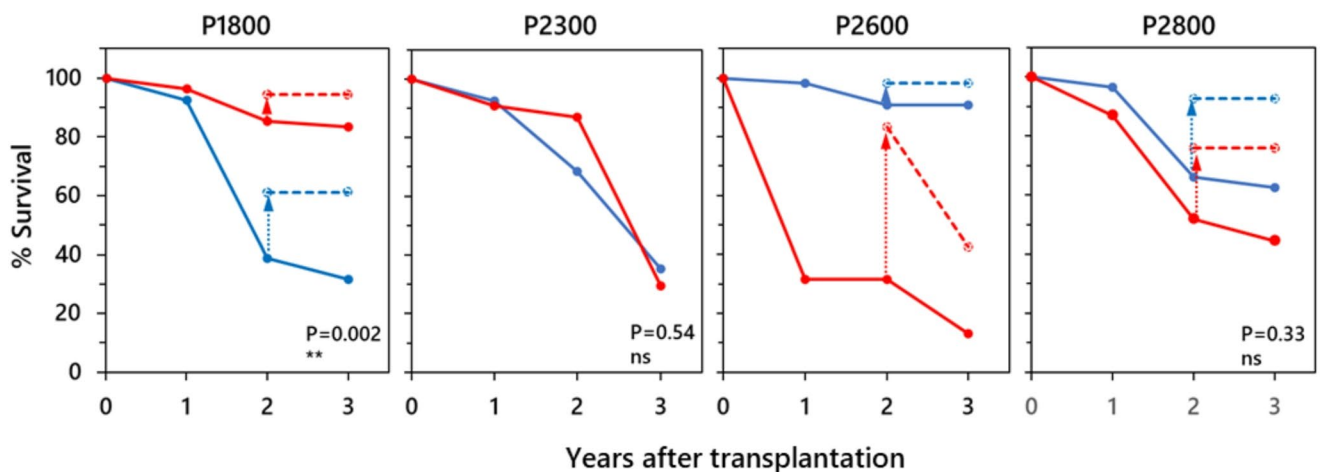


**Fig. 2** Survival rates of 2x (blue) and 4x (red) *Ranunculus kuepferi* plants in the transect experiment during 3 years following transplantation. Closed symbols and full lines: % survival of originally planted individuals. Losses were partly replaced (arrows), increasing the total percentage of living individuals (open symbols, broken lines).  $P$ -val-

ues and asterisks indicate significant differences between originally planted individuals in the third year (\*\* $P \leq 0.01$ , ns not significant, GLMM). Due to the loss of all 4x individuals at two out of three plots because of vole feeding, no statistical test was applied at P2600

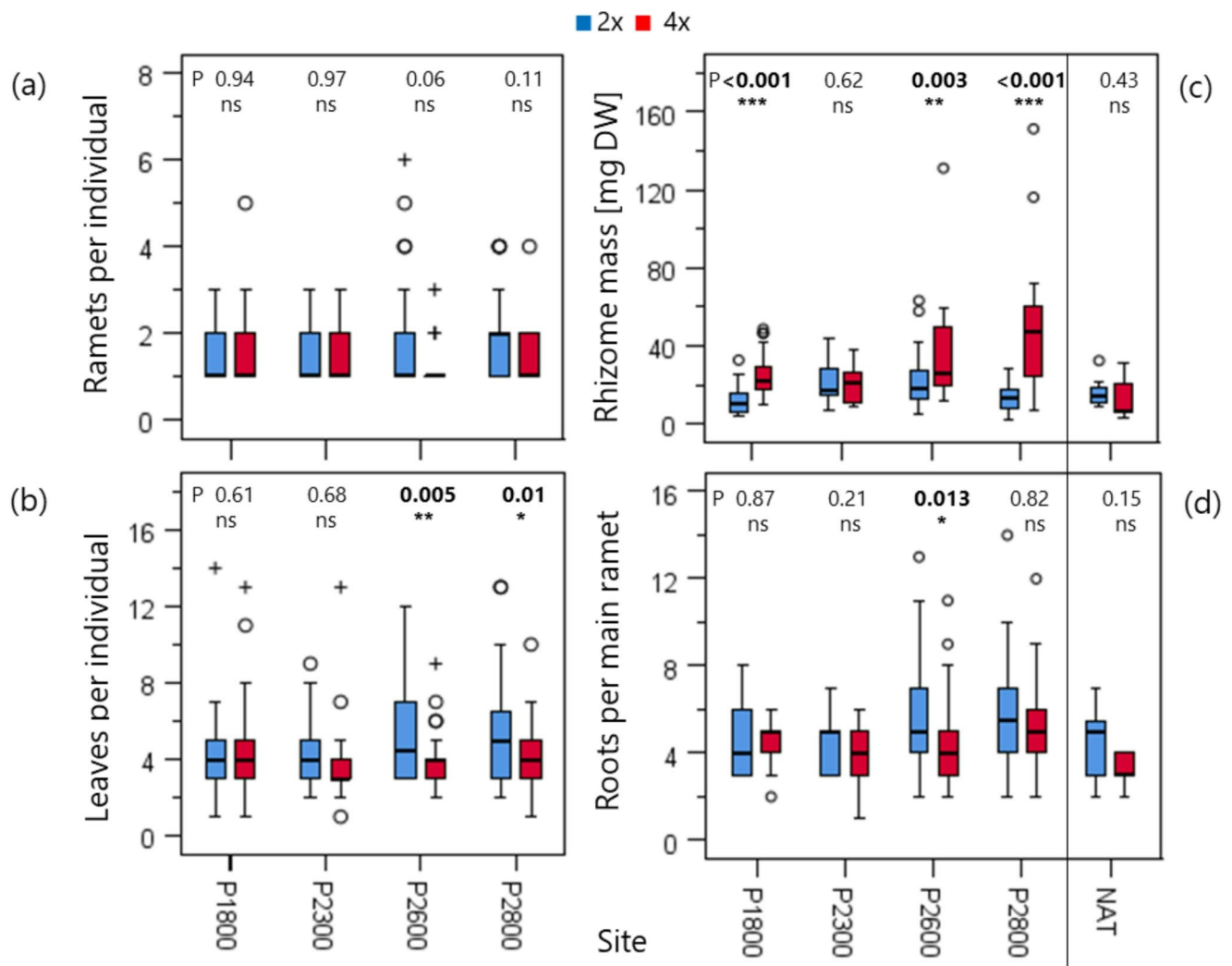


**Fig. 3** Vegetative growth of 2x (blue) and 4x (red) *Ramunculus kuepferi* plants in the transect experiment in the third year following transplantation, and at the natural sites (NAT). Number of **a** ramets per individual, **b** leaves per individual, **c** rhizome mass (mg dry weight DW) of the main ramet, and **d** number of thicker roots per main ramet. For

