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Obligate diapause and its termination shape the life-cycle seasonality of an Antarctic insect

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The Antarctic midge, *Belgica antarctica*, is a unique insect endemic to Antarctica. It has a 2-year life cycle, with larvae overwintering in two different instars and adults emerging the following summer. This seasonality is crucial for adaptation to Antarctica's harsh climates and ephemeral growing seasons; however, the underlying mechanisms remain unclear. We found that, under summer-like conditions, larvae could develop from egg to the fourth-instar larval stage without interruption, but they never pupated. Spontaneous developmental arrest at this stage suggests that they overwinter in obligate diapause, a genetically determined period of dormancy. The winter cold can terminate this diapause, and long-term cold exposure is more effective. Although this species can utilise two alternative cold tolerance strategies with diapause for overwintering, freezing was more successful than cryoprotective dehydration in allowing survival and developmental resumption in our experimental conditions. In contrast, the first three larval instars continued their development under the same conditions as the fourth-instar larvae. Although we do not exclude the possibility of facultative diapause, they likely overwinter in a quiescent state, an immediate developmental arrest in response to adversity, to maximise exploitation of the short Antarctic summer. Diapause and quiescence ensure developmental and reproductive success in this extremophile insect.

Keywords Cryoprotective dehydration, Dormancy, Freezing, Life history, Overwintering, Seasonal adaptation

Dormancy is the ability of an organism to arrest growth, development, and reproduction temporarily[1]. Dormancy includes hibernation and torpor in mammals and birds[2], embryonic developmental arrest in mammals, birds, reptiles, bony fish, and elasmobranchs[3–10], as well as developmental arrest in nematodes, insects, crustaceans, and rotifers[8,9,11,12]. Dormancy minimises metabolic activity; therefore, many organisms overwinter in a dormant state to reduce energy consumption. Invertebrate dormancy can be divided into two categories, quiescence and diapause, which have been extensively studied in insects[13,14].

Both quiescence and diapause are crucial for seasonal adaptation[15]. Quiescence is an immediate developmental arrest in response to adversity and is broken as soon as favourable conditions return[16,17]. For example, many invertebrates placed under cold conditions enter quiescence, halting their activity, development, and reproduction, but then resume these processes almost immediately when warmed to more favourable temperatures[15]. Quiescence enables a swift transition between active and inactive lifestyles, making it possible to maximally exploit periods when temperatures exceed a certain threshold[15]. Diapause is a more tightly regulated stage-specific strategy commonly adopted by temperate insect species to survive winter[18,19]. This occurs in anticipation of an upcoming harsh environment, allowing insects to accumulate additional energy reserves and seek suitable overwintering sites before diapause entry[15]. Insects obligately or facultatively enter diapause. In the less common obligate diapause, insects require no external cues to initiate developmental arrest because it represents a fixed component of the ontogenetic program[14]. In the more widespread facultative diapause, insects

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require specific stimuli (most commonly photoperiod) to initiate developmental arrest, where individuals can switch between two ontogenetic alternatives: direct development or diapause[15,20].

In contrast to quiescence, insects in diapause must experience a certain time interval or specific environmental conditions prior to resuming development and reproduction. These conditions may include chilling, moisture, food availability, and daylength[21]. The latency period of diapause acts as a safeguard, preventing the untimely initiation of development and reproduction when conditions are or will soon become prohibitive for development and reproduction. This delayed recovery from a dormant state distinguishes diapause from quiescence[15]. Prolonged low temperatures in late autumn and early winter stimulate diapause termination in many insect species. However, as long as temperatures during mid-winter to early spring remain below the developmental threshold, then the individual remains dormant and enters a phase known as post-diapause quiescence[20,21]. The cold serves as a mechanism for arresting all individuals in the post-diapause stage, which are then fully competent to develop synchronously when the temperature rises above a certain threshold in spring. This simple mechanism promotes synchronous emergence under favourable conditions for development and reproduction[15]. Thus, quiescence and diapause are complementary. Quiescence is an immediate response to unpredictable environmental challenges, but on its own, it is unable to synchronize development. In contrast, diapause offers developmental synchronisation but may result in missed opportunities for growth and reproduction.

The Antarctic midge, *Belgica antarctica* (Diptera: Chironomidae), is the southernmost insect inhabiting Antarctica[22–25]. This species has four larval instars, in common with most other chironomids[25–27]. The relative frequency of cohorts of different instars during the Antarctic seasons indicates that a 2-year lifespan is typical[27]. Although this species can overwinter in all four larval instars, field evidence suggests that larvae mostly spend their first winter in the second instar and the second winter in the fourth instar[27]. The habitat of this species is covered by snow and ice during long (6–9 months) winters, and larvae spend the winter at low temperatures ranging from −2 to −10 °C in a dormant state[28,29]. Pupation and adult emergence occur synchronously shortly after snowmelt[27]. Adult females lay eggs immediately after mating[27,30,31], and females that fail to mate die before reproduction because of their very short adult longevity (only a few days), further indicating the importance of synchronous adult emergence[31,32]. *Belgica antarctica* must coordinate its life cycle with the Antarctic seasons to exploit a very short summer for development and reproduction[28,33].

