Cold treatment benefits Mediterranean orchid seedlings cultivated *in vitro*

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Conflict of interest

The authors have nothing to disclose.

Author contributions

GD and AG conceived the ideas and designed methodology; GD, VC, AT collected the data; GD analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

The data supporting the results and the code used are freely available on Zenodo.org (https://doi.org/10.5281/zenodo.20120715). The DOI represents all versions and will always resolve to the latest one.

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Abstract

1. Effective ex situ propagation is increasingly critical for the conservation and restoration of terrestrial orchids threatened by habitat loss, over-collection and climate change. However, large-scale propagation remains constrained by developmental bottlenecks during the transition from protocorms to established plantlets with storage organs. Cold exposure is often recommended to improve propagation success in temperate orchids, yet the thermal conditions required to maximize survival and post-dormancy performance remain poorly understood.
2. We experimentally exposed protocorms of four Mediterranean terrestrial orchids (*Anacamptis coriophora*, *A. laxilora*, *Himantoglossum robertianum*, *H. hircinum*) to eight thermal environments for eight weeks, including controlled laboratory conditions and outdoor treatments distributed along a steep altitudinal gradient. We quantified survival, shoot growth, and tuber development before and after summer dormancy, and evaluated the relative importance of multiple temperature descriptors using an information-theoretic model selection approach.
3. 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) controlled survival and reserve accumulation before dormancy, while chilling below 6–7 °C promoted post-dormancy shoot growth, indicating delayed carry-over effects on subsequent development. These responses generated a positive feedback between winter chilling and growth during the following season. Thermal requirements differed markedly among species, suggesting species-specific climatic adaptations that may

influence propagation success and restoration outcomes under future warming conditions.

4. *Synthesis and applications.* Our results demonstrate that winter chilling is a key determinant of propagation success in Mediterranean terrestrial orchids and identify temperature thresholds that can be directly integrated into ex situ cultivation and restoration protocols. Species-specific chilling requirements further suggest that climate warming may alter the suitability of current propagation practices and reintroduction strategies. Optimizing cold treatments could substantially improve the efficiency of orchid conservation programmes and support climate-adapted restoration planning.

Keywords: *Anacamptis*; asymbiotic propagation; climate change; dormancy; geophytes; *Himantoglossum*; Orchidaceae; protocorm development.

1. Introduction

Orchidaceae is one of 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

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

106 A critical stage during *in vitro* propagation of terrestrial orchids is the transition from
107 protocorm to plantlet, during which the shoot apical meristem (SAM) is established (Yeung,
108 2017). The protocorm describes the life stage from germination until the formation of a shoot
109 tip with primordial leaves but no roots, during which mycorrhizal fungal association is
110 established (Rasmussen, 1995). Cold exposure treatment has been recommended to induce the
111 transition from protocorm to plantlet in temperate orchids including representatives of *Aceras*,
112 *Anacamptis*, *Dactylorhiza*, *Himantoglossum*, *Neotinea*, *Orchis* and *Spiranthes,* with
113 recommendations for temperature and duration ranging from 2-12 °C over 6-12 weeks (Hadley,
114 1970; Beyrle et al., 1985; Rasmussen, 1995; Malmgren, 2004; Ponert et al., 2012; Malmgren
115 and Nyström, 2026). Differences appear in cold treatment requirement that are likely to depend
116 on individual species cold tolerance and population geographic origin (e.g. elevation or
117 latitude) (Kauth et al., 2008b, 2011; Rasmussen et al., 2015). Better definition of temperature
118 requirements of orchid seedlings is necessary in order to improve *in vitro* propagation
119 protocols, for restoration programmes (including plant translocation) and to model species
120 response to climate change (Garbisch et al., 1995; Kauth et al., 2011; Shefferson et al., 2017).

121 This study explores the influence of cold treatment on *in vitro* orchid seedling survival
122 and growth, from protocorm until the end of summer dormancy. Protocorms of the terrestrial
123 orchids *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum hircinum* and *H. robertianum*
124 grown asymbiotically *in vitro* were subjected to varying temperature regimes. We hypothesized
125 that there would be a quantifiable, species-specific, relationship between organ growth (tubers
126 and shoots) and temperature of the cold treatment.

127

128 **2. Materials and Methods**

129 2.1. Seed collection

130 Mature seeds of *Anacamptis coriophora*, *A. laxiflora* and *Himantoglossum robertianum*
131 were collected from healthy and robust parent plants between 2016 and 2020 on the island of
132 Cephalonia, Greece. Mature seeds of the hardier *H. hircinum* were collected in 2020 from the
133 island of Ré, France. The selected species, while having localized distributions, were abundant
134 in their locations, such that seed collection would not jeopardize the natural populations.
135 Foreign materials such as capsule fragments were removed by sieving through a 500 μm sieve.
136 The seeds were dried in a desiccator with silica gel, sealed in labelled glass vials, and stored at
137 –20 °C until sowing.