details on the boxplots see legend of Fig. 1a. Circles indicate outliers and crosses indicate extreme values. *P*-values indicate statistical differences between cytotypes at the same site (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , ns not significant, **a** GLMM, **b**, **d** GLM, **c** LME)

between chlorophyll fluorescence (expressed as a percentage of the ratio  $F_v/F_m$  before the frost treatment) and the extent of visible damage (no damage above 90% of the initial value, partial damage between 90% and 40%, and full damage below 40%). First visual injury and  $F_v/F_m$ -values below the control range were observed at  $-5^\circ\text{C}$  at all sites. Most leaves were fully damaged at  $-9^\circ\text{C}$  at the lower sites, but beyond  $-12^\circ\text{C}$  at the higher sites. LT50, i.e. the temperature at which  $F_v/F_m$  was 50% of the initial value before the freezing treatment, was significantly lower in tetraploids than in diploids at all transect sites ( $P < 0.001$ ).

### Vegetative traits at natural sites

Since *R. kuepferi* forms clusters of individually rooted ramets, it was not possible to delimit genetic individuals in the natural populations (NAT). For the measurements, flowering ramets were randomly chosen within the stand. Mean rhizome mass and mean root number did not significantly differ between cytotypes at the natural sites (Fig. 3c, d, NAT). Values for 2x NAT plants were in the range of the 2x transect values, while those for 4x NAT plants were generally lower than in the transect.

Leaves were significantly smaller and lighter and had a significant lower SLA in 2x NAT plants than in 4x NAT plants ( $P < 0.001$ ; Table 1; Fig. 4a, b, c). 2x NAT leaves were also significantly smaller ( $P \leq 0.001$ ) and lighter ( $P \leq 0.03$ ),

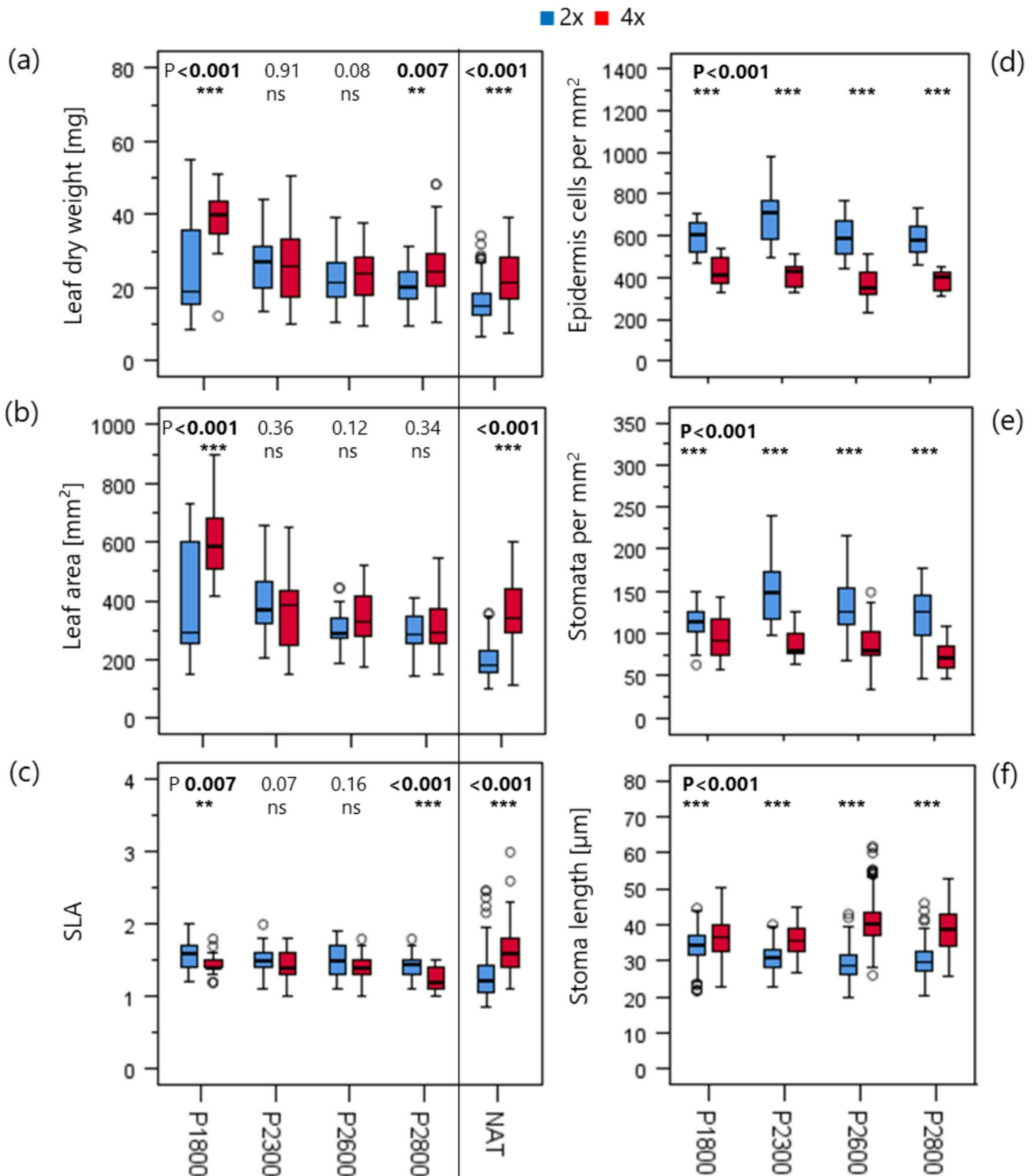


**Fig. 4** Leaf traits of 2x (blue) and 4x (red) *Ranunculus kuepferi* plants in the transect experiment in the 3rd year after transplantation and from random measurements in natural populations (NAT). **a** leaf dry weight, **b** leaf area, **c** specific leaf area (SLA, leaf area/dry weight), **d** number of epidermis cells per mm<sup>2</sup> epidermis, **e** number of stomata

per mm<sup>2</sup> epidermis, and **f** stoma size in the two largest leaves of the main ramet. For details on the boxplots see legend of Fig. 1a. P-values and asterisks (\*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ ) indicate statistical differences between cytotypes (**a**, **b**, **c**, **f** t test, **d**, **e** GLM); ns not significant