Although diapause is a major overwintering strategy used by temperate and Arctic species, it appears uncommon in Antarctica[34–36]. In Antarctica, the unpredictability of freezing and desiccation makes it difficult to determine the appropriate timing for diapause initiation, and the fitness cost of inappropriate initiation is high[34,36]. Alternatively, flexibility and opportunism that allow growth and development whenever conditions are favourable seems to be more common[34–37]. In *B. antarctica*, photoperiod, which is the most influential environmental cue for facultative diapause in temperate insects[38], does not affect larval cold tolerance[39]. Furthermore, behavioural and gene expression analyses revealed that the circadian clock, a prerequisite system for facultative diapause[38,40], does not function in this species[41]. These results support the idea that *B. antarctica* does not anticipate upcoming seasons from environmental changes, at least from photoperiod[34,36]. Convey (1996) hypothesised that the seasonality observed in the life cycle of this species could be based simply on direct developmental arrest, i.e. quiescence[34]. However, developmental rates can vary greatly with larval nutritional and temperature conditions[42], indicating that developmental stages at the end of summer may be widely scattered in the field. If the larvae only overwinter in a quiescent state, then asynchronous development continues. Thus, the synchronous adult emergence observed in the field cannot likely be explained solely by quiescence[43].

Here, we propose that *B. antarctica* adopts obligate diapause to adapt to the Antarctic seasons. If this species enters diapause, this can be terminated by low temperatures during overwintering. *Belgica antarctica* has been known to adopt two distinct ecophysiological overwintering strategies: freeze tolerance and cryoprotective dehydration[33]. This species tolerates the formation of ice in their body tissues and fluids, as reported in various insect species[44]. Cryoprotective dehydration is a type of freeze avoidance whereby ice formation is prevented via the removal of freezable water to environmental ice through a permeable cuticle[28,45–47]. In *B. antarctica*, slow cooling to −3 °C in an environment at equilibrium with the vapour pressure of ice in the laboratory reduces larval water content by ~40% and depresses the body fluid melting point more than threefold to −2.6°C[33]. The depression in melting point is due to both the concentration of preexisting solutes (i.e. reduction of body water) and the initiation of osmolyte synthesis[33]. These laboratory experiments revealed that freezing and cryoprotective dehydration are both effective in promoting the survival of larvae, at least after 32 days of chilling, with surprisingly little difference in energetic costs[28].

In the present study, we carefully examined larval development in the laboratory to clarify the physiological strategies for seasonal adaptation of *B. antarctica*. We found that the newly hatched larvae could develop to the fourth-instar larval stage without interruption and then spontaneously arrested their development at the middle (L4-2 stage) of that instar. These results suggest that fourth-instar larvae spend their second winter in obligate diapause. Next, we examined the environmental conditions required to terminate diapause and identified a critical overwintering period for diapause termination. We also assessed the effect of freezing within ice and cryoprotective dehydration on survival and developmental resumption[24,41,42].

Results

Larvae of *B. antarctica* were collected on several islands near Palmer Station, Anvers Island, in 2018 and 2020. These larvae became pupae and adults in the laboratory between late May and September 2018 and June and September 2020. The adults laid egg masses within 1–2 days after emergence. As reported in previous studies, first-instar larvae emerged within approximately 16 days at 4 °C[32,42].

Larval development

First-instar larvae from the 2018 collection were maintained at 4 °C under L: D 18:6 h, as previously described[32,33,42,48]. The larvae successfully developed into fourth-instar larvae without interruption at 4 °C (Fig. 1A_1). The time required for half of the population to develop into the second-, third-, and fourth-instar larval stage was 40, 70, and 190 days, respectively. However, the fourth-instar larvae did not pupate by the time the experiment was concluded on day 360 (Fig. 1A_1). We further maintained the larvae from the 2020 collection at 10 °C under L: D 18:6 h. Although this temperature was higher than that used in previous studies[32,33,49], larval development and survival of this colony were better at this temperature than at 4 °C. A high rearing temperature accelerated larval development; thus, the time required for half of the population to develop into second-, third-, and fourth-instar larval stages was 20, 30, and 40 days, respectively (Fig. 1A_2). Again, the fourth-instar larvae did not pupate by the end of the experimental period (day 240) (Fig. 1A_2).