138

139 2.2. Laboratory method

140 Seeds were sown asymbiotically *in vitro* in March, August and November 2020. In
141 nature, orchids require an association with symbiotic fungi to germinate, as they supply
142 nutrients to the seeds (Rasmussen et al., 2015; Çiğ et al., 2018). However, many practitioners
143 opt for asymbiotic germination (Kauth et al., 2008a; Yam and Arditti, 2009; Lee and Yeung,
144 2018), whereby the nutrients are furnished by a nutrient medium and the seeds are scarified
145 using a disinfecting solution. At the onset of the experiment, four species had well developed
146 seedlings, *Anacamptis coriophora* R.M.Bateman, Pridgeon & M.W.Chase, *A. laxiflora*
147 R.M.Bateman, Pridgeon & M.W.Chase, *Himantoglossum robertianum* Loisel., and *H.*
148 *hircinum* (L.) Spreng (Table 1).

149

150 *2.2.1. Preparation of solutions*

151 The culture substrate was a modified version of the "SM-organic" medium (Table S1).
152 This is a time-tested formula developed by Svante Malmgren, also published by Rasmussen
153 (1995), which has proved successful in previous studies (Katsalirou et al., 2017, 2019; Calevo

et al., 2020; Deconninck and Gerakis, 2021). The term "organic" refers to the N source, an amino acid mixture sold under the trade name Vaminolac® (Fresenius Kabi, Uppsala, Sweden). After mixing, pH was adjusted to 5.5–6.0 with a few drops of 3 M H_2SO_4 solution. The seeds were scarified and disinfected with a 0.5% NaClO solution to which a drop of Tween® 20 (Merck, Darmstadt, Germany) was added to reduce surface tension. Disinfection times, specific to each orchid species (Katsalirou et al., 2017, 2019; Deconninck and Gerakis, 2021), were chosen based on past experiments (Table 1).

2.2.2. Sowing

Sowing was performed inside a laminar flow cabinet (Esco®, EQU/04-EBC-2A, Singapore). All surfaces and materials were disinfected (for details, see Deconninck and Gerakis, 2021). For each species, 90 mg of seeds were scooped up with a spatula and placed in 15 mL Sarstedt® test tubes. The test tubes were nearly topped with 0.5% NaClO disinfecting solution, capped, and shaken vigorously to remove any air bubbles in contact with the seed. The shaking was repeated every 15 min thereafter until the end of the disinfection period. Then, the suspensions were decanted into Erlenmeyer flasks fitted with a funnel lined with filter paper to recover the seeds. The filter paper had been pre-soaked in the same disinfecting solution. After filtering the suspensions, each filter paper was rinsed five times with 7 ml of sterile deionized water. Each culture vessel was filled with 16 mL of sterile nutrient medium and a potato cube. The seeds were scraped with a spatula from the filter paper and distributed evenly within the culture vessel, approximately 4 mg of seeds per vessel. The tips of the steel tools were heat sterilized with alcohol burner between sowings. After sowing, the vessels were incubated in a dark room at an ambient temperature of 22 °C. At regular intervals, all vessels were visually examined for the development of fungal and bacterial infections.

179 *2.2.4. Reflasking*

180 At the protocorm stage, the seedlings were thinned and reflasked in vessels with fresh
181 medium. Appropriately sized protocorms were defined as round protocorms that had developed
182 a small shoot tip. The vessels containing the protocorms were externally disinfected with 70%
183 v/v ethanol solution before being exposed to a UVC germicidal lamp (Philips TUV 36W/G36
184 T8). Vessels for reflasking were filled with 30 mL of sterile nutrient medium and a potato cube.
185 After solidification, the protocorms were transferred with pliers from the old to the new
186 medium. Between transfers, the tips of the pliers were wiped clean and heat sterilized using an
187 alcohol burner. Each new vessel hosted five randomly selected protocorms. The number of
188 vessels for the experiment depended on the number of protocorms available for each species:
189 1960 for *A. coriophora*, 1600 for *A. laxiflora* and *H. robertianum*, and 320 for *H. hircinum*;
190 resulting in 392, 320, 320, and 64 vessels, respectively (Table 1). Some vessels appeared to
191 have more than five protocorms at the end of the experiment, likely a result of being attached
192 together when transferred. Final protocorm counts per treatment are detailed in Table S2.
193
194 2.3. Experimental setup

195 To ascertain whether temperature influences seedling development, the seedlings were
196 subjected to different temperature treatments as follows: (1) Ambient lab temperature of ~18
197 °C; (2) Refrigerated at ~7 °C; and (3) Natural temperature fluctuations at six outdoor stations
198 along a steep altitudinal gradient (Table 2). The vessels of each species were randomized and
199 distributed equally among eight crates covered with a mesh of chicken wire and wrapped in
200 plastic bags to protect them from rain and feral animals. The bags were semi-translucent to
201 provide weak light exposure, as advised for young seedlings (Svante Malmgren, personal
202 communication, 2020). Inside the refrigerator, illumination was provided by cool white,
203 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.