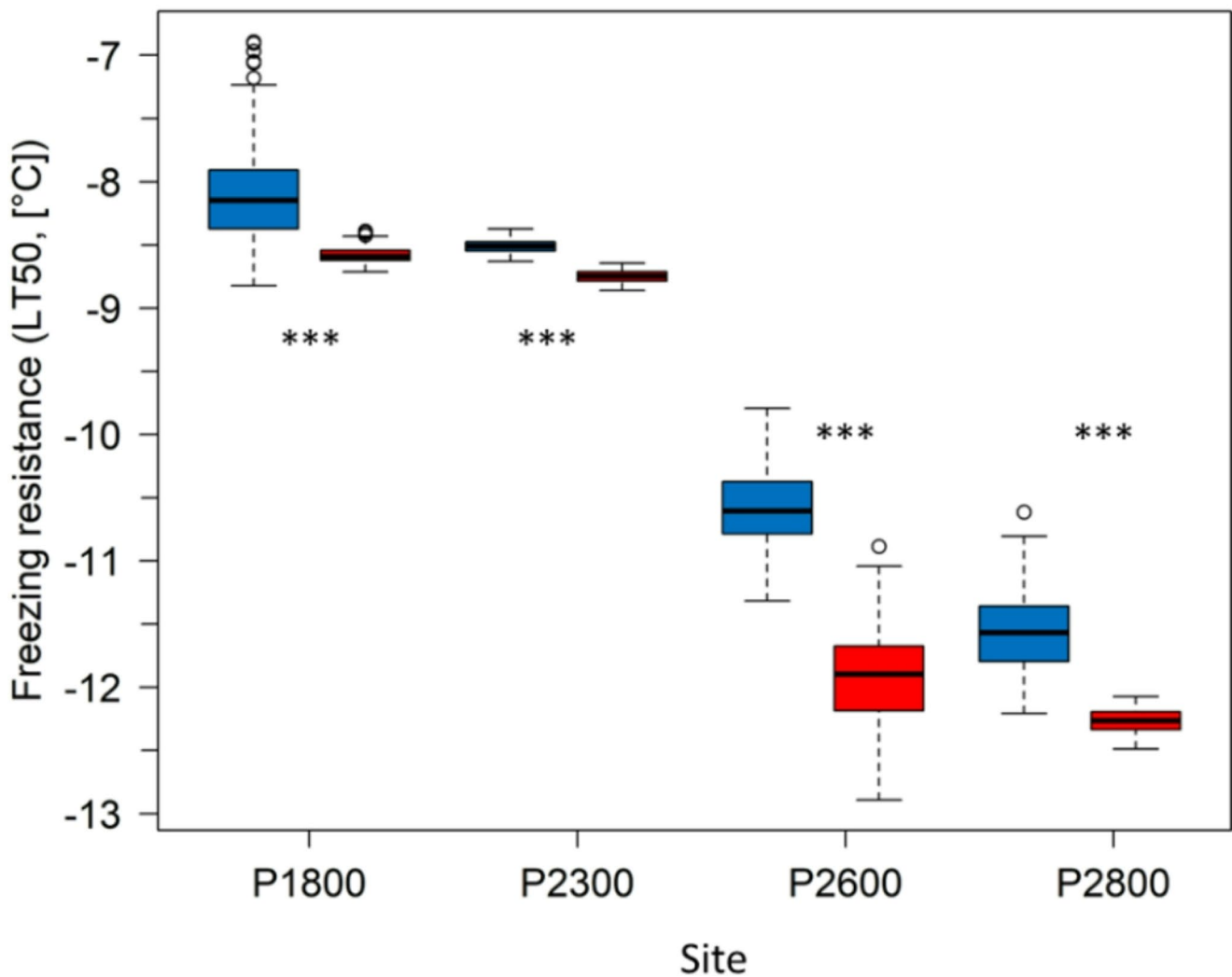


**Fig. 5** Leaf freezing resistance in 2x (blue) and 4x (red) *Ranunculus kuepferi* plants in the transect experiment in the 3rd year after transplantation expressed as the lethal temperature LT50 calculated from the chlorophyll fluorescence (Fv/Fm) after the frost treatment related

and had a significant lower SLA ( $P \leq 0.003$ ) compared to 2x leaves in the whole transect. The size of 4x NAT leaves was in the range measured in the transect from P2300 upwards (Fig. 4a, b NAT), and SLA was remarkably high (Fig. 4c NAT).

### Flowering and reproductive success

In the first year after transplantation, the fraction of flowering individuals did not differ greatly in both cytotypes over the whole transect (Fig. 6a, b, open bars). Since flowers are initiated in the year before flowering, flowering frequency in the first year was influenced by the floral status that plants had acquired at the original natural sites before transplantation into the plots. In the study years 2 and 3, flowering frequency significantly differed between cytotypes in

to the initial value immediately before the treatment (= 100%) in single leaves. For details on the boxplots see legend of Fig. 1a. Asterisks indicate significant differences between cytotypes at the same elevation (\*\*\*)  $P < 0.001$ , Wilcoxon rank sum test)

dependence of elevation (cytotype\*elevation interaction,  $P = 0.007$ , Table 2); with increasing elevation, the percentage of flowering individuals increased in diploids, whereas no clear trend was discernible in tetraploids (Fig. 6a, b). The number of inflorescences per flowering individual (ESM\_1, Fig. S6a), flowers per inflorescence (ESM\_1, Fig. S6b) and flowers per flowering individual (Fig. 6c 2nd and 3rd year pooled; ESM\_1, Fig. S6c single years) varied greatly and therefore did not significantly differ among cytotypes and did not show a significant impact of elevation (Table 2).

Depending on the cytotype, year and elevation, 50 to 80% of flowers matured aggregate fruits (Fig. 6d). The three-year averages were about the same in 2x and 4x plants along the transect, except for the highest site where the fruit/flower ratio in 4x plants dropped markedly below the value