The fourth-instar larval stage can be subdivided into three developmental stages, as reported in a previous study[50]: L4-1, L4-2, and prepupa (Fig. 1B). L4-1 is the earliest stage, with a short body length and small imaginal leg discs. The thick L4-2 larvae had round, enlarged discs. The prepupae were further thickened, their second and third thoracic segments were swollen, and their leg discs developed into a complex form. At the end of the experimental period, all fourth-instar larvae that had not pupated were at the L4-2 stage. These results indicate that early instar larvae have no ability to arrest their development, whereas fourth-instar larvae spontaneously arrest development at the L4-2 of the fourth-instar larval stage with no external cues.

Developmental resumption after chilling

In many cases, diapause can be terminated by low-temperature exposures, as commonly reported in temperate species[15]. To investigate this, the L4-2 fourth-instar larvae from the 2020 collection were transferred to peat moss (a soil substrate), which was rehydrated with water at 0.9 g water/g dry mass to simulate a winter habitat[33]. The soil containing larvae was maintained under winter conditions of − 5 °C and constant darkness for 3 and 6 months. We confirmed that the soil containing larvae was frozen. Other larvae were continuously maintained under summer conditions of 4 °C under L: D 18:6 h, as a reference. After exposure to − 5 °C, the larvae were transferred to the summer conditions.

Under summer conditions without low-temperature exposure, survival was 85.7% (30 out of 35 individuals) by the end of the experimental period (Fig. 2A). None of these larvae developed into prepupae. Survival of fourth instar larvae after 3- and 6-month exposures to − 5 °C was 97.3% (73 out of 75 individuals) and 66.7% (92 out of 138 individuals), respectively (Fig. 2B,C). A small number of surviving larvae that had been maintained at − 5 °C for 3 months developed into prepupae (11.0%), pupae (11.0%), and adults (8.2%). The proportions of continuing development further increased after exposure for 6 months at − 5 °C: 43.5% prepupae, 34.8% pupae, and 28.3% adults. These proportions of larvae advancing to each developmental stage were statistically significant for all comparisons (Tukey-type multiple comparisons for proportions, $P < 0.05$). These results suggest that fourth-instar larvae enter obligate diapause, which can be terminated by prolonged chilling.

Adults (15 females and 11 males) that emerged after the 6-month exposure could mate and laid 10 egg masses containing 38–214 eggs with a median fertility of 88.9% (0–100%). The egg masses contained 19–196 fertilised eggs with a median hatchability of 97.6% (82.6–100%). Thus, individuals that had terminated diapause by 6 months at − 5 °C developed into fertile adults.

The effect of freezing and cryoprotective dehydration on diapause termination

We found that chilling was effective for diapause termination, but we still do not know which overwintering strategy, freezing or cryoprotective dehydration, is effective for diapause termination. To approach this, we collected fourth-instar larvae at the L4-2 stage and placed them in water containing ice to induce inoculative freezing or in cryoprotective dehydration conditions with environmental ice. They were kept at two temperatures (− 3 °C and − 5 °C) for 6 months, as described in a previous study[29]. The supercooling points (SCPs) of summer and winter larvae were − 10 and − 15 °C, respectively, indicating that larvae would require inoculation by environmental ice to initiate freezing[51–53]. We confirmed that the larvae were encapsulated in ice under freezing conditions. We also verified the status of the larvae during cryoprotective dehydration based on their body mass. Larvae undergoing cryoprotective dehydration at − 3 and − 5 °C for 6 months lost 76.3% and 51.4% of their body mass, respectively, because they remained unfrozen and were, therefore, dehydrated (Fig. S1). Although we did not measure the dry mass, the weight reduction at − 5 °C corresponded to a 68.0% water loss when we assumed an initial water content of 75.6%[54]. Cryoprotective dehydration at − 3 °C was substantially more severe in the current experiment, resulting in the removal of nearly all body water. After these 6-month winter treatments, the larvae were transferred to summer conditions (4 °C under L: D 18:6 h).

Lower survival rates were observed in cryoprotective dehydration compared to freezing within ice (Fig. 3). The proportions of fourth instar L4-2 larvae that survived were 54.5% and 41.8% for freezing within ice at − 3 °C and − 5 °C, respectively, and 5.5% and 9.1% for cryoprotective dehydration at − 3 °C and − 5 °C, respectively ($n = 55$ for each). Small proportions of prepupae (12.7%), pupae (7.3%), and adults (5.5%) appeared after exposure to freezing conditions at − 3 °C ($n = 55$) (Fig. 3A). The proportions increased after exposure to freezing conditions at − 5 °C (Fig. 3B): prepupae (29.1%), pupae (29.1%), and adults (23.6%) ($n = 55$). In contrast, these proportions were very small in cryoprotective dehydration at both temperatures (5.1% or lower; Fig. 3C,D). These results indicate that freezing within ice at − 5 °C is the most effective treatment for the combination of survival and diapause termination in our experimental conditions.

We collected four females and nine males that emerged after exposure to freezing conditions at − 5 °C and observed their reproductive success. Five egg masses containing 23–50 eggs were laid, with a median fertility of 94.5% (0–100%). Egg masses contained 41–50 fertilised eggs, with a median hatchability from fertilised eggs of 91.1% (90.2–100%). Thus, individuals that had terminated diapause by freezing developed into fertile adults.