2.4. 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, signaled by shoots starting to wither and tubers turning from white to cream color. 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 caliper 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 signaled 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. The length of each plantlet (tuber plus shoot) and its mass were recorded.

2.5. 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. For each species, we identified the minimal tuber volume associated with the production of a functional shoot, defined as reaching at least 10% of the maximum shoot length observed for that species. To avoid spurious classification due to outliers or measurement noise, this threshold was required to be met by at least two individuals. The resulting species-specific tuber volume thresholds (2.0, 1.0, 2.0 and

253 10.5 mm³ for *A. coriophora*, *A. laxiflora*, *H. robertianum* and *H. hircinum*, respectively)
254 corresponded to minimum shoot lengths of 14, 12.5, 6.5 and 8.3 mm. Individuals with tuber
255 volumes below these thresholds were considered non-viable prior to dormancy. Post-dormancy
256 survival was directly assessed depending on the emergence of a shoot.

257 *Plant performance.* To identify which components of thermal regime best explained
258 variation in plant performance, we used an information-theoretic model selection approach
259 (Burnham & Anderson, 2002). We used the five continuous descriptors of the thermal regime
260 experienced during the 8 weeks of cold treatment (minimum, maximum, median, mean
261 temperature, temperature range) and the 13 cumulative chilling indices (C_0–C_{12}), where C_x
262 represents the number of hours with temperature below x °C. These variables capture both the
263 intensity and duration of cold exposure. For each response variable (pre-dormancy and post-
264 dormancy survival; pre-dormancy shoot length, tuber volume and tuber mass per vessel; post-
265 dormancy tuber mass and shoot length), we fitted a set of competing generalized linear or linear
266 models in which each thermal variable was entered separately, together with species identity
267 and their interaction, allowing for species-specific thermal responses (R package 'lme4'; Bates
268 et al., 2015). For each thermal predictor, we fitted both linear and quadratic equations to
269 account for monotonic effects as well as non-linear (optimum-type) physiological responses.
270 Binomial error distributions were used for survival models, and Gaussian errors were used for
271 continuous traits after appropriate variance-stabilizing transformations (see Supplementary
272 Tables for details). All models were compared using AICc (Akaike Information Criterion
273 corrected for small sample size) as implemented in the MuMIn package (Burnham &
274 Anderson, 2002). For each trait, we ranked all candidate models and identified the most
275 parsimonious predictors based on ΔAICc and Akaike weights. This procedure allowed us to
276 determine which thermal thresholds and temperature descriptors best explained each biological
277 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).

3. Results

3.1. Temperature

The six outdoor stations were chosen based on the assumption that temperature decreases with altitude (Ackerman and Knox, 2007). Accordingly, 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) and 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. The Refrigerated treatment was excluded from subsequent analyses as plantlets grown in this treatment generated outlier results that are likely a consequence of exposure to nearly constant temperature and photoperiod (Stewart and Kane, 2006; Calevo and Bazzicalupo, 2020; Nabieva et al., 2025) and prevent direct comparison to the other treatments. 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.

303 3.2. Plant survival

304 Overall, pre-dormancy survival was high and differed among species (P<0.0001; Table
305 S4A) averaging (mean ± SE) 80.6 ± 0.9%, 84.3 ± 0.9%, 77.4 ± 1.0% and 85.2 ± 1.7% for *A.*
306 *coriophora*, *A. laxiflora*, *H. robertianum* and *H. hircinum*, respectively. Survival was primarily
307 controlled by cumulative exposure to moderately low temperature rather than by extreme cold.
308 Model selection identified cumulative hours below 10-11 °C (C_{10}-C_{11}) as the best predictor of
309 survival (P<0.0001; Table S3). Survival increased significantly with increasing C_{10} in *A.*
310 *laxiflora* and *H. robertianum* (P=0.02 and P<0.0001, respectively), while no significant effect
311 was detected in *A. coriophora* or *H. hircinum* (P=0.64 and P=0.51, respectively; Figure 1A,
312 Table S4A). Accordingly, the global model showed a significant C_{10} × Species interaction
313 (P=0.0058), indicating divergent chilling sensitivities among taxa.