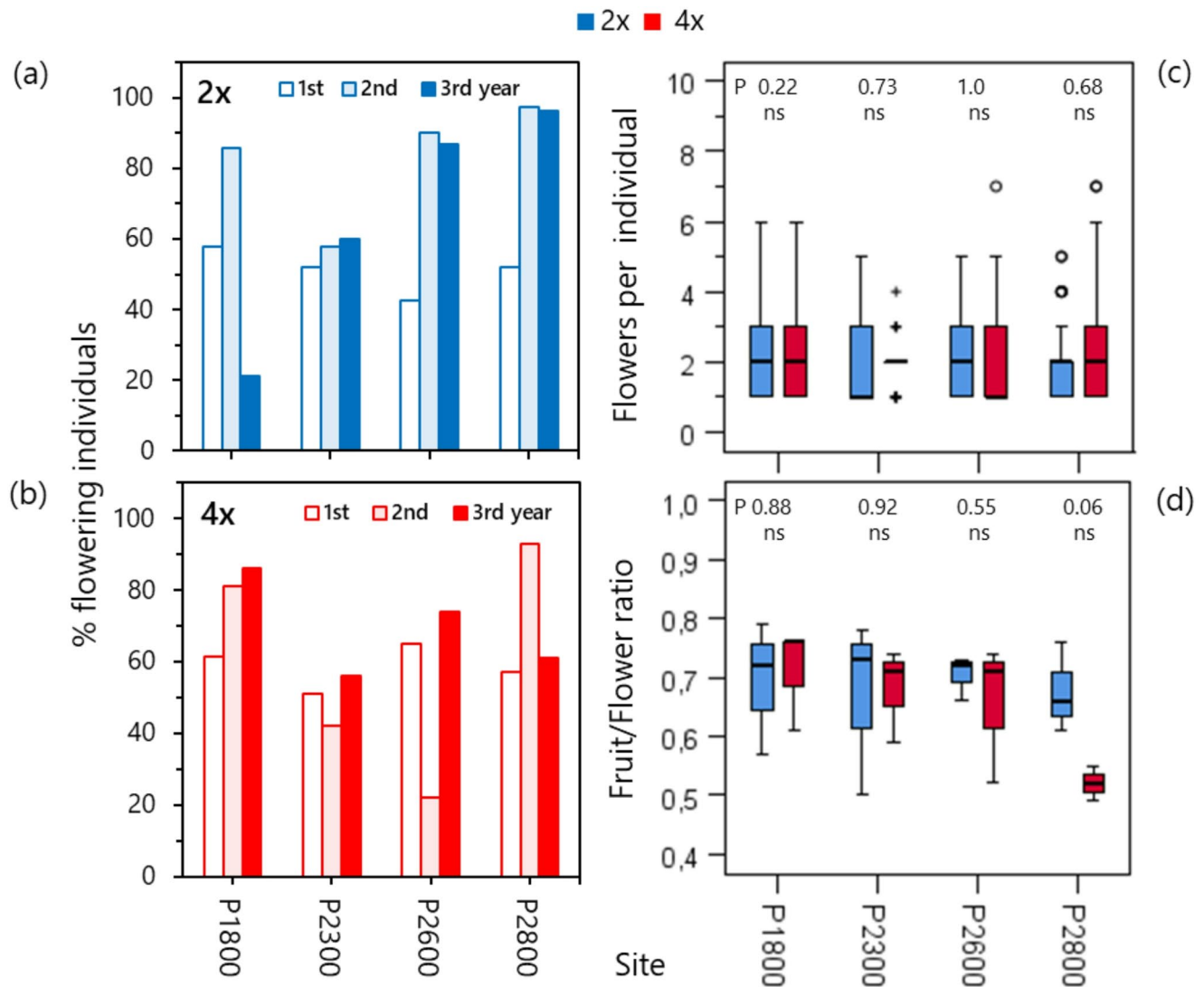


**Fig. 6** Flowering and fruiting in 2x (blue) and 4x (red) *Ranunculus kuepferi* plants in the transect experiment. **a**, **b** percent flowering individuals in the 1st (open bars), 2nd (light colored bars), and 3rd study year (dark colored bars), **c** number of flowers per flowering individual (2nd and 3rd year pooled), and **d** Fruit/Flower ratio in three years

in 2x plants. Because of the low number of repetitions ( $n=3$  years) the difference is not significant.

Tetraploids developed about twice as much carpels per flower than diploids both in the plots and at the natural sites. Since carpels of *R. kuepferi* are uniovular these values automatically apply to the number of ovules per flower (Fig. 7a). Ovule number increased with elevation particularly in tetraploids, resulting in a highly significant cytotypic\*elevation interaction ( $P<0.001$ , Table 2). At the natural sites, ovule numbers corresponded to those found in the lower transect plots up to P2600 (2x) and P2300 (4x).

Seed number per aggregate fruit increased in 2x plants but decreased in 4x plants with elevation (Fig. 7b; cytotypic\*elevation interaction  $P<0.001$ , Table 2). Though

after transplantation. For details on the boxplots see legend of Fig. 1a. Circles indicate outliers and crosses indicate extreme values. P-values in **c** and **d** indicate no statistical differences between cytotypes (ns, c GLMM, d LME)

diploid flowers formed far less carpels and thus ovules (Fig. 7a) they matured an equal number (P1800) or even significantly more seeds (P2600, P2800) than tetraploid flowers. Interestingly, in the natural populations, the mean seed output of a single flower did not differ between cytotypes and was higher than in the transect (Fig. 7b NAT).

Achene mass did not show a uniform trend along the transect in both cytotypes: the weight of P2300 fruitlets was in the range of the P2800 fruitlets and that of P1800 in the range of the P2600 fruitlets (Fig. 7c). Tetraploids produced significantly heavier achenes than 2x plants both at the natural sites and in the transect, with the exception of the highest site (Fig. 7c NAT).

**Table 2** Effect of cytotype and elevation and their interaction on reproductive traits of diploid sexual and tetraploid apomictic *Ranunculus kuepferi* plants in the transect experiment and at natural sites (NAT). Measures of explanatory power (i.e. Eta-square, McFadden's  $R^2$ , Nakagawa's  $R^2$ ) are summarized in supplementary material ESM\_4, table S2)

