Winter chilling regulates seedling development in Mediterranean terrestrial orchids cultivated *in vitro*

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Running title: Cold regulates terrestrial orchid growth

Supporting information

Table S1. Composition of modified "SM-organic" nutrient medium.

Table S2. Sample sizes (Refrigerator treatment excluded).

Table S3. Model selection for various traits.

Table S4. Logistic regression models of the influence of cumulative hours below 10 °C and minimum temperature on survival.

Table S5. Linear regression models of the effect of median temperature on pre-dormancy shoot length after the onset of cold treatment.

Table S6. Linear regression models of the effect of cumulative hours below 12 °C on pre-dormancy tuber volume and tuber mass per vessel.

Table S7. Linear regression models of the effect of cumulative hours below 10 °C and 6 °C on post-dormancy tuber mass and shoot length.

Figure S1. Cumulative hours below a temperature threshold at the end of the cold treatment as a function of altitude above sea level.

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Author contribution

GD and AG conceived and designed the study. GD conducted the experimental work, analysed the data and wrote the original manuscript draft. VC and AT contributed to the experimental work. AG and EK supervised the research, secured funding and provided resources. All authors reviewed and approved the final manuscript.

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Data availability statement

The data supporting the results and the code used are freely available on Zenodo.org (https://doi.org/10.5281/zenodo.22030723). The DOI represents all versions and will always resolve to the latest one.

Ethics approval

The species sampled are not endangered or protected. Fieldwork was conducted in locations where no permission was required.

Consent for publication

Not applicable.

Competing interests

The authors have no conflict of interest to declare.

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Abstract

Effective *ex situ* propagation is critical for the conservation and restoration of threatened terrestrial orchids. However, large-scale propagation remains constrained by developmental bottlenecks including the transition from protocorms to plantlets with storage organs. Although cold exposure is often recommended to improve propagation success, the thermal conditions governing survival and post-dormancy performance remain poorly understood. We experimentally exposed protocorms of the Mediterranean terrestrial orchids *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum hircinum* and *H. robertianum* to eight thermal treatments for eight weeks, including controlled laboratory conditions and outdoor sites along an altitudinal gradient. Survival, shoot growth, and tuber development were quantified before and after summer dormancy, and their relationship with temperature descriptors were analyzed using a model selection approach. Plant performance was consistently better explained by cumulative chilling exposure than by mean temperature alone, with different developmental processes responding to distinct thermal thresholds. Exposure to moderately low temperatures (10–12 °C) determined survival and reserve accumulation before dormancy, while chilling below 6–7 °C doubled post-dormancy shoot length in responsive species, revealing carry-over effects across growing seasons. Species differed markedly in their thermal responses; increased chilling reduced post-dormancy survival by ~50% in *A. coriophora* but increased it twofold in *A. laxiflora*, whereas *H. robertianum* developed leaves only following cold exposure. Winter chilling therefore appears to be a key regulator of seedling development in Mediterranean terrestrial orchids. The identified thermal thresholds provide a basis for improving *ex situ* propagation and restoration protocols, while highlighting species-specific vulnerabilities and responses to future climate change.

Keywords: chilling requirement; *ex situ* propagation; Mediterranean orchids; Orchidaceae; protocorm; restoration; seedling development; temperature threshold; terrestrial orchids; winter chilling

Introduction

Orchidaceae are among the most diverse and widespread angiosperm families (Swarts and Dixon 2009; Chase *et al.* 2015; Zhang *et al.* 2018). However, many orchid species are increasingly threatened due to habitat degradation, collection for commercial trade, and climate change (Vogt-Schilb *et al.* 2015; Gerakis *et al.* 2016; Hirth 2016; Kreziou *et al*. 2016; Popova *et al.* 2016; Fay 2018; Hinsley *et al.* 2018; Hutchings *et al.* 2018; Masters *et al.* 2020). Conservation and restoration efforts are urgently needed (Ponert *et al.* 2012). *In vitro* propagation represents a promising approach (Katsalirou *et al.* 2017; Phillips *et al.* 2020; Jolman *et al.* 2022), provided that standardized, scalable protocols are developed for adoption by plant propagation laboratories to enable mass seedling production for conservation and restoration initiatives (Stewart and Kane 2006; Lee and Yeung 2018; Deconninck and Gerakis 2021; Jolman *et al.* 2022).

In natural environments, temperate terrestrial orchids experience seasonal temperature fluctuations that regulate growth and developmental transitions (Arditti 1992). Replicating these cues during *in vitro* propagation is therefore essential to ensure completion of the plant life cycle. Cold stratification, defined as the exposure of mature seeds to low temperature, breaks orchid seed dormancy and promotes germination (Rasmussen 1995; Kauth *et al.* 2008a; Jevšnik and Luthar 2015). Germination typically occurs at ambient temperatures of approximately 24 °C, although optimal temperatures may vary among species according to their distribution ranges (Rasmussen 1995; Rännbäck 2007; Kauth et al. 2008a; Godo *et al.* 2010; Zhang *et al.* 2018). In some cases, fluctuating rather than constant temperatures have been shown to enhance germination in European orchids (Calevo and Bazzicalupo 2020; Nabieva *et al.* 2025). Prolonged cold exposure can also initiate or accelerate flowering (vernalization; Chouard 1960; Michaels and Amasino 2000; Kim *et al*. 2009). However, while germination and flowering induction are relatively well understood, temperature requirements

during intermediate stages, particularly seedling growth and development, remain poorly characterized (Stewart and Kane 2006).

A critical stage during *in vitro* propagation of terrestrial orchids is the transition from protocorm to plantlet, during which the shoot apical meristem (SAM) is established (Yeung 2017). The protocorm describes the life stage from germination until the formation of a shoot tip with primordial leaves but no roots, during which mycorrhizal fungal association is established (Rasmussen 1995). Cold exposure treatment has been recommended to induce the transition from protocorm to plantlet in temperate orchids including representatives of *Anacamptis* Rich., *Dactylorhiza* Neck. ex Nevski, *Himantoglossum* Spreng., *Neotinea* Rchb.f., *Orchis* Tourn. ex L. (including the former genus *Aceras* R.Br.) and *Spiranthes* Rich., with recommendations for temperature and duration ranging from 2-12 °C over 6-12 weeks (Hadley 1970; Beyrle *et al.* 1985; Rasmussen 1995; Malmgren 2004; Ponert *et al.* 2012; Malmgren and Nyström 2026). Differences appear in cold treatment requirement that are likely to depend on individual species cold tolerance and population geographic origin (e.g. elevation or latitude) (Kauth *et al.* 2008b, 2011; Rasmussen *et al.* 2015). Better definition of temperature requirements of orchid seedlings is necessary in order to improve *in vitro* propagation protocols, for restoration programmes (including plant translocation) and to model species response to climate change (Garbisch *et al.* 1995; Kauth *et al.* 2011; Shefferson *et al.* 2017).

This study explores the influence of cold treatment on orchid seedling survival and growth, from protocorm until the end of summer dormancy. Protocorms of the terrestrial orchids *Anacamptis coriophora* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Anacamptis laxiflora* (Lam.) R.M.Bateman, Pridgeon & M.W.Chase, *Himantoglossum hircinum* Spreng. and *Himantoglossum robertianum* (Loisel.) P.Delforge grown asymbiotically *in vitro* were subjected to varying temperature regimes. We hypothesized that there would be a quantifiable,

153 species-specific, relationship between organ growth (tubers and shoots) and temperature of the
154 cold treatment.
155
156 **Materials and Methods**
157 Seed collection
158 Mature seeds of *A. coriophora*, *A. laxiflora* and *H. robertianum* were collected from
159 healthy and robust parent plants between 2016 and 2020 on the island of Cephalonia, Greece
160 (Table 1; Figure 1A-D). Mature seeds of the hardier *H. hircinum* were collected in 2020 from
161 the island of Ré, France (Table 1). The selected species, while having localized distributions,
162 were abundant in their locations, such that seed collection would not jeopardize the natural
163 populations. Foreign materials such as capsule fragments were removed by sieving through a
164 500 µm sieve. The seeds were dried in a desiccator with silica gel, sealed in labelled glass vials,
165 and stored at –20 °C until sowing.
166
167 Laboratory method
168 *Preparation of solutions*
169 Seeds were sown asymbiotically *in vitro* in March, August, and November 2020 (Table
170 1). In asymbiotic propagation, instead of relying on symbiotic fungi for nutrition, the seeds are
171 scarified using a disinfecting solution and the nutrients are furnished by a nutrient medium
172 (Kauth *et al.* 2008a; Yam and Arditti 2009; Rasmussen *et al.* 2015; Çiğ *et al.* 2018; Lee and
173 Yeung 2018). The culture substrate was a modified version of the "SM-organic" medium
174 (Table S1). This is a time-tested formula developed by Svante Malmgren, also published by
175 Rasmussen (1995), which has proved successful in previous studies (Katsalirou *et al.* 2017,
176 2019; Calevo *et al.* 2020; Deconninck and Gerakis 2021). The term "organic" refers to the N
177 source, an amino acid mixture sold under the trade name Vaminolac® (Fresenius Kabi,