314 Post-summer dormancy survival differed strongly among species (P<0.0001; Table
315 S4B). While survival was still relatively high for *Anacamptis* species (28.4 ± 1.0% and 15.6 ±
316 0.9% for *A. coriophora* and *A. laxiflora*, respectively), it was close to zero for *Himantoglossum*
317 species (0.7 ± 0.2% and 0.9 ± 0.5% for *H. robertianum* and *H. hircinum*, respectively). Post-
318 dormancy survival was best explained by minimum temperature experienced during the cold
319 treatment rather than by cumulative chilling (Table S3). Species differed strongly in their
320 responses (interaction min temperature × Species, P<0.0001; Figure 1B, Table S4B): low
321 temperature reduced survival in *A. coriophora* and *H. robertianum* (P<0.0001 and P=0.0240,
322 respectively), increased survival in *A. laxiflora* (P<0.0001) but had no effect on *H. hircinum*
323 (P=0.71). The thermal stress induced by cold treatment impacted species in contrasting ways
324 even under identical post-dormancy growing conditions.

325

326 3.3. 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*, *H. robertianum* and *H. hircinum* responded mainly linearly (Table S5A, Figure 2A). By the end of week 16, growth accelerated and non-linear responses were observed for all species except *H. hircinum* (Table S5B, S5C, Figure 2A). For *Anacamptis* species, the shoot length difference between treatments was less pronounced in week 16 than in week 8 (Figure 2A), indicating that cold treatment initially slowed growth and that species differed in their capacity to compensate during the acclimatization period.

3.5. Pre-dormancy tuber growth

At the onset of summer dormancy, tubers of *H. hircinum* were the largest, with mean volume of 99.3 ± 6.6 mm^3 (mean ± SE) and mean mass per vessel of 1.060 ± 0.107 g; tubers of *A. coriophora* were of intermediate size, with mean volume of 76.0 ± 1.5 mm^3 and mean

mass per vessel of 0.464 ± 0.010 g and tubers of *A. laxiflora* and *H. robertianum* were the smallest, with mean volume of 30.3 ± 0.8 and 26.7 ± 0.8 mm^3 and mean mass per vessel of 0.217 ± 0.006 and 0.192 ± 0.009 g, respectively ($P < 0.0001$; Table S4A, S4B).

Tuber volume ($P<0.0001$) and mass ($P<0.0001$) before dormancy were primarily controlled by cumulative exposure to moderate cold. For both traits, cumulative hours below 12 °C (C_{12}) provided the best fit (Table S3), with strong quadratic effects and significant species interactions ($P<0.0001$ for both traits; Table S6A, S6B). Cold generally induced a reduction in tuber volume and mass that was more pronounced for *A. coriophora* and *H. hircinum*, with a reduction of 43.8% and 55.9% in volume and 36.7% and 28.6% in mass, respectively (Figure 2B, 2C). In *A. coriophora*, *A. laxiflora* and *H. robertianum*, tuber volume and mass followed non-linear response curves, indicating an optimal range of chilling for reserve accumulation (Figure 2B, 2C). In contrast, *H. hircinum* exhibited only weak or linear responses with a decrease of tuber volume ($P<0.0001$) and mass ($P=0.0985$) induced by the accumulation of chilling (Figure 2B, 2C; Table S6A, S6B).

3.6. 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 3A). 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 3B). *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*=0.2806), mostly due to very low survival (Figure 3B).

4. Discussion

Cold treatment of orchid protocorms has been suggested to promote their transition to plantlets. However, the recommended temperatures differ among studies, and there has been limited understanding of the effect of cold on orchid growth (Hadley, 1970; Beyrle et al., 1985; Rasmussen, 1995; Malmgren, 2004; Kauth et al., 2008b, 2011; Ponert et al., 2012; Malmgren and Nyström, 2026). 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.

4.1. 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 *Neotinia ustulata*, *Cypripedium parviflorum*, *Cleistes bifaria* and *Ophrys sphegodes* (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.

4.2. 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* var. *tuberosus* (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

452 geographical origin. Future research should compare the response of orchid species to cold
453 treatment along latitudinal gradients to better decipher species *versus* local environment
454 effects.