| Reproductive traits                        | Transect          |                        |                   |                        | NAT                             |                        |                                 |                            |                                          |
|--------------------------------------------|-------------------|------------------------|-------------------|------------------------|---------------------------------|------------------------|---------------------------------|----------------------------|------------------------------------------|
|                                            | Cytotype<br>df=1  |                        | Elevation<br>df=3 |                        | Cytotype *<br>Elevation<br>df=3 |                        | Comparison<br>2x-4x<br>transect | Comparison<br>2x-4x<br>NAT |                                          |
|                                            | Test<br>statistic | P                      | Test<br>statistic | P                      | Test<br>statistic               | P                      | P                               | Test<br>statistic          | P                                        |
| Individuals flowering y/n                  | $\chi^2$ 3.878    | <b>0.049</b><br>*      | $\chi^2$ 12.27    | <b>0.007</b><br>**     | $\chi^2$ 26.01                  | <b>9.47E-06</b><br>*** | <b>2x ≤ 4x</b>                  | -                          | -                                        |
| Inflorescences<br>per flowering individual | $\chi^2$ 1.497    | 0.221                  | $\chi^2$ 0.687    | 0.876                  | $\chi^2$ 0.64                   | 0.887                  | 2x=4x                           | -                          | -                                        |
| Flowers per inflorescence                  | $\chi^2$ 0.944    | 0.331                  | $\chi^2$ 1.344    | 0.719                  | $\chi^2$ 3.67                   | 0.210                  | 2x=4x                           | -                          | -                                        |
| Flowers per flowering<br>individual        | $\chi^2$ 0.029    | 0.864                  | $\chi^2$ 0.994    | 0.803                  | $\chi^2$ 3.21                   | 0.360                  | 2x=4x                           | -                          | -                                        |
| Fruit/flower ratio                         | F 1.688           | 0.4297                 | F 1.7614          | 0.2006                 | F 1.39                          | 0.2866                 | 2x=4x                           | -                          | -                                        |
| Ovules per flower                          | $\chi^2$ 2062.1   | <b>2.20E-16</b><br>*** | $\chi^2$ 232.54   | <b>2.20E-16</b><br>*** | $\chi^2$ 53.6                   | <b>1.40E-11</b><br>*** | <b>2x &lt; 4x</b>               | $\chi^2$ 488.7<br>(1,163)  | <b>2x &lt; 4x</b><br><b>&lt;0.001***</b> |
| Seeds per flower                           | $\chi^2$ 81.448   | <b>2.20E-16</b><br>*** | $\chi^2$ 27.303   | <b>5.09E-06</b><br>*** | $\chi^2$ 261.7                  | <b>2.20E-16</b><br>*** | <b>2x ≥ 4x</b>                  | $\chi^2$ 0.449<br>(1,177)  | 2x=4x<br>0.50                            |
| Achene mass [mg]                           | F 24.843          | <b>9.53E-07</b><br>*** | F 23.895          | <b>3.37E-14</b><br>*** | F 1.65                          | 0.178                  | <b>2x ≤ 4x</b>                  | F 7.6<br>(1,106)           | <b>2x &lt; 4x</b><br><b>0.007**</b>      |
| Seed germination<br>1st year after sowing  | $\chi^2$ 125.12   | <b>2.20E-16</b><br>*** | $\chi^2$ 29.012   | <b>2.23E-06</b><br>*** | $\chi^2$ 1.49                   | 0.806                  | <b>2x ≤ 4x</b>                  | -                          | -                                        |
| Seed germination<br>2nd year after sowing  | $\chi^2$ 111.48   | <b>2.00E-16</b><br>*** | $\chi^2$ 10.081   | <b>0.01789</b><br>*    | $\chi^2$ 1.49                   | 0.685                  | <b>2x ≤ 4x</b>                  | -                          | -                                        |

Notes: Statistics for flowering data (individuals flowering y/n, inflorescences per flowering, flowers per inflorescence, flowers per flowering individuals) refer to the 2nd and 3rd year, for fruit/flower ratio, ovules per flower, seeds per flower to year 1–3, and achene mass to the 1st year after transplantation. Statistics for seed germination refers to the 1st and 2nd year after sowing

$\chi^2$  indicates Wald Chi-squared test statistics. Significance is indicated in bold, \*\*\* $P \leq 0.001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ . Bold letters in the column Comparison 2x-4x transect indicates significant difference between cytotypes at least at one site (details are shown in the figures); ≤ means that depending on the site, trait values were smaller, equal, or larger in diploids than in tetraploids. NAT test statistic: df values in brackets, – not determined

Seeds of 2x plants germinated to a high percentage already in the first year after sowing at P1800 (76%), P2300 (77%) and P2600 (71%), but only to 48% in P2800 (Fig. 7d, dark-blue bars). One year later, percent of germination increased to 69% in P2800 but did hardly change at the other sites. Thus, the final percentage of seed germination only insignificantly decreased with elevation in 2x plants. Seeds of 4x plants germinated significantly poorer than those of 2x plants ( $P < 0.001$ ); in the first year, percent germination was between 27, 26 and 24% in the plots up to P2600, and only 6% at P2800. In the second year, 30% more seeds germinated at the lower sites, but 67% more at P2800.

## Discussion

As outlined in the introduction, previous studies aiming at reconstructing Holocene range dynamics of diploid sexual and tetraploid apomictic *R. kuepferi* plants give evidence

that the niche optima of tetraploids have shifted towards cooler summer temperatures compared with those of the diploids (Kirchheimer et al. 2016). This suggests that growth and reproduction of tetraploids are adapted to a lower temperature regime. A direct comparison of the cytotypes in the elevation-dependent temperature gradient should show whether tetraploids and diploids differ in functional traits relevant for growth, survival and reproduction and whether such traits might have contributed to their different elevational ranges. As it was not possible to grow a sufficient number of flowering *R. kuepferi* individuals of the same age from seeds, ramets of flowering individuals from different populations had to be transplanted into the plots (for more information see Material and Methods). Thus, the study was not designed to disentangle cytotype-level responses (the reproduction mode included) from provenance-level variation caused by the respective natant environment.

Most traits did not show a clear linear elevation trend, very likely because the microclimate near the ground is very