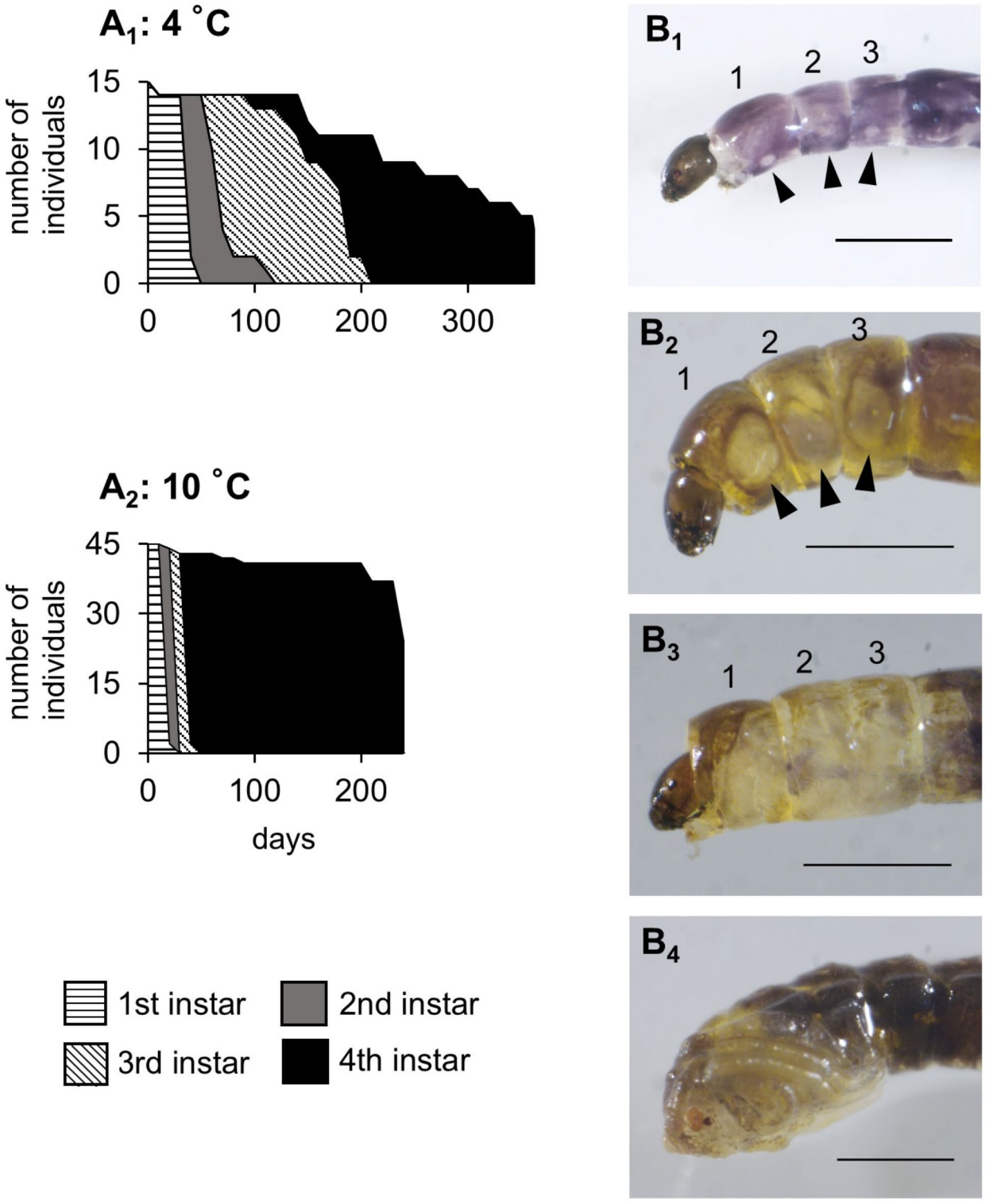

Fig. 1. Survival and development of larvae (**A**) and morphology of larvae and pupae (**B**) of *Belgica antarctica*. (**A**) Survival and development of larvae at 4 °C (A_1; $n = 15$) and 10 °C (A_2; $n = 45$). The larvae of A_1 and A_2 were from the 2018 and 2020 collections, respectively. (**B**) Morphology of the fourth-instar larvae and pupae. (B_1) L4-1 larvae with small imaginal discs. (B_2) L4-2 larvae with large imaginal discs. (B_3) Prepupae (L4-3), in which round-shaped imaginal discs further developed into complex forms and their thoraxes were swollen. (B_4) Pupae. The numbers indicate the thoracic segments. Arrowheads indicate imaginal leg discs. *Scale bars* 500 μm.