455 The beneficial effect of cold treatment was mostly visible post-dormancy with a strong
456 carry-over effect on growth. Shoot length increased steeply with cumulative exposure below
457 6–7 °C in *A. coriophora*, *A. laxiflora* and *H. robertianum*, and only cold-exposed plantlets of
458 *H. robertianum* and *H. hircinum* grew leaves. This demonstrates that chilling does not suppress
459 growth but, rather, promotes subsequent shoot development in most species, consistent with a
460 physiological chilling requirement for release of dormancy and efficient mobilization of
461 reserves. Post-dormancy, tuber mass declined with increasing cumulative exposure below 10
462 °C in *A. coriophora* and *A. laxiflora*, indicating that stronger chilling led to greater mobilization
463 of reserves for shoot growth. Together, the increase of shoot growth and decline in tuber mass,
464 indicate that cold exposure promotes translocation of stored carbon into above-ground growth
465 rather than directly increasing tuber size. The volume and mass of the tubers are directly related
466 to the leaf surface, as tuber storage (and therefore size) is a result of photosynthesis (Tissue et
467 al., 1995). It is likely that cold-exposed tubers enter a positive loop where a longer shoot will
468 promote higher carbon storage leading to longer shoots during the following season. Together,
469 these results indicate that chilling primarily enhances carbon storage before dormancy and
470 stimulates its mobilization for shoot growth after dormancy. The positive effects of cold
471 treatment are therefore expressed most clearly in the following growing season, rather than
472 during the chilling period itself.

473

474 **5. Conclusions**

475 This study focused on the influence of temperature on the growth of temperate
476 terrestrial orchids cultivated *in vitro*. Our data demonstrate that cold temperatures regulate

477 growth through species-specific, non-linear physiological responses rather than exposure to
478 extreme cold. Cumulative exposure to moderately low temperatures (10–12 °C) controls
479 survival and reserve accumulation before dormancy, while chilling below 6–7 °C promotes
480 post-dormancy shoot growth by enhancing reserve mobilization. These carry-over effects
481 generate positive feedback between winter chilling and growth during the following season.
482 Importantly, thermal responses differed strongly among species, indicating that chilling
483 requirements reflect species-specific physiological strategies. Besides their relevance for *in*
484 *vitro* culture, these results suggest that warmer winters may disrupt both survival and post-
485 dormancy development, with consequences that vary among taxa. This has direct implications
486 for conservation, restoration and *ex situ* propagation of orchids in the face of ongoing climate
487 change.
488
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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 SP.	LOCATION	COLLECTION	SOWING DATE	DISINF. (MIN)	REFLASK. DATE	#PROTOCORMS	#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).

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

#	LOCATION	COORDINATES	ALTITUDE (m)	ALTITUDE DIFF. (m)	MIN OF DAILY TEMP. (°C)	MAX OF DAILY TEMP. (°C)	MDN OF DAILY TEMP. (°C)	MEAN OF DAILY TEMP. (°C) ± SD	MEAN OF DAILY RANGE (°C) ± SD	CUM HOUR < 6°C
1	Refrigerator	34SDH5587824633	6	-	5.2	7.7	6.8	6.9 ±0.7[a]	1.6 ±0.9[a]	96
2	Laboratory	34SDH5587824633	6	-	16.0	20.8	18.3	18.4 ±1.6[b]	3.7 ±1.7[b]	0
3	Vlachata	34SDH6726319739	238	232	5.0	15.2	12.4	11.9 ±3.2[c]	7.5 ±1.9[c]	42
4	Lakomatia	34SDH6310023485	492	254	2.5	12.7	8.4	8.2 ±3.1[d]	6.5 ±2.3[d]	316
5	Reservoir	34SDH6591928049	707	215	2.9	11.1	8.1	7.9 ±2.7[e]	6.0 ±2.5[e]	300
6	Road Bend	34SDH6754527061	916	209	0.4	10.3	7.0	6.7 ±2.6[a]	4.4 ±2.2[g]	457

7	Yupari	34SDH6622926477	1122	206	0.0	9.5	6.3	5.9 ±3.0[f]	6.2 ±2.5[e]	663	
8	Ainos	34SDH6785422948	1291	169	1.2	9.8	5.8	5.9 ±2.6[f]	4.1 ±2.6[f]	703	

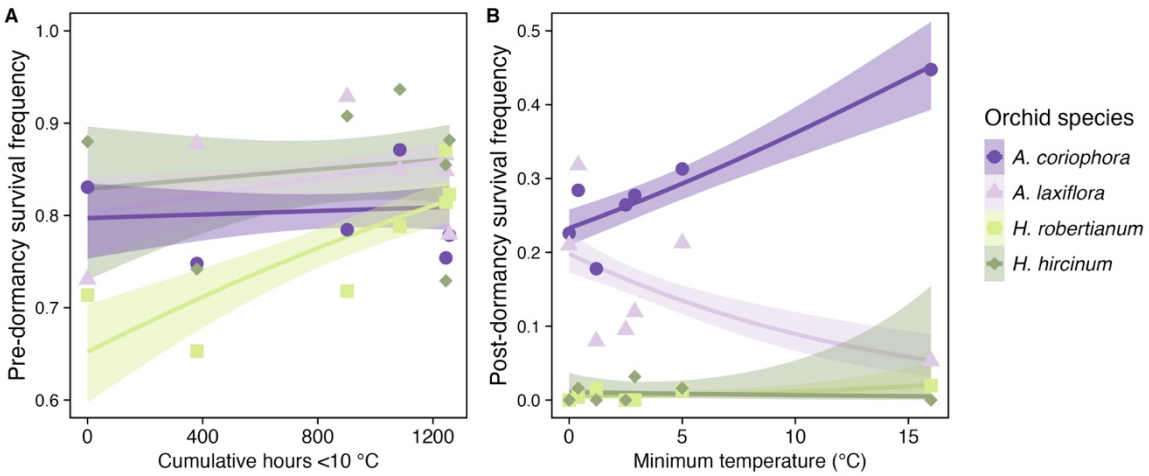
Note: Coordinates are in the Military Grid Reference System. Means with the same letter are not significantly different at $a = 0.05$ (pairwise t-test).