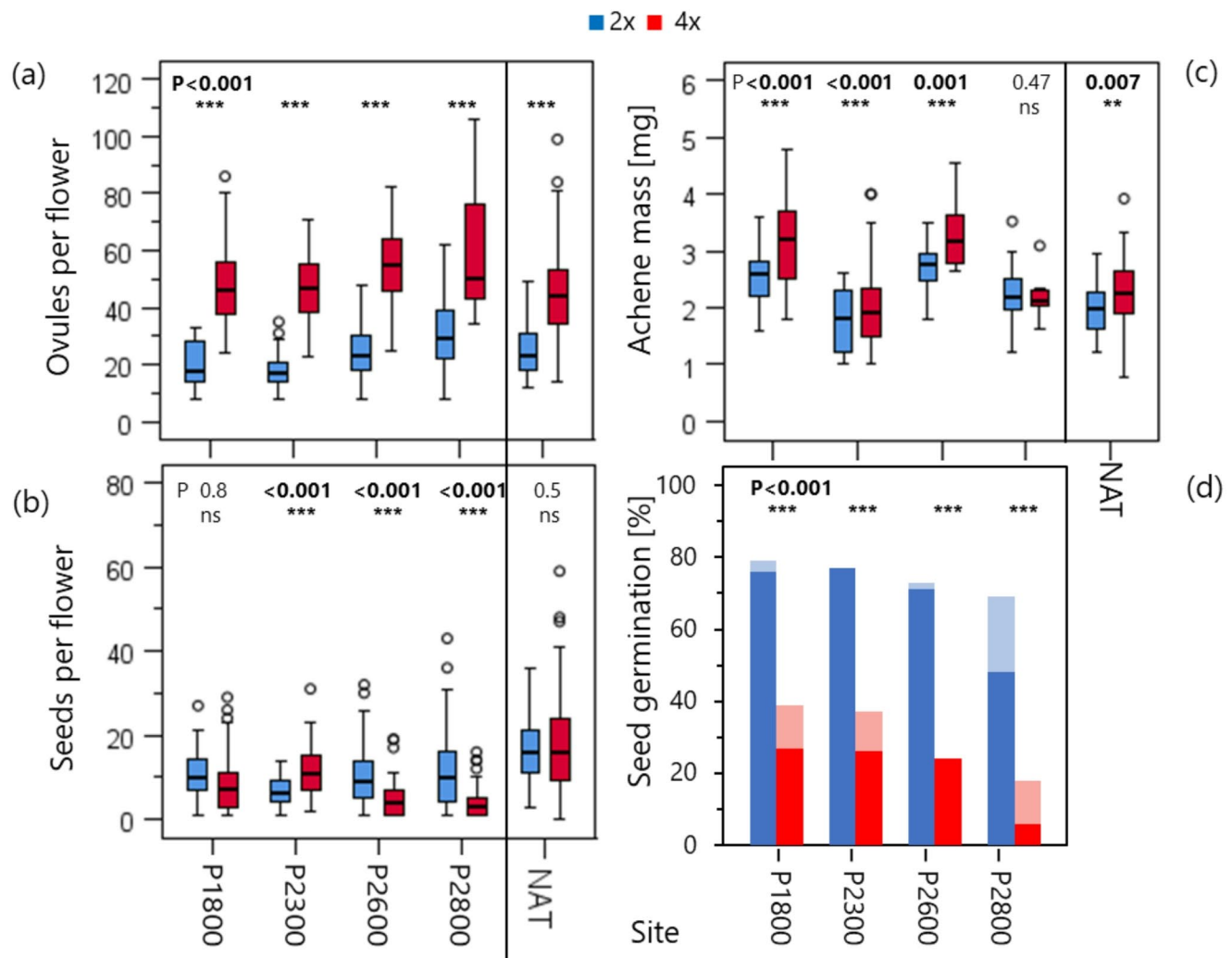


**Fig. 7** Reproductive fitness of 2x (blue) and 4x (red) *Ranunculus kuepferi* plants in the transect experiment and at the natural sites (NAT). **a** number of ovules per flower, **b** number of seeds per aggregate fruit, **c** achene mass, and **d** percent seed germination one year after sowing (dark colored bars), and the increase in the germination rate in the second year (light colored bars). Boxes in **a** and **b** show pooled data of three years in the transect and of a single year in NAT plants; achene

mass in **c** was determined in a single year at all sites. For details on the boxplots see legend of Fig. 1a. P-values and asterisks indicate significant differences between the cytotypes at the same site (\*\* $P \leq 0.01$ , \*\*\*  $P \leq 0.001$ , ns not significant, **a**, **b** GLMM, **c** LME). In **d** differences between cytotypes were significantly different ( $P < 0.001$ , GLM) in both years

different from the macroclimate and temperatures do not follow the linear adiabatic lapse rate of free air temperature (Körner 2021). A further key factor is the timing of winter snowmelt and growth period at different elevations. The probability of cold spells with snow falls is higher at early melting lower alpine sites than at higher sites where the growing season falls during the summer months. Thus, along the experimental transect, the range of daily mean canopy temperatures during the growth period of *R. kuepferi* did not significantly differ at the subalpine and alpine sites. Only at the upper alpine and subnival sites, mean temperatures at the plant level significantly decreased (cf. Figure 1). Interestingly, plant temperatures in the studied natural diploid population in the Western Alps were significantly higher

than in the whole experimental transect, apparently due to the a relatively late snowmelt date and summer temperatures during the growth period of *R. kuepferi* rarely falling below 5 °C. In contrast, daily mean canopy temperatures in the studied natural tetraploid population in the Eastern Alps were in the range of the subnival plot site. The microclimatic differences between both natural sites are consistent with the findings of Kirchheimer et al. (2016, 2018) that tetraploids inhabit cooler ecological niches.

### Survival and vegetative growth

Surprisingly, there was not clear overall response which would point to a general superiority of polyploid apomicts

over diploid sexuals at higher elevations and lower temperatures. For most traits, sexuals performed equal or even better than apomicts also at the highest site. Survival rates of both cytotypes were about the same at the alpine sites and the subnival site. The exceptionally high failure rates during winter for diploids at P1800 and tetraploids at P2600 are less due to a lack of adjustment capacity to the respective site conditions than to artificial effects in single plots such as snow vole feeding.

In some traits, tetraploids outperformed diploids. Tetraploid plants showed a significantly higher rhizome mass than diploids at higher elevations. Alpine plants generally do not allocate more biomass to special below-ground storage organs (such as rhizomes, tubers, and tape roots) as compared to low elevation plants (Körner and Renhardt 1987). However, as documented by a number of studies in different mountain systems of the world, alpine forbs invest less in stems and more into the fine root system, which changes the overall above-/below-ground biomass ratio in favor of below-ground structures (Körner 2021 and ref. therein). *R. kuepferi* only partly confirms this general trend. Root number tended to increase with elevation in both cytotypes, particularly in diploids. Rhizome mass was highly variable in tetraploids and reached particularly high values at the uppermost sites in a part of the ramets. Conversely in diploids, rhizome mass did not substantially change along the elevation transect but remained within the range of the values in natural populations. All in all, there is the tendency of tetraploids to invest more in rhizome storage than diploids.

Leaf number per individual correlated with the number of ramets and therefore increased with elevation in diploids and, because of feeding losses, less distinct in tetraploids. Leaf parameter values were clearly cytotype specific, but also revealed highly significant interactions between cytotype and elevation. In the natural population, diploids developed significantly smaller leaves than tetraploids. In the transect, however, these differences became only apparent at the lowest site but not higher up. This result corresponds to that of Schinkel et al. (2016) who found no heterosis effect in respect of leaf length for tetraploid individuals of *R. kuepferi* but rather a negative correlation to elevation. Leaf expansion starts immediately after winter snow melt and is completed with the beginning of anthesis. Low temperatures during this period strongly impact cell differentiation and maturation (Körner 2015; Nagelmüller et al. 2017) and, in the case of *R. kuepferi*, obviously counteracted the ploidy effect. Indeed, the probability of days with spring snow at the time of leaf growth is particularly high in the alpine zone, whereas higher up cooler night temperatures slow down development (Larcher and Wagner 2009; Ladinig et al. 2013).

Contrary to leaf size, leaf thickness usually increases with elevation mainly because of an increase in mesophyll layers (Körner et al. 1989; Bertel et al. 2017; 2022). In addition, epidermis cell walls become thicker and thus make leaves more robust and protect them against high solar/UV radiation. More mesophyll and thicker cell walls lead to an increase in leaf weight per unit area and, as a consequence, decrease in specific leaf area (SLA, area/dry weight) with elevation (Körner et al. 1989; Scheepens et al. 2010; Körner 2021 and references therein). SLA is an important indicator which reflects functional adjustment to the local environment during plant growth (Poorter et al. 2009; Körner 2021). Within a species, SLA is partly genetically determined (ecotype-specific), whereas intraspecific variation bases mainly on plastic adjustment of a population to the local environment (Prock and Körner 1996; Bertel et al. 2022). In *R. kuepferi*, SLA did not change along the elevation transect in diploids but decreased towards the highest site in tetraploids. This means that tetraploids are more flexible in adjusting leaf structures to the site climate than diploids.