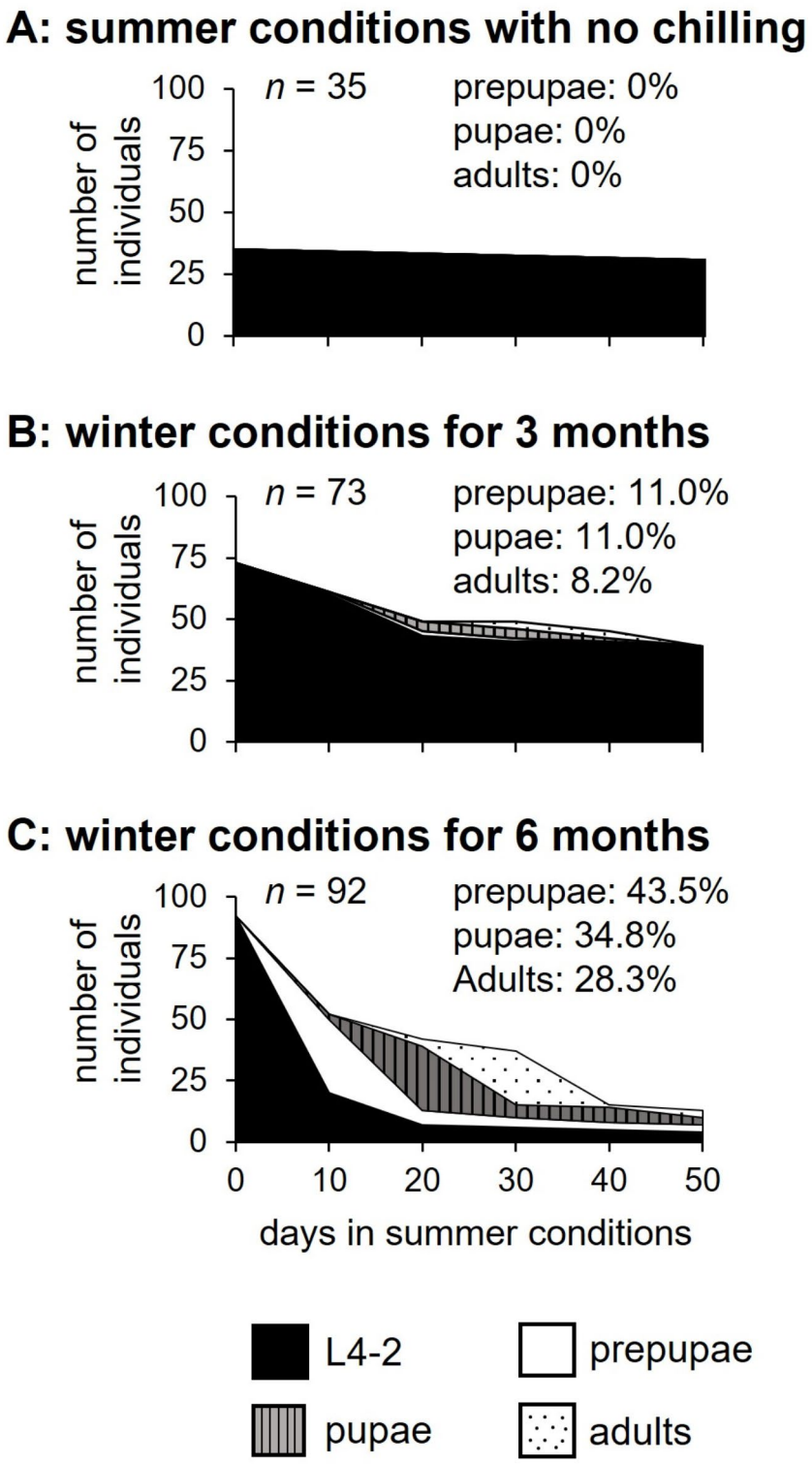

Fig. 2. Survival and development of *Belgica antarctica* larvae that had been exposed to winter conditions of − 5 °C under constant darkness for 3 and 6 months in a soil substrate and returned to summer conditions of 4 °C under L: D 18:6 h. (**A**) Larvae under summer conditions at 4 °C with no chilling. (**B**) Larvae exposed to winter conditions for 3 months. (**C**) Larvae exposed to winter conditions for 6 months. *n*, number of individuals. The proportions of prepupae, pupae, and adults until day 50 are shown.