642

643

644 **Figures**

645



646

647 **Figure 1.** Effect of (A) cumulative hours below 10 °C on pre-dormancy survival and (B)
648 minimum temperature (°C) on post-dormancy survival of *Anacamptis coriophora*, *A. laxiflora*,
649 *Himantoglossum robertianum* and *H. hircinum* (sample sizes in Table S2). Solid lines are
650 logistic regression for each species with 95% confidence intervals. Chilling increased pre-
651 dormancy survival in *A. laxiflora* and *H. robertianum*, while no significant effect was detected
652 in *A. coriophora* or *H. hircinum*. Low temperature reduced survival in *A. coriophora* and *H.*
653 *robertianum*, increased survival in *A. laxiflora* but had no effect on *H. hircinum* (see Table S4
654 for details).

655

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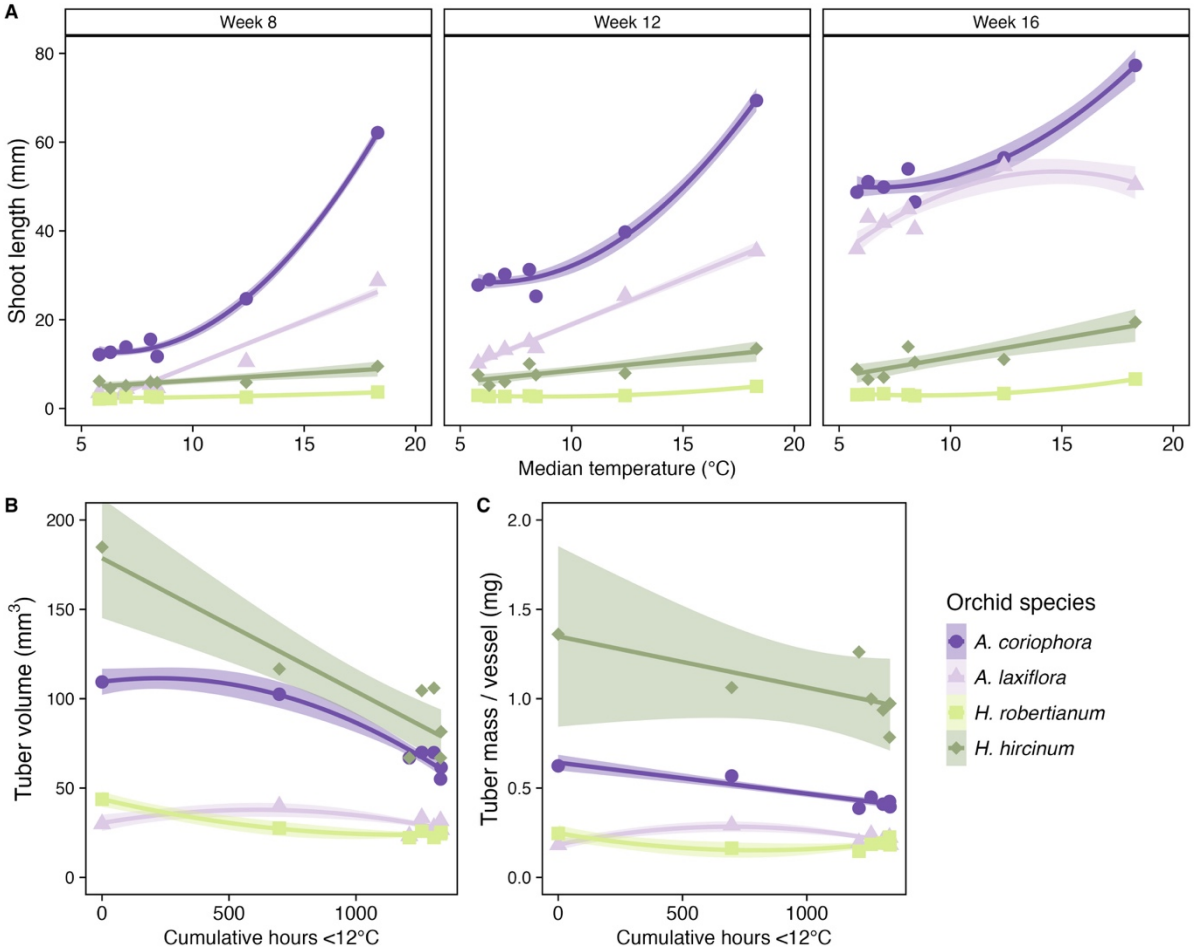
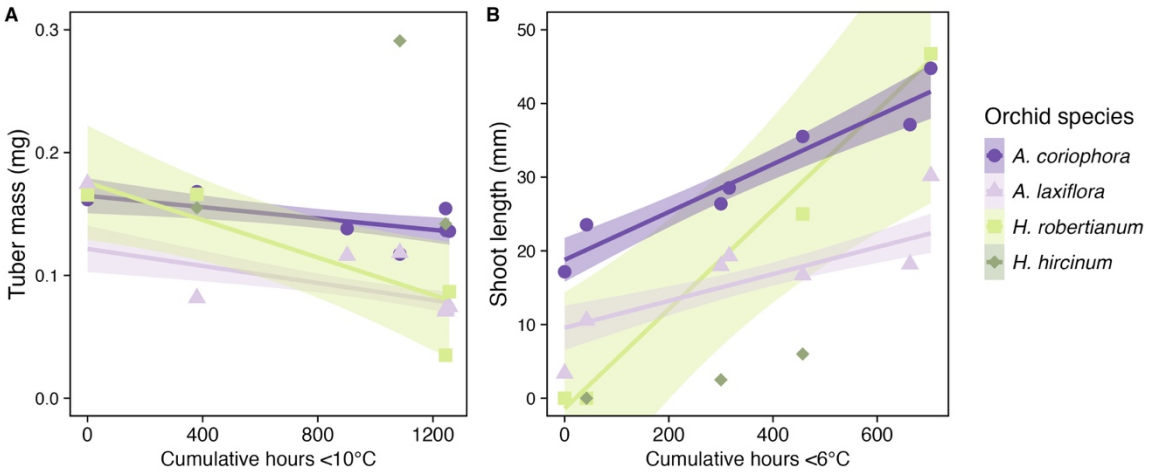