Within a species, the final size of a plant organ is rather determined by cell number than by cell size (Gasquez and Beemster 2017). This was also confirmed for a number of herbaceous perennial mountain plants, whose leaf size decreased with elevation because of a decrease of the cell number at a largely constant cell size (Körner et al. 1989). Epidermis cell number is a key factor in a leaf's size control in forbs (Dale 1988). This is due to the fact that epidermis cell division in young leaves ceases long before mesophyll cell division and thus determines leaf size at an early stage of development (Körner and Pelaez Menendez-Riedl 1989). In *R. kuepferi*, tetraploids formed significantly less epidermis cells per unit area and thus larger cells than diploids in the whole transect, which can be seen as cytotype effect. Generally, cell size correlates with genome size whereby cells with bigger genomes take longer to duplicate and show an enhanced post-mitotic expansion growth (Jones et al. 2017). In leaves, the positive correlation between genome size and cell size is most pronounced in epidermis cells, but less apparent in mesophyll cells (Tsukaya 2019). According to the general rule of intraspecific/intracytotypic cell size constancy in differently sized leaves, epidermis cell number per unit area was expected to remain rather constant along the elevation transect in *R. kuepferi*— which was not the case. Cell number slightly decreased with elevation in tetraploids, but highly varied in diploids being lowest both at the sub-alpine and at the subnival site. We do not have a reasonable explanation what might have caused this elevation dependent pattern in cell number and thus cell size in diploids. A possible reason could be different endoreduplication levels

in epidermal cells under the influence of site climate and stress (Van de Peer et al. 2021).

According to the higher genome size, tetraploids developed significantly larger stomata with lower density than diploids, but as with epidermal cell number the environmental effect superimposed the cytotype effect. Stomatal density showed the same elevation pattern as epidermis cell density, decreasing with increasing elevation in tetraploids, and peaking in the alpine zone in diploids. Stoma size, conversely, increased in tetraploids and tended to decrease in diploids. Generally, stomatal density and consequently the maximum leaf diffusive conductance increases with elevation, obviously as a consequence of a thicker mesophyll and thus a higher photosynthetic capacity per unit leaf area, which may partly compensate for the reduction of the leaf area with elevation (Körner 2021). Against this background, relationships between different leaf parameters were divergent between diploid and tetraploid *R. kuepferi* plants. With increasing elevation, diploid leaves decreased in size, but as evident from a constant SLA, did not increase in weight and thus thickness. Also, stomatal density did not positively correlate with elevation but rather suggests leaf diffusive conductance to be optimal in the middle region of the transect. Tetraploids, however, followed the general elevation trend towards a decrease in SLA, and thus an increase in leaf thickness, and a significant increase in stomatal conductance by increasing stoma size (which might compensate for the decrease in stomatal density).

### Leaf freezing resistance

High-mountain plants have to cope with unpredictable summer cold spells which may be accompanied by snow and frost. The probability of such short-time frost events increases with elevation and is highest at the beginning and at the end of the growing season (Taschler and Neuner 2004; Ladinig et al. 2013). Accidental cold spells have to be distinguished from regular night frosts in the glacier zone (Larcher and Wagner 2009). Thus, the precondition for colonizing higher elevations is that aboveground organs are sufficiently freezing tolerant at any time, i.e. they tolerate extracellular freezing and freeze-dehydration to a certain extent also in the metabolically active state (Hacker et al. 2011; Ladinig et al. 2013; Stegner et al. 2020; Neuner et al. 2020). The risk of suffering from frost damage strongly depends on the developmental state of the respective plant structure and its current frost resistance (Larcher et al. 2010). Floral structures have turned out to be most endangered during bolting and anthesis, and leaves during expansion growth (Ladinig et al. 2013). Generally, floral structures—because of their complexity in structure and function—are more vulnerable to frost than leaves. Repeated losses in flowers and reduced

offspring can be balanced out over the years, repeated losses of the photosynthetically active leaves as basis for the production of biomass and storage compounds, however, would endanger the survival of the whole individual. According to the distribution pattern of *R. kuepferi*, we expected tetraploids to be less susceptible to subzero temperatures than diploids. We found a distinct effect of elevation in both cytotypes, in that individuals in the subalpine and lower alpine plots experienced functional disturbance of photosynthesis and frost damage at higher temperatures than in the upper alpine and subnival plots. Evidently, *R. kuepferi* as species has a general potential for structural and physiological adjustments to low temperatures. The extent of adjustment, however, was more pronounced in tetraploids than in diploids. In the same direction point epigenetic patterns and gene expression profiles obtained under warm and cold conditions and reciprocal temperature change in growth chamber experiments on *R. kuepferi* (Syngelaki et al. 2020a, b, 2021).

### Flowering and reproductive success

Generally, diploids showed a better or at least equally good reproductive performance as tetraploids. Flowering frequency strongly varied among years in both cytotypes at all sites because single individuals did not flower every year but alternately—an observation which was also made by Klatt et al. (2018) and Syngelaki et al. (2020a) when cultivating *R. kuepferi* in climate growth cabinets. Flowering frequency and the number of inflorescences tended to increase with elevation in diploid individuals but not in tetraploids. The number of flowers per individual, however, was statistically the same in both cytotypes. Since sexual diploids are out-crossers, and thus rely on mating partners and pollen vectors in contrast to the self-fertile apomicts, one would expect a negative elevation effect on diploids but not on apomicts. However, the contrary was the case. The proportion of sexual flowers which matured fruits and—within a single flower—carpels that matured fruitlets was about the same along the whole transect which does not indicate any pollen limitation and/or developmental constraints. Tetraploid apomicts, in contrast, tended to perform poorer than diploids, particularly at the higher sites of the transect, even though they developed about twice as much carpels than diploids. This result is in accordance with earlier assessments of seed set (Cosendai and Hörandl 2010; Schinkel et al. 2016; Klatt et al. 2018). One of the main reasons is that developmental disturbances in apomicts during ovule formation considerably increased with elevation (Ladinig et al. 2024).