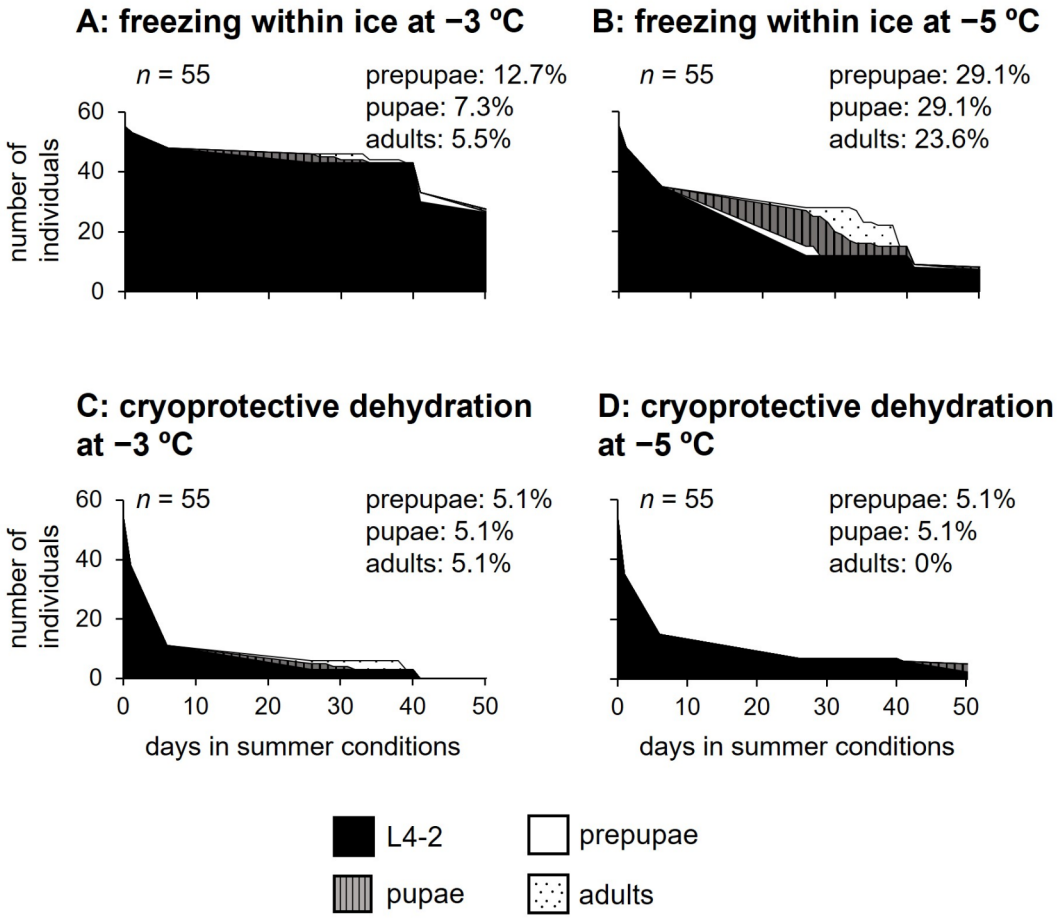

Fig. 3. Survival and development of *Belgica antarctica* larvae that had been frozen within ice or in cryoprotective dehydration at − 3 to − 5 °C for 6 months. Proportions of prepupae, pupae, and adults until day 50 are shown.

Discussion

Belgica antarctica is remarkably well adapted to the severe environmental conditions of Antarctica. This adaptability is a testament to the resilience of this species in the face of extreme challenges[55,56]. Although its stress tolerance has been extensively studied, the physiological mechanisms by which this species enters winter dormancy and synchronises its life cycle with Antarctic seasons have received less attention. In the present study, we found that larval development was uninterrupted at the earlier developmental stages (first- through to third-instar larvae) but was arrested spontaneously (obligate diapause) at the L4-2 stage of fourth-instar larvae. This diapause could then be terminated by chilling at − 5 °C and freezing within ice at both − 3 °C and − 5 °C, with the lower temperature being more effective. The long-term cryoprotective dehydration in the current experimental conditions was severe and thus less effective for overwintering (survival and diapause termination).

All larval stages of *B. antarctica* can overwinter, but the second and fourth instars are the predominant overwintering stages in the field[27]. The present study revealed that spontaneous developmental arrest did not occur in the early instar larval stages, suggesting that early instar larvae likely undergo quiescence in the field, an immediate interruption of growth due to unfavourable conditions with no preparatory phase. Quiescence offers the capacity to quickly stop and restart development multiple times at any stage in response to certain environmental challenges[15]. Adequately warm temperatures that permit development and feeding are at a premium in challenging polar environments. Thus, the lack of a spontaneous developmental arrest in the early instar stages may reflect an adaptation to fully exploit permissive but unpredictable thermal conditions during the brief austral summer. The significance of warm temperatures during the growing season is also supported by the larval locomotor activity rhythm, which is shaped by temperature but not by the circadian clock[41]. Therefore, early instar larvae appear to avoid preprogrammed behaviour to maximise their ability to grow and develop when temperatures are permissible. However, we still do not exclude a possibility that early instar larvae enter facultative diapause in response to Antarctic autumnal short daylengths and low temperatures. Further experiments under various conditions are required to see whether all or some of these larval instars have the capacity to enter facultative diapause.