178 Uppsala, Sweden). After mixing, pH was adjusted to 5.5–6.0 with a few drops of 3 M H_2SO_4
179 solution.
180
181 *Sowing*
182 Sowing was performed inside a laminar flow cabinet (Esco®, EQU/04-EBC-2A,
183 Singapore). All surfaces and materials were disinfected (for details, see Deconninck and
184 Gerakis 2021). For each species, 90 mg of seeds were scooped up with a spatula and placed in
185 15 mL Sarstedt® test tubes. The test tubes were nearly topped with 0.5% NaClO disinfecting
186 solution and a drop of Tween® 20 (Merck, Darmstadt, Germany) to reduce surface tension,
187 capped, and shaken vigorously to remove any air bubbles in contact with the seed. The shaking
188 was repeated every 15 min thereafter until the end of the disinfection period. Disinfection
189 times, specific to each orchid species (Katsalirou *et al.* 2017, 2019; Deconninck and Gerakis
190 2021), were chosen based on past experiments (Table 1). Then, the suspensions were decanted
191 into Erlenmeyer flasks fitted with a funnel lined with filter paper to recover the seeds. The filter
192 paper had been pre-soaked in the same disinfecting solution. After filtering the suspensions,
193 each filter paper was rinsed five times with 7 ml of sterile deionized water. Each culture vessel
194 (100 mL urine samplers made of polypropylene) was filled with 16 mL of sterile nutrient
195 medium and a sterile potato cube cut from the inner tissue of an organic potato tuber (*Solanum*
196 *tuberosum* L.). It is believed that potato is a natural supplement that promotes seed germination
197 and seedling growth in orchid tissue culture (Arditti 1967; Utami *et al.* 2020). The seeds were
198 scraped with a spatula from the filter paper and distributed evenly within the culture vessel,
199 approximately 4 mg of seeds per vessel. The tips of the steel tools were heat sterilized with
200 alcohol burner between sowings. After sowing, the vessels were incubated in a dark room at
201 an ambient temperature of 22 °C. At regular intervals, all vessels were visually examined for
202 the development of fungal and bacterial infections.

Reflasking

At the protocorm stage, the seedlings were thinned and reflasked in culture vessels (identical to those used for seed sowing) with fresh medium. Appropriately sized protocorms were defined as round protocorms that had developed a small shoot tip (Figure 1E-H). The vessels containing the protocorms were externally disinfected with 70% v/v ethanol solution before being exposed to a UVC germicidal lamp (Philips TUV 36W/G36 T8). Vessels for reflasking were filled with 30 mL of sterile nutrient medium and a potato cube. After solidification, the protocorms were transferred with forceps from the old to the new medium. Between transfers, the tips of the forceps were wiped clean and heat sterilized using an alcohol burner. Each new vessel hosted five randomly selected protocorms. The number of vessels for the experiment depended on the number of protocorms available for each species: 1960 for *A. coriophora*, 1600 for *A. laxiflora* and *H. robertianum*, and 320 for *H. hircinum*; resulting in 392, 320, 320, and 64 vessels, respectively (Table 1). Some vessels appeared to have more than five protocorms at the end of the experiment, likely a result of being attached together when transferred. Final protocorm counts per treatment are detailed in Table S2.

Experimental setup

To ascertain whether temperature influences seedling development, the seedlings were subjected to different temperature treatments as follows: (1) Ambient lab temperature of ~18 °C; (2) Refrigerated at ~7 °C; and (3) Natural temperature fluctuations at six outdoor stations along a steep altitudinal gradient (Table 2). The six outdoor stations were chosen based on the assumption that temperature decreases with altitude (Ackerman and Knox 2007). The vessels of each species were randomized and distributed equally among eight crates covered with a mesh of chicken wire and wrapped in plastic bags to protect them from rain and feral animals.

The bags were semi-translucent to provide weak light exposure, as advised for young seedlings (Svante Malmgren, personal communication, 2020). Inside the refrigerator, illumination was provided by cool white, fluorescent tubes (Osram Fluora L36W/77, Germany) with a 12 h photoperiod. At the outdoor stations, the crates were placed away from direct sunlight to avoid excessive warming due to any greenhouse effect. Temperatures were monitored at hourly intervals with miniaturized portable temperature loggers (Elitech®, Milpitas, CA, USA) enclosed in an empty culture vessel placed in the middle of each crate. In addition, two of the outdoor locations were equipped with weather stations. Every 10 d, the vessels were randomized within each crate so that they received equal amounts of light. The cold temperature treatment started on 25 January 2021 and lasted eight weeks, until 23 March 2021. At the end of week 8, all crates were assembled back to Lakomatia, a station of intermediate altitude (492 m a.s.l.), to be gradually acclimatized to spring temperatures.

Measurements of plantlet growth

During the ensuing eight weeks of acclimatization, shoot length was monitored with a ruler three times, i.e., at the end of 8, 12 and 16 weeks after the onset of cold treatment. The crates remained shaded at the Lakomatia station until the onset of summer dormancy, signalled by shoots starting to wither and tubers turning from white to cream colour. The crates were assembled back into the laboratory and tuber measurements started on 9 August 2021, 28 weeks after the onset of the cold treatment, as follows: The plantlets were extracted from the medium, washed in a 2 mm sieve under running water and patted dry with paper towels. The length of the longest leaf was recorded. The height and width of each tuber were measured with a vernier calliper and used to approximate its volume (volume of ellipsoid = $4/3\ \pi\ a\ b\ c$, where a, b, and c are the radii along each axis). Tubers shorter than 1 mm were discarded as non-viable. The tubers from each vessel were weighed on a laboratory scale (precision ± 0.001 g), returned to

their vessels, photographed, covered with slightly moistened soil and stored loosely capped in a dark, non-airconditioned room to simulate summer dormancy conditions in nature.

By 23 November 2021, 43 weeks after the onset of the cold treatment, the indoor environment had substantially cooled. The end of summer dormancy was signalled by most tubers sprouting a new shoot. The plantlets were extracted from the soil, washed with a thin jet of deionized water in a 2 mm sieve and patted dry with paper towel (Figure 1I). The length of each plantlet (tuber plus shoot) and its mass were recorded.

Statistical analyses

Temperature. Temperature was plotted throughout the cold treatment and subsequent acclimatization period. For each station, minimum, maximum, median and mean temperature, temperature range and cumulative hours below 13 temperature thresholds (< 0 °C to < 12 °C) were calculated to be used as explanatory variables in the statistical analyses. The thresholds were chosen according to the range of temperatures reported in the literature to relieve protocorm dormancy (2–12 °C, in Hadley 1970; Beyrle *et al.* 1985; Ponert *et al.* 2012; Malmgren and Nyström 2026), plus the thresholds of 0 and 1 °C that are believed harmful to plant tissues (Pfeifer *et al.* 2006). Linear regression was used to explore the relationship between the cumulative hours below selected temperature thresholds and altitude. Mean temperatures and mean daily temperature fluctuations at the eight stations were compared with paired t-tests.