Figure 2. Effect of median temperature on (A) pre-dormancy shoot growth at various intervals, and of cumulative hours below 12°C on pre-dormancy (B) tuber volume and (C) tuber mass per vessel of *Anacamptis coriophora*, *A. laxiflora*, *Himantoglossum robertianum* and *H. hircinum* (sample sizes in Table S2). Solid lines are regressions for each species with 95% confidence intervals. Shoot length (mm) measured from the medium surface to the tip of the longest leaf. Warmer temperature during the cold treatment generally induced a faster shoot growth even though the response differed between species, while chilling generally induced a reduction in tuber volume and mass that was more pronounced for *A. coriophora* and *H. hircinum* (see Tables S5 and S6 for details).



669

670 **Figure 3.** Effect of (A) cumulative hours below 10 °C on post-dormancy tuber mass and (B)
671 cumulative hours below 6 °C on post-dormancy shoot length of *Anacamptis coriophora*, *A.*
672 *laxiflora*, *Himantoglossum robertianum*, and *H. hircinum* (sample sizes in Table S2). Solid
673 lines are linear regression curves for each species with 95% confidence intervals. While
674 chilling negatively impacted tuber mass of *A. coriophora* and *A. laxiflora*, it strongly and
675 positively impacted shoot growth in all species, except *H. hircinum* for which the relationship
676 could not be assessed due to low sample size (see Table S7 for details).

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

678

679 **Supplementary Material**

680 **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

681

682 **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

683

684

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 (A) cumulative hours below 10 °C (C_{10}) on pre-dormancy survival and (B) minimum temperature (Temp_min) on post-dormancy survival 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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692 **Table S5.** Linear regression models of the effect of the median temperature (Temp_med) on pre-dormancy shoot length in mm of *Anacamptis*
693 *coriophora, A. laxiflora, Himantoglossum robertianum* and *H. hircinum* at the end of (A) 8 weeks, (B) 12 weeks, and (C) 16 weeks after the onset
694 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 the cumulative number of hours below 12 °C (C_{12}) on pre-dormancy (A) tuber volume in mm^3 and (B) tuber mass per vessel in g of *Anacamptis coriophora, A. laxiflora, Himantoglossum robertianum* and *H. hircinum* (sample size in Table S2).