Spacht et al. reported a coordinated decrease in oxygen consumption during March, the end of summer, in field-collected fourth-instar larvae of *B. antarctica*[57]. This metabolic depression occurred irrespective

of environmental conditions, such as shortened daylength, dehydration, and chilling. Combined with the spontaneous developmental arrest at the fourth-instar (L4-2) larval stage observed in the current study, this suggests an anticipatory response to seasonal changes, regardless of environmental cues. This represents an intrinsic pre-programmed obligate diapause mechanism that requires winter chilling or encapsulation in ice to be terminated to facilitate synchronous adult emergence and reproduction at the beginning of the very brief austral summer.

In most temperate insects, a certain time interval must elapse before diapause can be terminated[15]. This safeguard prevents the untimely initiation of development when the conditions are or will soon become prohibitive. The term diapause development captures the idea that events occurring during diapause lead to its eventual termination[58]. Chilling accelerates diapause development in many insect species[15,21]. In our experiments with *B. antarctica*, low-temperature exposure was also required for diapause termination to effectively induce developmental resumption after transferring to summer conditions. This further suggests that the observed developmental arrest is diapause. It is also noteworthy that larvae discriminated the duration of chilling, i.e. exposure for 6 months at − 5 °C was more effective for developmental resumption than exposure for 3 months. Complete diapause development may require a longer duration of cold exposure, suggesting that it functions as a safeguard.

Freeze tolerance and cryoprotective dehydration are cold tolerance strategies used by various invertebrate species in polar regions and indeed, *B. antarctica* utilises both for overwintering[28,59]. Larvae that are frozen in ice and cryoprotectively dehydrated readily survived 32 days of simulated overwintering[46]. Unlike many insects restricted to highly specific microhabitats, *B. antarctica* larvae inhabit a remarkably diverse range of substrates that differ in vegetation, substrate type, slope, drainage, and thermal and hydric conditions[56]. Whether larvae overwinter by freezing or by cryoprotective dehydration is likely determined by moisture levels within their hibernaculum[28,33], a strategy also employed by an Arctic enchytraeid worm[60]. The flexibility to use either strategy is considered to account, in part, for their extreme southern distribution. However, the present study indicates that long-term cryoprotective dehydration, losing 68.0% or more of boy water, is less effective strategy for overwintering (survival and diapause termination) in *B. antarctia*. Further experiments are needed to investigate the adequacy of freezing and dehydration for overwintering because the experimental conditions in this study were still unrealistic in detail (encased within ice and gradual decrement of temperature with subsequent constant temperature exposure).

The present study further reveals an unexplored feature in diapause development, with developmental resumption occurring most effectively after freezing at − 5 °C compared to − 3 °C. *Belgica antarctica* larvae may be able to proceed with biochemical reactions that promote diapause development in a frozen state and discriminate between temperature conditions. How this occurs is largely unknown, but a similar result was reported in the freeze-tolerant gall fly *Eurosta solidaginis*[61]. This species discriminates the duration of exposure in a frozen state at − 20 °C. Investigating the physiological mechanisms underlying diapause development under such low-temperature conditions will be of great interest.

Diapause has long been assumed to be uncommon in Antarctic species[34], but the present study reveals that *B. antarctica* utilises diapause for seasonal adaptation, as in many temperate species[15]. Bartlett et al. reported in a sister species of *B. antarctica*, the sub-Antarctic endemic midge *Eretmoptera murphyi*, that progression from fourth-instar larvae to pupae was infrequent and unpredictable in the laboratory at 4 °C under constant darkness[62]. This may also indicate the possibility of obligate diapause in *E. murphyi*. Both species were separated from their closest known relatives in Patagonia ~ 68.5 million years ago[63]. The ability of *B. antarctica* to enter obligate diapause may have been inherited from its common ancestor. Obligate larval diapause has been reported in other chironomid species inhabiting high latitudes and altitudes[18,19,64]. In summary, *B. antarctica* likely adopts two distinct strategies for overwintering, quiescence and obligate diapause. Chilling or freezing within ice completes its diapause development, and the resulting competent larvae resume synchronous development at the onset of the austral summer when temperatures exceed their developmental threshold. This, in turn, enables synchronous adult emergence, which is critical to ensuring successful reproduction given both the narrow window of favourable conditions offered by the austral summer and the limited longevity of the adult life stage. These physiological mechanisms shape the phenology of this midge in Antarctica. The present study further proposes that semivoltine or long-lived invertebrates inhabiting harsh environments may also utilise quiescence and diapause to exploit their benefits.

Materials and methods

Belgica antarctica larvae were collected on several islands near Palmer Station, Anvers Island (64° 46′ S, 64° 04′ W), along with the substrate, which included small rocks, detritus, algae, and mosses, from their habitat. The larvae and substrates were transported with ice under constant darkness at 0–4 °C to Osaka City University via Miami University (OH) and the University of Kentucky (permission no. 24K134; Plant Protection Station, Ministry of Agriculture, Forestry and Fisheries, Japan). Larvae in the Antarctic substrates were maintained in sealed plastic boxes (width 26.0 cm, depth 18.5 cm, height 6.0 cm) at 4 °C under L: D 18:6 h to approximate the summer conditions of their habitat[32,42].