Survival assessment. Because individual plantlets could not be tracked across developmental stages, pre-dormancy survival was inferred from functional thresholds linking belowground reserve accumulation to aboveground growth. The use of tuber volume thresholds was preferred over visual assessment of shoot browning (commonly used to assess survival; Van Waes, 1984), as some individuals classified with browned shoots still possessed viable

278 tubers (data not shown). For each species, the threshold was determined empirically by
279 identifying the smallest tuber volume from which at least two individuals produced a functional
280 shoot, defined as reaching at least 10% of the maximum shoot length observed for that species.
281 This criterion was chosen to avoid defining the threshold from isolated individuals that could
282 represent measurement error or atypical development. Although empirical, this approach
283 provided a conservative criterion aimed at minimizing false classification of living individuals
284 as dead. The resulting species-specific tuber volume thresholds (2.0, 1.0, 2.0 and 10.5 mm^3 for
285 *A. coriophora*, *A. laxiflora*, *H. robertianum* and *H. hircinum*, respectively) corresponded to
286 minimum shoot lengths of 14, 12.5, 6.5 and 8.3 mm. Individuals with tuber volumes below
287 these thresholds were considered non-viable prior to dormancy. Post-dormancy survival was
288 directly assessed depending on the emergence of a shoot.

289 *Plant performance.* To identify which components of thermal regime best explained
290 variation in plant performance, we used an information-theoretic model selection approach
291 (Burnham and Anderson 2002). We used the five continuous descriptors of the thermal regime
292 experienced during the 8 weeks of cold treatment (minimum, maximum, median, mean
293 temperature, temperature range) and the 13 cumulative chilling indices (C_0–C_{12}), where C_x
294 represents the number of hours with temperature below x °C. These variables capture both the
295 intensity and duration of cold exposure. For each response variable (pre-dormancy and post-
296 dormancy survival; pre-dormancy shoot length, tuber volume and tuber mass per vessel; post-
297 dormancy tuber mass and shoot length), we fitted a set of competing generalized linear or linear
298 models in which each thermal variable was entered separately, together with species identity
299 and their interaction, allowing for species-specific thermal responses (R package 'lme4'; Bates
300 *et al.* 2015). For each thermal predictor, we fitted both linear and quadratic equations to account
301 for monotonic effects as well as non-linear (optimum-type) physiological responses. Binomial
302 error distributions were used for survival models, and Gaussian errors were used for continuous

traits after appropriate variance-stabilizing transformations (see Supplementary Tables for details). All models were compared using AICc (Akaike Information Criterion corrected for small sample size) as implemented in the MuMIn package (Burnham and Anderson 2002). For each trait, we ranked all candidate models and identified the most parsimonious predictors based on ΔAICc and Akaike weights. This procedure allowed us to determine which thermal thresholds and temperature descriptors best explained each biological process, and whether responses were best described by linear or non-linear relationships. When a given predictor was strongly supported by model selection, we refitted the corresponding model and its species-specific components to estimate coefficients and visualize thermal response curves. All analyses were performed in R version 4.1.3 (R Core Team 2021).

Results

Temperature

Mean temperature at the outdoors stations varied from 11.9 ± 3.2 °C (mean ± SD) in Vlachata to 5.9 ± 2.6 °C in Ainos (238 and 1291 m a.s.l., respectively; Table 2), consistent with temperature decreasing with altitude (Ackerman and Knox 2007). Laboratory was the warmest with mean temperature of 18.4 ± 1.6 °C. Except for Yupari and Ainos (5.9 ± 3.0 °C and 5.9 ± 2.6 °C, respectively), mean temperatures were significantly different among stations (Table 2). Refrigerator, Laboratory and Ainos stations experienced the lowest mean daily fluctuations of 1.6 ± 0.9, 3.7 ± 1.7 and 4.1 ± 2.6 °C, respectively, while the highest fluctuations were at Vlachata, Lakomatia and Yupari stations, of 7.5 ± 1.9, 6.5 ± 2.3 and 6.2 ± 2.5 °C, respectively (Table 2). The total number of hours below a temperature threshold throughout the cold treatment increased with altitude following a linear relationship (Figure S1). Laboratory, with a minimum temperature of 14 °C, was the only station without any hours below the temperature

thresholds. During the eight weeks of the acclimatization period at Lakomatia, hourly temperatures ranged between 2.8 °C and 27.7 °C with a mean of 14.1 ± 4.8 °C.

Temperature was likely the main factor influencing orchid growth in our study. Although light effects cannot be entirely excluded, all outdoor stations experienced the same natural photoperiod, were located in close geographical proximity, and seedlings were maintained in identical semi-translucent bags protected from direct sunlight, resulting in similar daylength and broadly comparable illumination. In contrast, the Refrigerated treatment was excluded from subsequent analyses because plantlets produced outlier responses, likely resulting from exposure to nearly constant temperature and photoperiod (Stewart and Kane 2006; Calevo and Bazzicalupo 2020; Nabieva *et al.* 2025), precluding direct comparison with the outdoor treatments.

Plant survival

Overall, pre-dormancy survival was high and differed among species (P<0.0001; Table S4A) averaging (mean ± SE) 80.6 ± 0.9%, 84.3 ± 0.9%, 77.4 ± 1.0% and 85.2 ± 1.7% for *A. coriophora*, *A. laxiflora*, *H. robertianum* and *H. hircinum*, respectively. Survival was primarily controlled by cumulative exposure to moderately low temperature rather than by extreme cold. Model selection identified cumulative hours below 10-11 °C (C_{10}-C_{11}) as the best predictor of survival (P<0.0001; Table S3). Survival increased significantly with increasing C_{10} in *A. laxiflora* and *H. robertianum* (P=0.0242 and P<0.0001, respectively), while no significant effect was detected in *A. coriophora* or *H. hircinum* (P=0.64 and P=0.51, respectively; Figure 2A, Table S4A). Accordingly, the global model showed a significant C_{10} × Species interaction (P=0.0058), indicating divergent chilling sensitivities among taxa.

Post-summer dormancy survival differed strongly among species (P<0.0001; Table S4B). While survival was still relatively high for *Anacamptis* species (28.4 ± 1.0% and 15.6 ±

0.9% for *A. coriophora* and *A. laxiflora*, respectively), it was close to zero for *Himantoglossum* species (0.7 ± 0.2% and 0.9 ± 0.5% for *H. robertianum* and *H. hircinum*, respectively). Post-dormancy survival was best explained by minimum temperature experienced during the cold treatment rather than by cumulative chilling (Table S3). Species differed strongly in their responses (interaction min temperature × Species, $P<0.0001$; Figure 2B, Table S4B): low temperature reduced survival in *A. coriophora* and *H. robertianum* ($P<0.0001$ and $P=0.0240$, respectively), increased survival in *A. laxiflora* ($P<0.0001$) but had no effect on *H. hircinum* ($P=0.71$). The thermal stress induced by cold treatment impacted species in contrasting ways even under identical post-dormancy growing conditions.

Pre-dormancy shoot growth

Shoot growth during the acclimatization phase (post cold treatment) was strongly temperature dependent ($P<0.0001$) and differed among species ($P<0.0001$). Overall, *A. laxiflora* had the highest growth rate, growing from a mean shoot length of 8.6 ± 0.3 to 44.5 ± 0.7 mm (growth rate = 517.4%), followed by *A. coriophora*, growing from 21.8 ± 0.5 to 54.8 ± 0.7 mm (growth rate = 251.4%). *Himantoglossum* species had the lowest growth rates: *H. hircinum* shoots grew from 6.2 ± 0.4 to 11.0 ± 0.8 mm (growth rate = 177.4%) and *H. robertianum* from 2.6 ± 0.4 to 3.7 ± 0.1 mm (growth rate = 142.3%). Across all weeks, growth was best described by quadratic functions of median temperature, with highly significant interactions between temperature, species, and time ($P<0.0001$). This indicates that shoot growth followed species-specific thermal reaction norms rather than monotonic responses to cold.