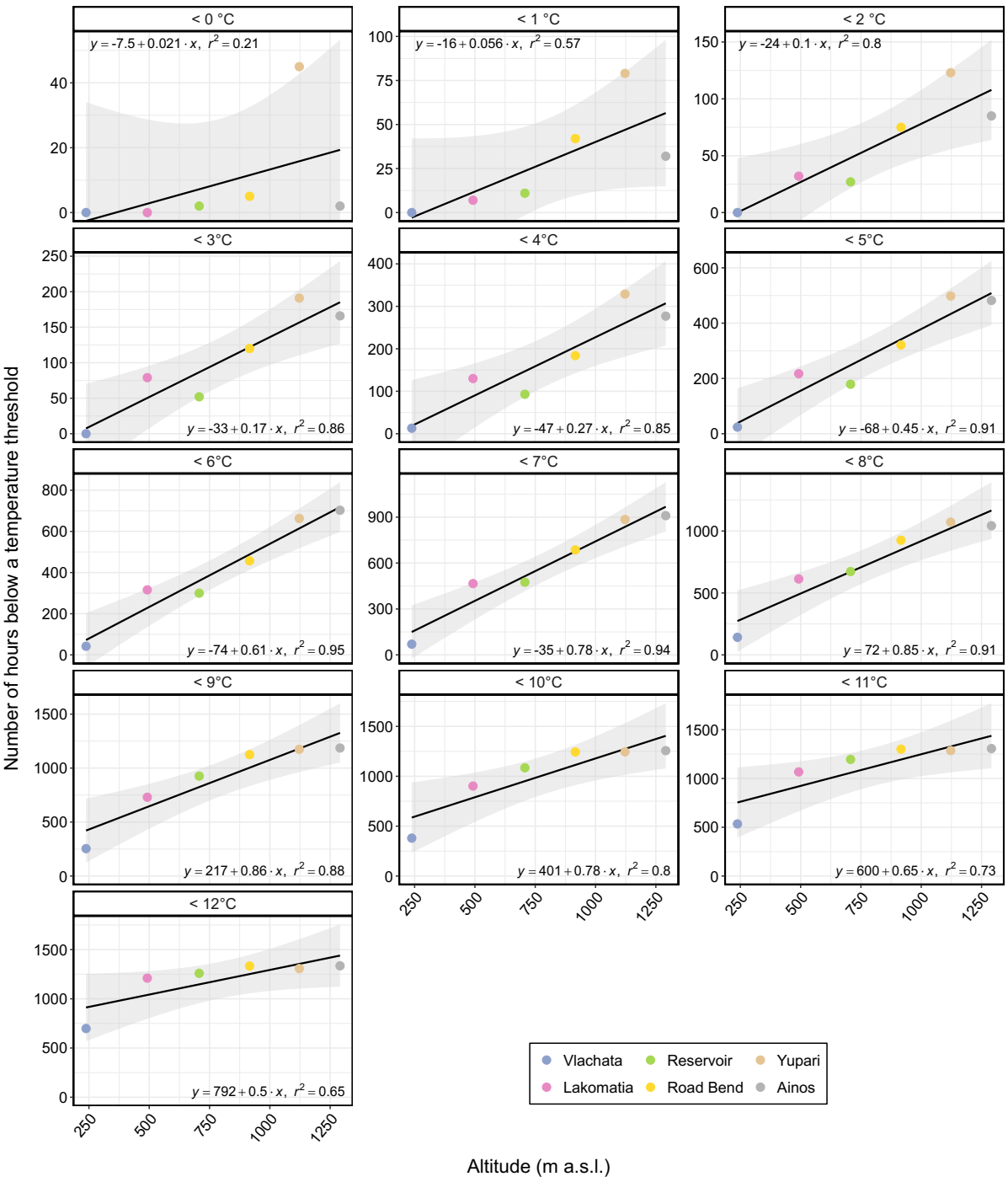
Species	Adj-R^2	Intercept	Lin term	Lin SE	Quad term	Quad SE	Lin *p*-value		Quad *p*-value		Effect
(A) Effect of cumulative hours below 12 °C on pre-dormancy tuber volume											
Global model											
C_{12}^2									**3.3e-16**	***	Strong nonlinear chilling effect
Species									**<2e-16**	***	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

Table S7. Linear regression models of the effect of (A) the cumulative number of hours below 10 °C (C_{10}) on post-dormancy tuber mass in g and of (B) the cumulative number of hours below 6 °C (C_6) on post dormancy shoot length in mm 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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708 **Figure S1.** Cumulative hours below a temperature threshold at the end of the cold treatment as

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

710 Lines are fitted models.