Spontaneous adult emergence occurred in the laboratory, and adults were transferred to Petri dishes (diameter 7.0 cm, height 1.0 cm) that contained a piece of wet paper towel. Dishes were sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, MN, USA) to maintain high humidity and maintained at 4 °C under L: D 18:6 h for mating. Females laid egg masses on paper towels within 1–2 days after emergence. Egg masses were placed in sealed Petri dishes at 4 °C under L: D 18:6 h, as described above. Hatching occurred approximately 16 days later[32,42].

The larvae were fed an artificial diet of 2.0% milk in 0.5% agar with a powdered phytophagous fish meal (Hikari Crest Pleco; Kyorin Company, Himeji, Japan), which contained marine algae and *Chlorella*[42], to adjust the nutritional conditions.

The fourth-instar stage can be divided into three substages: L4-1, L4-2, and prepupae[50]. The prepupae metamorphosed into pupae.

Larval development

The dish and diet were replaced every 5–10 days, and the number of surviving individuals and their developmental stages were recorded for 360 and 240 days at 4 °C and 10 °C, respectively. Any period of developmental arrest was noted. Larval stages were distinguished by the width of the head capsule[27]. Developmental stages within the fourth-instar larval stage were also recorded[50].

Developmental resumption after chilling

The soil containing larvae was maintained under winter conditions of − 5 °C and constant darkness in a plastic seal dish (diameter 6.0 cm, height 3.0 cm) for 3 or 6 months. We confirmed that the soil was frozen. After 3- or 6-month exposure to − 5 °C, the larvae were transferred to summer conditions of 4 °C under L: D 18:6 h. As a reference, other larvae were continuously maintained under summer conditions without exposure to − 5 °C. To avoid mould growth, an artificial diet was not provided. We replaced the plastic seal dishes every 10 days and recorded the number of survivors (the number of individuals exhibiting voluntary movement) and their developmental stages.

Adult emergence was monitored daily. Newly emerged adults were collected and transferred to Petri dishes for mating. Egg masses were collected and maintained at 4 °C under L: D 18:6 h to observe their development. Fertilised eggs gradually turn yellowish within a few days[32]. The proportion of fertilised eggs was calculated by dividing the number of fertilised eggs by the total number of eggs in the mass. Hatchability was calculated by dividing the number of hatched larvae by the number of fertilised eggs.

The effect of freezing within ice and cryoprotective dehydration on diapause termination

For the freezing treatment, the L4-2 fourth-instar larvae were placed in groups of 5–10 in tap water in a 0.6-mL plastic tube and placed directly at either − 3 °C to − 5 °C for 6 months. We confirmed that the water remained frozen under these conditions. For cryoprotective dehydration, groups of 5–10 L4-2 fourth instar larvae were placed in a 0.6-mL plastic tube covered with nylon mesh and placed in a glass tube containing ice. The tube was tightly closed using a cotton ball. Larvae were exposed to gradual cooling from either 0 to − 3 °C for 5 days or 0 to − 5 °C for 6 days (− 0.6 °C/day)[49]. The larvae were subsequently exposed to a stable temperature of − 3 °C to − 5 °C for 6 months[29].

To determine the status of the larvae that had been frozen within ice or under cryoprotective dehydration, the body masses of 10 larvae were individually recorded. After recording the body mass of larvae, samples were kept in water for 1 day at 4 °C under L: D 16:8 h for rehydration. After that, we placed the larvae into peat moss (0.9 g water/g dry mass) and observed larval development every 10 days. No artificial diet was provided to avoid mould growth. The number of adults was recorded daily. The proportion of fertilised eggs and hatchability of eggs laid by the emerging adults were also recorded, as described above.

Statistical analysis

The proportions of prepupae, pupae, and adults and survival rates were analysed using Tukey-type multiple comparisons for proportions. Body weights were analysed using Student's *t*-test.

Data availability

Data is provided within the manuscript and supplementary information file. You can see all the raw data in the "data.xlsx".

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M.Y.: conceptualisation, methodology, formal analysis and investigation, writing original draft preparation, review and editing, and funding acquisition; P.C.: writing review and editing, and funding acquisition; S.A.L.H.: writing review and editing, and funding acquisition; R.E.L.: writing review and editing, and funding acquisition; D.L.D: writing review and editing, and funding acquisition; N.M.T.: writing review and editing, and funding acquisition; S.G.G.: conceptualisation, methodology, writing review and editing, and supervision. All authors provided final approval for publication and agreed to be held accountable for the work performed therein.

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Declarations

Competing interests
The authors declare no competing interests.

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