By the end of week 8, growth increased strongly with temperature in all species ($P<0.0001$), but the shape of the response differed (interaction median temperature × Species: $P<0.0001$). *Anacamptis coriophora* showed a steep non-linear response, whereas *A. laxiflora*,

377 *H. robertianum* and *H. hircinum* responded mainly linearly (Table S5A, Figure 3A). By the
378 end of week 16, growth accelerated and non-linear responses were observed for all species
379 except *H. hircinum* (Table S5B, S5C, Figure 3A). For *Anacamptis* species, the shoot length
380 difference between treatments was less pronounced in week 16 than in week 8 (Figure 3A),
381 indicating that cold treatment initially slowed growth and that species differed in their capacity
382 to compensate during the acclimatization period.
383
384 Pre-dormancy tuber growth
385 At the onset of summer dormancy, tubers of *H. hircinum* were the largest, with mean
386 volume of 99.3 ± 6.6 mm^3 (mean ± SE) and mean mass per vessel of 1.060 ± 0.107 g; tubers
387 of *A. coriophora* were of intermediate size, with mean volume of 76.0 ± 1.5 mm^3 and mean
388 mass per vessel of 0.464 ± 0.010 g and tubers of *A. laxiflora* and *H. robertianum* were the
389 smallest, with mean volume of 30.3 ± 0.8 and 26.7 ± 0.8 mm^3 and mean mass per vessel of
390 0.217 ± 0.006 and 0.192 ± 0.009 g, respectively (P<0.0001; Table S4A, S4B).
391 Tuber volume (P<0.0001) and mass (P<0.0001) before dormancy were primarily
392 controlled by cumulative exposure to moderate cold. For both traits, cumulative hours below
393 12 °C (C_{12}) provided the best fit (Table S3), with strong quadratic effects and significant
394 species interactions (P<0.0001 for both traits; Table S6A, S6B). Cold generally induced a
395 reduction in tuber volume and mass that was more pronounced for *A. coriophora* and *H.*
396 *hircinum*, with a reduction of 43.8% and 55.9% in volume and 36.7% and 28.6% in mass,
397 respectively (Figure 3B, 3C). In *A. coriophora*, *A. laxiflora* and *H. robertianum*, tuber volume
398 and mass followed non-linear response curves, indicating an optimal range of chilling for
399 reserve accumulation (Figure 3B, 3C). In contrast, *H. hircinum* exhibited only weak or linear
400 responses with a decrease of tuber volume (P<0.0001) and mass (P=0.0985) induced by the
401 accumulation of chilling (Figure 3B, 3C; Table S6A, S6B).

Post-dormancy growth

Post-dormancy, tuber mass was best predicted by cumulative hours below 10 °C (C_{10}; Table S3). In *A. coriophora, A. laxiflora* and *H. robertianum*, tuber mass linearly decreased with increasing C_{10} (P=0.0004, P=0.0002 and P=0.0097, respectively), while in *H. hircinum* there were insufficient survivors to assess any relationship (Table S7A; Figure 4A). In contrast, shoot growth responded strongly and positively to chilling. Shoot length increased linearly with C_6 in *A. coriophora*, *A. laxiflora* and *H. robertianum* (P<0.0001 for all species), with intensity differing between species (interaction C_6 × Species: P=0.0025; Table S7B; Figure 4B). *Himantoglossum robertianum* showed the most extreme response, with little or no shoot development under warm conditions but strong leaf emergence after prolonged chilling (P<0.0001). *Himantoglossum hircinum* showed no detectable response (P=0.28), mostly due to very low survival (Figure 4B).

Discussion

The effect of cold on orchid growth has received limited attention (Hadley 1970; Beyrle *et al.* 1985; Rasmussen 1995; Malmgren 2004; Kauth *et al.* 2008b, 2011; Ponert *et al.* 2012; Malmgren and Nyström 2026). Nevertheless, several studies have suggested that exposure to low temperatures promotes the transition of orchid protocorms to plantlets, although the temperatures reported to be effective vary considerably among species and studies. We exposed protocorms of *Anacamptis* and *Himantoglossum* species to different thermal regimes and showed that plant responses were best explained by cumulative chilling exposure rather than by mean temperature alone, with species-specific responses and distinct thermal thresholds controlling different developmental processes.

Survival response to cold is species-specific

Pre- and post-dormancy survival following cold treatment differed between species, likely due to differences in cold tolerance (Garbisch *et al.* 1995; Rasmussen 1995; Delforge 2006). Pre-dormancy survival was high overall (>60%) and, when chilling had an effect, it increased survival. Moderate winter chilling seems therefore to improve physiological preparedness for dormancy for some species without inducing physiological stress in other species. On the contrary, post-dormancy survival was drastically reduced in all species, dropping to less than 50% regardless of temperature treatment. Dormancy can induce increased mortality in some orchids, including *Neotinea ustulata* (L.) R.M.Bateman, Pridgeon & M.W.Chase, *Cypripedium parviflorum* Sims, *Cleistes bifaria* (Fernald) Catling & Gregg and *Ophrys sphegodes* Mill. (Hutchings 1987; Shefferson *et al.* 2003; Gregg and Kéry 2006; Shefferson and Tali 2006). Post-dormancy survival of *Himantoglossum* species was extremely low, even though they are considered cold hardy (Garbisch *et al.* 1995; Kauth *et al.* 2011). Their shoots also remained relatively small compared to those of *Anacamptis* species. *Anacamptis* species were more advanced in development at the onset of the experiment compared to *Himantoglossum* species (they had developed the first leaf while *Himantoglossum* were still at the protomeristem stage; stage 5 *vs* stage 4, according to Swarts and Dixon 2017). The smaller protocorms of *Himantoglossum* species might have not been ready to be exposed to cold, explaining the difference in post-dormancy survival. In *Anacamptis* species, cold induced either an increase (*A. coriophora*) or a decrease in survival (*A. laxiflora*), indicating strong differences in species sensitivity to winter thermal stress. Overall, the results indicate that seedling survival was not controlled by exposure to extreme cold, but by species-specific responses to winter thermal regimes. Moderate chilling can enhance survival in some taxa, while low minimum temperatures may become detrimental or beneficial depending on species.

Cold slows pre-dormancy orchid growth but has a carry-over effect post-dormancy

Cold stress, including chilling (0–15 °C) and freezing (<0 °C), restrict growth, development and geographical distribution of plants (Liu *et al.* 2018; Ding *et al.* 2020). Accordingly, in this study, pre-dormancy shoot and tuber growth was strongly controlled by temperature; all species exhibited non-linear responses to temperature, with mostly reduced shoot length and tuber size at low temperature and species-specific responses. Although low temperature can hinder plant growth, a longer growing period can compensate for the decline in growth rate (Zhang *et al*. 2018). This was most evident in *Anacamptis* species, for which the size gap in shoot length between cold-treated and control plantlets decreased with time. Stabilization of tuber volume and mass is consistent with shoot growth eventually catching up. *Anacamptis* species also grew much longer shoots than *Himantoglossum* species, likely due to differences in growth rates (Poorter 1989), although the small size of *Himantoglossum* tubers protocorms at the onset of experiment could also explain the difference in final shoot length (see section 4.1 above). Control plantlets in the laboratory also grew faster and taller than cold-treated ones, allowing them to start photosynthesis earlier and therefore develop storage organs faster (Tissue *et al*. 1995). Growing larger tubers more rapidly ensures the development of storage organs during shortened growing season and increases winter survival, as reported for *Calopogon tuberosus* (L.) Britton, Sterns & Poggenb. (Kauth *et al*. 2008b).

Some studies suggest an optimum temperature for orchid growth, at which the rate of progress toward a particular developmental event is maximal (e.g. Zhang *et al.* 2018). This optimal temperature was apparent in *A. laxiflora* shoot growth, that was optimized at an intermediate median temperature of 14 °C, and in tuber volume and mass of *A. coriophora* and *A. laxiflora*, that were maximized at intermediate cumulative hours of cold, indicating an optimal chilling range for reserve accumulation. The optimal temperature, and the requirement of cold treatment for growth, while species-specific, is also likely to be influenced by the

environment from which the seeds originate (Rasmussen *et al.* 2015). For instance, northern populations of *Calopogon tuberosus* require a minimum chilling period of eight weeks for maximum shoot emergence, likely due to the experience of a longer winter in the field compared to southern plants (Kauth *et al.* 2011). Similarly, most studies reporting benefit of cold treatment on orchid growth used seeds of specimens originating from cold climates (Hadley 1970; Beyrle 1985; Ponert *et al.* 2012; Malmgren and Nyström 2026). However, contrary to our expectation that *H. hircinum* seeds would benefit more from a cold treatment than seeds from the other species that were harvested from lower latitudes in the thermomediterranean climatic zone of Cephalonia, cold impaired growth and development of this species. This result was consistent with a 26-year survey of *H. hircinum* populations in a natural reserve in Germany, where colder winter conditions reduced survival and growth rates of the plants (Pfeifer *et al.* 2006). It is therefore likely that the differences between species seen in our study reflect species-specific cold tolerance and requirements rather than geographical origin. Future research should compare the response of orchid species to cold treatment along latitudinal gradients to better decipher species *versus* local environment effects.