Cytotypic differences became also apparent during seed germination. Not only that the percentage of germination was twice as high in seeds from the 2x sexual plants than in

seeds from the 4x apomictic plants, there were also significant differences with regard to the elevation and year of germination. A high percentage of 2x seeds germinated already in the first year after sowing, except for the highest transect site where about a third of seeds germinated only in the second year. In 4x seeds, the proportion of seeds that remained dormant ranged between one third and two thirds at the highest site. There were clear cytotype-specific differences in the level of seed dormancy which is - according to Baskin and Baskin (2004) - of the physiological type in *R. kuepferi*. Generally, environmental cues such as temperature, day length and soil moisture influence the depth of dormancy during seed maturation (primary dormancy) and after seed dispersal (secondary dormancy); (Penfield and MacGregor 2017; Klupczyńska et al. 2021; Finch-Savage and Leubner-Metzger 2006). In the case of *R. kuepferi*, maturation of the collected seeds in the sexual and apomictic NAT population occurred at about the same temperatures and daylengths in July of the same year. Thus, germinability and the state of primary dormancy depended rather on intrinsic factors and the reproductive mode (sexual versus apomictic), and was less likely influenced by environmental cues. After sowing in the transect plots, the climatic conditions at the highest sites seemed to have further stimulated secondary dormancy both in sexual seeds and particularly in apomictic seeds. Germination was observed for two years only, so it is unknown whether the final status was reached or more seeds would have germinated in the following years. Seeds with different dormancy levels may establish soil seed banks which is advantageous in disturbed habitats (Cooper et al. 2004; Schwienbacher et al. 2011), particularly under the unpredictable climatic conditions at higher elevations (Cavieres and Arroyo 2001; Cotado et al. 2020). In case seedlings get damaged, new offspring can arise from the belowground reserves. Seed longevity, in general, does not correlate with seed size in the alpine flora (Schwienbacher et al. 2010), because of phylogenetic constraints (Pluess et al. 2005). This means that in a multi-species comparison it depends on the taxa studied, whether seed size matters or not. However, larger seeds evidently have a higher emergence success under stressful conditions as they provide more reserves for initial seedling growth (Moles and Westoby 2004). In *R. kuepferi*, achenes of both cytotypes that had matured at different elevations in the transect varied greatly in size, whereby in addition to the ploidy effect and different maternal resource allocation in individual flowers, also the climatic conditions during histogenesis obviously had influenced the achene mass. Despite the high variability, mean achene weight was significantly higher in apomictic individuals than in diploid individuals at most sites because of a higher proportion of heavier achenes. This difference was also reported by Kirchheimer et al. (2018) and could

be due to different seed mass, which relates in general to different ploidy levels in the endosperm between sexual and apomictic seeds (Paczesniak et al. 2022). In tetraploid pseudogamous *R. kuepferi*, endosperm is 10x or 12x with a 2:5 or 2:6 ratio of embryo: endosperm, whereas in diploid sexuals endosperm is just 3x, with a 2:3 ratio of embryo and endosperm (Cosendai and Hörandl 2010; Schinkel et al. 2016; Klatt et al. 2018). In apomictic *Boechea*, seed mass influenced various reproductive traits (Paczesniak et al. 2022). Higher ploidy levels in the endosperm likely have positive effects on embryo development via increased gene expression and higher stress tolerance of polyploid tissues (e.g., Fox et al. 2020). Higher seed mass and a better seed bank are probably major benefits for tetraploids, balancing or even overcoming disadvantages of lower seed set.

### Relevance of results for the geographical parthenogenesis pattern

The transect experiment on diploid sexual and tetraploid apomictic individuals of *R. kuepferi* originating from different populations in the Alps did not show a general advantage of polyploid apomicts over diploid sexuals also at the highest, coolest site. Instead, diploids performed equal or even better than tetraploids with respect to vegetative growth and reproductive fitness. Some traits showed a pronounced cytotype-specific response pattern, such as leaf epidermal traits and the number of ovules per flower. In most of the traits, the cytotypes differed in their response along the transect resulting in significant cytotype\*elevation interactions. Changing directions in the response within a single trait point to differences in the potential for adjustment to the respective site conditions. Tetraploids showed some features, which could be advantageous in a cold climate: (1) SLA (leaf area per dry weight) decreased and thus the robustness of leaves significantly increased at the highest plot site. (2) Freezing resistance of leaves increased with elevation in both cytotypes but was higher in tetraploids than in diploids. (3) At higher elevations, tetraploids invested more into rhizome mass than diploids. (4) Tetraploids had heavier achenes and showed a greater tendency to establish seed banks than diploids.

Nevertheless, the question remains why diploids performed equal or better in reproductive traits, even at the highest site close to the glacier. This result appears to contradict the general geographical parthenogenesis pattern with the much larger distribution area of tetraploids, also at higher elevations, as observed in *R. kuepferi*. In the light of previous studies, the causality of this pattern appears to be complex, with historical, genetic, reproductive and ecological factors contributing to GP, similar to other model systems (Hörandl 2023). Models of the postglacial recolonization

history of *R. kuepferi* clearly showed a much faster migration of tetraploids (Kirchheimer et al. 2018), which can be best explained by reproductive assurance due to uniparental, apomictic reproduction of tetraploids (Cosendai et al. 2013). Minority cytotype disadvantages might have hindered diploids to colonize the area already occupied by tetraploids. Better colonization abilities of tetraploids were combined with a better cold acclimation of tetraploids in the higher parts of the Alps (Kirchheimer et al. 2016, 2018; Schinkel et al. 2016; Syngelaki et al. 2020a, 2021). Here we show specifically that cold acclimation relies on various phenotypic features and cannot rely on a few fitness measures. Robustness and better freezing tolerance of leaves in tetraploids, and a more robust rhizome, are probably crucial features for long-term survival of a perennial plant under alpine conditions, which guarantees successful sprouting in every season. Reproductive fitness, as measured with flower and seed set, however, appears to be more flexible and opportunistic in tetraploids. In fact, for diploids the observed higher reproductive fitness requires more resources, which might become disadvantageous at higher elevations at sites with increasing nutrient-poor and acid soils (Kirchheimer et al. 2016). High reproductive fitness might be at expense of vegetative adaptations to cold acclimation. Hence, tetraploids might have developed a better long-term trade-off strategy of investment into the essential vegetative fitness and a more opportunistic, cost-reducing reproductive fitness strategy. Physiological trade-offs are often observed in alpine plants (Körner 2021), but have not yet been studied so far in the context of geographical parthenogenesis. Our study provides comprehensive data for a better understanding of ecological niche shifts of tetraploids, which is in general an important factor of GP (Hörandl 2023).

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**Data availability** All data supporting the findings of this study are available in the Supplementary Information file ESM\_3.

## Declarations

**Conflict of interest** The authors declare that they have no competing interests.

**Research involving human and / or animals** Not applicable.

**Informed consent** Not applicable.

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