The beneficial effect of cold treatment was mostly visible post-dormancy, revealing a carry-over effect on growth (O'Connor *et al.* 2014). In other words, thermal conditions experienced before dormancy continued to influence plant performance during the following growing season through persistent physiological changes, notably reserve accumulation. Shoot length increased steeply with cumulative exposure below 6–7 °C in *A. coriophora*, *A. laxiflora* and *H. robertianum*, and only cold-exposed plantlets of *H. robertianum* and *H. hircinum* grew leaves. This demonstrates that chilling does not suppress growth but, rather, promotes subsequent shoot development in most species, consistent with a physiological chilling requirement for release of dormancy and efficient mobilization of reserves. Post-dormancy, tuber mass declined with increasing cumulative exposure below 10 °C in *A. coriophora* and *A.*

laxiflora, indicating that stronger chilling led to greater mobilization of reserves for shoot growth. Together, the increase of shoot growth and decline in tuber mass, indicate that cold exposure promotes translocation of stored carbon into above-ground growth rather than directly increasing tuber size. The increase of leaf surface will subsequently influence the volume and mass of the tubers, as tuber storage (and therefore size) is a result of photosynthesis (Tissue *et al.* 1995). It is likely that cold-exposed tubers enter a positive loop where a longer shoot will promote higher carbon storage leading to longer shoots during the following season. Together, these results indicate that chilling primarily enhances carbon storage before dormancy and stimulates its mobilization for shoot growth after dormancy. The positive effects of cold treatment are therefore expressed most clearly in the following growing season, rather than during the chilling period itself.

Conclusions

This study focused on the influence of temperature on the growth of temperate terrestrial orchids cultivated *in vitro*. Our data demonstrate that cold temperatures regulate growth through species-specific, non-linear physiological responses rather than exposure to extreme cold. Cumulative exposure to moderately low temperatures (10–12 °C) controls survival and reserve accumulation before dormancy, while chilling below 6–7 °C promotes post-dormancy shoot growth by enhancing reserve mobilization. These carry-over effects generate positive feedback between winter chilling and growth during the following season. Importantly, thermal responses differed strongly among species, indicating that chilling requirements reflect species-specific physiological strategies. Besides their relevance for *in vitro* culture, these results suggest that warmer winters may disrupt both survival and post-dormancy development, with consequences that vary among taxa. In natural populations, orchid growth also depends on associations with mycorrhizal fungi, whose seasonal dynamics

may further influence carbon acquisition and storage in response to winter temperatures (Calevo and Duffy, 2022). Understanding how temperature and mycorrhizal interactions jointly shape orchid performance will therefore be important for conservation, restoration and *ex situ* propagation of orchids in the face of ongoing climate change.

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Tables

Table 1. Location of seed donors, collection year, sowing date, seed disinfection duration, reflasking date, total number of protocorms and number of experimental vessels for the four studied orchid species.

Orchid species	Location	Collection year	Sowing date	Disinfection time (min)	Reflasking date	Number of protocorms	Number of vessels
Anacamptis coriophora	34SDH8074312878	2017	10 Nov 2020	60	22 Jan 2021	1960	392
Anacamptis laxiflora	34SDH5698823917	2018	5 Aug 2020	85	10 Jan 2021	1600	320
Himantoglossum robertianum	34SDH6148321415	2016	5 Aug 2020	85	5 Jan 2021	1600	320
Himantoglossum hircinum	30TXS1881618746	2019	11 Mar 2020	85	31 Dec 2020	320	64

Coordinates are in the Military Grid Reference System (MGRS; https://en.wikipedia.org/wiki/Military_grid_reference_system; verified 20 Jan. 2026).

709 **Table 2.** Location and altitude above sea level of the temperature treatments with minimum, maximum, median, and mean ± SD of hourly
710 temperature (°C), mean ± standard deviation (SD) of daily temperature range (°C), and cumulative hours below 6, 10 and 12 °C (C_6, C_{10}, C_{12})
711 inside the vessels during the eight weeks of cold treatment.

Location	Coordinates	Altitude (m)	Min daily temp (°C)	Max daily temp (°C)	Median daily temp (°C)	Mean daily temp (°C) ± SD	Mean of daily range (°C) ± SD	C_6	C_{10}	C_{12}
Refrigerator	34SDH5587824633	6	5.2	7.7	6.8	6.9 ±0.7[a]	1.6 ±0.9[a]	96	1351	1353
Laboratory	34SDH5587824633	6	16.0	20.8	18.3	18.4 ±1.6[b]	3.7 ±1.7[b]	0	0	0
Vlachata	34SDH6726319739	238	5.0	15.2	12.4	11.9 ±3.2[c]	7.5 ±1.9[c]	42	380	698
Lakomatia	34SDH6310023485	492	2.5	12.7	8.4	8.2 ±3.1[d]	6.5 ±2.3[d]	316	902	1210
Reservoir	34SDH6591928049	707	2.9	11.1	8.1	7.9 ±2.7[e]	6.0 ±2.5[e]	300	1085	1259
Road Bend	34SDH6754527061	916	0.4	10.3	7.0	6.7 ±2.6[a]	4.4 ±2.2[g]	457	1244	1333
Yupari	34SDH6622926477	1122	0.0	9.5	6.3	5.9 ±3.0[f]	6.2 ±2.5[e]	663	1245	1307
Ainos	34SDH6785422948	1291	1.2	9.8	5.8	5.9 ±2.6[f]	4.1 ±2.6[f]	703	1257	1335

Note: Coordinates are in the Military Grid Reference System. Means with the same letter are not significantly different at $a = 0.05$ (pairwise t-test).

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713

714 **Figures**

715

716 Figure 1. Inflorescences (top) and protocorms (middle) of *Anacamptis coriophora* (A,E), *A.*

717 *laxiflora* (B,F), *Himantoglossum hircinum* (C,G), and *H. robertianum* (D,H). Bottom:

718 Sprouting tubers of *A. laxiflora* after summer dormancy (I).

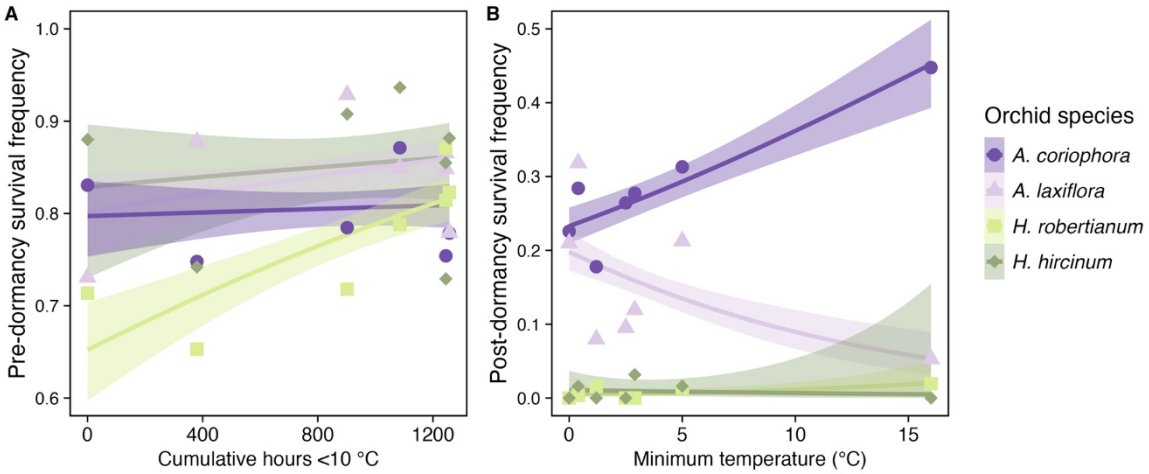
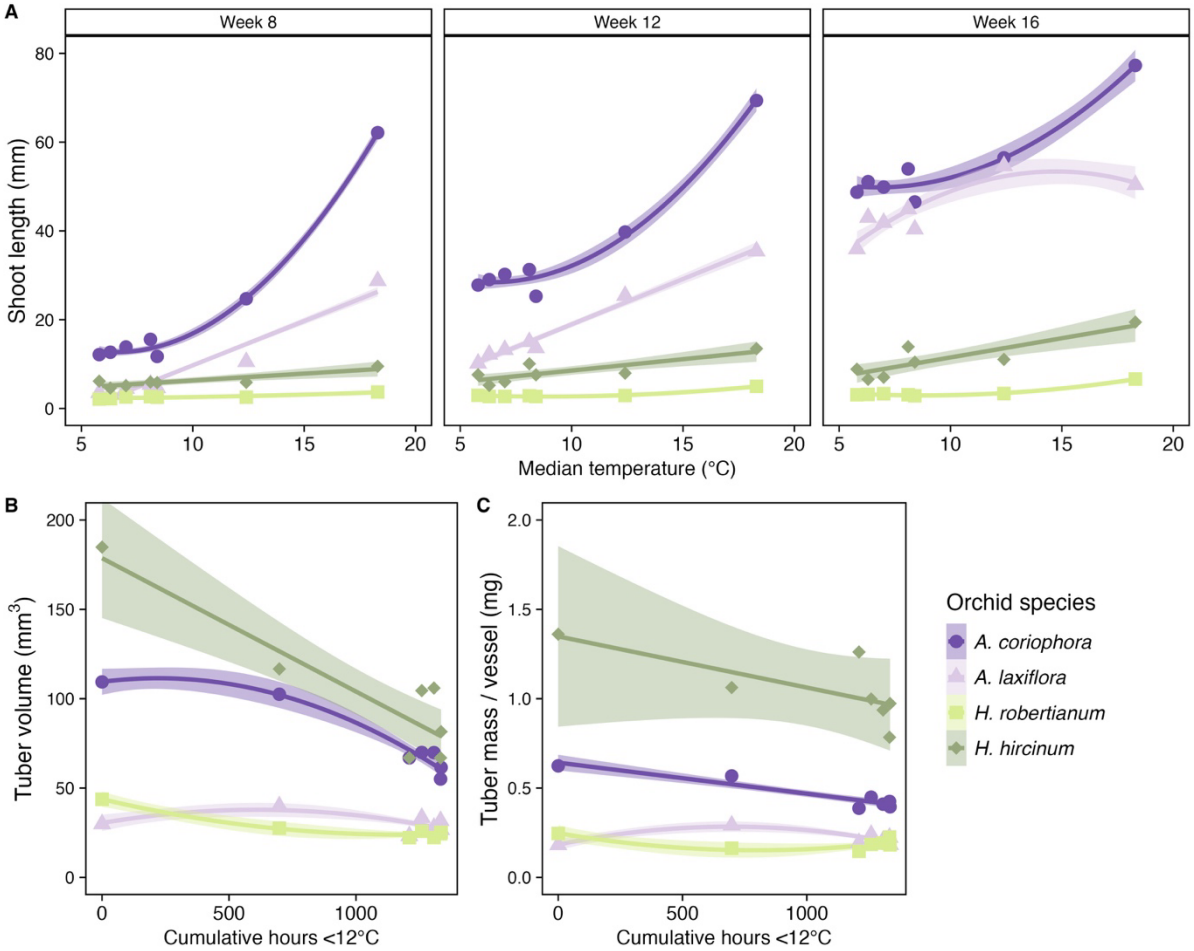


Figure 2. Effect of cumulative hours below 10 °C on pre-dormancy survival (A) and minimum temperature (°C) on post-dormancy survival (B) of *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum robertianum* and *H. hircinum* (sample sizes in Table S2). Solid lines are logistic regression for each species with 95% confidence intervals. Chilling increased pre-dormancy survival in *A. laxiflora* and *H. robertianum*, while no significant effect was detected in *A. coriophora* or *H. hircinum*. Low temperature reduced survival in *A. coriophora* and *H. robertianum*, increased survival in *A. laxiflora* but had no effect on *H. hircinum* (see Table S4 for details).



731

732 **Figure 3.** Effect of median temperature on pre-dormancy shoot growth (A) at 8, 12 and 16
733 weeks, and of cumulative hours below 12°C on pre-dormancy tuber volume (B) and tuber mass
734 (C) per vessel of *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum robertianum* and *H.*
735 *hircinum* (sample sizes in Table S2). Solid lines are regressions for each species with 95%
736 confidence intervals. Shoot length (mm) measured from the medium surface to the tip of the
737 longest leaf. Warmer temperature during the cold treatment generally induced a faster shoot
738 growth even though the response differed between species, while chilling generally induced a
739 reduction in tuber volume and mass that was more pronounced for *A. coriophora* and *H.*
740 *hircinum* (see Tables S5 and S6 for details).

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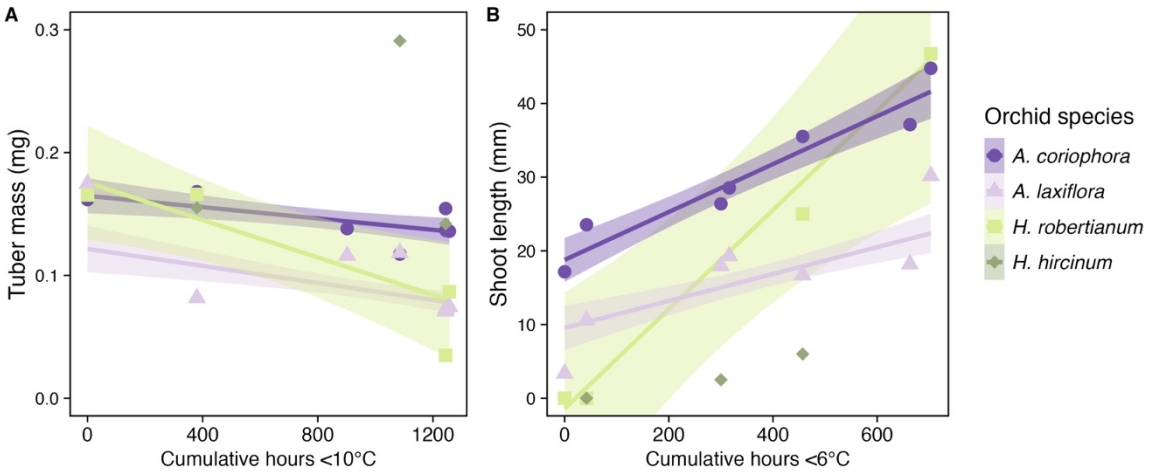

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744 **Figure 4.** Effect of cumulative hours below 10 °C on post-dormancy tuber mass (A) and
745 cumulative hours below 6 °C on post-dormancy shoot length (B) of *Anacamptis coriophora*,
746 *A. laxiflora*, *Himantoglossum robertianum*, and *H. hircinum* (sample sizes in Table S2). Solid
747 lines are linear regression curves for each species with 95% confidence intervals. While
748 chilling negatively impacted tuber mass of *A. coriophora* and *A. laxiflora*, it strongly and
749 positively impacted shoot growth in all species, except *H. hircinum* for which the relationship
750 could not be assessed due to low sample size (see Table S7 for details).

751 **Cold treatment benefits Mediterranean orchid seedlings cultivated *in vitro***

752

753 **Supplementary Material**

754 **Table S1.** Composition of modified "SM-organic" nutrient medium.

Ingredients	**Quantity**
Mineral water	1 L
Saccharose (sucrose)	12.5 g
KH_2PO_4	75 mg
$MgSO_4 \cdot 7H_2O$	150 mg
$CaHPO_4$	100 mg*
H_3BO_3	10 mg
Activated charcoal	750 mg
Danish agar	10 g
Vaminolac® (Fresenius Kabi, Uppsala, Sweden)	4.6 mL
Pineapple juice	20 mL
3 M H_2SO_4 for pH adjustment	~ 10 drops
Potato (*Solanum tuberosum*) tuber	1 cm^3 per culture vessel

*Equivalent to 75 mg $Ca_3(PO_4)_2$ in the original formula

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Table S2. Sample sizes (Refrigerator treatment excluded).

Species	Number of hours <6°C	Initial number of protocorms	Pre-dormancy				Post-dormancy
			Shoot length	Tuber volume	Tuber mass per vessel	Survivors	Survivors, tuber mass and shoot length
A. coriophora	0	248	245	206	48	206	111
	42	246	245	184	45	184	77
	300	256	245	224	48	223	71
	316	246	245	194	49	193	65
	457	257	245	223	49	223	73
	663	248	245	187	48	187	56
	703	253	245	197	47	197	45
A. laxiflora	0	208	245	152	38	152	11
	42	221	245	195	38	194	47
	300	226	245	193	37	192	27
	316	252	245	234	40	234	24
	457	223	245	193	38	193	71
	663	224	245	190	37	190	47
	703	213	245	166	38	166	17
H. robertianum	0	206	200	154	38	147	4
	42	219	200	155	37	143	3
	300	250	200	211	39	197	0
	316	234	200	184	38	168	0
	457	263	200	232	39	229	1
	663	253	200	213	40	206	0
	703	254	200	214	38	209	4
H. hircinum	0	50	40	46	8	44	0
	42	62	40	52	7	46	1

300	63	40	63	8	59	2
316	65	40	64	8	59	0
457	62	40	62	8	53	1
663	48	40	37	6	35	0
703	76	40	75	8	67	0

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759 **Table S3.** Model selection for various traits. Models retained for analyses are highlighted in bold.

Trait	Model	AICc	delta	weight
Survival pre-dormancy	**C_{10}^2**	**5187.8**	**0**	**0**
	C_{11}^2	5187.9	0	0
	C_9^2	5192.1	4.2	4.2
	$Temp\_fluc^2$	5192.6	4.8	4.8
	C_{12}^2	5193.9	6	6
Growth	**$Temp\_med^2$**	**38217.3**	**0**	**0**
	$Temp\_mean^2$	38226.7	9.5	9.5
	C_{12}^2	38239.3	22.1	22.1
	C_{11}^2	38246	28.7	28.7
	C_{10}^2	38260.1	42.9	42.9
Tuber mass pre-dormancy	**$C12^2$**	**-799.2**	**0**	**0**
	$Temp\_med^2$	-796.8	2.3	2.3
	$Temp\_mean^2$	-796.7	2.5	2.5
	C_{11}^2	-796.5	2.6	2.6
	C_{10}^2	-793.7	5.5	5.5
Tuber volume pre-dormancy	**C_{12}^2**	**12815.7**	**0**	**0**
	C_{11}^2	12827.9	12.2	12.2

	Temp_med^2	12838.5	22.7	22.7
	C_{10}^2	12839.8	24	24
	Temp_mean^2	12840.5	24.8	24.8
Survival post-dormancy	**Temp_min**	**3579**	**0**	**0**
	Temp_min^2	3584.4	5.4	5.4
	Temp_med^2	3587.1	8.2	8.2
	Temp_max	3587.7	8.8	8.8
	Temp_mean	3589	10	10
Shoot length post-dormancy	**C_6**	**3176.6**	**0**	**0**
	C_7	3177.1	0.6	0.6
	Temp_med^2	3178.9	2.3	2.3
	C_5	3179.6	3	3
	C_8	3180.5	4	4
Tuber mass post-dormancy	Temp_max	-1247.8	0	0
	C_{10}	**-1246.6**	**1.2**	**1.2**
	Temp_min	-1246.5	1.3	1.3
	C_{11}	-1246.5	1.3	1.3
	Temp_mean	-1246.1	1.7	1.7

Abbreviations: C_0–C_{12}: cumulative chilling indices, where C_x represents the number of hours with temperature below x °C; Temp_min, Temp_max, Temp_mean and

Temp_med: minimum, maximum, mean and median temperature; Temp_fluc: mean of daily temperature range.

Table S4. Logistic regression models of the influence of cumulative hours below 10 °C (C_{10}) on pre-dormancy survival (A) and minimum temperature (Temp_min) on post-dormancy survival (B) of *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum robertianum* and *H. hircinum* (sample size in Table S2).

	Intercept	Slope	SE	*z*-value	*p*-value		Effect
(A) Effect of cumulative hours below 10 °C on pre-dormancy survival							
Global model							
C_{10}					**1.88e-06**	***	Overall C_{10} effect
Species					**5.26e-07**	***	Species differences
C_{10}:Species					**0.0058**	**	Species-specific response
Species model							
A. coriophora	1.369	0.00006	0.00013	0.46	0.6429		No chilling effect
A. laxiflora	1.394	0.00033	0.00015	2.25	**0.0242**	**	Positive chilling effect
H. robertianum	0.629	0.00069	0.00012	5.52	**3.42e-08**	***	Strong positive chilling effect
H. hircinum	1.576	0.00020	0.00030	0.66	0.5125		No chilling effect
(B) Effect of minimum temperature (°C) on post-dormancy survival							
Global model							
Temp_min					**0.0083**	**	Overall cold temperature effect
Species					**<2e-16**	***	Species differences

Temp_min:Species					**2.68e-14**	***	Species-specific cold response
Species model							
A. coriophora	-1.193	0.063	0.010	6.45	**1.12e-10**	***	Lower temperature reduces survival
A. laxiflora	-1.399	-0.092	0.020	-4.62	**3.86e-06**	***	Lower temperature increases survival
H. robertianum	-5.435	0.098	0.043	2.26	**0.0240**	*	Lower temperature reduces survival
H. hircinum	-4.499	-0.050	0.134	-0.37	0.7105		No temperature effect

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Table S5. Linear regression models of the effect of median temperature (Temp_med) on pre-dormancy shoot length in mm of *Anacamptis coriophora, A. laxiflora, Himantoglossum robertianum* and *H. hircinum* at the end of 8 (A), 12 (B), and 16 (C) weeks after the onset of cold treatment (sample size in Table S2).

Species	**Adj-R^2**	**Intercept**	**Lin term**	**Lin SE**	**Quad term**	**Quad SE**	**Lin *p*-value**		**Quad *p*-value**		**Effect**
(A) Effect of median temperature on pre-dormancy shoot length - Week 8											
Global model											
$Temp\_med^2$									**<2e-16**	***	Strong nonlinear thermal control
Species									**<2e-16**	***	Strong species differences
$Temp\_med^2$:Species									**<2e-16**	***	Species-specific thermal response
Species model											
A. coriophora	0.49	4.165	59.644	1.504	15.499	1.502	**<2e-16**	***	**<2e-16**	***	Strong accelerating response
A. laxiflora	0.47	0.510	0.140	0.004	NA	NA	**<2e-16**	***			Strong linear thermal response
H. robertianum	0.07	1.014	0.022	0.002	NA	NA	**<2e-16**	***			Weak linear response
H. hircinum	0.04	1.520	0.028	0.008	NA	NA	**2.3e-04**	***			Weak linear response
(B) Effect of median temperature on pre-dormancy shoot length - Week 12											
Global model											
$Temp\_med^2$									**<2e-16**	***	Strong nonlinear thermal control
Species									**<2e-16**	***	Strong species differences
$Temp\_med^2$:Species									**<2e-16**	***	Species-specific thermal response

Species model											
A. coriophora	0.21	5.509	44.296	2.131	12.165	2.128	**<2e-16**	***	**1.3e-08**	***	Strong optimum-type response
A. laxiflora	0.26	1.735	0.221	0.010	NA	NA	**<2e-16**	***			Linear thermal response
H. robertianum	0.07	0.919	5.832	0.627	2.847	0.638	**<2e-16**	***	**8.9e-06**	***	Optimum-type response
H. hircinum	0.07	1.365	0.0468	0.010	NA	NA	**7.0e-06**	***			Linear thermal response
(C) Effect of median temperature on pre-dormancy shoot length - Week 16											
Global model											
Temp_med^2									**<2e-16**	***	Strong nonlinear thermal control
Species									**<2e-16**	***	Strong species differences
Temp_med^2:Species									**<2e-16**	***	Species-specific thermal response
Species model											
A. coriophora	0.10	54.785	375.094	28.893	116.501	28.854	**<2e-16**	***	**5.6e-05**	***	Strong accelerating response
A. laxiflora	0.03	44.440	172.335	27.690	-106.561	28.185	**6.4e-10**	***	**1.6e-04**	***	Clear thermal optimum
H. robertianum	0.08	1.735	7.687	0.772	3.658	0.786	**<2e-16**	***	**3.6e-06**	***	Optimum-type response
H. hircinum	0.07	1.373	0.059	0.013	NA	NA	**5.8e-06**	***			Linear thermal response

Table S6. Linear regression models of the effect of cumulative hours below 12 °C (C_{12}) on pre-dormancy tuber volume in mm^3 (A) and tuber mass per vessel in g (B) of *Anacamptis coriophora, A. laxiflora, Himantoglossum robertianum* and *H. hircinum* (sample size in Table S2).

Species	Adj-R^2	Intercept	Lin term	Lin SE	Quad term	Quad SE	Lin *p*-value		Quad *p*-value		Effect
(A) Effect of cumulative hours below 12 °C on pre-dormancy tuber volume											
Global model											
C_{12}^2									**3.3e-16**	***	Strong nonlinear chilling effect
Species									**<2e-16**	***	Strong species differences
C_{12}^2:Species									**<2e-16**	***	Species-specific thermal response
Species model											
A. coriophora	0.10	8.104	-40.930	3.389	-15.422	3.500	**<2e-16**	***	**1.1e-05**	***	Strong concave chilling response
A. laxiflora	0.02	2.961	2.549	1.136	-4.766	1.080	**0.0249**	*	**1.1e-05**	***	Weak optimum-type response
H. robertianum	0.02	2.801	-5.013	1.212	5.103	1.237	**3.7e-05**	***	**3.9e-05**	***	Strong convex chilling response
H. hircinum	0.05	4.584	-0.001	0.000	NA	NA	**8.5e-06**	***			Linear chilling effect
(B) Effect of cumulative hours below 12 °C on pre-dormancy tuber mass per vessel											
Global model											
C_{12}^2									**2.4e-07**	***	Strong nonlinear chilling effect
Species									**<2e-16**	***	Strong species differences
C_{12}^2:Species									**1.9e-09**	***	Species-specific thermal response
Species model											

A. coriophora	0.18	0.642	-0.000	0.000	NA	NA	**<2e-16**	***			Strong linear chilling response
A. laxiflora	0.09	0.453	0.167	0.250	-1.306	0.253	0.5042		**5.0e-07**	***	Weak optimum-type response
H. robertianum	0.03	0.405	-0.487	0.393	1.104	0.400	0.2165		**0.0062**	**	Convex chilling response
H. hircinum	0.03	0.166	-0.000	0.000	NA	NA	0.0985				No clear chilling effect

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Table S7. Linear regression models of the effect of cumulative hours below 10 °C (C_{10}) on post-dormancy tuber mass in g (A) and of cumulative hours below 6 °C (C_6) on post dormancy shoot length in mm (B) of *Anacamptis coriophora, A. laxiflora, Himantoglossum robertianum* and *H. hircinum*. Sample size in *H. hircinum* was insufficient to build a regression model (sample size in Table S2).

Species	**Adj-R^2**	**Intercept**	**Lin term**	**Lin SE**	**Lin *p*-value**		**Effect**
(A) Effect of cumulative hours below 10 °C on post-dormancy tuber mass							
Global model							
C_{10}					**3.9e-07**	***	Strong chilling effect
Species					**<2e-16**	***	Strong species differences
C_{10}:Species					0.3202		No species-specific sensitivity
Species model							
A. coriophora	0.02	0.394	-3.7e-05	1.0e-05	**0.0004**	***	Strong negative chilling effect
A. laxiflora	0.05	0.334	-5.2e-05	1.4e-05	**0.0002**	***	Negative chilling effect
H. robertianum	0.45	0.422	-1.3e-04	3.9e-05	**0.0098**	**	Negative chilling effect
H. hircinum	-0.38	0.389	7.5e-05	1.8e-04	0.7187		No chilling effect
(B) Effect of cumulative hours below 6 °C on post-dormancy shoot length							
Global model							
C_6					**<2e-16**	***	Strong chilling effect
Species					**<2e-16**	***	Strong species differences
C_6:Species					**0.0025**	**	Species-specific sensitivity

Species model							
A. coriophora	0.10	3.987	0.003	0.000	**<2e-16**	***	Strong positive chilling response
A. laxiflora	0.12	2.730	0.003	0.000	**1.4e-08**	***	Positive chilling response
H. robertianum	0.81	-0.124	0.009	0.001	**4.1e-05**	***	Very strong chilling response
H. hircinum	0.28	-0.387	0.006	0.004	0.2806		No detectable effect

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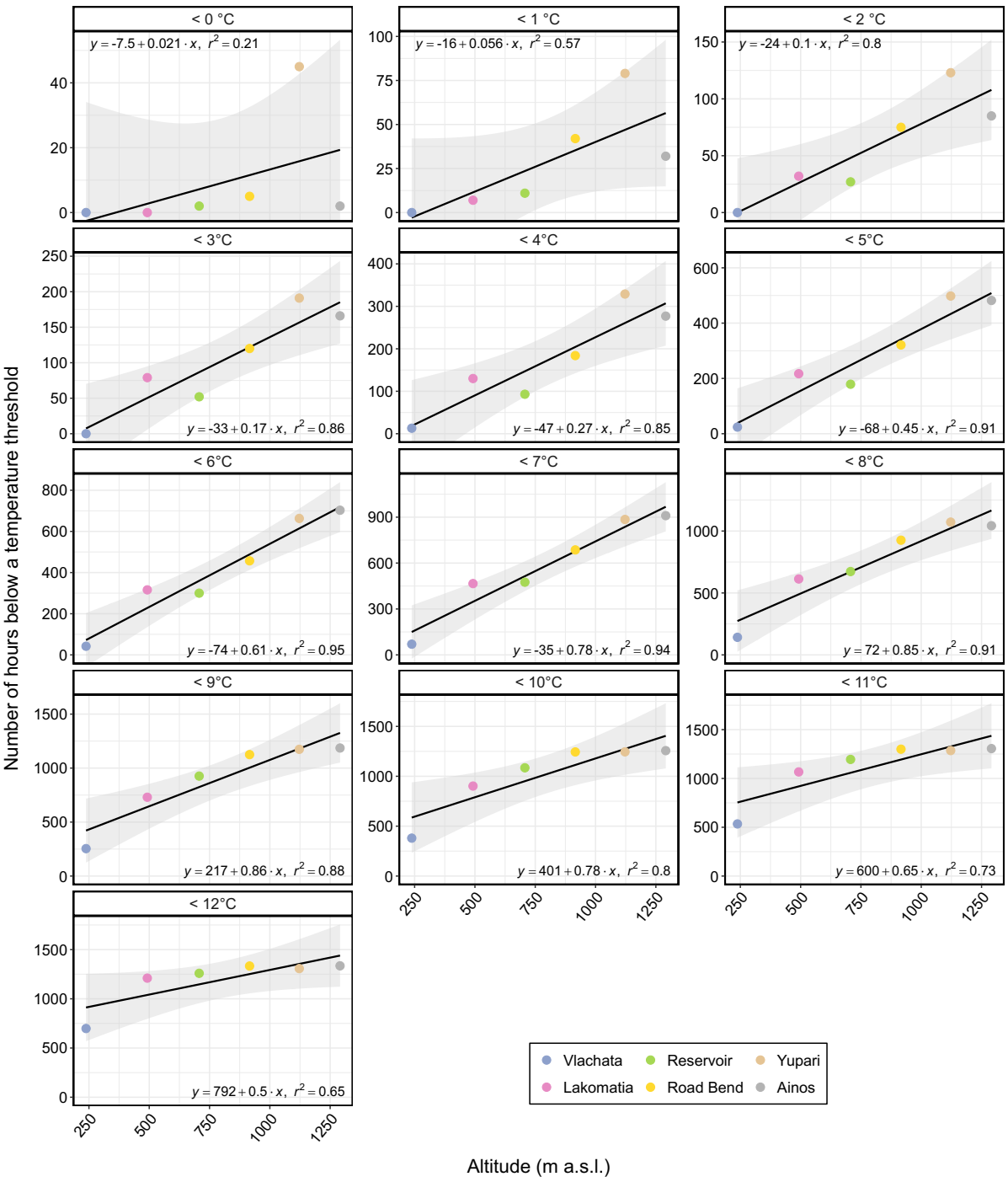

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781 **Figure S1.** Cumulative hours below a temperature threshold at the end of the cold treatment as

782 a function of altitude above sea level. Each colored point corresponds to an outdoor station.

783 Lines are fitted models.